Enterohepatic Circulation of Tetracycline in Rats

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Abstract □ The absorption of tetracycline hydrochloride excreted in the bile of rats was evaluated using the *in situ* intestinal preparation. For comparative purposes, the absorption of the drug from an aqueous solution having the same pH as that of the bile was also determined. After 4 hr, the amounts of tetracycline absorbed from the bile and aqueous solutions were 72.92 and 77.34%, respectively. There was no significant difference in the amount of drug accumulated in the gut tissue. The disappearance of the drug from the intestinal lumen was biexponential, and the kinetic parameters appeared to be similar. It was concluded that tetracycline excreted in the bile is readily absorbed from the rat intestine. Accordingly, biliary excretion does not seem to account for a significant elimination of this antibiotic from the body.

Keyphrases □ Tetracycline—intestinal absorption from bile and aqueous solutions, elimination, rats □ Absorption—intestinal, tetracycline from bile and aqueous solutions, rats □ Excretion, biliary—role in tetracycline elimination, rats □ Biliary elimination—tetracycline, rats

Studies on the disposition of tetracycline in humans and animals indicate that 50–70% of the dose is eliminated unchanged in the urine after both intravenous and oral administrations (1–5). Since no significant metabolism of tetracycline has been observed (6), excretion *via* the bile is presumed to be the other route of elimination (7–10). The observed high levels of tetracycline in the bile (11–13) and the detection of high percentages of the administered dose in feces (3, 4, 14) supported this notion.

Recently, von Wittenau and Twomey (8) reported that doxycycline excreted in dog feces could not be totally extracted by the same solvent system employed to extract the unchanged drug from other fecal excreta. Accordingly, it was speculated that the positively charged doxycycline may form a complex with the negatively charged bile salts and that this complex is unavailable for reabsorption from the intestine (8).

The purposes of this investigation were to determine whether tetracycline excreted in the bile is reabsorbed from the small intestine and, eventually, to delineate the role of biliary excretion in the overall elimination of this drug from the body.

EXPERIMENTAL

Chemicals—^{3}H-7-Tetracycline hydrochloride powder was obtained commercially in a sealed borosilicate glass container[1]. The radioactive material (specific activity of 2.9 mCi/mg) was dissolved in methanol and stored at −20°. Nonlabeled tetracycline hydrochloride was used as obtained[2]. Anhydrotetracycline was synthesized according to the method of McCormick *et al.* (15), and 4-epitetracycline was synthesized according to the method of Simmons *et al.* (16).

Purity of Samples—The radiolabeled tetracycline was claimed by the manufacturer to be 98% pure according to TLC analysis on silica gel developed in 10% citric acid solution saturated with *n*-butanol and by paper chromatography on Whatman P20 cellulose phosphate cation paper in 0.1% NH_4Cl. To ascertain this purity, TLC purity and UV absorption of both labeled and nonlabeled materials were determined. Glass plates, 20 × 20 cm, were coated with a 0.25-mm layer of a slurry composed of 50 g of Kieselguhr G[3] and a mixture of 95 ml of 0.1 *M* edetate sodium in distilled water and 5 ml of 20% (v/v) polyethylene glycol 400 in glycerol (17). After air drying, the plates were developed in methyl ethyl ketone saturated with citric acid–phosphate buffer at pH 4.7 (17).

Twenty-five microliters of the methanolic solution of the labeled tetracycline was spotted and developed. After drying in air, the different spots were located by fluorescence under long wavelength UV light. The spots were identified by comparing their R_f values with those observed with methanolic solutions of tetracycline, anhydrotetracycline, and 4-epitetracycline. The plates were then radioscanned, and the area under the peaks was determined by the cut-and-weigh method.

UV absorption wavelengths were determined by scanning[4] a methanolic solution of each compound. The concentration of anhydrotetracycline was determined by absorption at 391 nm. Tetracycline was quantitated by the difference in absorption at 357 and 391 nm, using an equation reported previously (18). 4-Epitetracycline could not be quantitated by UV absorption.

Biliary Excretion Studies—Male Sprague–Dawley rats, 380–510 g, were anesthetized with urethan (1.3 g/kg im). A midline abdominal incision was made, and the common bile duct was cannulated with polyethylene (PE 10) tubing. After a steady flow of bile was observed, a 10-mg/kg dose of tetracycline (specific activity of 34.7 μCi/mg) was injected in the femoral vein over 5 min. The injectable dose was freshly prepared by evaporating the methanol from the tritiated tetracycline solution and adding nonlabeled aqueous drug solution to yield the desired specific activity.

The exteriorized bile was collected under a gentle stream of nitrogen in covered dark vials to avoid decomposition of its constituents. After 8–15 hr of collection, the bile solution was frozen and then mixed with bile collected from other rats treated in a similar way (total of six rats). The pH of the pooled bile from rats was determined, and an aliquot was used for qualitative and quantitative analyses. No attempt was made to calculate the percent of dose excreted in the bile of individual rats.

***In Situ* Absorption Studies**—Overnight fasted, male Sprague–Dawley rats, 280–380 g, were used. After urethan anesthesia, an abdominal midline incision was made. An 80–100-cm segment of the small intestine was isolated and cannulated with Tygon tubing about 2 cm distal to the common bile duct junction and proximal to the cecal end. The loop was washed thoroughly with 0.9% NaCl at 37° to remove any residual food and flushed with a mixture of 95% oxygen and 5% carbon dioxide to expel excess saline solution. Each cannula was attached to a 20-ml syringe to allow sampling of the loop content.

Five milliliters of the pooled bile solution was introduced, and a 0.1-ml aliquot was removed at 10-min intervals for the 1st hr, every 15 min for the 2nd hr, and every 30 min for the next 2 hr. Sampling was alternated between the two syringes. The volume loss due to water absorption from the drug solution was replenished with distilled water, adjusted to the same pH as the bile solution (8.75). At the end of the experiment, the intestinal segment was excised and its length was measured. It then was homogenized and incubated with a tissue solubilizer[5], and an aliquot was used for quantitative analysis. The *in situ* intestinal preparation was similar to that reported previously (19).

For comparative purposes, the absorption of tetracycline from

[1] Batch 10, Amersham/Searle Corp., Arlington, Ill.
[2] Lederle Laboratories, batch 48355-177, American Cyanamid Co., Pearl River, N.Y.
[3] E. Merck AG., Darmstadt, Germany.
[4] Beckman 25, Beckman Instruments Corp., Fullerton, Calif.
[5] Soluene TH 100, Packard Instrument Co., Downers Grove, Ill.

Table I—Quantitative Analysis of Tetracycline in the Bile and Aqueous Solutions

Solution	R_f[a]	Composition, % Labeled: Radioscanning	Labeled: UV Absorption	Nonlabeled, UV Absorption
Aqueous				
Unknown	0.09 (0.0)	0.9	N.D.[b]	N.D.
4-Epitetracycline	0.24 (0.26)	2.5	N.D.	N.D.
Tetracycline	0.56 (0.62)	89.1	75.6[c]	84.4[c]
Anhydrotetracycline	0.98 (1.00)	7.2	10.4[d]	N.P.[e]
Bile				
Unknown[f]	0.03	94.9	N.D.	N.D.
Anhydrotetracycline	0.98	5.1	N.D.	N.D.

[a] R_f values given in parentheses were reported by Lanman *et al.* (20). [b] N.D. = not determined. [c] Calculated by the equation of Pernarowski *et al.* (18). [d] Determined from Beer's law plot of anhydrotetracycline solutions. [e] N.P. = not present. [f] See text for explanation.

an aqueous solution having a pH and specific activity similar to the bile solution was determined as already described.

Partition Coefficient Studies—The partitioning characteristics of the bile and aqueous solutions of tetracycline were determined by mixing equal volumes of either solution and isoamyl alcohol in a shaker[6] at 37°. The partition coefficient is defined as the equilibrium ratio of radioactivity of the isoamyl alcohol phase to that of the test solution.

Assay—The level of radioactivity in the bile and aqueous and tissue homogenate samples was quantitated in 15 ml of a scintillation counting solution consisting of 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 5.5 g of 2,5-diphenyloxazole, and 250 ml of octoxynol[7] and diluted to 1 liter with toluene. The counting[8] efficiency of each sample was determined by the internal standard method using tritiated toluene.

RESULTS

Table I shows the composition of the tetracycline samples. In spite of the presence of some degradation products, the composition of the labeled and nonlabeled samples was similar. About 85–90% of the sample was present as pure tetracycline according to radioscanning and UV absorption analyses. However, analysis of the bile solution indicated that the major portion of radioactivity (95%) was attributed to an unknown species which remained at the origin of the thin-layer chromatogram. Further disparity between the properties of the bile and aqueous solutions is indicated by the partition coefficients. The partition coefficient of the bile solution (0.573) was more than twice that of the aqueous solution (0.213), as indicated by the equilibrium ratio of the radioactivity of the isoamyl alcohol phase to that of the test solution.

The results of the *in situ* absorption studies of tetracycline from the bile and aqueous solutions are shown in Table II. A negligible percent of the dose initially introduced in the intestinal loop accumulated in the gut wall. Furthermore, there was no statistical difference in the amount of drug found in the gut walls between the bile (2.24%) and aqueous (2.44%) solutions. Accordingly, the disappearance of the drug from the intestinal lumen could be construed to indicate its absorption into the systemic circulation. On the average, 72.92% of the drug in the bile solution was absorbed compared with 77.34% absorbed from the aqueous solution. The difference in the percent absorbed was statistically significant ($p < 0.05$).

Figure 1 shows the time course of the disappearance of tetracycline from the rat intestinal lumen expressed as the ratio of the amount of drug present at a given time to the amount introduced at zero time. Only the values obtained at and beyond the 75-min sample were statistically greater ($p < 0.05$) for the bile than for the aqueous tetracycline solutions. However, the disappearance of the drug from both solutions was rapid initially. On the average, 40–50% of the initial dose disappeared from the lumen during the first 0.5-hr.

[6] Dubnoff incubator, Precision Scientific Co., Chicago, Ill.
[7] Triton X-100.
[8] Packard Tri-Carb 2425 liquid scintillation spectrometer, Packard Instrument Co., Downers Grove, Ill.

Table II—Absorption of Tetracycline in the Bile and Aqueous Solutions from the Rat Intestine[a]

Solution	Loop, Length, cm	Percent Absorbed[a]	Percent in Tissue
Aqueous			
Mean (five rats)	87.8	77.34	2.44
SD	10.8	2.73	0.52
Bile			
Mean (five rats)	86.2	72.92	2.24
SD	8.2	3.20	0.27
p[b]	>0.8	<0.05	>0.6

[a] Obtained by subtracting the amount of drug found in the lumen and tissue at 240 min from the initial amount placed in the lumen. [b] Unpaired t test.

DISCUSSION

The report by Lanman *et al.* (20) on the purity of tritiated tetracycline hydrochloride samples available commercially instigated a thorough examination of the purity of the samples used in the present study. These workers found that two commercially available radioactive samples contained only 50–60% pure tetracycline hydrochloride; the rest consisted of the degradation products, anhydrotetracycline and 4-epitetracycline. Another marketed sample consisted mainly of anhydrotetracycline (20).

It is obvious that the presence of substantial quantities of the degradation products of tracers could lead to serious misinterpretations. Usually, these tracers are mixed with nonlabeled, chemically pure materials which may behave differently in the biological system. Therefore, it was essential to verify the purity of the labeled tetracycline, although the sample employed in the present study was obtained from a different source than the samples tested by Lanman *et al.* (20). The findings of the present study indicate that both the labeled and nonlabeled samples contained 85–90% pure tetracycline.

Analysis of the tetracycline bile solution showed that except for one spot corresponding to anhydrotetracycline, 95% of the radioactivity remained at the origin. Therefore, the tetracycline compounds existed in a polar form which could not migrate in the solvent system employed in the development of the thin-layer plates. It is possible that this polar form is a complexation product of the charged tetracycline species and bile constituents. Similar inference was made by von Wittenau and Twomey (8) with respect to the extraction of oxytetracycline from dog fecal excreta. On the other hand, this complex appears to possess a greater lipid solubility than the uncomplexed drug, as indicated by its greater tendency to partition into the isoamyl alcohol from the test solution. Further investigations are needed to characterize more fully the properties of this complex.

Although the difference between the amount of tetracycline absorbed from the bile and aqueous solutions is statistically signifi-

Table III—Kinetic Parameters of the Disappearance of Tetracycline in the Bile and Aqueous Solutions from the Rat Intestine[a]

Solution	A	α, min^{-1}	B	β, min^{-1}
Aqueous				
Mean (five rats)	0.648	0.062	0.361	0.0025
SD	0.004	0.013	0.040	0.0003
Bile				
Mean (five rats)	0.447	0.061	0.443	0.0025
SD	0.117	0.028	0.092	0.0010
p[b]	<0.01	>0.95	>0.2	>0.95

[a] The ratios of the amount of tetracycline at a given time to the amount initially introduced were fitted to Eq. 1 (see text). [b] Unpaired t test.

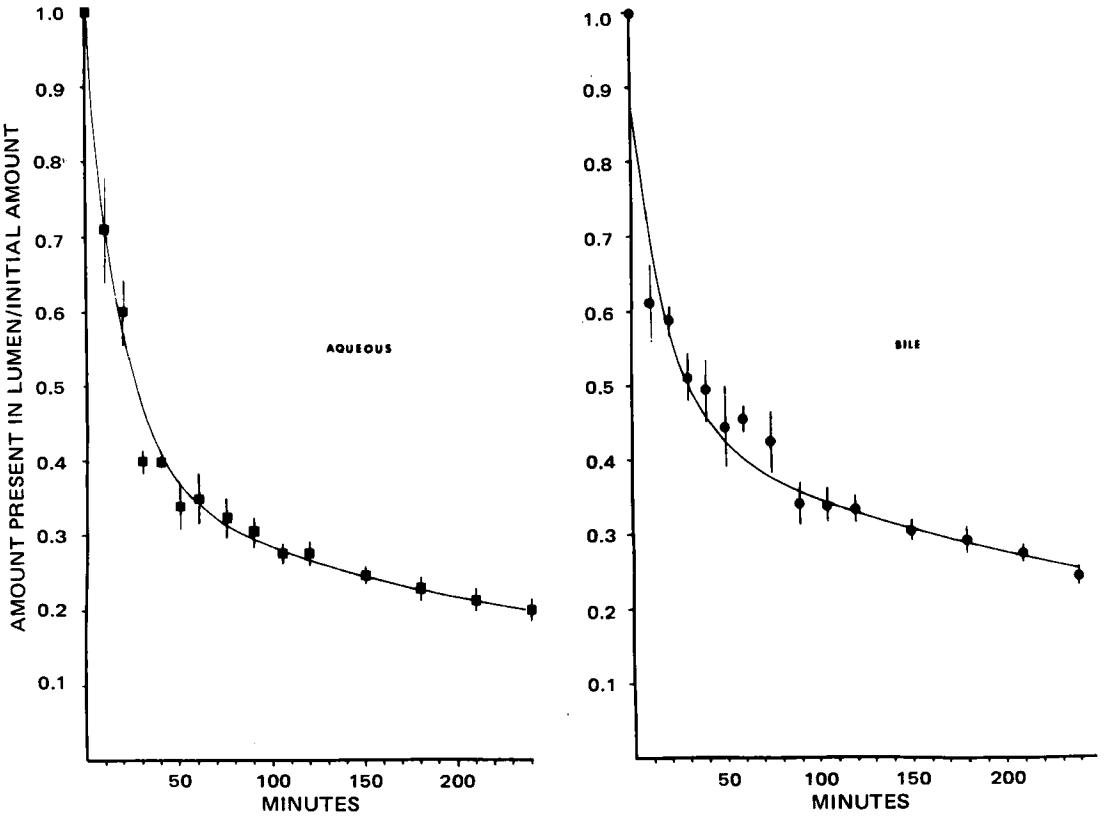

Figure 1—*Disappearance of tetracycline hydrochloride in the bile and aqueous solutions from the rat intestine as a function of time. The ordinates represent the ratio of the amount of the drug present at a given time to the amount introduced initially. Each point represents the mean of five rats, and vertical lines indicate the standard error of the mean. The continuous curves denote computer-fitted lines to the mean data.*

cant (Table II), it may not be biologically important unless it is accompanied by a similar difference in the rate of absorption of the drug from the two solutions. The time course of the disappearance of tetracycline from the rat intestinal lumen was plotted on semilogarithmic graph paper and found to be biphasic. Accordingly, data of each rat were fitted to the following biexponential equation using the SAAM 25 digital program (21):

$$R = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

where R is the ratio of amount of radioactivity present at a given time, t, to the amount initially introduced; α and β are the rate constants (minutes^{-1}) characterizing the rapid and more slowly declining phases, respectively; and A and B are constant coefficients, the sum of which represents the value of R at $t = 0$. The derived kinetic parameters are listed in Table III. It is clear that there are no statistical differences between the kinetic parameters B, α, and β whereas the value of A is significantly less for the bile solution than for the aqueous solution ($p < 0.01$). This may be explained by the greater tendency of the former to partition onto the gut wall, as indicated by its higher partition coefficient compared to the aqueous solution.

The findings of this investigation raise questions regarding the other routes of elimination of tetracycline in addition to excretion in urine. In a well-designed study, von Wittenau *et al.* (22) demonstrated that diffusion of doxycycline from the blood into the lumen of the small intestine and its ultimate excretion in feces constitute the other major route of elimination of this antibiotic in the rat. Other investigators detected tetracycline in the lower intestinal lumen of mice at various times after the intravenous administration of the drug (23, 24). Pindell *et al.* (25) demonstrated that tetracycline is rapidly absorbed from the duodenum but that negligible absorption takes place from the colon.

It is quite possible, therefore, that because of the poor absorption from the lower intestine and colon, tetracycline excreted into these areas is eventually eliminated in the feces. In fact, the biphasic disappearance of tetracycline from the isolated loop observed in the present study may be explained by the back-diffusion of drug absorbed from the duodenum and jejunum into the lower small intestine. The intestinal segment used in this investigation was sufficiently long to include the major portion of the ileum.

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Complexation in Formulation of Parenteral Solutions: Solubilization of the Cytotoxic Agent Hexamethylmelamine by Complexation with Gentisic Acid Species

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Abstract □ The apparent solubility of hexamethylmelamine in aqueous solutions suitable for intravenous use was increased by complexation with gentisic acid. Studies were carried out in the pH 0–8 range. Unprotonated hexamethylmelamine did not form complexes with the gentisate ion, while the hexamethylmelammonium ion appeared to form several different complexes with both the gentisate ion and gentisic acid. Two different solid complexes were isolated and characterized. The solubility increases observed at pH 3.5–5.0 are described by mathematical relationships involving the stability constants of some postulated complex species. From these results, suitable formulations for use as parenteral solutions are proposed. The increase in the apparent aqueous solubility of hexamethylmelamine in such formulations may range from five- to 90-fold, depending upon the pH and total gentisate-ion concentrations.

Keyphrases □ Complexation—hexamethylmelamine–gentisic acid species, pH 0–8, effect on solubility □ Solubilization—hexamethylmelamine by complexation with gentisic acid species, pH 0–8, intravenous formulations □ Cytotoxic agents—hexamethylmelamine, solubilization by complexation with gentisic acid species, intravenous formulations □ Hexamethylmelamine—solubilization by complexation with gentisic acid species, intravenous formulations □ Triazine derivatives—hexamethylmelamine, solubilization by complexation with gentisic acid species, intravenous formulations

Hexamethylmelamine[1], a cytotoxic triazine, was evaluated in extensive phase I and II clinical trials (1). Although the efficacy is not particularly striking in any tumor type, clinical interest in hexamethylmelamine continues in view of its consistent, although low, response rate in several tumors including bronchogenic carcinoma (2, 3).

In phase I and II trials, hexamethylmelamine was given by the oral route (1), and GI side effects such as nausea and vomiting were encountered in such studies (4, 5). To avoid such problems, an intravenous dosage form was developed. Because of the weakly basic nature of the drug and its low aqueous solubility (<0.1 mg/ml), the only parenteral formulation used was an aqueous hydrochloric acid solution (pH ~2) of the drug[2]. The clinical use of this solution has been restricted by the occurrence of thrombophlebitis and local irritation. To determine whether these untoward reactions at the injection site are inherently due to hexamethylmelamine or to the very acidic formulation, a less acidic (pH > 3) formulation of hexamethylmelamine at a concentration of 2–5 mg/ml was desired[2].

Previous experience with the complexation tendencies of various hydroxybenzoic acids with substances exhibiting characteristics akin to those of hexamethylmelamine (6–9) suggested that the apparent solubility of this drug probably could be increased through complexation. Such complexation necessitated the identification and use of a suitable ligand. Preliminary studies with gentisic acid as the ligand demonstrated a significant increase in the apparent solubility of hexamethylmelamine.

This report describes the qualitative and quantitative nature of the interactions of hexamethylmelamine with gentisic acid species, with particular attention being paid to those pH and concentration condi-

[1] 2,4,6-Tris(dimethylamino)-*s*-triazine; NSC 13875.

[2] J. P. Davignon, National Cancer Institute, personal communication.