New 6-substituted bile acids: physico-chemical and biological properties of 6α-methyl ursodeoxycholic acid and 6α-methyl-7-epicholic acid

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Abstract New analogs of ursodeoxycholic acid and 7-epicholic acid containing a 6α-methyl group were synthesized, and their physico-chemical properties were studied and compared with those of their natural analogs. The 6α-methyl group slightly increases the lipophilicity and slightly lowers the critical micellar concentration with respect to the corresponding natural analogs. Simulated bile 50% enriched with 6α-methyl ursodeoxycholic acid, with a total bile acid/phospholipid ratio of 10/1, demonstrated a higher cholesterol-holding capacity and a faster cholesterol gallstone dissolution rate with respect to ursodeoxycholic acid, while 6α-methyl-7-epicholic acid and 7-epicholic acid were much less efficient in these processes. The 6α-methyl analogs were highly stable toward 7-dehydroxylation when incubated with human stool in anaerobic conditions. Their transport, metabolism, and effect on biliary lipid secretion were evaluated both in rats and hamsters after acute intravenous and intraduodenal infusion at a dose of 10 μmol/min per kg. In both species, 6α-methyl ursodeoxycholic acid is efficiently secreted in bile, with a cumulative recovery similar to that of ursodeoxycholic acid. The only metabolites of 6α-methyl ursodeoxycholic acid identified were its glycine and taurine amidated forms. 6α-Methyl-7-epicholic acid was efficiently secreted into bile when infused intravenously, and to a lesser extent when infused intraduodenally, in both rats and hamsters; it was secreted in bile as amidate and also as free acid. When 6α-methyl ursodeoxycholic acid, 6α-methyl-7-epicholic acid, ursodeoxycholic acid, and 7-epicholic acid were chronically administered to hamsters (for 3 weeks, at a dose of 50 mg/kg per day) their accumulation in gallbladder bile was, respectively, 25.1%, 4.0%, 15.2%, and 3.4% of the total bile acids. In conclusion, of the two analogs, only 6α-methyl ursodeoxycholic acid shows potential as a cholesterol gallstone-dissolving agent. In this regard, its most important properties are moderate lipophilicity, good metabolic stability, and better conservation in the enterohepatic circulation, with respect to ursodeoxycholic acid.—**Roda, A., R. Pellicciari, C. Cerrè, C. Polimeni, B. Sadeghpour, M. Marinozzi, G. Cantelli Forti, and E. Sapigni.** New 6-substituted bile acids: physico-chemical and biological properties of 6α-methyl ursodeoxycholic acid and 6α-methyl-7-epicholic acid. *J. Lipid Res.* 1994. **35:** 2268–2279.

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The extensive use of ursodeoxycholic acid (UDCA) as a cholesterol gallstone-dissolving drug (1–4) and its recent application for treatment of cholestatic liver diseases (5–8) has created the need for developing new, more potent synthetic analogs. One reason for this need is that recent studies have indicated that UDCA is only partially accumulated in bile because of incomplete intestinal absorption (9, 10). Indeed, many such UDCA synthetic analogs have already been developed (11–22), the structure of which increases the biological half-life of the molecules by preventing intestinal metabolism, and thus increasing their absorption.

Bile acid (BA) analogs resistant to 7-dehydroxylation by intestinal bacteria have already been reported; these include 7-hydroxy-methyl derivatives of cholic acid (CA) and UDCA (11, 12), as well as 6-hydroxy-methyl-substituted BA (18). Side chain-modified BA, such as UDCA sulfonates (23) and cholylsarcosine (24), have also been demonstrated to be more stable than their natural analogs towards 7-dehydroxylation.

When designing a new UDCA analog, it is important not only to make the molecule more stable to 7-dehydroxylation but also to increase its biological half-life and to enhance its activity. In the case of UDCA, increased activity has been partially attributed to the fact that it is less detergent than any of the endogenous BA present in human bile (25, 26).

In previous studies, we reported development of a series

Abbreviations: BA, bile acid; CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; 6-MUDCA, 3α,7β-dihydroxy-6α-methyl-5β-cholan-24-oic acid; CHOL, cholesterol; PL, phospholipid; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; CMC, critical micellar concentration; IV, intravenous; ID, intraduodenal; CMpH, critical micellar pH.

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of side chain-modified UDCA analogs (27–29) including 23-methyl (30) and 22,23-cyclopropyl analogs (31, 32), both in free and taurine amidated forms; their relevant physico-chemical and biological properties, as well as the procedures for their synthesis were also described. Various diastereoisomers of the above compounds and their properties were also reported (33, 34).

The main advantages offered by these new analogs, as demonstrated in the rat, are the lack of side-chain amidation in the case of unconjugated analogs (or deamidation of the corresponding taurine conjugate forms), and simultaneous prevention of 7-dehydroxylation by intestinal flora. The main drawback of the unconjugated 23-methyl and some cyclopropyl diastereoisomer analogs is poor biliary secretion due to lack of conjugation in the liver, a process which is impeded by the presence of the 23-methyl group or the cyclopropane ring, respectively, both of which hinder amide bond formation. A similar behavior has also been described by other authors for nor (C-23) and homo (C-25) derivatives of some dihydroxy BA (14, 27). On the contrary, the corresponding taurine amidated forms of these molecules behaved like natural conjugated BA, and so were efficiently secreted in bile. When the four diastereoisomers of 22,23-cyclopropilogs of UDCA were isolated and studied separately, they each behaved differently, suggesting that not only the side chain structure, but also its configuration are important features, particularly as regards hepatic amidation and intestinal active transport (33).

In this work we describe the physico-chemical and biological properties of new 6α-methyl analogs of UDCA and 7-epicholic acid. The rationale for adding the methyl group in position 6 was to prevent, by steric hindrance, 7-dehydroxylation or epimerization, and thus to stabilize the molecule during exposition to intestinal bacteria. We also reasoned that, despite this alteration, the molecule should be amidated by the liver, following a pharmacokinetic pattern similar to that of conventional amidated BA, and then efficiently absorbed by the intestine, either as a free or an amidated BA. UDCA and 7-epicholic acid were chosen in order to study 6α-methyl analogs with different lipophilicity.

The more important physico-chemical properties in aqueous solution, including pKa, critical micellar concentration, lipophilicity, and solubility versus pH were measured and compared with those of the parent natural analogs UDCA and 7-epicholic acid.

In vitro studies were performed to assess their stability in human intestinal microflora in anaerobic conditions, and to evaluate their efficacy in dissolving cholesterol gallstones in simulated bile enriched with these substances. Studies of acute effects were performed in bile fistula rats and hamsters; in these studies the methyl derivatives of both analogs were administered both intravenously and intraduodenally at a dose of 10 μmol/min per kg, after which their biliary secretion and hepatic metabolism were determined. Their effect on biliary lipid secretion was also evaluated. Finally, the analogs were administered chronically (3 weeks at 50 mg/kg per day) to hamsters in order to quantify their metabolism and accumulation in bile as compared with that of natural analogs.

MATERIAL AND METHODS

Chemicals

3α,7β-Dihydroxy-6α-methyl-5β-cholan-24-oic acid (6-MUDCA) and 3α,7β,12α-trihydroxy-6α-methyl-5β-cholan-24-oic acid (6-methyl-7-epicholic acid) were synthesized and purified as described in detail elsewhere. Briefly, 6-methyl analogs of UDCA and 7-epicholic acid were prepared from their respective 7-oxo-derivatives in which the 3α-hydroxyl and the 3α,12α-hydroxyls, in the case of 7-epicholic acid, were protected with tetrahydropyranyl groups. Methylation was carried out under controlled conditions, using methyl iodide and an appropriate base-solvent system. The reaction was carried out at −78°C, using lithium diisopropylamide as a base and 1,2-dimethoxyethane, containing hexamethylphosphoramide, as solvent. The 6-methyl derivatives thus obtained were then deprotected and the 7-keto group was selectively reduced to give the corresponding 7β-hydroxy derivatives. The final compounds were purified by column chromatography.

UDCA and 7-epicholic acid were kindly supplied by Giuliani Spa, Milan, Italy. Chenodeoxycholic acid (CDCA), cholic acid (CA) and deoxycholic acid (DCA) were purchased from Sigma (St. Louis, MO).

All the experiments were carried out using the sodium salts of the synthesized bile acids. The sodium salts were prepared by adding an equimolar amount of $NaHCO_3$ to the aqueous suspension of the free acid, which was heated to 80°C, agitated in an ultrasound bath, and then freeze-dried.

Analytical methods

Total bile acids (BA), cholesterol (CHOL) and phospholipids (PL), were determined in bile using conventional enzymatic spectrophotometric procedures as previously described (35–37). The qualitative-quantitative composition of BA in bile, or in other matrices such as stool, was also determined by different combined chromatography techniques. The biological fluids were submitted to a preliminary clean-up procedure using conventional C-18 reversed-phase extraction (C-18 Bond Elut, Analytichem International, Harbor City, CA). The sample was diluted (1:5) with NaOH 0.1 M and applied to a column previously activated with methanol and water. The retained BA were eluted with 4 ml of methanol after a washing step with water. The eluted methanol solution was

dried under vacuum and reconstituted with an appropriate amount of methanol or HPLC mobile phase, according to the expected BA concentration.

Thin-layer chromatography (TLC), using silica gel 0.25 μm thickness plates (Merck, Darmstadt, Germany), was selected as a first screening test. The solvent system used for the separation of conjugated BA was composed of propionic acid–isoamyl acetate–water–N-propanol 3:4:1:2 (v/v, solvent I); that used for the unconjugated BA consisted of acetic acid–carbon tetrachloride–isopropyl ether–isoamyl acetate–N-propanol–benzene 1:4:6:8:2:2 (v/v, solvent II). A small quantity of bile was spotted onto TLC plates and, after development, the plate was immersed in 5% phosphomolybdic acid in ethanol solution, removed, and heated at 120°C for about 10 min.

The BA were also evaluated by high performance liquid chromatography (HPLC) (38) using a Waters 600E multisolvent delivery system equipped with an autosampler injector (Waters 717). The apparatus was connected to an evaporative light-scattering detector ELSD II (Varex Corporation, Burtonsville, MD). The signal was recorded using a Waters 746 data module. A Nova-Pak C-18 Waters steel column was used (3.9 mm × 300 mm); particle size was 4 μm. The column temperature was kept at 37 ± 0.2°C, controlled by a Waters TCM thermostat.

For the separation of glycine and taurine amidated BA, a mobile phase was used, composed of aqueous methanol 65% v/v containing 15 mM ammonium acetate, with an apparent pH of 5.4 ± 0.1 (solvent A) under isocratic conditions (0.9 ml/min). Unconjugated BA were separated using aqueous methanol 75% v/v containing 15 mM ammonium acetate, with an apparent pH of 5.4 ± 0.1 at a flow rate of 0.9 ml/min (solvent B).

Physico-chemical properties

Critical micellar concentration (CMC). This value was determined by surface tension measurements using a maximum bubble-pressure method (39). The tensiometer was a Sensadyne 6000 (Chem-Dyne Research Corp., Milwaukee, WI) equipped with two glass probes of 0.5 and 4.0 mm diameters connected to a source of compressed air. The bubble frequency was 1 bubble/second in distilled water at 25°C and the calibration was made with double-distilled water and methanol. The surface tension of aqueous solutions at various concentrations (range 0.5–150 mM) of the BA sodium salts was measured at 25°C. The surface tension values were plotted against the logarithm of the bile salt concentration; the regression lines corresponding to the two parts of the curve (monomeric and micellar phases) were calculated using the method of least squares, and the intersection of the lines was taken as the CMC value.

Water solubility. BA were suspended in 100 ml of 0.01 M $HClO_4$ and the saturated solutions were transferred to a thermostat-equipped water bath maintained at 25°C. After incubation for 1 week, the solutions were filtered on a Millipore filter (0.22 μm) and the concentration of BA was measured enzymatically using 3α-hydroxysteroid dehydrogenase (40).

Hydrophilicity. The relative hydrophilicity of the bile salts was measured by reversed phase HPLC (41). The analyses were carried out under isocratic conditions (0.9 ml/min), using a mixture of methanol–water 65:35 (v/v) as the mobile phase, with the addition of ammonium acetate 2 mM at pH 7, in order to ensure a complete ionization of all the BA. A retention factor (K') was calculated from the relative mobilities of the separated bile acids using the formula:

$$K' = (t_v - t_o)/t_o$$

where t_o = retention of the solvent and t_v = retention time of bile salts. Data were expressed as relative retention factor rK' (relative to UDCA).

Octanol/water partition coefficient. 1-Octanol/water partition coefficient (log P) was evaluated using a conventional shake-flask procedure as previously described (41). The experiments were carried out on 1 mM initial bile salt solution buffered at pH 7.4 with 0.1 M potassium phosphate buffer to ensure complete ionization of the BA; the log P values refer to the BA in the ionized form, not to the protonated species, and the initial concentration of each BA was below its own CMC value.

Acidity. Acidity constants were determined by potentiometric measurements in solutions of aqueous methanol at different mole fractions (42). The pKa values, estimated in water by means of previously established correlations at pKa values in mixed solvents, were in close agreement with each other. All the measurements were carried out at 25 ± 0.1°C.

Critical micellar pH (CMpH). The estimation of the pH-solubility relationship of each bile acid and its salt was carried out by aqueous acidometric titration of the bile salt in water with a strong mineral acid (HCl); 20 ml of 50 mM solution of each bile salt studied was titrated with HCl 0.1 M. The CMpH is the pH at which precipitation of the protonated form is first visualized by turbidimetry at 500 nm. This value was confirmed by back titration with NaOH 0.1 M until the suspension became transparent.

Albumin binding. Albumin binding was evaluated by equilibrium dialysis at a fixed BA-albumin ratio (43, 44). BA was dissolved at a concentration of 100 μM in 5% bovine serum albumin-saline solution and left to stand for 24 h at 25°C. Two ml of this solution was dialyzed in cellulose sacs having a molecular weight cut-off of 12–14,000 (Spectra/Por, Spectrum Medical Industries Inc., Los Angeles, CA) against 25 ml of saline solution. The system was equilibrated by mechanical shaking for 72 h at 25°C. BA concentrations of the dialyzed solution and of the starting solution were determined by enzymatic method,

and the relative percent of albumin binding was calculated by their ratio.

In vitro studies

Bacterial 7-dehydroxylation. Immediately after evacuation, human fresh stools were homogenized with water 1:1 (v/v) under nitrogen stream, and 500 mg was transferred into sterile vials to which 5 ml of sterilized chopped meat-glucose medium (Scott Lab., Fiskville, RI) was added. BA were then added to this medium at a concentration ranging from 0.01 to 0.1 mM. Control analyses were performed using UDCA and 7-epicholic acid. All the experiments were carried out under nitrogen in capped vials. The anaerobic conditions were verified with a disposable anaerobic indicator (Gas Pac, Becton Dickinson Co., Orangeburg NY). Vials were incubated at 37°C; then, at 0, 4, 8, 16, 24 and 72 h after the addition of the BA, the reaction was stopped with 150 μl of 30% KOH. The samples were centrifuged at 3500 rpm for 10 min. The BA were isolated from 2 ml of the supernatant by solid-phase extraction, and separated using TLC (solvent I) and HPLC (solvent B) techniques.

Cholesterol gallstone dissolution. The kinetics of cholesterol gallstone dissolution were evaluated in a simulated bile solution composed of 100 mM total BA and 10 mM phospholipids (purified egg lecithin, Lipid Products, South Nutfield, Surrey, UK). The BA were a 50:50 mixture of taurocholic acid (TCA) and one of the studied 6α-methyl analogs or its natural parent compound. Control solution consisted of 100% TCA. To 100 ml of these solutions a synthetic cholesterol gallstone (monohydrate cholesterol-calcium carbonate 90:10 (w/w) 10 mm diameter) was added and the solution was kept at 37°C under constant agitation. Every day for 3 weeks an aliquot of 0.5 ml was taken and filtered with a Millipore filter (0.22 μm); the concentration of cholesterol in solution was determined enzymatically. From the plot of cholesterol concentration versus time, the rate of dissolution (slope of the first part of the curve) and the cholesterol holding capacity (concentration at plateau) were calculated.

In vivo study

Acute study: hepatic metabolism and biliary secretion in rats and hamsters. The study was performed both in male Sprague-Dawley rats (300–330 g) and Syrian Golden hamsters (90–125 g). The animals were anesthetized with ethyl carbamate and the bile duct was cannulated with PE-10 tubing (Clay Adams, Becton Dickinson, Parsippany, NJ).

After 1 h of baseline steady-state, the BA were administered as sodium salts through the femoral vein or through the duodenum (six animals in each group), at a dose of 10 μmol/min per kg, and bile was collected for 3 h at 30-min intervals. Controls consist of UDCA, 7-epicholic acid, and saline solution. The bile flow was measured gravimetrically taking the density of bile as one; the concentrations of BA, CHOL, and PL in the samples were determined by enzymatic methods. BA composition was evaluated by the HPLC method described above in Analytical Methods using solvent A. Biliary lipid secretion, calculated from the volume of secreted bile and from the biliary lipid concentration, was expressed as μmol/min per kg. From the plot of biliary secretion versus time, the following parameters were calculated: mean ± SD maximum secretion rate and relative time (S_{max} and T_{max}), mean residual secretion rate (R_s) and mean cumulative percent recovery in bile.

Chronic study: 3 weeks oral administration by gavage in hamsters. Male young Golden Syrian hamsters (100–120 g) were designated to five groups (6-MUDCA, 6-methyl-7-epicholic acid, UDCA, 7-epicholic acid, and control) by computerized stratified randomization. The BA (sodium salt form) were administered to hamsters (six for each group) by oral gavage at a dose of 50 mg/kg per day. Control animals received the vehicle (distilled water) alone at the same volume. The animals were treated once a day, for 3 weeks. At the end of treatment, gallbladder bile was collected and total BA, CHOL, and PL concentrations were determined by enzymatic methods. BA composition was determined by HPLC method (solvent A, see above).

Statistical analysis

Statistical analysis was carried out using analysis of variance to demonstrate differences among the various groups studied. An addition, the *t*-test of Bonferroni was used to identify and isolate the agents producing significant results. All values are expressed as mean values ± SD; calculations were performed using the PROC ANOVA of the Statistical Analysis System (SAS).

RESULTS

Physico-chemical properties

Table 1 shows the physico-chemical properties of 6-MUDCA and 6-methyl-7-epicholic acid in aqueous solution as compared with those of unconjugated BA of similar steroid structure or other naturally occurring dihydroxy and trihydroxy BA. 6-MUDCA did behave differently from UDCA in water. While the thermodynamic pK_a values were similar and the water solubility values of the acid forms were in the same order of magnitude, 6-MUDCA lipophilicity and detergency were greater than those of the natural analog. While the methyl group increases the value of logP only slightly, the CMC is significantly lower; however, the surface tension of 6-MUDCA at the CMC was similar to that of UDCA.

A similar trend was observed when 6-methyl-7-epicholic acid was compared with 7-epicholic acid: 6-methyl-7-epicholic acid was still highly hydrophilic (low logP) and poorly detergent (high CMC).

TABLE 1. Physico-chemical properties of the two 6-methyl analogs and those of natural occurring bile acids

Bile Acid	Water Solubility	CMC	Surface Tension	pKa	CMpH	C-18 rK'	$logP_A^-$	Albumin Binding
	μM	*mM*	*dyne/cm*					%
6-MUDCA	28	17	47.1	5.0	7.8	1.32	2.30	80
6-MUCA	1270	36	48.3	5.0	6.5	0.46	0.52	10
UDCA	8	26	48.2	5.0	8.4	1.00	2.21	91
UCA	2560	52	48.8	5.0	6.3	0.33	0.10	20
CA	273	10	55.0	5.0	6.7	1.20	1.10	40
CDCA	27	6	51.3	5.0	7.6	3.88	2.25	90
DCA	28	7.5	50.2	5.0	7.3	4.10	2.65	94

The binding capacity with albumin was stronger with DCA, CDCA, UDCA, or 6-MUDCA than with CA, 7-epicholic acid or 6-methyl-7-epicholic acid. With CMpH the lower values were 7-epicholic acid, 6-methyl-7-epicholic acid and CA (pH 6.3–6.7) in comparison with DCA, CDCA, 6-MUDCA, and UDCA with higher pH (7.3–8.4).

In vitro study

Bacterial 7-dehydroxylation. 6-MUDCA and 6-methyl-7-epicholic acid were stable when incubated in anaerobic conditions with human stools, i.e., they were not 7-dehydroxylated or metabolized to other compounds as verified by TLC and HPLC analysis. **Figure 1** shows the kinetics of biotransformation of 6-MUDCA and 6-methyl-7-epicholic acid as compared with those of UDCA and 7-epicholic acid: the latter analogs were rapidly metabolized, mainly to lithocholic and deoxycholic acid, respectively.

Cholesterol gallstone dissolution. As shown in **Table 2** and **Figure 2,** in the BA-phospholipid micellar solution containing 6-MUDCA, the rate of cholesterol monohydrate dissolution was higher than that in the UDCA solution, as was the cholesterol holding capacity. On the contrary, in the micellar solution containing 6-methyl-7-epicholic acid, the rate of gallstone dissolution as well as the cholesterol holding capacity were very low in comparison with those of UDCA solution.

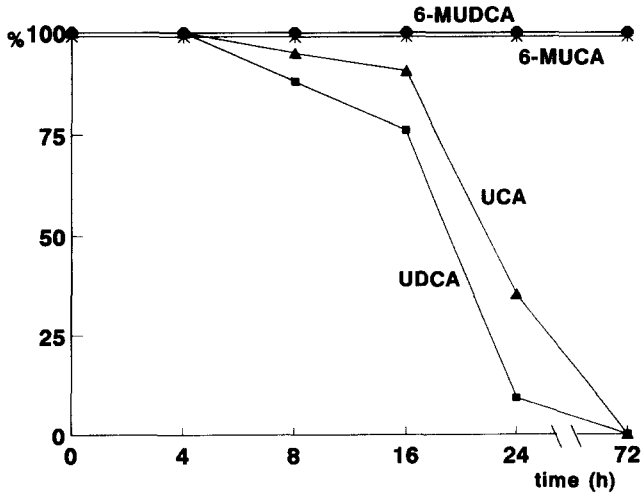

Fig. 1. Metabolism of the studied bile acids when incubated in human stool under anaerobic conditions. Results are expressed as quantity of original molecule recovered as a percentage of the total bile acids present after incubation.

In vivo study

Acute study: hepatic metabolism and biliary secretion in rats. **Table 3** shows the kinetic parameters of biliary secretion after intravenous and intraduodenal administration in rats and hamsters of the studied BA. When infused intravenously, 6-MUDCA was taken up by the liver and secreted in bile as taurine and glycine conjugates, as shown in **Figure 3;** the maximum secretion rate of total BA (S_{max}) was significantly lower than that of UDCA ($P < 0.01$), as the result of a slightly delayed secretion rate (longer T_{max}). The cumulative recovery of 6-MUDCA in bile over a 3-h period was significantly lower than that of UDCA, while the R_s was slightly higher; its choleretic effect was lower than that of UDCA, as result of delayed secretion of the molecule (Table 3).

After intravenous infusion, 6-methyl-7-epicholic acid also showed an S_{max} slightly lower than that of UDCA and significantly lower than that of 7-epicholic acid ($P < 0.002$). 6-Methyl-7-epicholic acid was found in bile as its taurine and glycine conjugates and also in relevant

TABLE 2. In vitro cholesterol monohydrate dissolution kinetic in simulated bile 50% enriched with the 6-methyl analogs and the parent compounds

Bile Acid	Dissolution Rate	Cholesterol Holding Capacity
	mmol/l/day	*mmol/l*
6-MUDCA	1.10 ± 0.20	4.0 ± 0.6
6-MUDCA	0.13 ± 0.02	2.1 ± 0.3
UDCA	0.42 ± 0.05	3.2 ± 0.2
UCA	0.10 ± 0.04	1.3 ± 0.1
Control	0.16 ± 0.03	2.0 ± 0.3

Each value is mean ± SD of five experiments.

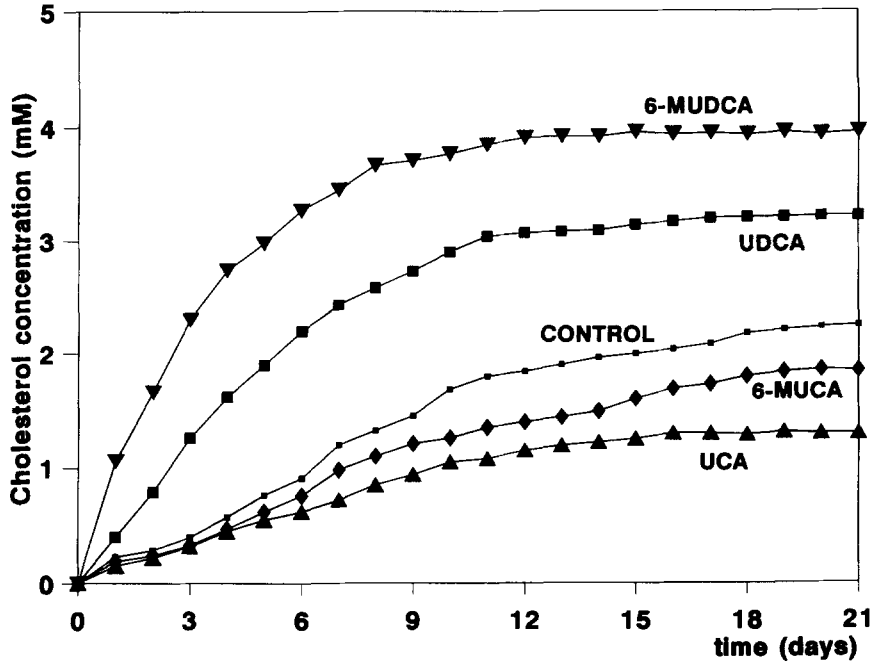

Fig. 2. Rate of cholesterol dissolution in simulated bile enriched with the studied bile acids.

amounts as a free form (Fig. 3). When infused intraduodenally, 6-MUDCA showed a significantly lower S_{max} than UDCA ($P < 0.05$), while 6-methyl-7-epicholic acid had an S_{max} similar to 7-epicholic acid. 6-MUDCA showed delayed secretion and a longer T_{max}, with a cumulative recovery significantly lower than that of UDCA ($P < 0.01$). While 6-methyl-7-epicholic acid was secreted as such and also as taurine and glycine conjugates, similar to 7-epicholic acid, 6-MUDCA was secreted in bile only as taurine and glycine conjugates, in equimolar amounts. The biliary recovery of 6-methyl-7-epicholic acid was significantly higher than that of 7-epicholic acid.

Hepatic metabolism and biliary secretion in hamsters. In **Figure 4,** secretion rates versus time of the administered compounds and their metabolites are reported. The maximum biliary secretion after intravenous administration of 6-MUDCA was similar to that of UDCA. 6-MUDCA was secreted in bile mainly in the form of taurine amidates, and to a lesser extent, as glycine amidate or free acid. The cumulative percent biliary recovery was higher than that of UDCA (Table 3). The behavior of intravenously administered 6-methyl-7-epicholic acid was different from that of 6-MUDCA: the S_{max} was slightly higher than that of UDCA but similar to that of 7-epicholic acid; also, a large amount of the 6-methyl-7-epicholic acid was secreted in bile as such (up to 70%), along with a small percentage of glycine and taurine conjugates. The secretion rate was very fast. The percent recovery of 6-methyl-7-epicholic acid was similar to that of 7-epicholic acid, but higher than those of the other bile acids.

When infused intraduodenally, 6-MUDCA and 6-methyl-7-epicholic acid showed an S_{max} slightly lower than that of UDCA. 6-MUDCA and 6-methyl-7-epicholic acid showed similar cumulative percent recovery that was higher than those of their parent analogs.

6-MUDCA was secreted in bile mainly as its taurine conjugate, although a small percentage was secreted as such or as its glycine conjugate. 6-Methyl-7-epicholic acid was secreted in bile mainly as glycine and taurine conjugates, but also as free acid.

Effects on biliary lipid secretion in rats and hamsters. The effects of the administered BA on PL and CHOL secretions are reported in **Table 4.** In rats, when administered IV or ID, 6-MUDCA induced PL secretion slightly less than did UDCA; the effect of 6-methyl-7-epicholic acid was lower than that of UDCA. Similar data were found in hamsters. The cholesterol maximum secretion rate of 6-methyl-7-epicholic acid was lower than that of UDCA and 7-epicholic acid in both rats and hamsters; whereas, regarding 6-MUDCA, the S_{max} was similar to that of its parent compound in the rat, but lower than this in the hamster.

In the rat, the greater choleretic effects were observed with intravenous 6-methyl-7-epicholic acid and 7-epicholic acid. In the hamster, all four analogs induced choleresis whether infused IV or ID, though the effect of UDCA was slightly lower with respect to the other three analogs.

TABLE 3. Biliary secretion parameters in bile fistula rat and hamster after intravenous (IV) or intraduodenal (ID) administration of the studied bile acids at a dose of 10 μmol/min per kg

Bile Acid	Rat								Hamster							
	S_{max}		T_{max}		RS		Recovery		S_{max}		T_{max}		RS		Recovery	
	ID	IV	ID	IV	ID	IV	ID	IV	ID	IV	ID	IV	ID	IV	ID	IV
	μmol/min/kg		*min*		*μmol/min/kg*		%		*μmol/min/kg*		*min*		*μmol/min/kg*		%	
6-MUDCA	1.40 ± 0.29	1.96 ± 0.55	90–180	90	0.91 ± 0.08	0.61 ± 0.12	49 ± 9	51 ± 8	1.47 ± 0.20	1.79 ± 0.77	90–150	120	1.19 ± 0.10	1.02 ± 0.15	52 ± 8	57 ± 7
6-MUCA	2.21 ± 0.23	2.69 ± 1.29		90	1.07 ± 0.18	0.57 ± 0.15	64 ± 7	62 ± 8	1.91 ± 0.37	3.78 ± 2.11		90	1.07 ± 0.22	0.34 ± 0.09	55 ± 6	75 ± 9
UDCA	2.68 ± 1.30	3.80 ± 1.30		60	0.80 ± 0.17	0.44 ± 0.11	68 ± 7	65 ± 9	2.25 ± 0.71	1.98 ± 0.55		60	0.69 ± 0.05	0.48 ± 0.13	41 ± 6	42 ± 7
UCA	2.50 ± 0.48	5.20 ± 0.65		90	0.95 ± 0.12	0.45 ± 0.12	35 ± 6	68 ± 7	2.28 ± 0.48	4.05 ± 1.05		90	0.70 ± 0.12	0.28 ± 0.08	36 ± 5	80 ± 6
Saline	1.52 ± 0.49	0.89 ± 0.18		60	0.50 ± 0.21	0.55 ± 0.23			0.47 ± 0.16	0.35 ± 0.08		60	0.18 ± 0.03	0.18 ± 0.03		

Chronic study: 3-weeks oral administration by gavage in hamsters. **Figure 5** shows the percent BA composition (determined by HPLC) of the gallbladder bile in the five groups of animals. The biliary accumulations of 6-MUDCA and UDCA (as glycine and taurine amidates) are, respectively, 25.1% and 15.2% of the total BA. 6-Methyl-7-epicholic acid was present mainly as the glycine conjugate, plus a small amount as free acid; its total percent accumulation (4.0%) was very low with respect to that of UDCA (15.2%), but similar to that of 7-epicholic acid (3.4%). While 6-MUDCA and 6-methyl-7-epicholic acid administration did not significantly modify the endogenous bile acid composition, with UDCA and 7-epicholic acid administration, amidated CDCA and CA increased significantly with respect to control animals.

DISCUSSION

This study provides new information about the effect on the physico-chemical and biological properties of 7-epicholic acid and UDCA by the introduction of a methyl group in the 6α position. Our aim was to establish a complete set of data regarding the new analogs, from basic information about their physico-chemical behavior in aqueous solution to pharmacological activity after chronic feeding in hamsters.

From structure-activity relationship studies (45–47) it is known that a $-CH_3$ is a lipophilic group ($\pi_{CH3} = +0.56$ vs. $\pi_H = +0.23$ or $\pi_{OH} = -1.64$); as would be expected, the two 6-methyl analogs studied are slightly more lipophilic than the respective natural analogs, UDCA and 7-epicholic acid. The presence of a methyl group in the 6α position does not sterically hinder micellar formation, and it increases the size of the lipophilic area of the steroid back (β face). As a result of an increased hydrophobic BA interaction, the CMC values of the two compounds are slightly lower than those of the natural analogs. Moreover, this increased detergency is still lower than that of UDCA or 7-epicholic acid 7α epimers such as CDCA or CA, suggesting that the topographic distribution of the polar substituents on the α or β face of the parent molecule is important in determining these properties. This represents an obvious advantage of these analogs over a previously described 7-methyl-7-hydroxy derivative (11–13) in which it was difficult to control the stereochemistry because both methyl and hydroxyl groups were in the same position.

The stability of these molecules towards bacterial 7-dehydroxylation (48) is very high, showing a strong protective effect by the 6-methyl group which is likely due to steric hindrance, as it has a much larger volume than -H, (31.7 vs. 7.2 $Å^3$). Indeed, a strong steric effect of the methyl group in 23 position was previously shown (30); 23-methyl analogs of UDCA (α and β) were not amidated by the liver and, in turn, taurine and glycine 23-methyl

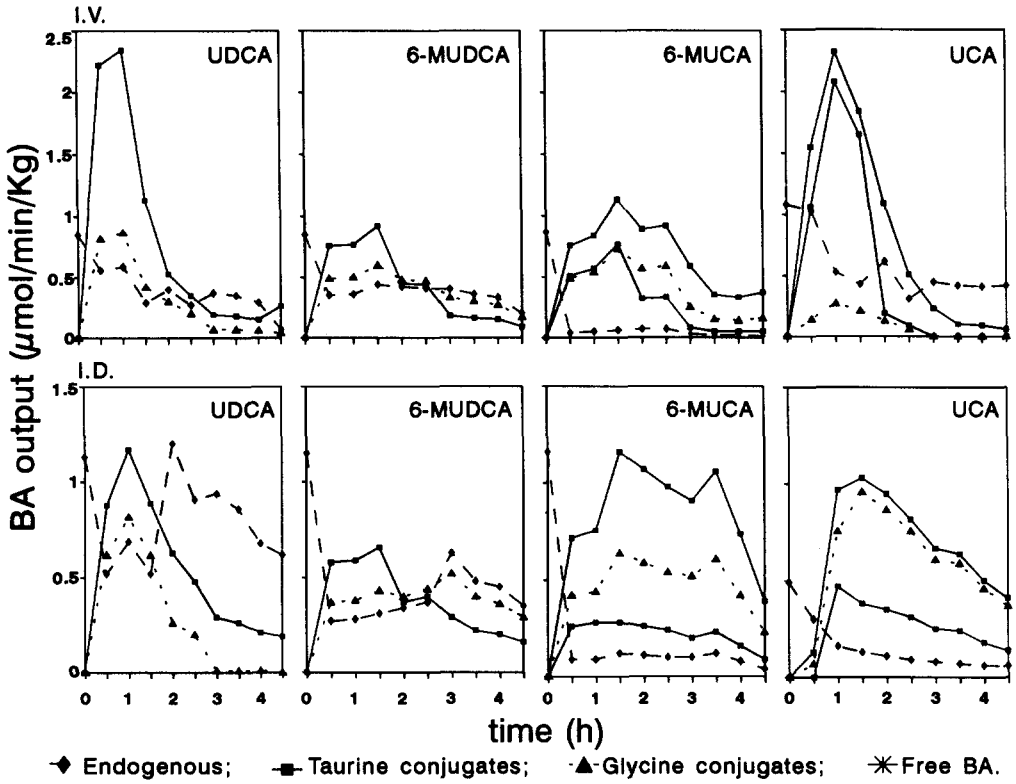

Fig. 3. Biliary secretion rate of the 6-methyl analogs, UDCA, UCA and their hepatic metabolites in bile fistula rat after I.V. and I.D. administration at a dose of 10 μmol/min per kg. Each point is a mean value of six animals.

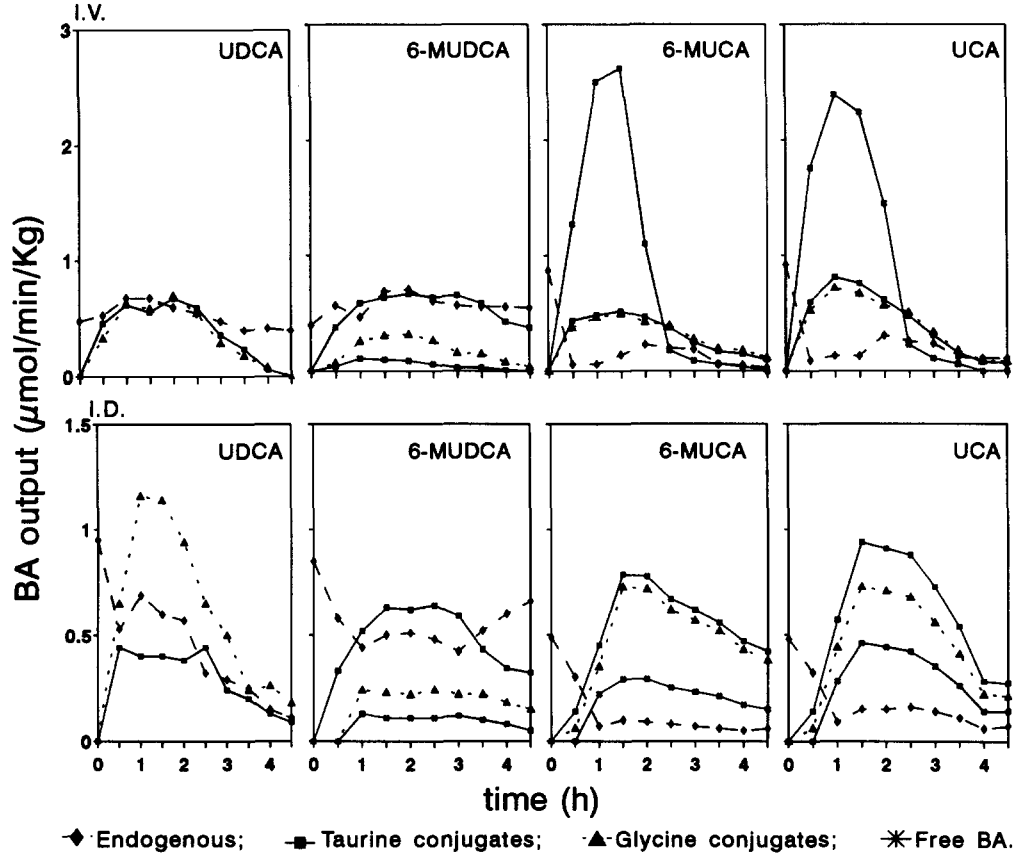

Fig. 4. Biliary secretion rate of the 6-methyl analogs UDCA, UCA and their hepatic metabolites in bile fistula hamster after I.V. and I.D. administration at a dose of 10 μmol/min per kg. Each point is a mean value of six animals.

TABLE 4. Effect in bile fistula rat and hamster of intravenous (IV) or intraduodenal (ID) administration of the studied bile acids on phospholipids (PL), cholesterol (CHOL) secretion, and bile flow

	Rat						Hamster					
	PL		CHOL		Bile Flow		PL		CHOL		Bile Flow	
Bile Acid	ID	IV	ID	IV	ID	IV	ID	IV	ID	IV	ID	IV
	μmol/min/kg		*nmol/min/kg*		*ml/min/kg*		*μmol/min/kg*		*nmol/min/kg*		*ml/min/kg*	
6-MUDCA	0.18 ± 0.02	0.19 ± 0.04	13.0 ± 8.4	19.0 ± 1.4	0.05 ± 0.01	0.07 ± 0.02	0.12 ± 0.09	0.16 ± 0.10	12.0 ± 6.2	14.0 ± 7.0	0.09 ± 0.01	0.12 ± 0.06
6-MUCA	0.10 ± 0.02	0.15 ± 0.07	12.0 ± 3.2	20.0 ± 11.0	0.09 ± 0.02	0.10 ± 0.05	0.09 ± 0.05	0.13 ± 0.11	8.0 ± 2.0	12.0 ± 3.3	0.07 ± 0.01	0.11 ± 0.03
UDCA	0.22 ± 0.09	0.21 ± 0.01	19.0 ± 2.1	24.7 ± 12.2	0.06 ± 0.03	0.08 ± 0.03	0.18 ± 0.08	0.23 ± 0.10	12.0 ± 6.2	15.3 ± 2.3	0.08 ± 0.04	0.08 ± 0.03
UCA	0.11 ± 0.02	0.18 ± 0.06	14.0 ± 2.5	20.0 ± 10.2	0.10 ± 0.04	0.10 ± 0.08	0.09 ± 0.06	0.15 ± 0.04	10.1 ± 2.5	15.0 ± 3.2	0.06 ± 0.02	0.14 ± 0.05
Saline	0.11 ± 0.01	0.17 ± 0.06	10.0 ± 6.2	15.1 ± 5.2	0.05 ± 0.01	0.05 ± 0.01	0.08 ± 0.01	0.05 ± 0.04	5.0 ± 1.2	2.6 ± 0.8	0.05 ± 0.01	0.05 ± 0.01

Values are the mean ± SD of six animals.

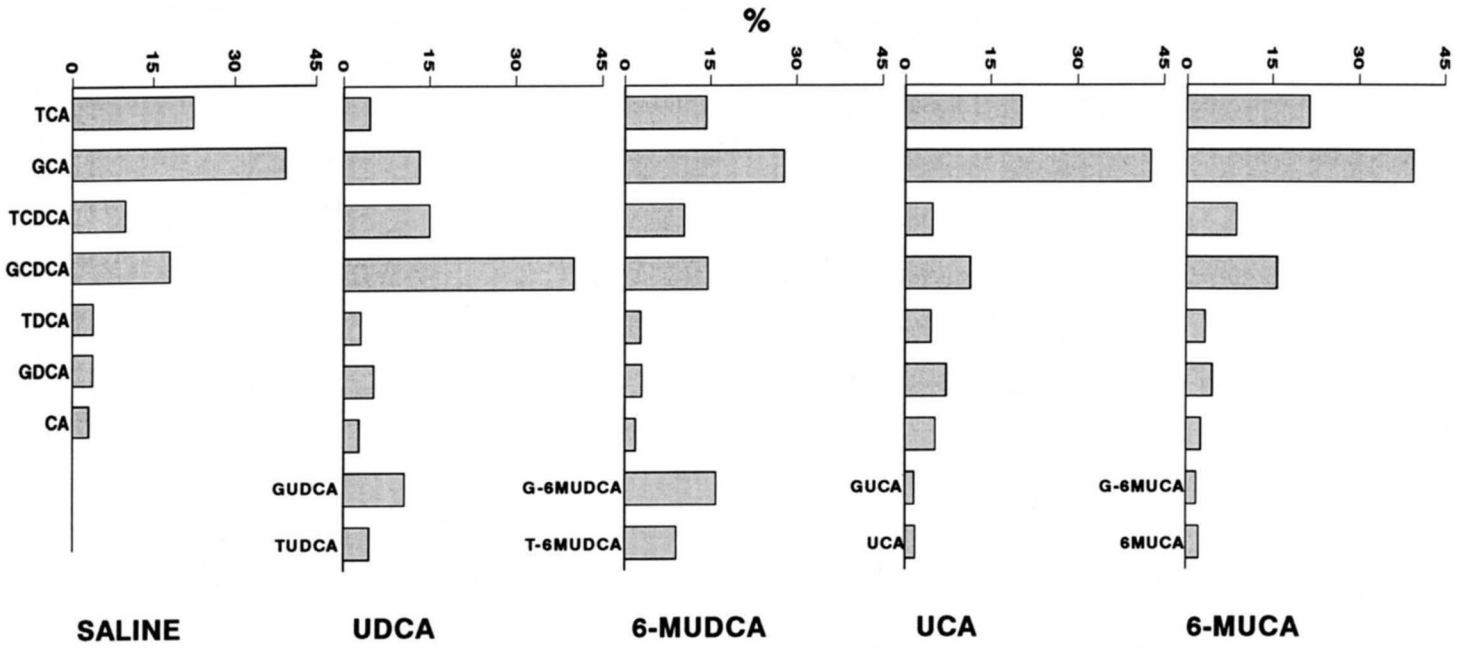

Fig. 5. Bile acid composition of hamster gallbladder bile after 3 weeks of chronic feeding at a dose of 50 mg/kg day of the 6-methyl analogs, UDCA and UCA. Each point is a mean value of six animals.

amidates were not deamidated (28).

Regarding in vivo study, biliary secretion rates after IV or ID administration showed different results between the two new analogs and, for each given analog, results also varied in the two animal species studied. In rats, 6-MUDCA presented a slightly delayed biliary secretion when administered either IV or ID. A similar S_{max} after IV or ID administration both in the hamster and the rat suggests efficient intestinal absorption, as confirmed by high accumulation of this analog after chronic feeding. On the other hand, a low S_{max} associated with an increased T_{max} and R_s for 6-MUDCA compared with UDCA suggests that this molecule has a rate-limiting metabolic step in biliary secretion due to hepatic amidation. 6-MUDCA is probably a poorer substrate than UDCA for the amidating process as its hepatic residence time is higher. Moreover, the ratio of the cumulative recovery of 6-MUDCA after ID and IV administration was 1:1 in both rats and hamsters, suggesting efficient intestinal and hepatic transport.

6-Methyl-7-epicholic acid behaved differently from 6-MUDCA; in the rat, its recovery in bile was similar in both IV and ID studies; in the hamster the recovery after IV infusion was much higher than when administered ID, in which respect its behavior was similar to that of 7-epicholic acid. This latter difference was due to the fact that a large amount of this compound was secreted in bile as such, with no need for amidation because of its high hydrophilicity. This process happened very quickly; the biliary secretion rate reached a high maximum value in a short time and, at the end of the experiment, only a very small amount remained to be secreted. However, the ratio of the total recovery after ID versus IV administration was almost 1 in rats and 0.75 in hamsters, suggesting a species difference for the intestinal transport of BA. This transport may be both active and passive in the ileum, as has been documented for other trihydroxy unconjugated BA (49–51).

The chronic studies carried out in hamsters give more complete information on the extent of accumulation of the new molecules in the enterohepatic circulation. The bile was enriched with the administered compound only in the glyco and tauro amidated forms and no other metabolites were present. These results are different from UDCA and 7-epicholic acid studies which show a poor accumulation of the molecule and an increased formation of their 7α epimers, CDCA and CA, with respect to untreated animals.

6-MUDCA accounted for 25% of the total BA in hamster bile, almost twice the percentage reached by UDCA; the latter was largely metabolized to CDCA which became the predominant BA. On the contrary, 6-methyl-7-epicholic acid was poorly accumulated, as was its analog 7-epicholic acid. This finding supports the principle that optimal intestinal absorption is a prerequisite for good accumulation, even for a molecule such as 6-methyl-7-epicholic acid which is not likely to be metabolized. Indeed, in a previous work, we showed that there is a critical lipophilic value for passive absorption (51).

In conclusion, the results of our study show the following. 6-MUDCA is very resistant to bacterial dehydroxylation; it is amidated by the liver and accumulates in the bile in this form. While its biliary accumulation is higher than that of UDCA administered at same dose, it is not as high as we expected. This is probably due to the rate of passive intestinal absorption of 6-MUDCA which is slowed by its relatively low lipophilicity. It may be supposed that, once amidated, the glycine and taurine forms would also not be efficiently absorbed, in this case by active transport. While species differences limit our ability to predict the behavior of this new analog in human bile, other properties demonstrated by 6-MUDCA in this study suggest that it has considerable potential as a pharmaceutic agent for use in humans. In particular, when compared with UDCA, 6-MUDCA shows a faster in vitro dissolution rate of cholesterol monohydrate, an obvious advantage for a cholesterol gallstone dissolving agent, and also higher cholesterol holding capacity.

6-Methyl-7-epicholic acid, like 6-MUDCA, is a bacteria-resistant molecule; however, despite the fact that acute studies in the rat and hamster give results similar to 6-MUDCA, chronic studies show that it is not well accumulated in the bile. This may be due to poor absorption of its glycine and taurine forms; whatever the reason, poor accumulation in the bile precludes its application as an orally administered drug.

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