Bile acids with a cyclopropyl-containing side chain. IV. Physicochemical and biological properties of the four diastereoisomers of 3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic acid

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Abstract To define the influence of the side chain modification on physicochemical and biological properties of bile acids, 3α, 7β-dihydroxy-22,23-methylene-5β-cholan-24-oic acid, a cyclopropyl analog of ursodeoxycholic acid (UDCA) was synthesized in both unconjugated and taurine-conjugated form. The presence of a cyclopropyl ring at C-22, C-23 position introduces chirality generating four diasteroisomers (A, B, C, and D) which greatly differ for the hydrophilicity and critical micellar concentration: A and B are more hydrophilic (K' = 0.21, 0.80 and CMC = 25,20 mM, respectively) than UDCA (K' = 0.95; CMC = 19 mM) while C and D are more hydrophobic and with lower CMC (K' = 1.30, 2.05; CMC = 14, 10 mM, respectively) than UDCA. Differences in these properties are related to the orientation of the C-25 carboxyl which in isomers A and B is oriented toward the back of the steroid body, reducing the continuity of the hydrophobic area. Using the isolated perfused rat liver we found that the isomers inhibited [^{3}H]UDCA uptake differently. Isomer D (noncompetitive) was the most potent (51%) while isomer A (competitive) was the least potent (15%). When infused intravenously to rats, only D isomer and UDCA were quantitatively recovered in bile. They were secreted predominantly as taurine and glycine conjugates. Isomers A, B, C are not conjugated and only partially recovered in bile as unconjugates (less than 15% of the administered dose). The increase in bile flow per unit increase in bile acid secretion induced by isomers A, B, and C, was much greater than that induced by isomer D which is similar to that of UDCA (0.32 ± 0.04 and 0.22 ± 0.01, respectively) while it is reduced during infusion of the other isomers. When infused as taurine conjugates they behaved similarly to tauroursodeoxycholic acid. When incubated in anaerobic conditions with human stools only isomer D is partially 7-dehydroxylated (t/2 = 18 hr) even though slower than UDCA (t/2 = 5 hr). The substrate specificity of the taurine conjugated toward cholylglycine hydrolase is very poor, only isomers C and D are partially deconjugated with a kinetics much slower than that of UDCA (10 hr vs. 0.2 hr). By using molecular models it is possible to explain these differences due to the conformation of the side chain that, in the case of isomer D, is quite similar to UDCA. These data are useful to explain the metabolism of dihydroxy bile acids and to design new analogs with enhanced cholelitholytic activity.—**Roda, A., B. Grigolo, R. Aldini, P. Simoni, R. Pellicciari, B. Natalini, and R. Balducci.** Bile acids with a cyclopropyl-containing side chain. IV. Physicochemical and biological properties of the four diastereoisomers of 3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic acid. *J. Lipid Res.* 1987. **28:** 1384 – 1397.

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In previous papers (1, 2), we reported the synthesis and preliminary physicochemical and biological properties of 3α, 7β-dihydroxy-22,23-methylene-5β-cholan-24-oic acid (CUDCA) in which the C_{22}-C_{23} side-chain bond forms one side of the three-membered ring. This cyclopropyl analog of ursodeoxycholic acid (UDCA) has been studied both in the unconjugated form and conjugated with taurine at C-24 (1, 2). The aim of our program is the development of structurally modified UDCA analogues in order to: *a*) prevent its degradation by intestinal bacteria by the introduction of steric and/or electronic hindrance on the side chain, and *b*) to increase its activity as a drug useful in the medical treatment of cholesterol gallstones (3–6).

Abbreviations: UDCA, ursodeoxycholic acid; CUDCA, 3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic acid; BA, bile acid(s); TLC, thin-layer chromatography; GLC, gas–liquid chromatography; CMC, critical micellar concentration; HPLC, high performance liquid chromatography; TCUDCA, taurine-conjugated CUDCA; HLB, hydrophilic/lipophilic balance.

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Preliminary studies were performed on a mixture of the four diastereoisomers obtained by a non-stereospecific reaction of a carbenoid with a bile acid olefin. The mixture of these new bile acids (BA) has properties similar to properties of UDCA in water: the sodium salt self-aggregates to form micelles and the pKa is similar to the natural analogs (1).

As far as the biological properties are concerned, the CUDCA was taken up efficiently by rat liver and partially secreted into bile. Bile flow was highly increased and biliary lipid secretion was reduced when it was administered to rats at a dose of 3 μmol/min per kg.

The presence of the cyclopropyl ring partially suppresses the glycine/taurine conjugation by the liver and the 7-dehydroxylation by intestinal bacteria. Moreover, the taurine conjugates are poorly deconjugated by cholylglycine hydrolase. The cyclopropyl ring introduces chirality in the BA side chain with the formation of four stereoisomers (A, B, C, D) in which the rotation about the bond C_α-$C\beta$ with respect to the C-24 carboxyl is restricted and the functionality is fixed in space.

The aim of this work was: *a*) to define the physicochemical properties in water of the four stereoisomers; and *b*) to study the major biological properties, especially the hepatobiliary BA transport system. In particular, once the hepatic uptake of the mixture of the four diastereoisomers was evaluated, we studied whether they inhibited the hepatic uptake of ^{3}H-labeled UDCA and whether this inhibition, if any, was different for each diastereoisomer.

The kinetics and chemical forms of bile secretion and the effect on bile flow and biliary lipid secretion were evaluated in bile fistula rats by intravenous administration of a dose of 3 μmol/min per kg body weight. The metabolism in terms of 7-dehydroxylation and the deconjugation of the corresponding taurine conjugates was studied in vitro by incubation with fresh stools in broth media for anaerobic cultures.

The distinct physicochemical and biological properties in relation to side chain configuration are also discussed.

MATERIALS AND METHODS

Chemical synthesis of CUDCA

We have previously reported (1, 2) the synthesis of CUDCA (3) by the dirhodium tetraacetate-catalyzed decomposition of ethyl diazoacetate in the presence of the Δ^{22}-24-norcholene derivative followed by alkaline hydrolysis of the cyclopropyl ester (2) thus obtained **(Fig. 1)**. TLC and GLC analyses of (3) **(Table 1)** showed that it was a mixture of four diastereoisomers (CUDCA A, B, C, D) as could have been anticipated by the formation of two new chiral centers at C_{22} and C_{23} and by the utilization of a nonstereospecific carbene addition reaction. Since preliminary physicochemical and biological studies carried out on the diastereomeric acid (3) showed promising results, a study of the four diastereoisomers' properties was considered to be necessary in order to ascertain the effect of subtle alterations in geometry around the 22,23-bond on physicochemical and biological properties. Our strategy for the separation of the diastereoisomeric mixture started with ethyl *cis*- and *trans*-cyclopropane carboxylate (2) which appeared as two spots by TLC, and which were separated very efficiently by medium pressure chromatography (Lobar column, Merck, light petroleum-diethyl ether 8:2). Hydrolysis of the more polar fraction gave two acids (CUDCA A and B) which were again separated by medium pressure chromatography (Lobar column, Merck, chloroform with 2.5% methanol) to give pure CUDCA A and CUDCA B with 60.5 and 30.4% yield, respectively. Application of the same separation sequence to the less polar ester fraction yielded pure CUDCA C and CUDCA D in 48.5 and 33.0% yield, respectively, CUDCA A, B, C, and D were identified as the 22S, 23S (3a), 22R, 23R (3b), 22S, 23R (3c), and 22R, 23S (3d) diastereoisomers respectively, mainly by use of ^{13}C NMR spectroscopy (Fig. 1). The stereochemistry assigned was confirmed by the stereoselective synthesis of CUDCA C and D. Details of the isolation procedure, spectroscopic properties, and synthesis of the CUDCA diastereoisomers are described elsewhere.

Direct conjugation of these bile acids with [^{14}C]taurine (sp. act 100 mCi/mmol) in aqueous ethanol in the presence of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline gave the sodium salt of taurine conjugated in 78–85% yield (2, 7). The taurine-conjugated BA were purified by TLC (silica gel 0.25 μm thickness) using a solvent system composed of $CHCl_3CH_3OH$-H_2O 75:25:3(v/v/v).

Physicochemical properties

Theoretical molecular models. A very good approximation of the three-dimensional structure of UDCA and the four stereoisomers of CUDCA was generated from a modified SMILES (8) description of the connectivity and chirality of the compounds using the program CONCORD (9). The geometries were then optimized by energy minimization using the AMI (10) Hamiltonian contained in the molecular orbital package MOPAC (11). The resulting molecular models were then displayed for visual inspection on a graphic terminal with commercially available molecular computer graphic software **(Fig. 2).**

Critical micellar concentration. The CMCs were measured in water by the dye solubilization method and, in particular, with orange OT as a water-insoluble dye (12). Different bile salt solutions were prepared by weight in concentrations ranging from 0.1 to 100 mmol/l, and the pH was adjusted to 8.00 with sodium hydroxide. A sample of the final solution was analyzed for bile acid content

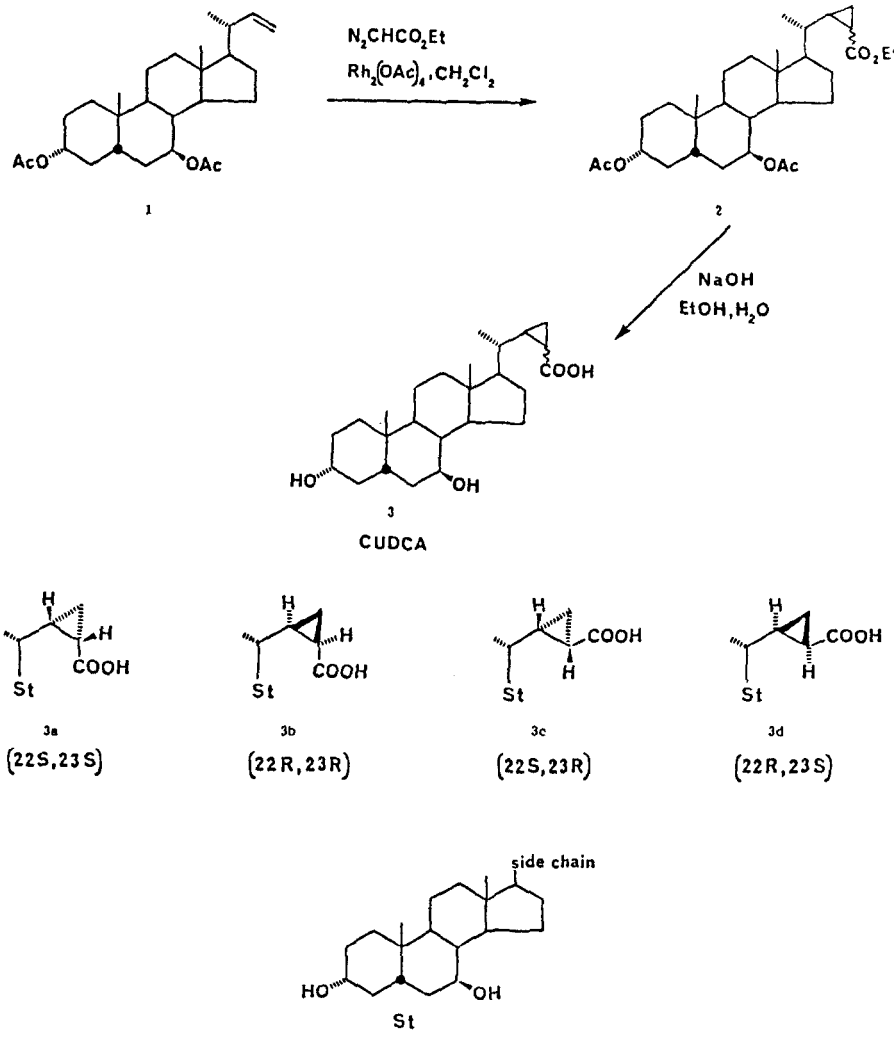

Fig. 1. Schematic synthesis of 3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic-acid (3) and representation of the four diasteroisomers A, B, C and D.

by an enzymatic method with 3α-hydroxysteroid deydrogenase (13). The sodium content was measured by atomic absorption spectroscopy. Bile salt solution, 2 ml, was rotated for 1 day at room temperature with an excess of crystalline dyes (kept in the dark). The solutions were then filtered through a 0.22 μm Millipore filter; absorbance was recorded at 490 nm and plotted against bile salt concentration.

Water solubility. Bile acids (0.2 mmol) were suspended in 100 ml of 0.01 M $HClO_4$ (14). The solutions were refluxed for 24 hr in order to achieve equilibrium. Two-ml aliquots of the saturated solutions were transferred to a thermostat maintained at 25°C. After 1 month the solutions were filtered on a Millipore filter (0.22 μm) and the concentration of BA was measured enzymatically using a 3α-hydroxysteroid dehydrogenase enzyme (13).

Hydrophilic–lipophilic balance. The hydrophilic-lipophilic properties of bile salts were measured by reverse phase high performance liquid chromatography (HPLC) (15, 16).

HPLC was performed using a Waters Inc. (Milford, MA) liquid chromatograph.

A C-18 reverse phase column of 5 μ pore size and 10 cm length was used. The analysis was carried out under iso-

TABLE 1. Chromatographic characteristics of the four CUDCA diasteroisomers

CUDCA	Thin-Layer Chromatography[a] R_f	Gas-Liquid Chromatography RRT
A	0.43	1.28
B	0.39	1.00
C	0.36	1.30
D	0.33	1.13

[a]Solvent system: $CHCl_3$–MeOH 9:1 (v/v); silica gel plates; 20°C.
[b]As ethyl ester 3,7-diacetoxy derivatives; 1% SP 2250; 265–275°C, rate 0.33°C/min; nitrogen flow rate 20 ml/min.

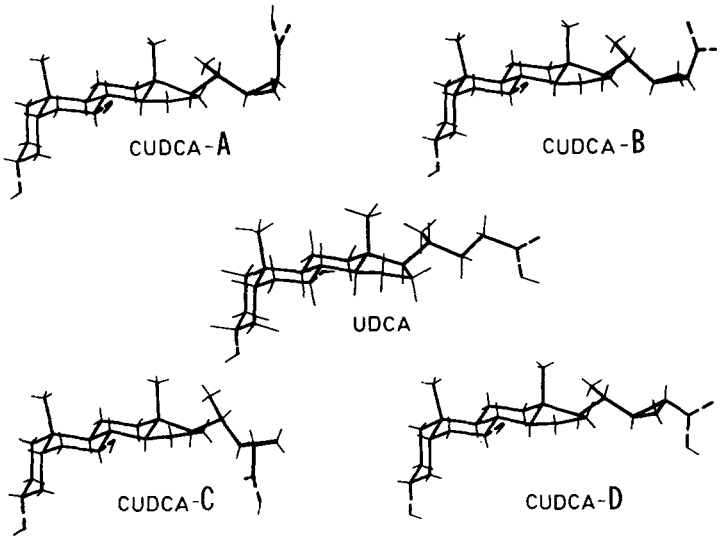

Fig. 2. Configuration of the four isomers of 3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic-acid (A, B, C, D) assigned by a molecular computer graphics technique.

cratic conditions. As a mobile phase a mixture of 2-propanol–water 8:17(v/v) pH 7.00, was used.

A "retention factor" (K') was calculated from the relative mobilities of the separated bile acids **(Fig. 3).**

Acidity. Acidity constants were determined by potentiometric measurements in solutions of aqueous methanol of different mole fractions. The pKa values, estimated in water by means of previously assessed correlations from the pKa values in mixed solvents, were in close agreement with each other (17).

All the measurements were carried out at 25 ± 0.01°C.

Biological properties: in vivo studies

Hepatic uptake. The hepatic uptake of TCUDCA and experiments on the inhibition of UDCA uptake by isomers A, C, and D were evaluated by means of rat liver perfusion experiments. Isolated livers from male Sprague-Dawley rats (200–250 g body weight) were perfused as described by Mortimore (18) using Krebs-Ringer bicarbonate solution (pH 7.4) containing glucose (5.55 mmol/l) with 3% (w/v) bovine serum albumin (Fraction V, essentially fatty acid-free, Sigma, St. Louis, MO). Perfusion was established without anticoagulant and the livers were perfused with 40 ml of Krebs-Ringer bicarbonate solution. While washing, complete blanching of all the liver lobes indicated satisfactory perfusion. Oxygenation was carried out as described by Hamilton (19) with 95% O_2 and 5% CO_2, using silastic tubing (Dow-Corning, Midland, MI). The flow rate was 2.5–2.8 ml/min per g of liver.

Temperature, flow rate, and perfusion pressure were kept constant and continuously monitored. The viability of the livers and the continuous monitoring of experimental conditions were performed as previously reported (20). The single pass hepatic extraction of [^{35}S]taurocyclopropyl ursodeoxycholic acid was evaluated by injecting a tracer (100 nCi) dose of the labeled bile acid into the portal vein and collecting the outflow samples from the hepatic veins immediately after injection. The inhibition experiments were as follows. In a first set of experiments, the hepatic single pass extraction of a tracer dose of labeled ursodeoxycholic acid was evaluated in the presence of different doses of each isomer. [11,12-^{3}H(N)]Ursodeoxycholic acid, (sp act 30–60 mCi/mmol) was supplied by NEN (New England Nuclear, Boston, MA). The radiochemical purity of the commercially available isotope was assessed by radiochromatography using a Berthol radiochromatograph and proved to be more than 99% pure. [^{3}H]Ursodeoxycholic acid (15 nCi), dissolved in 300 μl of the above described perfusion mixture, together with 0, 0.3, 3.0, 30.0 μmol of isomers A, C, D, respectively, were pulse-injected into the portal vein and the outflow from the liver was collected from the suprahepatic veins.

A total of nine livers were perfused, three for each isomer; each liver was injected with all four doses of the inhibiting isomer, randomly administered. A 5-min rest was allowed between each injection.

In a second set of experiments, we aimed at defining the type of inhibition of each isomer of CUDCA on the kinetics of the hepatic uptake of UDCA. Once the effective inhibiting dose had been assessed, the kinetics of hepatic uptake of UDCA was evaluated in the absence and presence of 3.0 μmol of each isomer; a total of 12 livers were used.

The hepatic uptake of UDCA was evaluated as follows. Each liver was perfused with a sequence of seven doses of

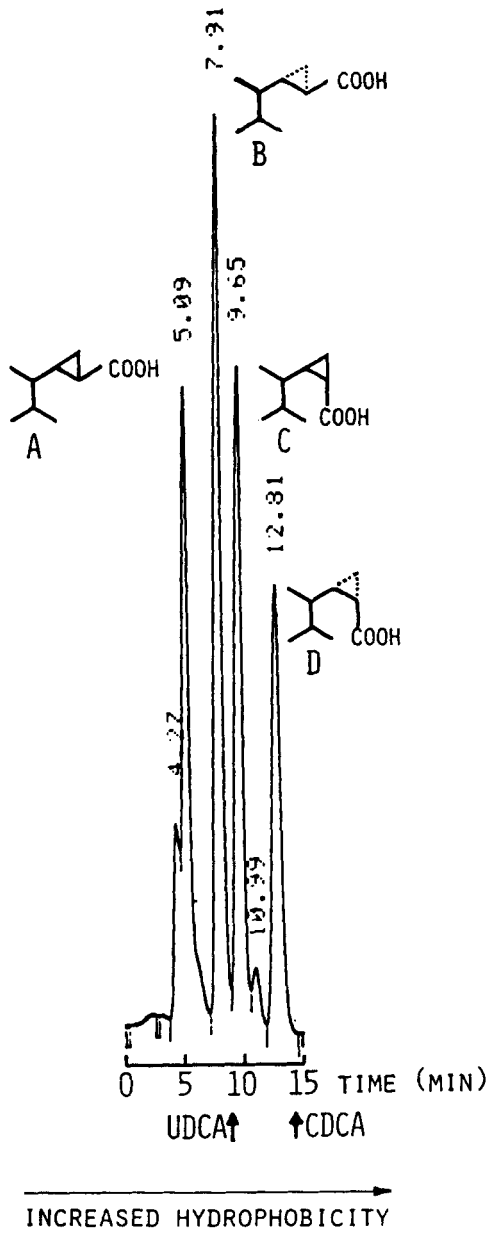

Fig. 3. HPLC separation of the four isomers (A, B, C, D) in respect to UDCA. The retention factor (K') is calculated from the mobilities of the separated bile acids.

labeled UDCA (15 nCi) together with increasing amounts of unlabeled UDCA (0.1; 0.3; 1; 5; 10; 20; 30 μmol), dissolved in 300 μl of the perfusion mixture, either with or without 3.0 μmol of each isomer. Evans blue was added as a marker of the albumin space (21).

The outflow from the inferior vena cava was collected at 5-sec intervals over 5 min after the injection of each mixture.

The hepatic single-pass extraction of taurine-conjugated CUDCA and UDCA was expressed as a percentage of the administered dose, corrected for the Evans blue recovery.

The kinetics of the uptake of UDCA by the liver was evaluated by means of the relationship between dose and removal rate of ursodeoxycholate.

We applied the model of liver uptake process proposed by Goresky (22). The differentiation into extravascular and intravascular spaces of the sinusoidal space was not taken into account and the vascular reference substance was not used.

The concentrations of UDCA and Evans Blue in the hepatic vein were expressed as outflow fraction of injected dose per ml of plasma. To evaluate the removal rate of UDCA we applied the procedure proposed by Reichen and Paumgartner (23). The natural logarithm of the ratio (Evans Blue outflow fraction)/(bile acid outflow fraction), recovered in each sample, was plotted versus time. The slope of the line, obtained for each experiment at fixed dose, gave the uptake rate relative to that dose.

The maximal uptake rate (V_{max}) and the half saturation constant (K_m) were evaluated according to Michaelis-Menten kinetics by linear regression analysis of the plot of the UDCA removal rate versus the ratio UDCA removal rate BA dose (Eeadie-Hofstee plot) (24). The intercept of this plot with the ordinate axis represents the V_{max}, while the slope represents the K_m.

Biliary secretion. Sprague-Dawley male rats (300–330 g) were used. The rats were anesthetized with ethyl carbamate, and the bile was collected. The isomers were administered intravenously as sodium salts through the femoral vein at a dose of 3 μmol/min per kg (five rats each group) over 1 hr. Bile samples were collected at 15-min intervals for a 2-hr period.

The concentrations of bile acids, cholesterol, and phospholipids in bile samples were determined by enzymatic procedures (13, 25, 26). Bile acid compositions were determined by an HPLC method and by TLC on silica gel G plates, 250 μm thickness.

The conjugated bile acids (glycine/taurine) were separated from the corresponding unconjugated BA by TLC using a solvent system of propionic acid–isoamyl acetate–water–1-propanol 15:20:5:1 (v/v/v/v).

Biliary lipid secretion, calculated from the volume of the excreted bile and from the biliary lipid concentration, was expressed as μmol/min per kg.

The variations in biliary lipid secretions induced by the different isomers were expressed as mean maximum secretion rates using:

$$SM = \frac{S_{15} + S_{30} + S_{45} + S_{60}}{4}$$

where S_{15}, S_{30}, S_{45}, S_{60} are the secretion rates 15, 30, 45, 60 min after the start of the infusion. SM values were calculated for secretion of bile (SMV_o) (μl/min per kg),

and BA (SMBA), cholesterol (SMCHOL), and PL (SMPL) (μmol/min per kg).

The plots of bile secretion, and cholesterol and phospholipid secretion versus bile acid secretion permit the calculation of the slope of the regression line.

In vitro studies

Cholylglycine hydrolase: substrate specificity. Fifty mg of enzyme (activity = 100–200 units/mg of protein) was suspended in 5 ml of water and sonicated in an ultrasound bath. The enzymatic activity in the reaction mixture was ~50 units/μl.

The incubation mixtures consisted of 200 μl of acetate buffer (0.3 M, pH 5.6), EDTA (0.2 M, 40 μl), mercaptoethanol (0.2%, 40 μl), enzyme (choloylglycine hydrolase, 50 μl), 10 μl of taurine-conjugated bile acid solution (10 mM) (TUDCA, isomers A, B, C, and D) conjugated with taurine.

After 10, 20, 30, 40, 50, 60 min, and after 15 hr the reaction was stopped by adding 200 μl of a 30% solution of KOH. Bile acids were isolated and analyzed using procedures described below.

Bacterial 7-dehydroxylase: substrate specificity. Immediately after evacuation, human fresh stools were homogenized with water (1/1, v/v) under a nitrogen stream, and 500 mg was transferred into sterile vials to which 5 ml of sterilized chopped meat-glucose medium (Scott Lab., Fiskeville, RI) was added. To this medium the different BA were added at a concentration ranging from 0.01 to 0.1 mM.

All the experiments were carried out under nitrogen in capped vials. The anaerobic conditions were maintained with a disposable anaerobic indicator (Gas Pac, Becton Dickinson Co., Orangeburg, NY).

The incubations were carried out at 37°C, and 4, 8, 16, 24, and 72 hr after the addition of the corresponding BA, the reaction was stopped with 150 μl of a 30% solution of KOH.

Tubes were centrifuged at 3500 rpm for 10 min, and 2 ml of the supernatant was transferred into a tube to which 8 ml of 0.1 M NaOH solution was added. The solutions were applied to a C_{18} Bond Elut cartridge which was eluted at a flow rate of 1 ml/min; the cartridge was washed with 10 ml of water and the BA were collected with 4 ml of methanol. The eluate was dried under a N_2 stream and reconstituted with 0.5 ml of CH_3OH. BA were separated using TLC and HPLC techniques. Conjugated BA were separated from the free BA by TLC on silica gel plates (0.25 μm) (Merck FRG) with the solvent system $CHCl_3CH_3OH$-water 75:25:3 (v/v/v).

The qualitative-quantitative compositions were obtained by HPLC with a 5-μm C-18 reverse-phase column (Waters Associates). The mobile phase was CH_3OH-KH_2PO_4 (0.01 M, pH 5.8, 130:70, v/v) (27).

The analysis was carried out under isocratic conditions at a flow rate of 0.3 ml/min with use of an ultraviolet detector at 200 nm.

RESULTS

Physicochemical properties

Critical micellar concentration. As shown in **Table 2** the four diastereoisomers greatly differ in the CMC values and the order is A>B>C>D ranging from 25 ± 1 to 10 ± 0.5 mM. The natural analog UDCA presents a CMC value (19 ± 1 mM) intermediate between the isomers A, B and C, D.

Hydrophilic-lipophilic balance. The "retention factor" values K', taken as an index of the hydrophilic-lipophilic balance (15, 16) of the four isomers are also very different. The order of hydrophilicity is A>B>C>D, (Table 2) and it is inversely related to the CMC values **(Fig. 4).** Isomer A is very hydrophilic with K' values similar to trihydroxy BA, while isomer D is very lipophilic with values similar to highly lipophilic BA.

Water solubility and acidity. The water solubility values of the protonated forms is reported in Table 2; no significant differences exist among the four isomers and the values resemble those of other dihydroxy BA (14).

Finally, the presence of the cyclopropyl ring at C-22–C-23 position does not modify the pKa of the acid, the values are similar to that of the natural analog (Table 2) (17).

TABLE 2. Physicochemical properties of the four cyclopropyl analogs of UDCA in comparison with UDCA

Bile Acid	Melting Point	$[\alpha]_D^{25}$	S_{wo}	CMC	K'	pKa
	°C		μM	μM		
UDCA			9 ± 2	19 ± 1	0.95 ± 0.02	5.03
CUDCA A	248–251	+56.4	2.5 ± 0.5	25 ± 1	0.21 ± 0.05	5.06
CUDCA B	252–255	+ 7.5	6 ± 1	20 ± 0.8	0.80 ± 0.06	5.02
CUDCA C	133–136	+14.5	6 ± 1	14 ± 0.5	1.30 ± 0.05	5.01
CUDCA D	168–170	+59.9	5 ± 1	10 ± 0.5	2.05 ± 0.06	5.02

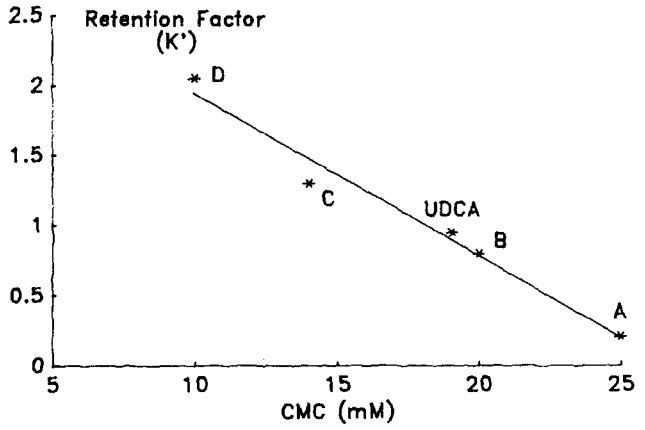

Fig. 4. Relationship between the hydrophobicity (K') of the different isomers and the self-association in water (CMC).

Biological properties: in vivo studies

Hepatic uptake. The single pass hepatic extraction of tauro-CUDCA ranged from 50% to 66% of the injected dose (62.80 ± 3.11%; mean ± SD) which is in the range of dihydroxy conjugated bile acids.

The isomer D proved to be the most potent inhibitor of UDCA single-pass hepatic clearance, since it was effective at as low a dose as 0.3 μmol. The hepatic uptake of UDCA decreased by 57% when isomer D was present at 30.0 μmol, while it decreased by percentages of 40% and 45% in the presence of the same doses, respectively, isomer A and C. Since all the bile acids were inhibiting at 3.0 μmol, this latter dose was chosen in further inhibition studies of full dose-response experiments of UDCA uptake, with an aim to defining the type of inhibition.

Both in the absence and presence of the inhibiting isomers, UDCA uptake showed saturation; each of the three isomers affected the kinetics parameters of UDCA uptake differently, as shown in **Table 3.**

Isomer A significantly ($P < 0.001$) increased the K_m of UDCA uptake, with no effect on the V_{max}; isomer D, on the contrary, decreased the V_{max} ($P < 0.001$), with no effect on the K_m.

Isomer C showed an intermediate behavior since it influenced both the V_{max} ($P < 0.001$) and the K_m ($P < 0.001$) **(Table 4).**

Biliary secretion. The kinetics of the biliary excretion of the four isomers was different. When administered in the unconjugated form, a low recovery in bile was observed for isomers A, B, and C (less than 15% of the administered dose) while isomer D was more efficiently recovered (50%) in bile, like UDCA **(Fig. 5).**

The chemical forms recovered in bile were also different: isomers A, B, and C were present in bile as free acids (less than 10%) and partially conjugated with taurine and glycine (less than 5%). On the other hand, isomer D was recovered in bile mostly conjugated with glycine and taurine (85%) and to a lesser extent as the free acid (10–15%). The kinetics of the biliary excretion of isomer D was similar to that of UDCA **(Fig. 6)**, left panel).

The maximum secretion rate (SMBA) achieved by the isomer D was 2.4 μmol/min per kg, slightly lower than that of UDCA (3.05 μmol/min per kg). The other isomers had very low SMBA values (less than 0.8 μmol/min per kg) and they were similar to SMBA values obtained during saline infusion (0.6 μmol/min per kg) **(Table 5)**.

The maximum secretion rate when the isomers were administered as taurine conjugates was much higher (3.18 μmol/min per kg) and similar to that of TUDCA (Fig. 6) and no differences were found among the four isomers.

Effect on bile flow. All the four isomers increased the net bile flow (SMV_o) in respect to baseline value (30 μl/min per kg) (Table 5). The order of potency expressed as maximum secretion rate was: isomer A > isomer B > isomer C > isomer D. Ursodeoxycholic acid administered at the same dose resulted in a value intermediate between isomer C and D. The choleretic effect was highly correlated to their hydrophilicity and to the liver's ability to conjugate it with taurine. The increase in bile flow per unit increase in bile acid secretion for isomer A (35 μl/mol), isomer B (30 μl/μmol), and isomer C (28 μl/μmol) was much greater than those for isomer D (16 ± 7 μl/μmol) and UDCA (12 μl/μmol) **(Table 6).**

The bile acid independent flow, obtained by extrapolation, was 20 μl/min per kg and similar for all the BA studied. When administered as taurine conjugates, the choleresis was drastically reduced and no significant differences among the taurine conjugates of the four isomers and TUDCA were found (Table 6, **Fig. 7**).

Effect on phospholipid and cholesterol secretion. The mean maximum secretion rates of phospholipids (SMPL) achieved during intravenous infusion of the four isomers and UDCA are reported in Table 5. Isomer D maintained

TABLE 3. Inhibition of the three isomers of tauro-UDCA on the single pass hepatic uptake of ^{3}H-labeled UDCA[a]

Inhibiting Isomer	[^{3}H]UDCA Single Pass Hepatic Extraction			
	0	0.3 μmol	3.0 μmol	30.0 μmol
A	68 ± 3%	58 ± 2%	42 ± 3%	41 ± 4%
C	68 ± 3%	54 ± 3%[b]	37 ± 2%[b]	37 ± 4%[b]
D	68 ± 3%	33 ± 4%[c,d]	33 ± 2%[c]	29 ± 3%[c,e]

[a]Single pass hepatic extraction of ^{3}H-labeled UDCA (% of the administered dose) in the presence of 0.3, 3.0, or 30.0 μmol of the inhibiting isomers.
[b]P versus A, not significant.
[c]P versus A: $P < 0.001$.
[d]P versus C: $P < 0.01$.
[e]P versus C: $P < 0.05$.

TABLE 4. Kinetics parameters of [^{3}H]UDCA uptake by the rat liver in the presence and absence of 3.0 μmol of tauro-UDCA, isomers A, C, D

Sample	V_{max}	K_m	r
	nmol/sec/g/liver	*nmol/g liver*	
UDCA			
1	15.1	354	0.94
2	15.9	325	0.89
3	16.2	321	0.95
Mean ± SD	15.7 ± 0.55	333 ± 18	0.92 ± 0.032
UDCA + isomer A (3,000 nmol)			
1	15.7	655	0.98
2	15.1	722	0.94
3	16.4	594	0.89
Mean ± SD	15.7 ± 0.65	657 ± 64	0.93 ± 0.04
(Versus UDCA)	(ns)	($P < 0.001$)	
UDCA + isomer C (3,000 nmol)			
1	13.7	462	0.93
2	12.6	444	0.88
3	12.1	473	0.92
Mean ± SD	12.8 ± 0.81	459 ± 15	0.91 ± 0.026
(Versus UDCA)	($P < 0.001$)	($P < 0.001$)	
UDCA + isomer D (3,000 nmol)			
1	9.7	257	0.95
2	10.6	354	0.89
3	8.5	321	0.94
Mean ± SD	9.53 ± 0.96	314 ± 53	0.92 ± 0.03
(Versus UDCA)	($P < 0.001$)	(ns)	

the highest PL secretion in bile, followed by UDCA. The infusion of the other three isomers (A, B, and C) separately did not affect the PL secretion in respect to baseline levels, nor did the taurine conjugates of UDCA and CUDCA modify the maximum secretion rate of PL in respect to baseline values.

The maximum secretion rate of cholesterol is shown in Table 5. All the isomers reduced the cholesterol secretion into bile and the order of potency was: isomer A (0.010 μmol/min per kg) > isomer B (0.012 μmol/min per kg) > isomer C (0.014 μmol/min per kg) = isomer C (0.010 μmol/min per kg). The maximum secretion rate during administration of UDCA was 0.012 μmol/min per kg. The reduction in cholesterol secretion induced by the corresponding taurine conjugates of both UDCA and CUDCA was much greater (Table 5).

The relationships between bile flow, cholesterol, phospholipids, and bile acid secretion rate, expressed as slope of the linear regression and correlation coefficients, are reported in Table 6.

Cholylglycine hydrolase: substrate specificity. All the taurine-conjugated isomers of CUDCA were poor substrates for cholylglycine hydrolase **(Fig. 8)**.

While tauroursodeoxycholic acid is quickly deconjugated (t <20 min), isomers A and B were not deconjugated for an incubation time up to 20 hr. On the other hand, isomers C and D were completely hydrolyzed after 10 hr of incubation with the enzyme with similar kinetics.

Bacterial 7-dehydroxylase: substrate specificity. The four isomers were 7-dehydroxylated with delayed kinetics compared to the natural UDCA **(Fig. 9)**.

Isomers A and B were poorly 7-dehydroxylated (with little difference between A and B); on the contrary, C and D were much more efficiently dehydroxylated even though the enzyme acted efficiently only on isomer D.

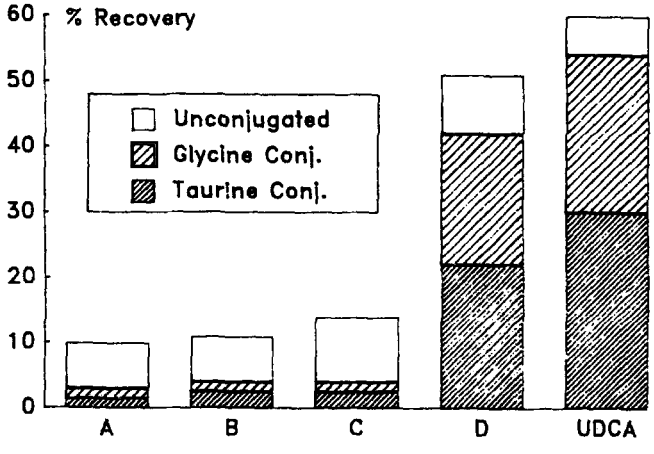

Fig. 5. Cumulative recovery in bile of the four diasteroisomers as compared with UDCA administered at same dose. The recovery is expressed as % recovery over 2 hr after the start of the intravenous infusion at a dose of 3 μmol/min per kg. 3α-Hydroxysteroid dehydrogenase-reactive bile acids were measured after TLC separation of unconjugated, glycine-, and taurine-conjugated BA.

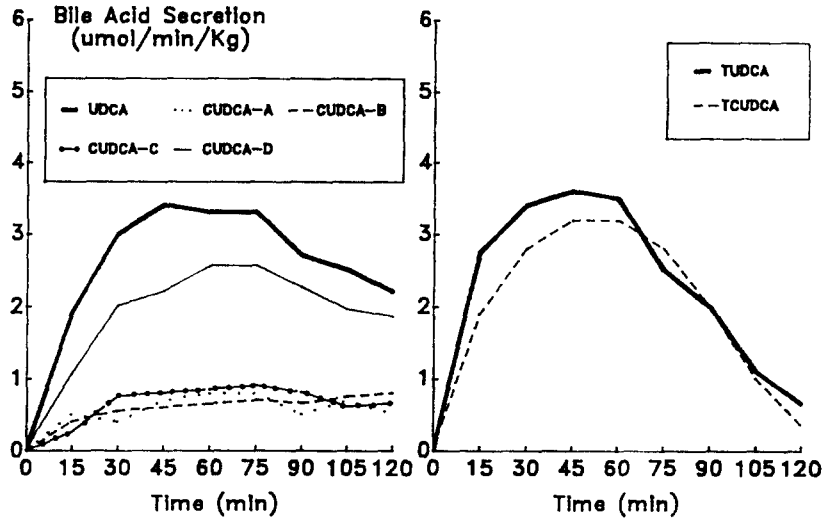

Fig. 6. Kinetics of biliary excretion of the four isomers of CUDCA when administered at a dose of 3 μmol/min per kg over 1 hr to rats. Left panel refers to the unconjugated form, compared with UDCA. Right panel, BA administered as taurine conjugates in comparison with TUDCA. The figure shows the behavior of the taurine conjugates of isomer A. The other isomers behaved identically. Bile acids were quantified enzymatically using 3α-HSD.

The time required to completely 7-dehydroxylate isomers A and B was very high: 65 hr of incubation compared to 5–7 hr necessary to 7-dehydroxylate UDCA. Isomer C requires 60 hr while the rate of dehydroxylation of isomer D was higher (less than 20 hr) and resembled that of homo-UDCA (18 hr) (28) (Fig. 9).

DISCUSSION

Molecular structure and physicochemical properties

The four diastereoisomers of CUDCA present peculiar and distinct physicochemical properties in water (Table 2). It is well established that the physicochemical properties of BA molecules are related to their structure (29); as amphipathic compounds they self-aggregate via a back-to-back interaction with formation of micelles. Any factor that reduces the continuity of the hydrophobic area of the molecule increases the hydrophilic/lipophilic balance (HLB) and the critical micellar concentration (12–17).

Previous studies have shown that both body and side-chain structure are key factors in determining these parameters. Number, position, and orientation of steroid hydroxyl groups affect the CMC and the HLB of C-24 bile acid; as far as the side-chain structure is concerned, the CMC increases as the side-chain is shortened, as usually observed for common detergents and this is the case of nor(C-23), bis-nor(C-22) analogs of C-24 BA (12).

TABLE 5. Maximum secretion rates of bile flow, BA, PL, and CHOL during intravenous infusion of UDCA, CUDCA (four isomers), and the corresponding taurine conjugates at a dose of 3 μmol/min per kg[a]

Bile Acid	n	SMV_o	SMBA	SMPL	SMCHOL
		μl/min/kg		*μmol/min/kg*	
UDCA	29	60 ± 10	3.05 ± 0.06	0.22 ± 0.01	0.012 ± 0.003
CUDCA A	28	70 ± 10	0.80 ± 0.05	0.16 ± 0.02	0.010 ± 0.0007
CUDCA B	26	68 ± 9	0.65 ± 0.05	0.14 ± 0.01	0.012 ± 0.0008
CUDCA C	30	65 ± 7	0.75 ± 0.08	0.14 ± 0.03	0.014 ± 0.001
CUDCA D	30	54 ± 8	2.40 ± 0.10	0.32 ± 0.04	0.014 ± 0.0008
TUDCA	25	38 ± 4	3.68 ± 0.08	0.12 ± 0.01	0.008 ± 0.0001
TCUDCA[b]	88	36 ± 2	3.18 ± 0.10	0.11 ± 0.01	0.009 ± 0.0001
Saline	50	30 ± 5	0.6 ± 0.02	0.12 ± 0.01	0.015 ± 0.0009

[a]The control experiment was intravenous infusion of saline. Each value denotes the mean ± SD of five rats. SMV_o, SMBA, SMPL, and SMCHOL are the mean maximum secretion rates of bile, bile acids, phospholipids, and cholesterol, respectively, over a 1-hr infusion.

[b]TCUDCA data were analyzed all together because no differences have been found among the four taurine-conjugated isomers.

TABLE 6. Relationship between bile flow, CHOL, PL secretion rate, and bile acid secretion expressed as slope of the linear regression and correlation coefficients

Bile Acid	n	Bile Flow vs. BA[a] Slope μl/μmol	Intercept y-axis for x = 0	r	CHOL vs. BA Slope · 10^3	r	PL vs. BA Slope · 10^3	r
UCDA	29	12	26	0.97	2.6	0.78	35	0.82
CUDCA A	28	35	21	0.87	21	0.88	280	0.86
CUDCA B	26	30	20	0.92	10	0.87	140	0.75
CUDCA C	30	28	22	0.88	15	0.86	180	0.68
CUDCA D	30	16	18	0.88	8.2	0.65	110	0.72
TUDCA	25	5	19	0.97	0.78	0.75	20	0.65
TCUDCA	88	6.4	20	0.98	0.85	0.68	25	0.72

[a]For the relationship between bile flow and BA output, the intercept with the y axis has also been calculated.

The differences in the CMC and HLB values observed for the four CUDCA analogs are a typical example of how the side-chain isomerism can modulate the HLB and the CMC values. As previously shown, the presence of a cyclopropyl ring makes the side-chain fixed in space and consequently the carboxy-group is differently oriented in space (Fig. 2).

According to the assigned configuration by NMR and to the visual inspection of the molecular computer graphic representation of the optimized molecular models, the four CUDCA diastereoisomers present four distinct side-chain orientations as reported in Fig. 2.

As compared with UDCA conformation, it is possible to distinguish two isomers (A and B) in which the carboxy group is oriented toward the back of the molecule (β face) thus reducing the hydrophobic area. In these cases the CMC is increased in respect to UDCA and differences between A and B are due to the distance between the carboxy group and the hydrocarbon area and the influence of the polar shell of the carboxy group.

On the other hand, isomers C and D are less hydrophilic than UDCA, with the carboxy group oriented on the α face of the molecule, thus facilitating the self-association of two molecules via back-to-back interaction. The continuity of the hydrophobic area is increased and it is similar to UDCA. The lower CMC values of isomers C and D in respect to UDCA are due to the fact that they possess one more carbon atom (C-25 bile acid) and the CMC and HLB values resemble those of homo-UDCA which has a CMC of 12 mM and $K' = 1.53$.

When data were compared with CMC values for other BA as previously measured by us using the same methodology (12), isomer D had a CMC similar to that of chenodeoxycholic (9 mM) and/or deoxycholic acid (10 mM), while isomer A was like a trihydroxylated BA such as cholic acid (13 mM) or hyocholic acid (17 mM).

With respect to the C-25 linear analog, homo-UDCA, all four isomers had higher CMC values.

Hepatic uptake

The hepatic uptake of TUDCA seems to be a highly efficient process, since the first-pass hepatic extraction ranges from 58 to 66% of the injected dose. The modification of the side-chain moiety, therefore, does not seem to impair the uptake by the liver.

The three isomers we have studied, however, seem to behave differently in terms of the inhibition of the hepatic UDCA uptake. Isomer D was the most potent inhibitor of UDCA uptake, and it was also effective when administered in a bolus of 0.3 μmol, while isomers A and C reached the highest degree of inhibition at a 3.0-μmol dose.

The type of inhibition was also different for the three isomers of CUDCA. Since isomer A inhibited UDCA uptake competitively, the K_m was increased, while the V_{max}

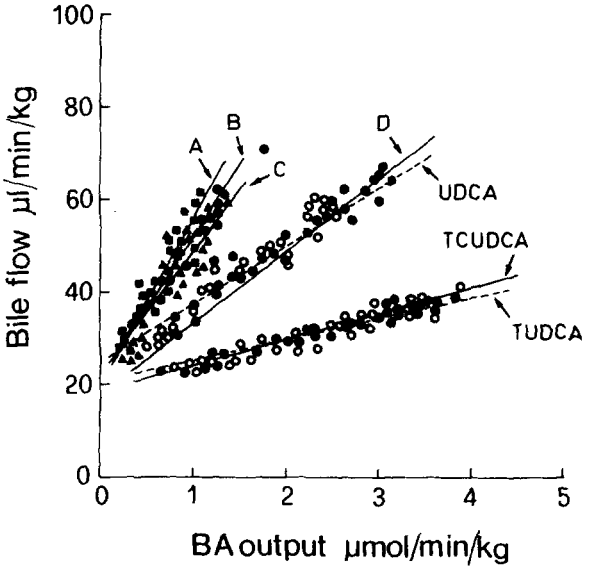

Fig. 7. Relationship between bile flow and total BA output during administration of the four isomers both free and taurine conjugates.

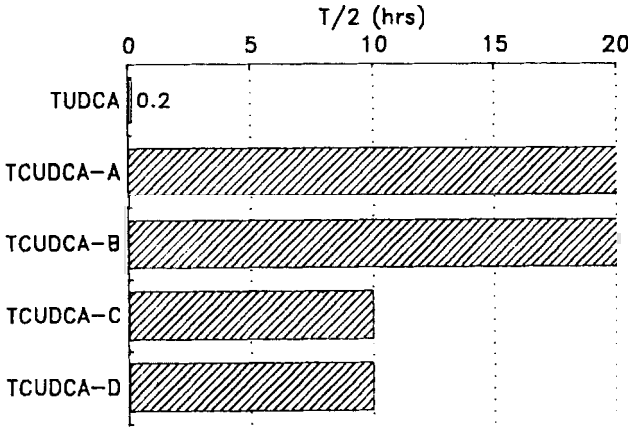

Fig. 8. Substrate specificity of the taurine conjugate isomers and UDCA for cholylglycine hydrolase enzyme.

was the same. A possible explanation is that the binding of the A isomer to the uptake sites is similar to that of UDCA and no binding sites are excluded from the transport of UDCA. Isomer D inhibits UDCA uptake in a noncompetitive fashion.

Isomer C shows an inhibition which is both competitive and noncompetitive. Quite interesting is the finding that, as far as the physicochemical properties of the BA are concerned (namely BA hydrophilicity), the A isomer, which is the most hydrophilic, inhibits competitively while the least hydrophilic (D), which is also the most powerful inhibitor, inhibits the UDCA uptake noncompetitively.

The differences in the degree and type of inhibition of UDCA hepatic uptake by isomers with different physicochemical properties may be explained in terms of different interactions with the cellular membranes (30, 31).

As an example, isomer D might absorb to the lipophilic cell membrane on both extra- and intracellular sides, thus reducing the mobility of solutes in the liquid layer next to the membrane surfaces. Moreover, membrane proteins involved in BA transport may be affected, and their mobility within the cell membrane may also be reduced. As a result, transport rates of UDCA are slowed down (V_{max} is reduced).

Biliary secretion

The rate and the efficiency by which the liver conjugates these isomers greatly affect their rate of secretion into bile as well as the transport of other lipids such as cholesterol and phospholipids (32).

Previous studies (33) have shown that bile acid CoA:amino acid-N-acyltransferase, the second and final step of bile acid amide conjugation by the liver, does not recognize nor-cholyl-CoA as substrate. More recently it has been shown that bile acid:CoA ligase, the initial step of bile acid amidation, is also reduced for C-23 nor-bile acids when compared to their corresponding C-24 homologues (34).

Isomers A, B, and C do not seem to be substrate for these enzymes, while the efficient recovery of isomer D as glycine and taurine conjugates into bile supports its efficient conjugation by the liver.

This can be explained by the peculiar molecular structure of this isomer which presents side-chain structural similarity to UDCA and can undergo activation with CoA with the formation of a CoA-thioester which is a substrate for the CoA:amino acid N-acyltransferase enzyme (Fig. 2).

Alternative conjugation mechanisms such as sulfation or glucuronidation have not been examined in the present study but it is possible that isomers A, B, and C are poorly conjugated on the side chain, but can be glucuronidated or sulfated in the steroid ring to facilitate their liver excretion (28, 35).

The lack of conjugation of isomers A, B, and C and their steroid structures (3α, 7β-dihydroxylated) play a major role in their biliary secretion.

Their low and delayed recovery into bile could be explained by a cholehepatic shunting pathway that these isomers can follow. A large fraction of the BA is secreted into canalicular bile and the unconjugated form, protonated by hydrogen ions derived via carbonic anhydrase enzyme, is passively absorbed.

Isomer D, like UDCA, is absorbed less because it is more efficiently conjugated with glycine or taurine. The conjugates are fully ionized (lower pKa), more hydrophilic, not passively absorbed, and completely into bile. Additional evidence derives also from the efficient recovery of all isomers when administered to rats as taurine conjugates (Fig. 6, right panel).

The kinetics of their biliary excretion is unique (Fig. 6): once the intravenous infusion is stopped, all the isomers, and particularly isomers A, B, and C, continue to be excreted for at least 2 more hours. Isomer D, even when delayed in respect to UDCA, disappears from bile 1 hr after the stop of the infusion. On the other hand, when

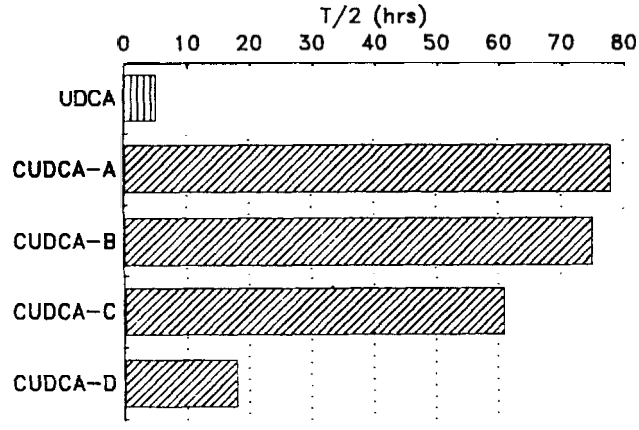

Fig. 9. Substrate specificity for bacterial 7-dehydroxylase on the four isomers (A, B, C, D) in respect to UDCA.

administered as taurine conjugates, all of the isomers behave in a similar fashion; they reach a high excretion rate and once the infusion is stopped they are no longer recovered in bile, suggesting a complete recovery. These data are additional evidence of the cholehepatic shunt pathway which nonconjugable dihydroxylated BA and secreted unconjugated BA can follow.

Effect on bile flow and biliary lipid secretion

Among the four isomers studied, the isomers A, B, and C exhibit extraordinary hypercholeresis, reaching values of specific choleretic activity as previously reported by Yoon et al. (36) for nor-bile acids. Isomer D behaves like a natural C-24 BA such as UDCA.

At the infused dose only UDCA and isomer D are efficiently conjugated with taurine and glycine and consequently efficiently secreted. The hypercholeresis is drastically reduced when both UDCA and the four isomers were administered as taurine conjugates. These data clearly show that the amidation at C-24 position renders the BA more hydrophilic and fully ionized at all pH values, thus facilitating their excretion as previously shown for other bile acids (31, 37).

The amplification of the apparent choleretic effect derives from the expression of these values; when we consider only the volume excreted versus time (SMV_o μl/min per kg), differences exist among the isomers as shown before as SMV_o values, which are related to their different hydrophilicities. Moreover, when we express the results as μl/μmol of bile acid secreted, the apparent hypercholeresis values increase for isomers A, B and C, which are not recovered in bile as unconjugates or glycine/taurine conjugates, contrary to isomer D or UDCA, which are conjugated and are efficiently recovered in bile.

We think that it is more appropriate to express the results as maximum secretion rates (SMV_o) at least at the present time, due to methodological limitation in defining quantitatively the biotransformation which these bile acids undergo within liver cells (sulfation and/or glucuronidation). These additional conjugating mechanisms can be induced, particularly in the case of isomers A, B, and C which are not conjugated with taurine or glycine, in order to facilitate their biliary excretion.

With the analytical methodologies (3α-HSD) we have used, and according to previous investigators (36), it is not possible to quantify BA which have the 3α-hydroxy groups esterified, thus rendering any conclusion which takes into account the total BA output ambiguous.

The underestimation of the total BA secretion can, in part, justify the apparent enormous hypercholeresis induced by isomers A, B, and C which probably follow this metabolic pathway with formation of 3-glucuronides or 3-sulfates.

Moreover, the delayed kinetics and the low recovery of these unconjugates in bile can also be explained, as previously suggested for other side-chain modified BA such as nor-BA or 23-methyl BA with a cholehepatic shunting pathway (36, 38).

The effect on phospholipid secretion is, in part, predictable by physicochemical properties. The SMPL values are partially related to their hydrophobicity. Isomer D, which is more hydrophobic, increases the PL secretion in respect to UDCA administration, while isomers A, B, and C do not modify the SMPL in respect to saline infusion. It seems that isomer D transports more PL because it is secreted into bile in a conjugated form, like UDCA.

When the isomers were administered as taurine conjugates, the SMPL was lower when compared with the corresponding unconjugated forms and similar to saline infusion and to TUCDCA. These data are in agreement with an increased lipophilicity of the molecule due to the presence of the amide bond and the fully ionized sulfonic acid.

The moles of phospholipids secreted per mol of bile acid are significantly higher (0.11–0.28) than those induced by UDCA (0.035). These results can reflect the underestimation of bile acid secretion (see above) but suggest also that other mechanisms of phospholipid transport can be present. In this stimulated and diluted bile enriched with BA with high CMC values and consequently highly hydrophilic, the physicochemical state of bile can be in favor of a formation of large liquid crystal vesicles rather than mixed micelles. This can also explain the transport of cholesterol in the presence of very low BA secretion. During infusion of all four isomers, the maximum cholesterol secretion (SMCHOL) similar to saline infusion and UDCA, but the moles of cholesterol secreted per mole of BA were much higher.

In vitro studies

The lack of hydrolysis of the taurine-conjugated isomers A and B and the low rate of hydrolysis of the isomers C and D indicate that there is an intrinsic resistance of the amide bond to enzymatic hydrolysis. We have previously shown that the taurine conjugates of nor- and homo-analogs of UDCA are poorly deconjugated by the same enzyme and this indicates the high selectivity of this enzyme for its substrate (38). One carbon atom less or more on the side chain is sufficient to abolish this process and this occurs also for the CUDCA isomers. It is interesting to note that the two isomers, A and B, that are not hydrolyzed present a side-chain structure completely different from that of UDCA, while in the isomers C and D, which are hydrolyzed, the C-25 carboxy group is oriented in space as the natural UDCA. These data suggest that the interaction with the active site of the enzyme is not possible when the carboxyl is oriented as in the isomers A and B, while isomers C and D are still recognized though to a lesser extent compared to C-24 UDCA.

The side-chain configuration and conformation also af-

fect the rate of 7-dehydroxylation of these UDCA analogs. The surprising relatively efficient 7-dehydroxylation of isomer D is further explained by its molecular structure; this isomer is more like natural UDCA according to the conformation analysis obtained by molecular computer graphics techniques. (Fig. 2).

These results are useful tools for explaining the substrate specificity and recognition of this enzyme and they are helpful as a molecular model for substrate-enzyme interaction.

In conclusion, these data indicate that the introduction of a cyclopropyl ring on the side chain of ursodeoxycholic acid significantly affects both its physicochemical and physiological properties. The four diastereoisomers present very different properties, thus suggesting an important role of the side chain in determining the hydrophilic/lipophilic balance and the self-association in water. The restricted orientation in space of the carboxyl group generates four structures that are recognized differently by enzymes responsible for conjugation, deconjugation of the taurine conjugates, and 7-dehydroxylation. As a consequence, the four isomers follow different biliary excretion pathways and metabolic routes. Moreover, their effect on bile flow and biliary lipid secretion are different. These data may be useful in the design of BA analogs with enhanced "activity" as cholelitholytic drugs. An optimal biliary excretion with reduced biotransformation by the intestinal bacteria associated with a mild hypercholeresis and reduced cholesterol secretion, such as observed for isomer D, will be an improvement in respect to the natural analog. The final result could be a new analog with a better conservation in the enterohepatic circulation and thus more effective at low doses. Further chronic studies in other species and in humans will be necessary to test this hypothesis.

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