# Hepatic Metabolism of $3\alpha$ -Hydroxy-5 $\beta$ -Etianic Acid ( $3\alpha$ -Hydroxy-5 $\beta$ -Androstan-17 $\beta$ -Carboxylic Acid) in the Adult Rat

JOANNA M. LITTLE, JAN ST. PYREK, and ROGER LESTER, Division of Gastroenterology, Department of Internal Medicine, University of Texas Medical School at Houston, Texas 77025

ABSTRACT Normal human meconium has been shown to contain short-chain ( $C_{20}$ - $C_{22}$ ) bile acids and, recently, these compounds have been identified in sera of patients with cholestasis. This suggests that shortchain bile acids may be secreted in bile. We have examined this point by studying the hepatic metabolism and biliary secretion of one naturally occurring  $C_{20}$ bile acid,  $3\alpha$ -hydroxy- $5\beta$ -etianic acid ( $3\alpha$ -hydroxy- $5\beta$ androstan- $17\beta$ -carboxylic acid).

 $[3-{}^{3}H]-3\alpha$ -hydroxy-5 $\beta$ -etianic acid was prepared and administered intravenously to rats prepared with an external biliary fistula.  $85.5\pm1.2\%$  of the administered dose was recovered in bile over 20 h with  $71.5\pm1.3\%$ appearing in the first hour.  $11.9\pm1.6\%$  of the dose was estimated to be distributed in body water and  $0.6\pm0.2\%$ was recovered as organic matter in urine. Total recovery of label was  $98.0\pm2.6\%$ . Administration of milligram quantities of  $3\alpha$ -hydroxy-5 $\beta$ -etianic acid produced an increase in bile flow ( $58.9\pm7.1\%$  over basal levels) within 20 min after injection of the steroid.

The radiolabeled material in bile was shown by thinlayer chromatography (TLC) to be a polar conjugate which, after  $\beta$ -glucuronidase hydrolysis, cochromatographed with authentic free  $3\alpha$ -hydroxy- $5\beta$ -etianic acid. After purification, and derivatization, the steroid moiety was proven by gas chromatography-mass spectrometry to be identical to  $3\alpha$ -hydroxy- $5\beta$ -etianic acid. Characterization of the conjugate by TLC and by  $3\alpha$ hydroxysteroid dehydrogenase assay, before and after  $\beta$ -glucuronidase hydrolysis, indicated that the steroid was secreted in bile as the 3-O- $\beta$ -glucuronide. It is concluded that  $3\alpha$ -hydroxy- $5\beta$ -etianic acid is cleared from the plasma, conjugated with glucuronic acid, and secreted into bile rapidly and in high concentration. The choleretic properties of this shortchain bile acid contrast with the cholestatic effects of lithocholic acid, its  $C_{24}$  analog. Both the form of conjugation of etianic acid and its effect on bile flow suggest that the shortened side chain of this steroid markedly alters its hepatic metabolism and physiology.

### INTRODUCTION

The bile acids are bioactive compounds that serve essential normal digestive functions and have disruptive effects when they accumulate in tissues in excess. Although bile acids frequently are considered a homogeneous group, each has peculiar physical characteristics and each is capable of exerting distinctive biological effects.

The application of sophisticated computerized gas chromatography-mass spectrometry  $(GC-MS)^1$  technology to the analysis of bile acids has increased the number and variety identified in biological materials. It is now recognized that the spectrum of naturally occurring compounds that have a hydroxylated steroid nucleus and an acidic side-chain is more complex than previously thought. The urine and plasma of patients with cholestasis contain atypical C<sub>24</sub> bile acids, and bile acids with longer (C<sub>27</sub>) and shorter (C<sub>23</sub>) side chains than those of conventional bile acids (1-9). A similar spectrum is found in normal human meconium (10). It has been shown recently that meconium contains a complex array of acidic steroids with short side chains (C<sub>20-22</sub>) (11-12), and most recently, a similar array has

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<sup>&</sup>lt;sup>1</sup> Abbreviations used in this paper: GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; TMS, trimethylsilyl.

been identified in cholestatic serum (13). In order to understand the role of these "short-chain bile acids"<sup>2</sup> in health and in states of liver disease, it will be necessary to explore their origin, metabolism, and physiologic behavior.

We have now examined the hepatic metabolism and biliary secretion of one of the  $C_{20}$  bile acids found in meconium,  $3\alpha$ -hydroxy- $5\beta$ -etianic acid, in the adult rat. The results show that this short-chain bile acid is efficiently cleared from plasma and secreted in bile as the  $3-O-\beta$ -glucuronide.

## **METHODS**

## Experimental design

The objective of the study was to examine the hepatic metabolism and biliary secretion of  $3\alpha$ -hydroxy- $5\beta$ -etianic acid  $(3\alpha$ -hydroxy-5 $\beta$ -androstan-17 $\beta$ -carboxylic acid). The structure of this C20 steroid is shown in Fig. 1; the structure of lithocholic acid  $(C_{24})$  is included for comparison. For these studies, the 3-3H-labeled steroid was prepared. In initial studies, a mixture of [3-3H]-3a-hydroxy- and [3-3H]-3B-hydroxy-5β-etianic acids (in a 7:1 ratio) was used. Later confirmatory experiments were carried out using purified [3-<sup>3</sup>H]-3 $\alpha$ -hydroxy-5 $\beta$ -etianic acid. The labeled steroid was administered intravenously, both in tracer amounts and with added carrier  $3\alpha$ -hydroxy- $5\beta$ -etianic acid, to rats prepared with an external biliary fistula. The biliary secretion of radiolabel was measured for 20 h, after which radiolabel was measured in plasma, urine, and tissues. The in vivo stability of the label was examined by measuring the amount of tritiated water formed and by determining total recovery. The nature of radiolabeled steroid in bile was determined, and

the steroid conjugate formed was characterized. Synthesis of  $[{}^{3}H]$ etianic acid. 5 $\beta$ -androstan-3-one-17 $\beta$ carboxylic acid methyl ester (Steraloids, Wilton, NH) was dissolved in iso-propanol and reduced with an excess of NaB<sup>3</sup>H<sub>4</sub> (sp act: 110 mCi/mmol; New England Nuclear, Boston, MA) dissolved in isopropanol containing 10% 0.025 N NaOH. The reduction yielded 3-3H-labeled etianic acid methyl ester (sp act: 100 mCi/mmol) that was >97% pure as judged by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). GC and HPLC established that 87.4% of label and mass were present as [3-<sup>3</sup>H]-3 $\alpha$ -hydroxy-5 $\beta$ -etianic acid and 12.6% as [3-<sup>3</sup>H]-3 $\beta$ -hydroxy-5 $\beta$ -etianic acid. The methyl esters were hydrolzed by dissolving the products of the reduction in methanol, adding 0.3 vol of 5 N NaOH and allowing the reaction to proceed for 3 h at 65°C. After hydrolysis, the free acids were extracted into ethyl ether from an acid medium, the ether extracts were pooled and backwashed with water, and the ether was removed by evaporation. This mixture of labeled

FIGURE 1 Structures of  $3\alpha$ -hydroxy- $5\beta$ -androstan- $17\beta$ -carboxylic acid ( $3\alpha$ -hydroxy- $5\beta$ -etianic acid) (A) and  $3\alpha$ -hydroxy- $5\beta$ -cholanoic acid (lithocholic acid) (B).

epimers was used for the initial experiments. Subsequently, the labeled  $3\alpha$ - and  $3\beta$ -epimers were separated by HPLC, as described below, and the purified (>98% purity) [3-<sup>3</sup>H]- $3\alpha$ -hydroxy- $5\beta$ -etianic acid was used.

The 3-O- $\beta$ -glucuronide of  $3\alpha$ -hydroxy- $5\beta$ -etianic acid was synthesized using established methods (15).

Surgical preparation and experimental protocol. Male Sprague-Dawley rats (200-400 g) were anesthetized with ether, the abdomen was incised in the midline, and a polyetheylene catheter (0.28 mm i.d. × 0.61 mm o.d.; Clay-Adams, Parsippany, NJ) was inserted into the common bile duct. The biliary catheter was exteriorized through a stab wound in the abdominal wall and the abdominal incision was closed. All rats were maintained under ether anesthesia for the first 2-3 h of the study. They were then allowed to regain consciousness in Bollman restraining cages in which they remained for the duration of the experiment. Labeled etianic acid (1-5  $\mu$ Ci of the mixture or of the purified 3a-hydroxy-steroid alone), with or without added cold carrier  $3\alpha$ -hydroxy- $5\beta$ -etianic acid (5-5.6 mg), was dissolved in 0.1-0.5 ml of 0.1 N NaOH and was brought to 10% albumin in saline in a final volume of 1.0-2.0 ml. In two studies, 1 µCi of [24-14C]lithocholic acid or [24-<sup>14</sup>Cjcholic acid (sp act: 52 mCi/mmol; New England Nuclear) was included in the dose. These solutions were administered intravenously to bile duct cannulated rats.

Bile samples were collected in preweighed  $10 \times 75$ -mm tubes at 10-min intervals beginning 1 h before injection of the dose and continuing for 1-2 h after injection. Subsequently, bile was collected at 30-min intervals for an additional 1-2 h and, thereafter, as a single sample until killing at 20 h.

In all studies, urine was collected during the experimental period in two fractions: 0-3 h and 3-20 h. At time of death, blood was collected from the abdominal aorta; plasma was separated and frozen. Liver, intestine, and kidneys were removed and frozen for later analysis.

### Analytical techniques

Determination of radioactivity. Bile volume was determined by weighing samples collected in tared tubes. Liver, intestine, and kidneys were homogenized in 2 vol of distilled water and aliquots of homogenates were extracted with 5 vol of methanol followed by centrifugation of the protein precipitate. Aliquots of bile, plasma, and the methanol extracts of tissue homogenates were analyzed for tritium and <sup>14</sup>C content in ACS scintillant (Amersham Corp., Arlington Heights, IL) in a Tracor Mark III, model 6882, liquid scintillation system (Tracor Analytic, Elk Grove Village, IL).

Tritiated water content of bile, urine, and plasma was determined by sublimation. An estimate of the amount of isotope distributed in total body water as <sup>3</sup>H<sub>2</sub>O was obtained

<sup>&</sup>lt;sup>2</sup> For the purpose of this paper, the term "bile acid" will be used to refer to the general class of carboxylated steroids, and will include bile acids with side-chains shorter and longer than those of conventional bile acids. Etianic acids ( $C_{20}$ ) have been classified as bile acids in standard chemical texts (14). The designation, however, implies nothing about the relation between their biosynthesis, metabolism, and function, and those of conventional bile acids.

by assuming that water constituted 65% of body weight and that  ${}^{3}H_{2}O$  in plasma was representative of that in body water.

Thin-layer chromatography. Whatman LK-5 thin-layer plates (Pierce Chemical Co., Rockford, IL) were used for all TLC analyses. Solvent systems were as follows: system 1 (for free bile acids): isooctane-ethyl acetate-glacial acetic acid (5:5:1); system 2 (for bile acid conjugates): chloroform-methanol-glacial acetic acid-water (65:20:10:5). Plates were developed to a distance of 15 cm from the origin and dried. Standards of free and conjugated C24 bile acids (Calbiochem-Behring, La Jolla, CA) and of free  $C_{20}$  bile acid (Steraloids) were visualized by spraying with phosphomolybdic acid (3.5% in isopropanol) and heating at 110°C for 20 min. Each sample lane was divided into 1-cm segments from origin to front and the gel in each segment was transferred to a scintillation vial. 1 ml of methanol was added to each vial, samples were allowed to stand for 30 min and were then counted in ACS

 $\beta$ -Glucuronidase hydrolysis. Bile samples were diluted with 0.075 M sodium phosphate buffer, pH 6.8, and 50 U of Escherichia coli  $\beta$ -glucuronidase (Sigma Chemical Co., St. Louis, MO) was added. Samples were incubated at 37°C for 24 h and were then acidified and extracted with ethyl ether as described above. For enzyme inhibition, incubation mixtures included 1.0 mM saccharo-1,4-lactone (Sigma Chemical Co.).  $\beta$ -Glucuronidase-treated samples of bile from animals given a load of etianic acid were diluted with methanol to dissolve crystalline material generated during the hydrolysis and were then assayed directly by TLC or with  $3\alpha$ -hydroxysteroid dehydrogenase for total bile acid content.

Total bile acid analysis. Total  $3\alpha$ -hydroxy bile acid concentrations in native bile and in bile subjected to enzymatic hydrolysis were assayed with  $3\alpha$ -hydroxysteroid dehydrogenase (Worthington Biochemical Corp., Freehold, NJ) using the method of Talalay (16) as modified at Admirand and Small (17).

High-pressure liquid chromatography. Separation of  $[3^{3}H]-3\alpha$ - and  $[3^{-3}H]-3\beta$ -hydroxy- $5\beta$ -etianic acid was accomplished by HPLC of the methyl esters on a Supelcosil LC-Si column (4.6 mm i.d., 150 mm length; Supelco, Inc., Bellefonte, PA). The solvent system consisted of 0.4% isopropanol in hexane; chromatography was run at a flow rate of 1.25 ml/min using an ISCO model 184 UV monitor (Instrumentation Specialties Co., Lincoln, NB) at 200 nm for detection. In this system, the retention volumes were 6.0 and 10.0 ml for the  $3\beta$ -hydroxy and  $3\alpha$ -hydroxy epimers, respectively.

Gas chromatography-mass spectrometry. Bile samples containing labeled etianic acid were subjected to hydrolysis with  $\beta$ -glucuronidase followed by ethyl ether extraction. The dried ether extracts were methylated with diazomethane. dried under nitrogen and the residues were dissolved in benzene. A 5-cm silica gel column (Woelm, 60-200 mesh) was deactivated by washing with acetone and was then washed with benzene. The methylated sample was applied in benzene; the column was washed with additional benzene and then eluted with 10 ml of benzene-acetone (95:5) collected in 0.5-ml fractions. An aliquot of each fraction was taken for measurement of radioactivity; those fractions containing isotope were taken to dryness. After conversion to the trimethylsilyl derivatives, the material was analyzed by capillary GC-MS (Finnigan 3300 GC-MS with Incos data system; Finnigan Instruments, Sunnyvale, CA) on a methyl silicone column (0.2 mm i.d., 12 m length; Hewlett-Packard Co., Inc., Avondale, PA) using GC and MS conditions previously described (12).

All solvents used in sample preparation for HPLC and GC-MS were redistilled in glass before use.

Statistical analysis. Data were analyzed using an H-P 9810 calculator (Hewlett-Packard) and Hewlett-Packard programs for t test, paired observation t test and two variable linear regression analysis (18).

#### RESULTS

# Biliary excretion and recovery of administered label

In initial studies, using the mixture of labeled  $3\alpha$ hydroxy- and  $3\beta$ -hydroxy- $5\beta$ -etianic acids, radiolabel was detected in bile within the first 10 min after injection and secretion continued throughout the period of study (Fig. 2). After 20 h of bile collection, 74.1±1.2% of the administered label had been recovered in bile (Table I). Small but measureable amounts of label were found in urine, plasma and tissue (1.2±0.2, 1.2±0.2, and 1.6±0.3% of the dose, respectively). Bile samples contained no measureable tritiated water while all the label in plasma and tissues was recovered as <sup>3</sup>H<sub>2</sub>O. Urine collected early in the study (0-3 h) contained a smaller proportion of total radiolabel in <sup>3</sup>H<sub>2</sub>O than did urine collected from 3-20 h (18.7±4.4% of urinary radiolabel in <sup>3</sup>H<sub>2</sub>O for 0-3 h urine vs. 63.6±1.3% for 3-20 h urine).  $16.0\pm1.3\%$  of the dose was estimated to be distributed in total body water and 0.7±0.2% was recovered from urine as radiolabel in organic material. The total recovery of administered label was 90.8±1.0%.

When purified  $[3-{}^{3}H]-3\alpha$ -hydroxy-5 $\beta$ -etianic acid was administered, the pattern of biliary excretion of label was similar to that of the mixture of 3-epimers (Fig. 2). Total biliary excretion of label was  $85.5\pm1.3\%$ of the administered dose (Table I), a figure which was significantly higher (P < 0.01) than in the previous series. Measureable amounts of isotope were again



FIGURE 2 Biliary secretion of administered isotope by rats given an epimeric mixture (ratio 7:1) of  $[3-{}^{3}H]-3\alpha$ - and  $3\beta$ -hydroxy- $5\beta$ -etianic acids ( $\bullet$ ) or pure  $[3-{}^{3}H]-3\alpha$ -hydroxy- $5\beta$ -etianic acid (X). Each point represents the mean of four experiments and the brackets give the SEM.

Metabolism of  $3\alpha$ -Hydroxy-5 $\beta$ -etianic Acid 75

Experiment no.	Rat wt	Compound	Dose	Isotope recovery			
				Bile	Urine	Body water*	Total
	g	g mg			%		
1	<b>387</b>	Mixturet	0.004	72.5	0.5	15.5	88.5
2	415	Mixturet	0.014	75.3	1.0	17.0	93.3
3	268	Mixture	0.018	76.9	0.3	12.7	89.9
4	207	Mixture‡	5.611	71.7	0.9	18.8	91.4
5	261	Pure§	5.007	85.2	1.0	13.9	100.1
6	325	Pure§	0.003	87.4	0.4	15.1	102.9
7	285	Pure§	0.006	82.1	0.3	8.3	90.7
8	420	Pure§	5.512	87.4	0.5	10.2	98.1

 TABLE I

 Experimental Conditions and Isotope Recoveries for Individual Animals

\* See text for method of estimation.

‡ Epimeric mixture of  $[3-^{3}H]-3\alpha$ - and  $[3-^{3}H]-3\beta$ -hydroxy-5 $\beta$ -etianic acid.

§ Purified [3-<sup>3</sup>H]-3 $\alpha$ -hydroxy-5 $\beta$ -etianic acid.

found in urine  $(1.3\pm0.4\%)$ , plasma  $(0.9\pm0.2\%)$ , and tissues  $(1.0\pm0.2\%)$ . As before, bile contained no detectable tritiated water, while radiolabel in plasma and tissues was all found in <sup>3</sup>H<sub>2</sub>O. The urinary excretion of <sup>3</sup>H<sub>2</sub>O was similar to that found when the mixture of 3-epimers was administered  $(14.7\pm6.2\%)$  of urinary radiolabel in <sup>3</sup>H<sub>2</sub>O for 0-3 h urine vs. 63.7±8.3\% for 3-20 h urine). The proportion of the dose estimated to be in total body <sup>3</sup>H<sub>2</sub>O was lower than when the mixture was given  $(11.9\pm1.6\%)$  but the difference was not statistically significant. Only  $0.6\pm0.2\%$  of the dose was recovered from urine as organic material. The overall recovery of the administered dose was  $98.0\pm2.6\%$ , significantly higher (P < 0.02) than that found with the mixture of both epimers.

In two experiments  $[24^{-14}C]$  lithocholic acid or  $[24^{-14}C]$  cholic acid was administered simultaneously with the dose of labeled etianic acid. The biliary excretion of these two C<sub>24</sub> bile acids compared to that of the C<sub>20</sub> acid is shown in Fig. 3. 94% of the  $[^{14}C]$  lithocholic acid and 101% of the  $[^{14}C]$  cholic acid given were recovered in bile after 20 h, as compared with the 85% of the etianic acid tritium excreted in the same period. No detectable  $^{14}C$  activity was found elsewhere.

Administration of  $3\alpha$ -hydroxy- $5\beta$ -etianic acid in milligram amounts had a profound effect on bile secretion (Fig. 4A). Bile flow increased from a rate of  $11.5\pm1.1 \ \mu$ /min, before injection of the steroid to a maximal rate of  $18.1\pm1.6 \ \mu$ /min, within 20 min after injection (P < 0.01). The mean increase in bile flow for all studies was  $58.9\pm7.1\%$  over preinjection values.

Identification of the steroid moiety of the labeled compound in bile. Greater than 95% of the radioactivity in acidified bile was ether extractable. TLC of whole bile, or the ether extract, in system 2 indi-

cated that >90% of the label was associated with a polar compound with a retardation factor  $(R_f)$  of 0.45, in the range of taurine conjugated  $C_{24}$  bile acids (R<sub>f</sub>: 0.41-0.57). Chromatography in system 1 revealed an additional spot containing < 2% of the label with a  $R_f$ of 0.58, identical to that of standard  $3\alpha$ -hydroxy-5 $\beta$ etianic acid. After alkaline hydrolysis, 70% of the label still chromatographed as the polar compound. However, after acid solvolysis or  $\beta$ -glucuronidase incubation, a single radioactive spot was found on chromatography using system 1. This spot contained >94% of the applied isotope and migrated with an Rf identical to that of authentic  $3\alpha$ -hydroxy- $5\beta$ -etianic acid. When 1.0 mM saccharo-1,4-lactone was included in the  $\beta$ -glucuronidase incubation mixture, <5% of the label migrated with the standard free etianic acid.



FIGURE 3 Comparison of biliary secretion of  $C_{20}$  and  $C_{24}$ bile acids. Secretion of radiolabel by rats given  $[3-^{3}H]-3\alpha$ hydroxy-5 $\beta$ -etianic acid ( $\bullet$ ) is compared to <sup>14</sup>C secretion by rats given  $[24-^{14}C]$ lithocholic acid ( $\times$ ) or  $[24-^{14}C]$ cholic acid (O).



FIGURE 4 A. Bile flow rate before and after intravenous administration of a load (5-5.6 mg) of  $3\alpha$ -hydroxy-5 $\beta$ -etianic acid. B. Concentrations of  $3\alpha$ -hydroxy steroid (as measured by  $3\alpha$ -hydroxysteroid dehydrogenase) in bile before ( $\bullet$ ) and after (O)  $\beta$ -glucuronidase hydrolysis. In both A and B, each point is the mean of four experiments and the brackets give the SEM. The arrow indicates the time of steroid injection.

To establish unequivocally the identity of the steroid moiety of the biliary conjugate, a  $\beta$ -glucuronidasetreated bile sample from a rat given 5.6 mg of etianic acid was extracted, methylated and purified by silica gel chromatography. Those fractions eluted that contained radiolabel were subjected to silvlation procedures and analyzed by capillary GC-MS. The GC-MS analysis of the principal fraction (Fig. 5) gave a single major GC peak (Fig. 5A) with a retention time identical to that of the trimethylsilyl (TMS) derivative of authentic  $3\alpha$ -hydroxy- $5\beta$ -etianic acid methyl ester. Comparison of the mass spectrum of this GC peak (Fig. 5B) with that of the authentic standard confirmed that  $3\alpha$ -hydroxy- $5\beta$ -etianic acid had been excreted in bile. No endogenous  $C_{20}$  bile acids could be detected by GC-MS in bile collected before injection of the etianic acid.

Characterization of the etianic acid conjugate excreted in bile. The chromatographic data presented in the previous section indicated that etianic acid was secreted in bile conjugated with glucuronic acid, most probably as the 3-O- $\beta$ -glucuronide conjugate. This assumption was supported by direct TLC comparison of the conjugate obtained from bile with the authentic

3-O- $\beta$ -glucuronide of  $3\alpha$ -hydroxy- $5\beta$ -etianic acid: the single radioactive spot in chromatographed bile had an R<sub>f</sub> of 0.48, identical to that of the standard. Moreover, the <sup>13</sup>C nuclear magnetic resonance (NMR) spectrum of radioactive etianic acid glucuronide (isolated from bile by preparative TLC) was identical to that of the synthetic glucuronide. Also, when the dimethyl ester triacetates of the biosynthetic glucuronide and of the synthetic compound were prepared, the two were proven to be entirely identical by TLC, HPLC, and <sup>1</sup>H-NMR spectra. Further evidence was obtained by enzymatic assay, with  $3\alpha$ -hydroxysteroid dehydrogenase, of total biliary bile acid content before and after  $\beta$ -glucuronidase hydrolysis of bile from rats given a load of etianic acid. After  $\beta$ -glucuronidase hydrolysis, mean  $3\alpha$ -hydroxy steroid concentrations in bile were significantly higher (paired t test) than those measured in native bile  $(32.5\pm0.6 \ \mu mol/ml$  after hydrolvsis as compared to  $22.3\pm2.1 \ \mu mol/ml$  in native bile; P < 0.01). The pattern of  $3\alpha$ -hydroxy bile acid concentrations of individual bile samples before and after  $\beta$ -glucuronidase hydrolysis is shown in Fig. 4B. The increases in post-hydrolysis bile acid concentrations parallel the increases in bile flow (Fig. 4A) and linear regression analysis demonstrated a significant correlation (r = 0.981, P < 0.01) between the two variables. The specific activity of the administered material and the radiolabel appearing in bile were used to calculate the theoretical concentration of etianic acid in bile. Comparison of these values with the measured differences between pre- and posthydrolysis bile acid concentrations demonstrated no significant difference (P > 0.2) between the two values by paired t test and a significant correlation (r = 0.972, P < 0.01) by linear regression analysis.

#### DISCUSSION

Meconium contains monohydroxylated, short-chain  $(C_{20-22})$  bile acids (11, 12). Etianic acids  $(C_{20})$  are a major component of the monohydroxylated bile acid fraction in meconium, and are present in quantities as large or larger than those of the two principal conventional bile acids in meconium,  $3\beta$ -hydroxy-chol-5-en-24-oic and lithocholic acids. The physical properties of short-chain bile acids extracted from meconium suggest that they are in the form of highly polar conjugates. More recently, a similar array of short-chain bile acids has been identified in cholestatic sera (13).

The presence of polar conjugates of short-chain bile acids in meconium and cholestatic sera raised the possibility that short-chain bile acids, like conventional bile acids, are metabolized by the liver and secreted in bile. To investigate this possibility, we administered one naturally occurring short-chain bile acid,  $3\alpha$ -hy-



FIGURE 5 GC-MS analysis of etianic acid isolated from rat bile. A. Reconstructed ion current (gas chromatographic record) of methyl ester, TMS derivatives. The major peak has the retention time (R<sub>1</sub>) of authentic  $3\alpha$ -hydroxy-5 $\beta$ -etianic acid methyl ester, TMS ether (R<sub>i</sub>: 6:01 min), while the R<sub>t</sub> of the second smaller peak corresponds to that of standard cholesterol TMS ether. B. Mass spectrum of the major peak in A. Ions at 406 (M<sup>+</sup>), 391 (M<sup>+</sup>-15), 375 (M<sup>+</sup>-31), 316 (M<sup>+</sup>-90), 301 (M<sup>+</sup>-90-15), and 215 (saturated ABC ring) identified the compound as a mono-carboxy, monohydroxy, saturated C<sub>20</sub> steroid. Comparison of the spectrum with that of the authentic standard confirmed the structure of the biliary steroid as  $3\alpha$ -hydroxy-5 $\beta$ -etianic acid.

droxy-5 $\beta$ -etianic acid, to adult rats prepared with an external biliary fistula. Secretion in bile, excretion in urine, and distribution in plasma and tissues were determined.

To accomplish this, it was necessary to prepare radiolabeled material.  $[3-^{3}H]-3-hydroxy-5\beta$ -etianic acid was prepared from 3-oxo-5 $\beta$ -etianic acid using tritiated borohydride reduction of the keto group. A mixture of  $[3-^{3}H]-3\alpha$ - and  $[3-^{3}H]-3\beta$ -hydroxy-5 $\beta$ -etianic acids was produced (proportions 7:1). Since the desired epimer predominated, initial studies were performed using the mixture. Subsequently, the epimeric pair was separated by preparative HPLC, and confirmatory studies were performed with radiochemically pure  $[3-^{3}H]-3\alpha$ -hydroxy-5 $\beta$ -etianic acid.

The lability of the <sup>3</sup>H-label in vivo was examined carefully, because of concern about the stability of the

tritium atom on the functionalized C3. In vivo lability proved to be minor, however. Even when calculations were based on complete equilibration of plasma and total body water, an assumption that maximalizes the derived value, <sup>3</sup>H<sub>2</sub>O formation averaged only 12% in those studies using radiochemically pure [3-<sup>3</sup>H]-3 $\alpha$ -hydroxy-etianic acid. The proportion of tritiated water to tritiated organic compounds in urine was greater in the 3-24-h collection than in the 0-3-h collection. This was probably due to the early spillover of organic material into urine shortly after bolus injection of the dose, and to the slower incorporation and distribution of tritium into the body water pool. Overall, >98% of the radiolabel was recovered and >80% was proven to be steroid-associated. The in vivo stability of the tritium atom at position 3 strongly suggests that the  $3\alpha$ -hydroxyl group of etianic acid did not undergo major oxidation and reduction in the course of hepatic metabolism.

The results show that  $[3-^{3}H]-3\alpha$ -hydroxy-5 $\beta$ -etianic acid was rapidly and efficiently secreted in bile. Half of the radiolabel appeared in bile within 30 min of intravenous administration, and secretion equalled >80% in 20 h. The radiolabeled polar conjugate in bile was hydrolyzed, and the steroid released was extracted and compared with authentic standards using TLC, HPLC, capillary GC, and capillary GC-computerized MS. The steroid moiety was identified as the administered  $3\alpha$ -hydroxy-5 $\beta$ -etianic acid. The steroid was not hydroxylated during the process of hepatic uptake, intracellular transport, and secretion of the administered steroid, and there was no isomerization. Secretion of the steroid was as efficient when either microgram or milligram quantities were administered. Unlike the behavior of its C24 homolog, lithocholic acid, which produces cholestasis (19-22), administration of milligram quantities of  $3\alpha$ -hydroxy-5 $\beta$ -etianic acid resulted in a pronounced choleresis, with a maximal increased rate of bile water formation of 59% over basal values, occurring within 20 min of injection. There was a direct correlation between the rate of  $3\alpha$ hydroxy-5 $\beta$ -etianic acid secretion and bile water secretion.

 $3\alpha$ -hydroxy- $5\beta$ -etianic acid was secreted in bile as a polar conjugate. Free steroid was liberated when the conjugate was subjected to hydrolysis with highly specific bacterial  $\beta$ -glucuronidase. Enzymatic hydrolysis was inhibited by saccharolactone and the conjugate was acid-labile, but alkali-stable. These properties are compatible with those that would be anticipated for a steroid 3-O-glucuronide (23). Further support for the hypothesis that the conjugate was linked through the 3-hydroxyl group of the native steroid was obtained using  $3\alpha$ -hydroxysteroid dehydrogenase (16, 17). Hydrolysis of a hypothetical 20-ester- $\beta$ -glucuronide with  $\beta$ -glucuronidase would not alter the quantity of  $3\alpha$ hydroxyl groups in bile available for interaction with  $3\alpha$ -hydroxysteroid dehydrogenase. On the other hand, hydrolysis of the posited steroid  $3-O-\beta$ -glucuronide would liberate free 3a-hydroxysteroid positions and increase the measured amounts of  $3\alpha$ -hydroxysteroid. As measured by  $3\alpha$ -hydroxysteroid dehydrogenase, 3a-hydroxysteroid concentrations increased after incubation of bile samples with  $\beta$ -glucuronidase. Biliary etianic acid, estimated using the specific activity of the administered dose and radioactivity in bile, was positively correlated with the difference between free  $3\alpha$ -hydroxysteroid groups measured enzymatically before and after hydrolysis with  $\beta$ -glucuronidase (r = 0.972, P < 0.01).

The relation between short-chain and conventional bile acids remains speculative. The behavior of shortchain bile acids after intravenous administration to adult rats, as partially characterized in these studies, has similarities to, and differences from that of conventional bile acids. Both are rapidly cleared from plasma, taken up by the liver and secreted at high concentrations in bile. Conventional bile acids are conjugated primarily with glycine and taurine (24), while minimal amounts of glucuronide conjugates are found normally, and bile acid glucuronides appear in quantity only in cholestatic liver disease (25-28). Thus, while the biliary secretory pattern observed for etianic acid is similar to that of conventional bile acids, secretion via mechanisms more closely related to those for bilirubin, bromsul phalein, and other organic anions (29, 30) is an alternative possibility. In this regard, studies examining competition between short-chain and conventional bile acids for hepatic uptake and/or secretion would be of interest.

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