Intestinal absorption, excretion, and biotransformation of hyodeoxycholic acid in man

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Abstract Five patients fitted with a biliary T-tube after cholecystectomy were given orally a tracer dose of [^{14}C]hyodeoxycholic acid and 500 mg of the same unlabeled acid. Intestinal absorption and biotransformation, liver metabolism, bile secretion, fecal and urinary excretions of this acid or of its metabolites were studied. Hyodeoxycholic acid was well absorbed by the human intestine. It was not subjected to intestinal transformations and, particularly, did not produce a significant amount of lithocholic acid, which does not support the existence of intestinal bacterial 6 α-dehydroxylases. The percentage of hyodeoxycholic acid and of its metabolites recovered in bile varied from 11.5 to 31%. Two major metabolites were isolated from bile: glycohyodeoxycholic acid and hyodeoxycholic acid glucuronide. Analysis of urinary bile acids showed that a large proportion (30–84%) of the administered hyodeoxycholic acid was excreted by the kidney as a glucuronide. The large extent of both glucuronidation and urinary excretion of hyodeoxycholic acid is a unique example of bile acid metabolism and excretion in man.—**Sacquet, E., M. Parquet, M. Riottot, A. Raizman, P. Jarrige, C. Huguet, and R. Infante.** Intestinal absorption, excretion, and biotransformation of hyodeoxycholic acid in man. *J. Lipid Res.* 1983. **24:** 604–613.

Supplementary key words bile salts • glucuronidation • fecal and urinary excretion

In many species, but particularly in man, the hydroxyl groups on the bile acids mainly occupy the 3, 7, 12 positions of the steroid nucleus. However, two common animal species, namely pigs and rats, also synthesize large amounts of bile acids hydroxylated in the 6 position. This leads to the presence of HDC and HC in pig bile (1) and to HDC (2–5) and (α,β,γ)-MC in rat bile, liver, and feces (6).

In healthy adult humans, only traces of 6α-hydroxylated bile acids have been found in the urine (7), although Greim et al. (8) have described 6α-hydroxylating enzyme in human liver microsomes, while Back and Walter (9) have isolated 6α-hydroxylated bile acids from human meconium.

The observations of Back and Walter (9) suggest that 6α-hydroxylation constitutes a major metabolic pathway during the prenatal period. After birth the activity of the 6α-hydroxylation enzyme is repressed. In cholestatic liver disease however (7, 10–12), and in patients with intestinal malabsorption (13), significant amounts of 6α-OH bile acids have been identified in urine, suggesting derepression of this enzyme. The mechanisms responsible for the disappearance and reappearance of 6α-hydroxylase activity, and thus for the formation of HDC, have not yet been established.

The only other study of HDC in man was published by Thistle and Shoenfield (14) as part of a pharmacological study on the effects of feeding different bile acids on bile lipid composition in gallstone patients; they gave 1.0 g of HDC/day for 4 months. Unlike CDCA with desaturated fasting duodenal bile, HDC neither modified bile lipid composition nor significantly increased biliary HDC. They did not comment on the fate of the administered bile acid.

All these results encouraged us to analyze more thoroughly the hepatic metabolism of HDC and to study its

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; LC, lithocholic acid; DC, deoxycholic acid; UDC, ursodeoxycholic acid; CDC, chenodeoxycholic acid; HDC, hyodeoxycholic acid; C, cholic acid; HC, hyocholic acid; TC, taurocholic acid; α,β,γ,-MC, α,β,γ-muricholic acid; TCDC, taurochenodeoxycholic acid; GC, glycocholic acid; GCDC, glycochenodeoxycholic acid. Trivial names: lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; deoxycholic acid, 3α-12α-dihydroxy-5β-cholan-24-oic acid; hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholan-24-oic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholan-24-oic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; α-muricholic acid, 3α,6β,7α-trihydroxy-5β-cholan-24-oic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholan-24-oic acid; ω-muricholic acid, 3α,6α,7β-trihydroxy-5β-cholan-24-oic acid; taurocholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid N-(2-sulfoethyl)-amide; glycocholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid N-(carboxymethyl)-amide; DMF, dimethylformamide.

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absorption and metabolism in the intestine. In particular, we wished to see if this 6α-hydroxylated compound was transformed by human intestinal bacteria into LC and other bile acids.

MATERIAL AND METHODS

Patients

Clinical details of the patients studied are summarized in **Table 1.** Three patients (B, F, and P) were women and two (G, Y) were men. B was 49 years old, F 86, P 66, G 64, and Y 39. They had all undergone cholecystectomy for gallstones during which T-tubes were inserted into the common duct. The studies were carried out between 8 and 15 days after the operation. Apart from the associated problems (Table 1), there were no other known diseases. Patients all gave their consent for the studies.

Experimental procedure

After an overnight (15–18 hr) fast, the patients ingested a gelatin-coated capsule containing 500 mg of HDC and 12 to 18 μCi of ^{14}C-labeled HDC. Samples of T-tube bile were collected at 2, 4, 6, and 24 hr after HDC administration in all patients. In two patients (F and G) 24-hr collections of bile and urine were controlled for 5 consecutive days. In patient Y, stools were collected over a 3-day period; in the remaining four patients, stools were collected over 5 days. All the samples were immediately frozen and stored at −80°C until analyzed.

Materials

The solvents used for extraction, chromatography, and synthesis procedures (analytical grade) were supplied by Merck and Prolabo. The various bile acids and particularly the HDC methyl ester were obtained from Steraloids. The HDC used for the absorption studies was supplied by Roussel Uclaf (Paris) and was 98% pure by GLC. [24-^{14}C]Sodium chenodeoxycholate (48.1 mCi/mol) was obtained from Amersham. Reagents used for silylating bile acids, hexamethyldisilazane and trimethylchlorosilane and the diazald used in the preparation of diazomethane, were supplied by Fluka and Aldrich, respectively. The enzymes used for the bile acids analyses were as follows: cholylglycine hydrolase (Sigma), 3α-hydroxysteroid dehydrogenase (Worthington), *Escherichia coli* β-glucuronidase (Sigma), and *Helix pomatia* extract, Helicase (Industrie Biologique Française, Clichy).

Preparation of [24-^{14}C]HDC

The ^{14}C-labeled HDC was prepared biologically. An inbred male rat of the Fischer 344 strain was given a solution of the sodium salt of [24-^{14}C]CDC through a stomach tube. Feces were collected over the next 9 days, extracted by boiling with ethanol in a Kumagawa apparatus for 48 hr, and bile acids were extracted according to the method of Grundy, Ahrens, and Miettinen (15). After methylation by diazomethane, the bile acids were fractionated by descending TLC on silica gel plates (60 cm in length) using chloroform–acetone–methanol 70:25:5 (v/v) (system I, 16). Pure bile acids were used as standards. Under these conditions, 55%

TABLE 1. Clinical data of patients and percentage of ingested radioactivity excreted each day[a]

Patient	Age	Sex	Pathology	Sample	% Excretion of Bile Acids					
					D1	D2	D3	D4	D5	Total
	yr									
Y	39	M	Gallstones, cholecystitis	Bile	30.2					30.2
				Feces	8.5	10.0	N[b]			18.5
B	49	F	Pancreatitis, common bile duct stones	Bile	11.6	0.8	0			12.4
				Feces	N	N	1.7	0.9	N	2.6
P	66	F	Cholecystitis	Bile	31.1					31.1
				Feces	8.3	12.3	9.1	Tr[c]	Tr	29.7
F	86	F	Common bile duct stones, extrahepatic cholestasis	Bile	8.5	0.9	2.5	0.8	0.5	13.3
				Feces	3.0	0.4	N			3.4
				Urine	8.3	7.8	6.3	5.2	5.0	32.5
G	64	M	Gallstones	Bile	10.3	1.2	Tr			11.5
				Feces	3.4	0.4	0.1	0.3		4.3
				Urine	50.1	22.8	5.9	3.6	1.9	84.2

[a] After ingestion of 500 mg of [24-^{14}C]HDC.
[b] N, no fecal excretion.
[c] Tr, trace.

of the radioactivity was recovered in the HDC methyl ester area. This material was then chromatographed on a column filled with 140 g of silicic acid (Mallinckrodt), 100 mesh, previously activated for 2 hr at 110°C; the mobile phase was benzene mixed with increasing amounts of acetone. The methyl ester of HDC was eluted with a benzene–acetone 77:23 (v/v).The radiochemical purity and the identity of the substance eluted with a sample of pure HDC methyl ester was determined using three methods: *1.* TLC in system I and examination of the plates by a Berthold radioscanner. *2.* GLC of the trimethylsilylated derivatives on a glass column (2 m × 2 mm ID) with 3% OV 17 on Gas Chrom Q, 100–120 mesh as stationary phase. The injector temperature was 250°C and that of the column 250°C; the carrier gas was nitrogen at a flow rate of 20 ml/min. *3.* Mass spectrometry of the silylated bile acids under the following conditions: a mass spectrometer (LKB 9000) was connected to a gas–liquid chromatograph. Columns were packed with 1% OV-1, the injector and separator temperatures were 240°C, the source temperature was 310°C, and the column temperature was 180°C, with an increase of 1° C/min. The carrier gas was helium at a flow rate of 30 ml/min. Spectra were detected at 22.5 eV.

The first two methods showed a single peak corresponding to HDC methyl ester. With the third method, there was no detectable difference between the fragmentation pattern of the sample obtained from the rat feces and that of standard HDC, and the mass spectra were comparable to those described in the literature (7, 13). Characteristic ions (m/e:relative intensity) were: 550:4, 535:3, 460:43, 445:17, 405:30, 370:100, 355:12, 323:15, 255:17 (**Fig. 1**). HDC methyl ester was hydrolyzed by 1 N sodium hydroxide and after acidification at pH 2, the free acid was extracted with chloroform. The specific radioactivity of an aliquot was determined by liquid scintillation counting in toluene containing dimethyl POPOP (0.1 g/l) and PPO (4 g/l) and by mass determination by GLC, as described above. We obtained 159 μCi of [24-^{14}C]HDC of specific activity 2.04 μCi/μmol; the yield was 54% of the radioactivity recovered from the rat feces.

Synthesis of [24-^{14}C]HDC 3-monosulfate and 3,6-disulfate

The sulfated derivatives of HDC were synthesized chemically according to the method described by Tserng and Klein (17) with modifications because of the special characteristics of this bile acid (reactivity and polarity). HDC (2.5 mmol, 2.5 μCi) was dissolved in 5 ml of anhydrous DMF. The complex of trioxide–triethylamine sulfur (1.1 g, 6 mmol) was added to the solution. The reaction was carried out at room temperature for 6 hr under nitrogen with magnetic stirring. After adding a further 6 mmol of complex, the reaction was continued for 24 hr and then stopped by the addition of three drops of water and heating at 40°C for 2 hr. The solution was poured into 65 ml of cold diethyl ether.

The colorless syrupy product obtained was separated from the ether phase and then washed several times with diethyl ether. After drying under nitrogen, the product was taken up in methanolic sodium hydroxide 0.2 N, to pH 9. The precipitate of sodium sulfate was then filtered. The filtrate, containing 2 μCi of radioactive product (yield 80%), was diluted with 1 volume of water and then small amounts of Dowex 50 H^+ resin were added until the pH reached 2.5. The resin was discarded by filtration. After evaporating the filtrate to dryness, an aliquot was analyzed by TLC on silica gel

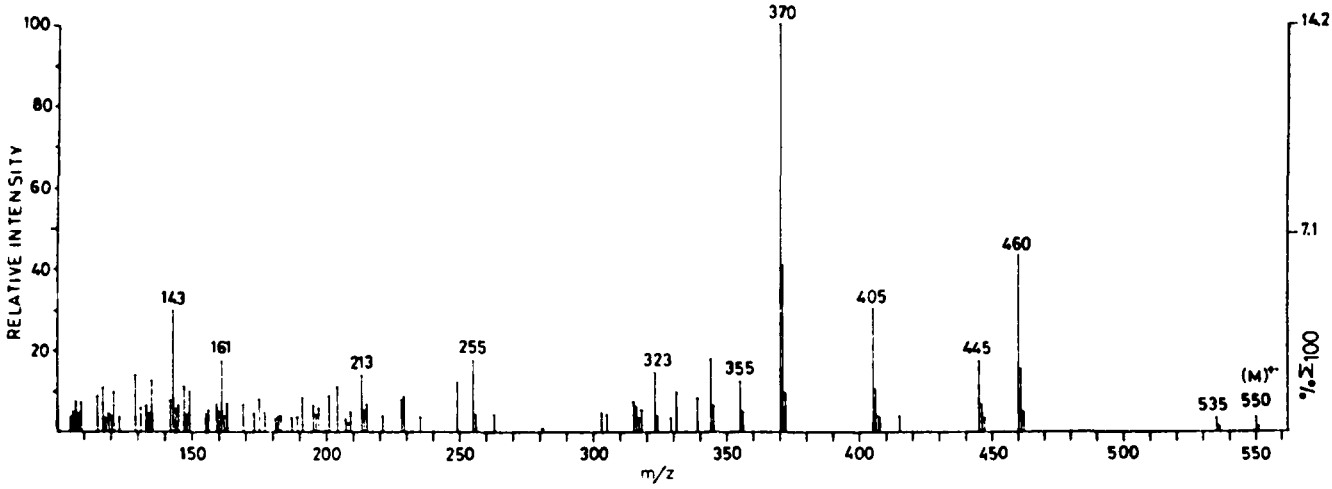

Fig. 1. Mass spectra of the silylated [24-^{14}C]hyodeoxycholic methyl ester. The labeled free bile acid was prepared biologically after administration of [24-^{14}C]chenodeoxycholic acid in rats. The fragmentation pattern of the sample extracted from rat feces was comparable to that of a standard hyodeoxycholic acid.

in butanol–acetic acid–water 10:1:1 (v/v) (18). This solvent was called system II. Spraying with phosphomolybdic acid (5%) in ethanol, then with sulfuric acid (10%), and heating at 100°C for 10 min showed spots I, II, and III, whose R_f values were 0.93, 0.63, and 0.25, respectively.

The three spots represented all the radioactivity deposited on the plate. A part of the mixture (0.9 μCi) was eluted on a silica gel column (80 g) in the same solvent mixture as that used for the TLC. Fractions of 5 ml were collected. Product I appeared in fractions 20 to 27, II in fractions 30 to 43, and III in fractions 43 to 140, representing 7%, 46%, and 47% of the recovered radioactivity, respectively.

After the methylation of product I by diazomethane, it migrated in TLC system I with the same R_f value as that of the known HDC methyl ester. After trifluoroacetylation or silylation of both the methylated product I and the HDC standard, analysis by GLC confirmed the identity of both products. Products II and III subjected to chemical solvolysis by dimethoxypropane (19) produced a single compound identified by TLC and GLC as HDC. *Helix pomatia* extract, which hydrolyzed 3-steroid sulfate in equatorial position (20) such as LC sulfate, also hydrolyzed product II and liberated HDC. It hydrolyzed substance III and did not produce HDC, but rather a substance that migrated in system II similar to substance II. We concluded that substance III was the 3α,6α-disulfate and substance II was the 3α-sulfate of HDC.

Analysis of bile, urine, feces samples

Bile and urine. To measure the radioactivity in bile and urine, 0.5- to 0.25-ml aliquots were placed in counting vials, the volume was adjusted to 3 ml with water and admixed with 10 ml Instagel. Bile samples were diluted 1/10 (v/v) in isopropanol, refluxed for a few minutes and then centrifuged. The supernatant was collected, desiccated, and taken up in water, with or without buffer, methanol, or ethanol according to the analyses to be performed. Urine samples were lyophilized and extracted by boiling with ethanol in a Kumagawa apparatus. Bile and urine samples were subjected to procedures as follows. *1.* Direct analysis of the alcoholic extracts by chromatography (see below). *2.* Separation of the bile acids after alkaline hydrolysis according to the methods of Grundy et al. (15) and van Berge Henegouwen, Allan, and Hofmann (21). *3.* Separation after enzymatic hydrolysis using the method of Nair et al. (22). *4.* Solvolysis with anhydrous methanolic HCl using dimethoxypropane (19), which at the same time methylated all the bile acids. *5.* Incubation with an extract of *Helix pomatia* which contains both sulfatases and β-glucuronidases (13). *6.* Action of *Escherichia coli* β-glucuronidase preparation (Sigma) which does not hydrolyze bile acid sulfates. System I was used with TLC (silica gel) for methyl esters, and system II or system III [chloroform–methanol–7 N ammonia 90:45:10 (v/v) (23)] was used for nonmethylated preparations. Systems II and III were used with TC, TCDC, GC, GCDC, C, LC, and mono- and disulfated HDC obtained by synthesis.

System III was adapted to column chromatography in the following way. Silica gel (Mallinckrodt) 100 mesh, was activated for 2 hr at 110°C, cooled, and admixed with distilled chloroform in order to constitute a column of gel topped by a large volume of chloroform. The sample was taken up in a minimum volume of ethanol and mixed with this large volume of chloroform. Chromatography was performed using chloroform to which was added increasing amounts of methanol–12 N ammonia 95:5 (v/v) (solvent A) until chloroform–solvent A mixture was 75:25 (v/v). Then the quantities of ammonia and water in the mixture were gradually increased. The levels of glucuronic acid in the isolated glucuronide fraction were determined according to the method of Nir (24).

Feces. The same method described for obtaining [24-^{14}C]HDC from rat feces was used to isolate fecal bile acid from patients. The radioactivity in the fecal extract, obtained by means of boiling with ethanol in the Kumagawa apparatus, was measured using Instagel (Packard) or toluene, POPOP, PPO, Triton (30%) admixed with 1 ml of water for 15 ml of scintillating mixture. The fraction containing the bile acids (obtained according to the method of Grundy et al. (15)) was methylated by diazomethane and chromatographed on TLC in system I, in the presence of the methyl esters of C, HC, β-MC, HDC, CDC, 3α,6β-dihydroxy-5β-cholanoic acid, UDC, 3α-hydroxy-6-keto-5β-cholan-24 oic acid, and LC.

The distribution of the radioactivity on the chromatograms was studied with a Berthold radioscanner and the radioactivity of the gel areas was estimated either by peak integration or, after recovery of the gel, by mixing with Cab-O-Sil and scintillation counting. An aliquot of the methylated extract was used to prepare trifluoroacetate derivatives which were then subjected to GLC on a 2 m × 2 mm (ID) column packed with 2% OV 210 fixed on Gas Chrom Q 100–120 mesh at 210°C. The injection temperature was 230°C; the carrier gas was nitrogen at a flow rate of 15 ml/min.

RESULTS

Balance of ^{14}C radioactivity excretion

The average T-tube flow rates (ml/hr) were 11.5 in patient Y, 12.9 in patient P, 6.3 in patient B, 11.8 in

TABLE 2. Fraction of administered [24-^{14}C]hyodeoxycholic acid in bile and urine[a]

Patient	Sample	Time (hr) 2	4	6	24	48	72	96	120
Y	Bile	691	1943	1845	646				
B	Bile	0	401	2085	745				
P	Bile	0	0	481	1641				
F	Bile	0	320	606	236	92	79	32	29
	Urine			94	151	157	116	106	94
G	Bile	193	237	153	88	19	0	0	0
	Urine	346	918	422	394	152	37	25	12

[a] Fraction of dose $\left(\frac{\text{dpm/ml of sample}}{\text{dpm ingested (initial dose)}}\right) \times 10^6$ in bile flowing through a T-tube and in the urine after ingestion of 500 mg of [24-^{14}C]HDC.

patient F, and 30 in patient G. The urine flow rates in patients F and G were 22 and 60 ml/hr, respectively. The highest radioactive elimination by these two routes was in patient G, whose excretion rate was three times that seen in the other patient. The fraction of the initial dose absorbed and recovered in bile, urine, and feces is shown in Table 1.

The percentage excretion by the different routes varied from patient to patient. Three patients (B, F, G) showed very low biliary and fecal excretion rates (15%, 16.7%, and 15.8%, respectively) of the ingested dose for both routes combined, whereas patients Y and P excreted 49 and 61% by these routes.

Fecal excretion was generally low since the largest value (in patient P over five days) was only 29.7% of the initial dose. In all five patients, the biliary secretion of radioactivity was highest during the first 24 hr of the collection and decreased rapidly thereafter. Two patients (F and G), whose urines were collected, excreted a major part of the ingested radioactivity by that route. Radioactivity reached 32.5% in patient F over the first 5 days of the collection and remained high on day 5. Patient G also showed high (84% of the dose over the 5 days) and early (4.5% within 2 hr; 9.8% within 4 hr; 17% within 6 hr, and 50% within 24 hr) urinary elimination.

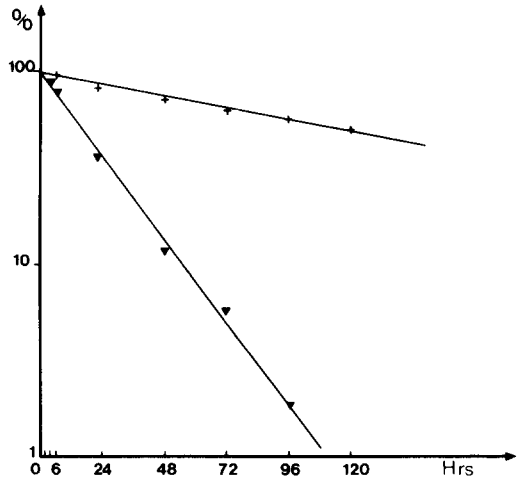

Fig. 2. Total excretion of radioactivity in patients F (+) and G (▲) after ingestion of [24-^{14}C]hyodeoxycholic acid. Y axis, % of the radioactivity remaining in the organism calculated by difference. X axis, time in hours.

Table 2 shows the fraction of the initial dose recovered per ml of bile or urine. These values correspond to the concentration of HDC, or of its metabolites, at the time of the collection. In four of the patients studied, the highest bile concentration was obtained in the early (4–6 hr) period of the collection. In two patients (Y and G), the fraction of the dose recovered in the bile reached high values within 2 hr of starting the study. Table 2 also compares urinary and biliary excretion. In patient G excretion by these routes followed a similar pattern with maximum concentrations being reached at the same time (hour 4 of the study) while the decrease in urinary concentration took place in parallel with the decrease in biliary excretion. Finally, in patients F and G, in whom excretion was studied by all three routes, the total excretion was mono-exponential with half-lives ($t_{1/2}$) of 120 and 16 hr, respectively (**Fig. 2**).

Metabolism of HDC

Identification of metabolites in bile and urine. Several methods for separation of free bile acids based on alkaline hydrolysis, the method of van Berge Henegouwen et al. (21) which combines solvolysis and alkaline hydrolysis, and simple methylation by diazomethane of a slightly acidified ethanolic extract of bile enabled us to observe many radioactive peaks by TLC. Subsequently, however, some of these appeared to be artefacts. Chromatographic analysis (in systems II and III)

of alcoholic extracts of the most radioactive bile samples showed the presence of two major components (**Fig. 3**).

The first component (A) migrated in the same position as the glycodihydroxylated acids (control GCDC). The second (B) migrated in system II, below TC which, in this system, migrated at the same place as disulfated HDC (B, 3.6 cm; TC, 2.5 cm). In system III component B remained close to the origin and separate from all the other bile acids visible in the system (running distance: B, 1.7 cm; GC, 4.7 cm; TC, 5.8 cm). TLC of urine extracts showed a single peak that migrated together with compound B in systems II and III. Chromatography of ethanolic extracts on silica gel columns separated these two peaks. The compound (A) was eluted in the chloroform–solvent A fraction 75:25 (v/v).

After incubation with cholylglycine-hydrolase and after methylation, chromatography in system I showed a single peak of radioactivity corresponding to the HDC methyl-ester. Substance B was eluted from the silica gel column in the chloroform–methanol–12 N ammonia–water 65:30:3:3 (v/v) fraction. Substance B, obtained either from bile or from urine, behaved similarly. The solvolysis in anhydrous medium, as well as the action of *Helix pomatia* extract or of *Escherichia coli* β-glucuronidase, followed by methylation and chromatography in system I, produced quantitatively HDC methyl ester. Column chromatography of urine extracts provided about 10 mg of substance B which, after treatment by acetic acid and lyophilization to eliminate the ammonium acetate produced, crystallized easily in the cyclohexane-methanol mixture. Its melting point was 175–177°C. Determination of glucuronic acid by the method of Nir (24) showed that this substance was composed of equimolar parts of glucuronic and hyodeoxycholic acids. The 3-OH steroid-dehydrogenase was inactive, suggesting that the hydroxyl group in position 3 was not free. All these observations enable us to conclude that substance A is glycohyodeoxycholic acid and substance B is 3α[β-D-glucuronyl]hyodeoxycholic acid.

Metabolite excretion in bile. The proportions of both substance A and substance B varied from patient to patient and from one time period to the next after ingestion. In patient G, for example, the proportions at 6 hr were 67% HDC conjugated with glycine, 33% with glucuronic acid, and traces of the free bile acid, while in patient F the corresponding proportions were 15%, 15%, and 70%, respectively. At 24 hr in patient F, there was only free acid while in patient P, 23% of the bile acid was conjugated with glycine, 67% with glucuronic acid, and 10% was in the form of the free acid. Thus, the capacity of the liver to metabolize HDC was highly variable from one patient to another.

Identification of metabolites in feces. In patients Y and P, the excretion of fecal radioactivity was high enough to permit chemical analysis of the bile acids. The chromatographic analysis, in system I, of the extracts obtained from the feces on day 2 of the study is illustrated in **Fig. 4.** The largest part of the radioactivity was located in the HDC methyl ester while there was a negligible amount of radioactivity in the LC methyl ester. GLC of the trifluoroacetates obtained from the feces on day 2 gave a pattern indicating the predominance of the secondary bile acids (LC and DC) and a peak having the retention time of HDC. The fecal bile acid composition was as follows: Patient Y, LC 32%, DC 36%, CDC 8%, HDC 13%, and C 11% (**Fig. 5**); and in patient P, LC 24%, DC 24%, CDC 13%, HDC 31%, and C 8%. The total fecal bile acid excretion was 370 mg in patient Y and 208 mg in patient P. Although the intestinal flora of these patients seemed to metabolize the endogenous bile acids normally, HDC was little affected; in particular there was no appreciable amount of LC formed.

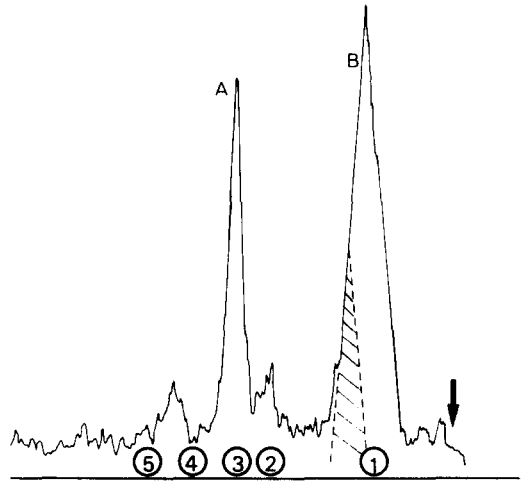

Fig. 3. Radioactivity scanning of a TLC silica gel plate of biliary bile acids (patient P). Solvent was butanol–acetic acid–water 10:1:1 (v/v). Arrow, origin. Circles correspond to the position of standards: 1, taurocholic and disulfated hyodeoxycholic acid; 2, monosulfated hyodeoxycholic acid; 3, glycochenodeoxycholic acid; 4, cholic acid; 5, lithocholic acid. Peak A was identified as glycohyodeoxycholic acid. Peak B was identified as 3α (β-D-glucuronyl)hyodeoxycholic acid. Hatched area corresponded to purified 3α (β-D-glucuronyl) hyodeoxycholic acid.

DISCUSSION

The results reported in this paper must be considered while taking into account some of the limitations resulting from the particular conditions of the study. For ethical reasons HDC was given by mouth in gelatin capsules instead of by a duodenal tube whose introduction would have given additional discomfort to patients recently operated on; thus, the kinetics of intestinal ab-

Definition of areas {1}

	Y	P
Origin	4.9 %	2.4 %
Cholic Hyocholic β-muricholic	1.5	1.8
Hyodeoxycholic	82.3	91.6
Chenodeoxycholic 3α, 6β-dihydroxy-5 β-cholanoic Ursodeoxycholic	5.0	0.8
3α-hydroxy-6 keto-5β-cholanoic	3.9	2.3
Lithocholic	1.5	0.6
Front	0.9	

{1} Methyl esters of control bile acids.

Fig. 4. Distribution of the radioactivity (%) in the thin-layer chromatograms. Fecal bile acids, obtained from feces of Y and P collected on day 2 after ingestion of [24-^{14}C]hyodeoxycholic acid, were extracted, methylated, and chromatographed on silica gel plates developed in chloroform–acetone–methanol 70:25:5 (v/v).

sorption varied from patient to patient by the sequence of gastric emptying. Also, collection of bile from the T-tube was not quantitative, an unestimated proportion of bile flowed into the duodenum. Bile acid enterohepatic circulation was thus maintained at a variable degree in each patient. These limitations, however, do not invalidate the main findings of the study, namely, the efficient intestinal absorption of HDC, the lack of intestinal transformation by 6α-hydroxylases, its preferential conjugation to glucuronic acids, and the urinary excretion of large amounts of HDC-glucuronide. Finally, urine was quantitatively collected in two out of the five patients studied; from the bile and fecal excretion it can be assumed, however, that in all patients urinary excretion of the HDC metabolite was quantitatively important.

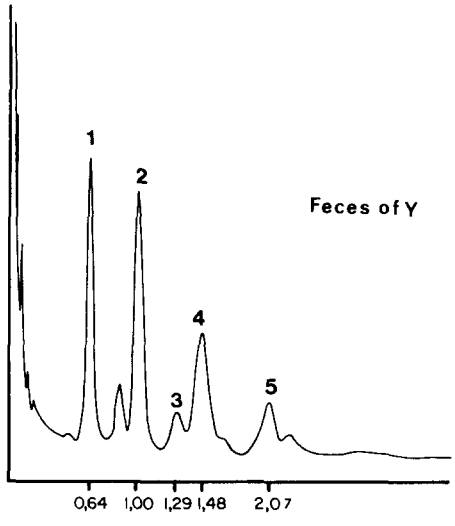

Fig. 5. Gas–liquid chromatography of trifluoroacetate derivatives of bile acid methyl esters obtained from feces of patient Y collected 48 hr after ingestion of hyodeoxycholic acid. Peaks identified by their retention time are: 1, lithocholic; 2, deoxycholic; 3, chenodeoxycholic; 4, hyodeoxycholic; and 5, cholic.

We checked that the bacterial transformation of endogenous bile acids was normal in those patients whose feces contained large amounts of secondary bile acids. In the two patients where fecal excretion was relatively high, HDC was only partially transformed by the intestinal bacteria, which did not change it into LC.

These results suggest that 6α-dehydroxylases cannot be present in human intestinal microflora. This enzyme activity does not exist in rat feces which contains large amounts of HDC and little LC. It remains possible, however, that HDC might undergo other transformations by the intestinal bacteria, particularly 6α-dehydrogenation. Tenneson, Owen, and Mason (25) observed in vitro the breakdown of the lateral chain of HDC by a

strain of *Escherichia coli* of human origin. The same group (26) also showed that a strain of *Pseudomonas* transforms HDC into various metabolites. The existence of such transformations in vivo is yet to be confirmed.

Since its fecal excretion remains low in all patients, we can conclude that HDC must be well absorbed from the human intestine. In patient G, where the combined excretion through the bile, fecal, and urinary routes reached 100%, more than 95% of the product passed through the intestinal barrier and was eliminated either through the T-tube or in the urine. In the human intestine, therefore, HDC seems to behave in the same way as most di- or trihydroxylated bile acids. Studies of the biotransformation of hyodeoxycholic acid by the intestinal bacteria were difficult because of the low excretion of this product.

After intestinal absorption of HDC, the next steps are its passage into the portal system, its transfer to the liver, and its uptake by the hepatocytes. At the present time, there is no information about the efficiency of the hepatic uptake of HDC.

In three patients, collection of T-tube bile for 3–5 days showed that the total recovery rate was low (B, 12.4%; F, 13.3%; G, 11.5%) for a bile acid and that most of the elimination into bile occurred on day 1 of the study (B, 93%; F, 64%; G, 89%). The low recovery of HDC and its metabolites in the bile and feces suggested that there must be some other route of elimination which, as discussed below, can only be urine.

Analysis of bile showed the presence of two main metabolites of HDC. The first was the result of conjugation of this acid with glycine in common with most bile acids in human bile. The second was identified as 3α [β-D-glucuronyl]-HDC for the following reasons. *1.* It was isolated by column chromatography, as described, and crystallized to a constant melting point (175–177°C); *2.* the presence of a glucuronyl group was demonstrated by Nir's reaction (24) with a 1:1 molar ratio of glucuronic acid–HDC; *3.* the glucuronyl group was in the 3-position on the molecule as evidenced by the absence of a reaction with 3α-OH dehydrogenase.

Identification of this second metabolite was obtained by solvolysis and enzymatic hydrolysis by β-glucuronidase (obtained from *Helix pomatia*), followed by identification of free HDC by TLC and GLC. As the β-glucuronidase preparation also contained sulfatases, we excluded the presence of sulfated HDC by comparing it with synthetic HDC sulfate (see Material and Methods). Finally, we also showed that β-glucuronidase from *Escherichia coli* (which does not contain sulfatases), also hydrolyzed the compound.

Glucuronidation is a process used by the liver for detoxification and elimination of many products of endogenous origin (27), particularly bilirubin, steroid hormones, and xenobiotics (27) such as some phenolic compounds. One of the first demonstrations of bile acid glucuronidation was described by Back, Spaczynski, and Gerok (28). This mode of conjugation of bile acids has been demonstrated several times in man, particularly in patients with cholestasis (29–31). In such patients, glucuronides of the main bile acids have been isolated both from the plasma and from urine, but the presence of bile acid glucuronides has never been described in the urine or plasma of healthy humans.

In the rat, the enzyme involved in the formation of bile acid glucuronides (UDP glucuronosyltransferase E.C.:2.4.1.17) was located in liver microsomal membranes (32, 33). This enzyme activity seems to be complex, multiple, and substrate-dependent. Very little information is available about UDP glucuronosyltransferase activity on bile acids in human liver. Matern et al. (34) showed that this activity was common to bile acids, bilirubin, and estradiol, whereas morphine, testosterone, and 4-nitrophenol were substrates for one of several other forms of UDP glucuronosyltransferase.

The appearance of very polar radioactive metabolites in bile had formerly led us to consider the formation of sulfated derivatives of HDC. It is well known that sulfation is a detoxification process largely used by the human liver. The existence of sulfated bile acids has frequently been described (35) in many pathological conditions. In order to test this possibility, we synthesized mono- and disulfated HDC and compared their properties with those of the HDC metabolites. The results of these studies enabled us to discard this hypothesis because the sulfates and glucuronides behaved differently as judged by silica gel TLC in various solvent systems as well as by the enzymatic tests. The most striking finding in the present study was the large elimination of this bile acid by the urine. This is distinctly unusual for this type of compound in patients who do not suffer from cholestasis, since their bile has drained freely several days through the T-tube. In patient G for example, in whom the metabolites were eliminated very rapidly (half-life for the total excretion, 16 hr), 0.63 mmol out of the 1.7 mmol of product ingested was excreted in the urine within 24 hr. The rapid elimination of this bile acid in the urine probably explains why it is not possible to enrich the bile acid pool with HDC. This may explain why Thistle and Shoenfield (14) did not find this product in the bile after 4 months of treatment with 1 g of HDC per day. The only metabolite isolated from urine was 3α[β-D-gucuronyl]-HDC, which was identical to that found in bile.

On the basis of the experimental protocol and the results obtained, it is possible to draw a diagram (**Fig. 6**) representing the possible movements of HDC and/

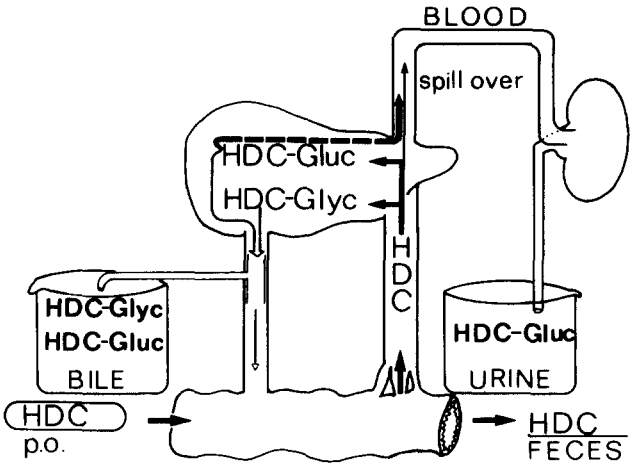

Fig. 6. Biotransformation and excretion of hyodeoxycholic acid in patients fitted with T-tubes. After intestinal absorption, most of HDC is probably taken up by the liver, a small spill-over allowing some HDC to circulate into the peripheral blood. From the two main products of hepatic metabolism, glycohyodeoxycholic acid (HDC-glyc) and 3α (β-D-glucuronyl)hyodeoxycholic acid (HDC-gluc), only the last one is released into the circulation and excreted in urine, although glucuronidation in the kidney of HDC cannot be excluded. HDC-gluc and HDC-glyc are excreted in bile, transformed into free bile acid by human intestinal bacteria, and finally excreted in feces.

or its metabolites between the different organs of the body. Although this metabolic and excretory pathway of HDC applies to studied gallstone patients, preliminary results from healthy subjects with intact enterohepatic circulation seem to confirm the glucuronidation and urinary excretion of HDC in normal man.

The diagram takes into consideration several hypotheses that might explain the appearance of the glucuronide in the urine. If the liver is the only organ responsible for the glucuronidation, the urinary and biliary elimination of the metabolite should result from a double secretion of the glucuronide into bile and plasma, followed by its urinary elimination. However, the kidney can also glucuronidate HDC; the substrate has to be carried to the kidney in the systemic circulation after escaping liver uptake from the portal blood. The demonstration of a large UDP-glucuronyl transferase activity in human kidney supports this hypothesis (36, 37).

However, a contribution of renal glucuronidation in man implies that the liver uptake capacity for HDC is much less than that described for other bile acids. Since these hypotheses are currently under investigation, the relative contributions of the liver and the kidney to the metabolism and excretion of HDC remain, at present, unknown.

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