SYNTHESIS, INTESTINAL ABSORPTION AND METABOLISM OF SARCOSINE CONJUGATED URSODEOXYCHOLIC ACID

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ABSTRACT

Sarcosine conjugated ursodeoxycholic acid (SUDC) was synthesized and its intestinal absorption and metabolism were studied in rat and hamster. Intestinal absorption study using bile fistula rat shows that more than 90% of SUDC administered intraduodenally was excreted in the bile within 24 hr. No change of the administered bile acid was seen during the absorption from the intestine, the passage of the liver, and the excretion into the bile. When [24-^{14}C]SUDC and [11,12-$^{3}H_2$]-ursodeoxycholic acid were administered orally to a hamster, more than 95% of both the admnistered ^{14}C and ^{3}H were recovered from the feces within 6 days. Most (77%) of the fecal ^{14}C-labeled compound was SUDC, whereas 95% of the fecal ^{3}H-labeled compound was unconjugated lithocholic acid. These results indicate that SUDC, unlike taurine or glycine conjugated bile acid, resists bacterial deconjugation and 7-dehydroxylation.

INTRODUCTION

Chenodeoxycholic acid (CDC) and ursodeoxycholic acid (UDC) have been used to dissolve cholesterol gallstones in humans (1,2). Administration of either compound to rabbit (3) or rhesus monkey (4,5) caused hepatotoxicity that is associated with hepatobiliary accumulation of a potential hepatotoxic compound, lithocholic acid (LC) (6), which is formed from either CDC or UDC in the colon by bacterial 7α- or 7β-dehydroxylation (7-9). Since humans sulfate LC and sulfation of LC produces a more excretable form of this toxic metabolite, they are believed to be protected from toxic accumulation of LC (10,11). However, many of the abnormalities seen in patients treated with CDC,

but not with UDC, have been attributed to the observed increase in biliary unsulfated LC (12,13). It should also be noted that LC has a promoting effect in N-methyl-N'-nitro-N-nitrosoguanidine-induced colon carcinogenesis in rats (14). Thus, it seems to be desirable to develop new cholelitholytic agents which, unlike both CDC and UDC, are not metabolized to LC by intestinal bacteria. The following report describes the synthesis and metabolism of sarcosine conjugated ursodeoxycholic acid (SUDC) (Fig. 1) which has been selected by us as a trial compound to develop such agents (15).

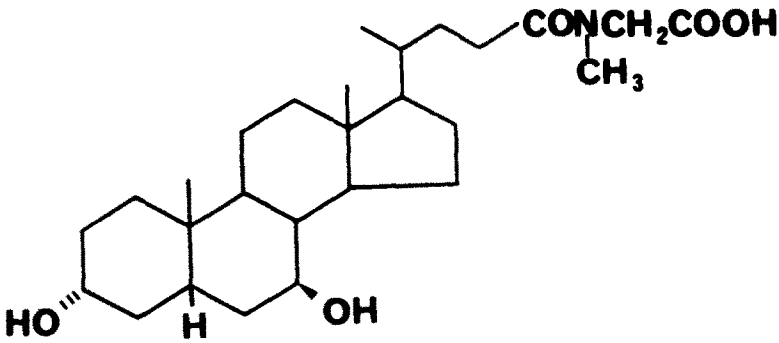

Fig. 1. Sarcoursodeoxycholic acid

MATERIALS AND METHODS

General

Melting points were determined with a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured in methanol on a Union Giken model PM-101 automatic polarimeter. Infrared (IR) spectra were taken on a JASCO IRA-1 spectrometer as KBr discs. Nuclear magnetic resonance (NMR) spectra were measured at 90 MHz on a Hitachi R-40 spectrometer. Chemical shifts (δ) are given in ppm downfield from tetramethylsilane internal standard. Radioactivity was measured using a toluene-based scintillation solution in a Packard Tri-Carb model 3320 liquid scintillation spectrometer. Suitable corrections were made for background counts and quenching.

Chromatography

Thin-layer chromatography (TLC) was carried out on precoated silica gel G plates (Merck) with a 10% solution of phosphomolybdic acid in ethanol as the detection reagent. For TLC separation of labeled compounds, samples were applied to the plate as a streak (1-3 cm).

Reference spots of known compounds were applied on either side of the streak. Pertinent bands were scraped from the developed plate according to the positions of the reference spots which were made visible by means of the phosphomolybdate reagent, and transferred to scintillation vials together with scintillation solution to determine radioactivity. In addition, the radioactivity of the origin, and of 5 mm sections between known bands was assayed.

High-performance liquid chromatography (HPLC) was carried out with a DuPont 830 liquid chromatograph equipped with a single-wavelength (254 nm) UV detector. The column was of a reversed-phase type (TSK Gel LS-410, 4 mm ID x 30 cm, Toyo Soda Co. Ltd.). All samples were analyzed as the p-bromophenacyl (PBP) ester derivatives which were prepared as described previously (16). The moving phase was 80% methanol and its flow rate was 0.7 ml/min. When labeled compounds were analyzed by HPLC, 0.7 ml fractions were collected in scintillation vials. After addition of scintillation solution, radioactivity of the collected fractions was measured.

Ion exchange chromatography on piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was performed according to the procedure reported by Goto et al (17). Preliminary experiments showed that by this method sarcosine conjugated bile acids were eluted together with glycine conjugated bile acids in the fraction eluted with 0.2 M formic acid in 90% ethanol.

Synthesis of Sarcoursodeoxycholate (SUDC)

To a solution of ursodeoxycholic acid (20 g) dissolved in 150 ml of dioxane containing 8 ml of tri-n-butylamine was added 4.6 ml of ethyl chlorocarbonate at 10°C. After 15 min, a solution of 6.1 g of sarcosine in 68 ml of 1N NaOH was added and the mixture was allowed to stand at room temperature for 2 hr. The reaction mixture was then evaporated to dryness under a reduced pressure. The residue was dissolved in 200 ml of 0.5N NaOH and acidified with dil-HCl. The resulting precipitate was filtered and dried. The dried precipitate was purified by silica gel column chromatography (500 g, Merck) with a mixture (30:10:1, by vol) of benzene, isopropanol, and acetic acid as the eluting solvent. Recrystallization from methanol-ethyl acetate gave crystals (15.1 g) of SUDC: mp 193-194°C; $[\alpha]_D$ = +55° (c=1.0, MeOH); Anal. Calcd for $C_{27}H_{45}NO_5$:C 69.9, H 9.7, N 3.0, Found C 70.0, H 9.7, N 2.9. IR ν_{max}^{KBr} (cm^{-1}): 3300 (OH), 1730 (COOH), 1630 ($CONCH_3$); NMR (δ): 0.70 (s, 3H, 18-CH_3), 0.96 (s, 3H, 19-CH_3), 0.99 (d, J=6 Hz, 3H, 21-CH_3), 3.22 (s, 3H, N-CH_3), 3.70 (m, 1H, 3β-H), 3.85 (m, 1H, 7α-H), 4.47 (d, J=6 Hz, 2H, N-CH_2-)

Labeled Bile Acids

[24-^{14}C]SUDC was prepared from [24-^{14}C]UDC (31.4 Ci/mmol, Daiichi Chemical Co.) in the same manner as described above for the unlabeled SUDC. The radioactive SUDC was then recrystallized together with the nonradioactive compound to give a final specific activity of 10.8 mCi/mmol. Its radiopurity checked by radio-HPLC was shown to be greater than 99%.

[11,12-$^3H_2$]UDC (37.0 Ci/mmol) was obtained from New England Nuclear Corp. and recrystallized together with unlabeled UDC to give a final specific activity of 17.6 mCi/mmol.

Reference Bile Acids

Nonradioactive CDC and UDC were supplied by Tokyo Tanabe Co. Ltd. (Tokyo, Japan). LC was a commercial product. 3α-Hydroxy-7-oxo-5β-cholan-24-oic acid (18) and 7β-hydroxy-3-oxo-5β-cholan-24-oic acid (19) were prepared from CDC and UDC, respectively, according to the reported procedures. Sarcosine conjugates of CDC (15), LC, and the two keto bile acids were prepared from corresponding unconjugated bile acids in the same manner as described above for SUDC: sarco-lithocholate, mp 192-193°C; IR ν_{max}^{KBr} (cm^{-1}): 3250 (OH), 1750 (COOH), 1735 (CO), 1625 ($CONCH_3$); NMR (δ): 0.63 (s, 3H, 18-CH_3), 0.92 (s, 3H, 19-CH_3), 0.98 (d, J=6 Hz, 3H, 21-CH_3), 3.22 (s, 3H, -N-CH_3), 3.91 (m, 1H, 3βH), 4.50 (d, J=6 Hz, 2H, N-CH_2-); sarco-3α-hydroxy-7-oxo-5β-cholan-24-oate, mp 202-203°C; IR ν_{max}^{KBr} (cm^{-1}): 3350 (OH), 1725 (COOH), 1700 (CO), 1625 ($CONCH_3$); NMR (δ) 0.63 (s, 3H, 18-CH_3), 0.97 (d, J=6 Hz, 3H, 21-CH_3), 1.14 (s, 3H, 19-CH_3), 3.22 (s, 3H, N-CH_3-), 3.84 (m, 1H, 3β-H), 4.51 (d, J=6 Hz, 2H, N-CH_2-); sarco-7β-hydroxy-3-oxo-5β-cholan-24-oate, amorphous powder, IR ν_{max}^{KBr} (cm^{-1}) 3470 (OH), 1720 (COOH), 1700 (CO), 1625 ($CONCH_3$); NMR (δ) 0.71 (s, 3H, 18-CH_3), 0.96 (s, 3H, 19-CH_3), 0.97 (d, J=6 Hz, 3H, 21-CH_3), 3.22 (s, 3H, N-CH_3), 3.74 (m, 1H, 7α-H), 4.51 (d, J=6 Hz, 2H, N-CH_2-)

Animal Experiments

Male rats of the Wistar strain weighing between 400g and 430g were fasted for a whole night prior to an experiment but water was allowed freely. Each rat was anesthetized with ether and a polyethylene cannula (0.61 mm ID) was introduced into the bile duct. A solution of [24-^{14}C]SUDC (0.1 mg, 2.3 μCi) dissolved in 1.5 ml of 40% aqueous ethanol was given by injection into the duodenum of each bile duct cannulated rat. The cannulated animals were kept individually in restraining cages with free access to food and water. Bile was collected for 72 hr.

In another series of experiments, a solution of [24-^{14}C]SUDC (0.6 mg, 14 μCi) and [11,12-$^{3}H_2$]UDC (0.6 mg, 27 μCi) in 1.5 ml of 40% aqueous ethanol was given orally to a male golden Syrian hamster weighing 90 g. The hamster was placed in a metabolic cage and feces and urine were collected separately.

RESULTS AND DISCUSSION

Synthesis of SUDC

SUDC was synthesized according to the procedure reported by Norman (20) for synthesis of taurine and glycine conjugated bile acids. A number of other methods have been reported for the preparation of conjugated bile acids (21). We have found that all the methods reported could be used for the synthesis of SUDC, but the method of Norman was more convenient to carry out and gave better yields than other

procedures.

Intestinal absorption and hepatic metabolism of SUDC

When [24-^{14}C]SUDC was given by injection into the duodenum of bile duct cannulated rats, the biliary excretion of the radioactivity is shown in Fig. 2. The maximum excretion occurred between 0 hr and 10 hr after the administration of the labeled SUDC. More than 96% of the administered radioactivity was excreted during the first 24 hr, and no radioactivity was excreted during the following days. The results show

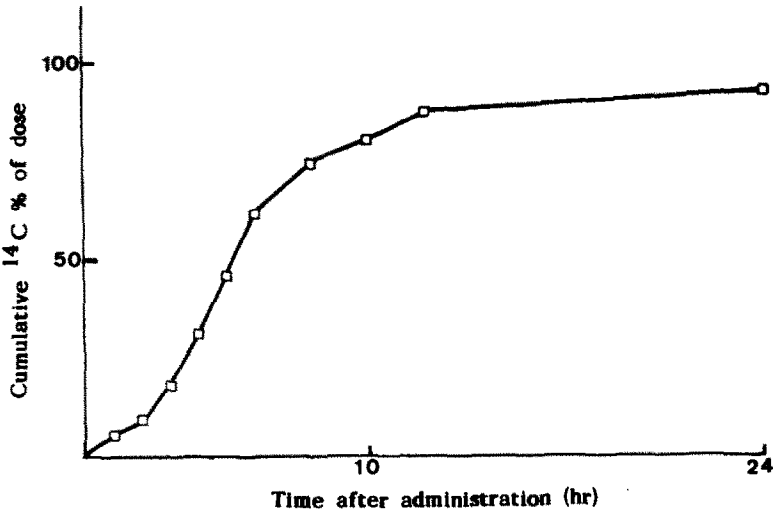

Fig. 2 Biliary excretion of radioactivity in bile fistula rats after administration of [24-^{14}]SUDC
Each value represents the mean of 3 rats

that SUDC, in spite that such a conjugate is not a normal constituent of mammalian bile, was efficiently absorbed from the intestine and quickly excreted in the bile, as are the usual bile acids (22). The mechanism of the absorption of SUDC was not known. Also, it is not known whether SUDC inhibited the absorption of endogenous bile acids.

Radio-TLC analysis of the biliary radioactive material showed only one radioactive band corresponding to SUDC. There was no evidence for

any biotransformation during the absorption from the intestine, the passage through the liver, and the excretion into the bile.

Metabolism of SUDC during the passage of intestinal tract

When [24-^{14}C]SUDC and [11,12-$^{3}H_2$]UDC were administered orally to an intact hamster, 95% of the administered ^{14}C and 97% of the administered ^{3}H were recovered from the feces within 6 days respectively. No radioactivity was found in the urine during this period of time. The fecal radioactive material was extracted and chromatographed on a column of PHP-LH-20. As shown in Fig. 3, nearly all ^{3}H radioactivity was eluted in the unconjugated bile acid fraction, whereas most (95%) of ^{14}C radioactivity was eluted in the

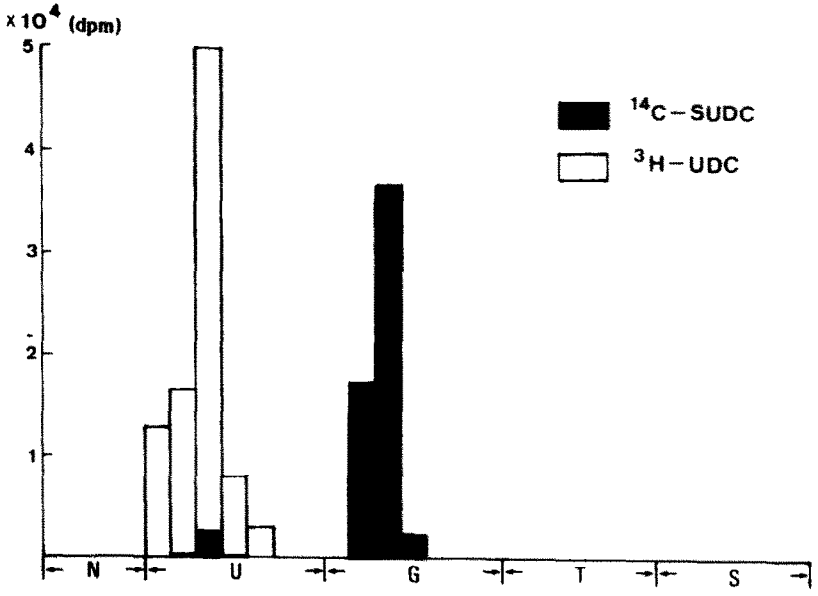

Fig. 3 Group separation of fecal radioactive bile acids by ion-exchange chromatography on PHP-LH-20 column
N : Neutral steroid fraction
U : Unconjugated bile acid fraction
G : Glycine-sarcosine conjugated bile acid fraction
T : Taurine conjugated bile acid fraction
S : Sulfated bile acid fraction

glycine-sarcosine conjugated bile acid fraction, and only 5% in the unconjugated bile acid fraction. TLC analysis of the radioactive material eluted in the unconjugated bile acid fraction showed two radioactive bands corresponding to UDC and LC, respectively. For further indentification, the radioactive material was converted to the *p*-bromophenacyl (PBP) ester, diluted with the PBP esters of unlabeled UDC and LC, and subjected to radio-HPLC. The radioactive compounds were eluted together with the unlabeled standards (Fig. 4) . Since preliminary radio-TLC analysis of the radioactive material eluted in the glycine-sarcosine conjugated bile acid fraction revealed a complex mixture of labeled compounds, the material was converted to the PBP ester and further fractionated into fractions I, II, and III on a column of silica gel using a system of ethyl acetate-benzene gradients

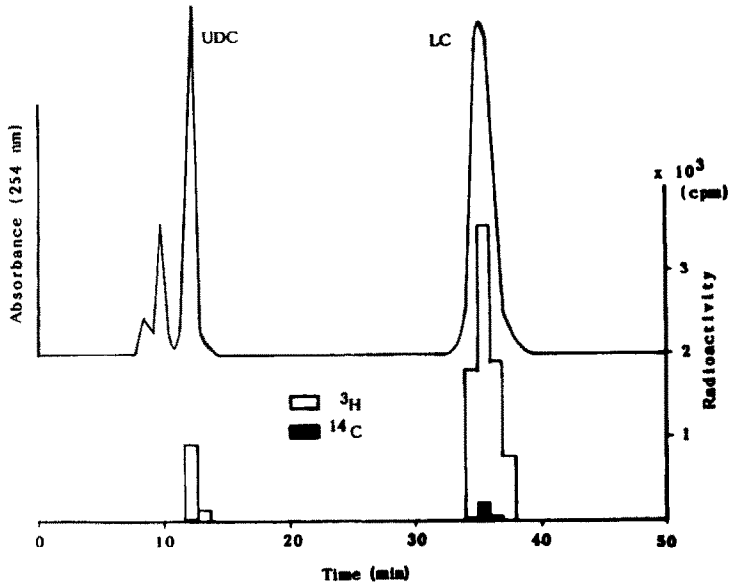

Fig. 4 High performance liquid chromatogram of labeled compounds in the unconjugated bile acid fraction

Table I. Separation and composition of PBP esters of labeled compounds in the sarcosine conjugated fraction by silica gel column chromatography

fraction	eluted solvent (% of benzene in AcOEt)	composition (% of total)
I[a)]	30	4.0
II[b)]	40	14.7
III[c)]	50	81.3

a): identified as sarcolithocholate
b): identified as sarco-7β-hydroxy-3-oxo-5β-cholan-24-oate and as sarco-3α-hydroxy-7-oxo-5β-cholan-24-oate
c): identified as sarcoursodeoxycholate

as the eluting solvent (Table I). Each fraction was analyzed by radio-HPLC, as was done with the unconjugated bile acid fraction. An aliquot of the fraction I was chromatographed along with the PBP ester of nonradioactive sarco-LC. The radioactivity was eluted together with the reference compound and the specific radioactivity of each column fraction remained constant throughout the carrier band. An aliquot of the fraction II was chromatographed along with the PBP esters of sarcosine conjugates of 7β-hydroxy-3-oxo-5β-cholan-24-oic acid and 3α-hydroxy-7-oxo-5β-cholan-24-oic acid. More than 96% of the radioactivity eluted with the PBP ester of sarco-7β-hydroxy-3-oxo-5β-cholan-24-oate and only 4% with the PBP ester of sarco-3α-hydroxy-7-oxo-5β-cholan-24-oate. An aliquot of the fraction III was chromatographed along with the PBP ester of unlabeled SUDC. Nearly all the radioactivity eluted with the reference compound.

Table II gives the approximate percentage of radioactive bile acids recovered from the feces. It is well known that most of bile acids excreted in feces are present in the deconjugated form and these unconjugated bile acids are formed from taurine and glycine conjugated bile acids by bacterial deconjugation during their passage through the

Table II. Percentage of radioactive bile acids recovered from the feces

	fraction	(%)	bile acid (% of total)					
			LC	UDC	SLC	S3K[a]	S7K[b]	SUDC
[^{3}H]	unconjugated	100	95.1	4.9				
	sarcosine conjugated	0						
[^{14}C]	unconjugated	4.4	3.5	0.9				
	sarcosine conjugated	95.6			3.8	13.4	0.6	77.7

a: sarco-7β-hydroxy-3-oxo-5β-cholan-24-oate
b: sarco-3α-hydroxy-7-oxo-5β-cholan-24-oate

intestinal tract (23). The present studies show that the fecal ^{3}H-labeled compounds were present in the unconjugated form, while most of the fecal ^{14}C-labeled compounds were present in the sarcosine conjugated form. These results indicate that sarcosine conjugated bile acids, unlike taurine or glycine conjugates, resists bacterial deconjugation.

It is generally accepted that bacterial 7-dehydroxylation takes place mainly with unconjugated bile acids (24). Indeed, the major part of the fecal ^{3}H-labeled compounds was identified as LC, the 7β-dehydroxylation product of UDC. Also, more than 75% of the unconjugated ^{14}C labeled compounds in the feces was LC. In contrast, only 4% of the sarcosine conjugated bile acids in the feces was sarco-LC. These results clearly indicate that SUDC is resistant to degradation by bacterial 7-dehydroxylation. The minimal transformation of SUDC into the 7-dehydroxylation products suggests that SUDC would be a more safe agent than the presently used cholelitholytic agents, CDC and UDC. Since SUDC was not toxic and its administration prevented gallstone formation in mouse (15), further studies of SUDC may be useful.

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* To whom all correspondence should be addressed.

The following trivial names and abbreviations have been used in this paper;
Lithocholic acid(LC) : 3α-hydroxy-5β-cholan-24-oic acid
Chenodeoxycholic acid(CDC) : 3α,7α-dihydroxy-5β-cholan-24-oic acid
Ursodeoxycholic acid(UDC) : 3α,7β-dihydroxy-5β-cholan-24-oic acid
Sarcosine : N-methylglycine

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