Physicochemical and biological properties of natural and synthetic C-22 and C-23 hydroxylated bile acids

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Abstract In order to define the effect of a side chain hydroxy group on bile acid (BA) physicochemical and biological properties, 23-hydroxylated bile acids were synthesized following a new efficient route involving the α-oxygenation of silylalkenes. 22-Hydroxylated bile acids were also studied. The synthesized bile acids included R and S epimers of 3α,7α,23-trihydroxy-5β-cholan-24-oic acid (23R epimer: phocaecholic acid), 3α,12α,23-trihydroxy-5β-cholan-24-oic (23R epimer: bitocholic acid), and 3α,7β,23-trihydroxy-5β-cholan-24-oic acid. A 3α,7α,22-trihydroxy-5β-cholan-24-oic acid (haemulcholic acid) was also studied. The presence of a hydroxy group on the side chain slightly modified the physicochemical behavior in aqueous solution with respect to common BA: the critical micellar concentration (CMC) and the hydrophilicity were similar to naturally occurring trihydroxy BA such as cholic acid. The pKa value was lowered by 1.5 units with respect to common BA, being 3.8 for all the C-23 hydroxy BA. C-22 had a higher pKa (4.2) as a result of the increased distance of the hydroxy group from the carboxy group. When the C-23 hydroxylated BA were intravenously administered to bile fistula rats, they were efficiently recovered in bile (more than 80% unmodified) while the corresponding analogs, lacking the 23- hydroxy group, were almost completely glycine- or taurine-conjugated. On the other hand, the C-22 hydroxylated BA were extensively conjugated with taurine and less than 40% of the administered dose was secreted without being conjugated. In the presence of intestinal bacteria, they were mostly metabolized to the corresponding 7-dehydroxylated compound similar to common BA with the exception of bitocholic acid which was relatively stable. The presence of a hydroxy group at the C-23 position increased the acidity of the BA and this accounted for poor absorption within the biliary tree and efficient biliary secretion without the need for conjugation. 3α,7β-23 R/S trihydroxy-5β-cholan-24-oic acids could improve the efficiency of ursodeoxycholic acid (UDCA) for gallstone dissolution or cholestatic syndrome therapy, as it is relatively hydrophilic and efficiently secreted into bile without altering the glycine and taurine hepatic pool. —**Roda, A., B. Grigolo, A. Minutello, R. Pellicciari, and B. Natalini.** Physicochemical and biological properties of natural and synthetic C-22 and C-23 hydroxylated bile acids. *J. Lipid Res.* 1990. **31:** 289–298.

Supplementary key words bile acid structure • activity relationship • bile acid analogs • cholesterol gallstones

Unconjugated bile acids (BA) are synthesized by the liver cells from cholesterol (CHOL) via steroid hydroxylating steps; they are subsequently amidated on the side chain with glycine and taurine and efficiently secreted into bile.

These metabolic pathways impart bile acid molecules with an optimum lipophilicity that is responsible for efficient biliary secretion and hepatic and intestinal uptake, and prevent their passive absorption in the biliary tree (1–4). Hepatic synthesis from cholesterol, conjugation, intestinal deconjugation, and the dehydroxylation process are in part species-dependent (5) and few vertebrates have similar bile acid compositions (6). Continuous improvements in analytical methodology have allowed identification and confirmation of the structure of many unknown bile acids partially isolated in the past from the bile of many animals. Haslewood (7), in his pioneer studies, collected a great deal of information on different animal species and stimulated studies of comparative physiology to account for these species differences. Moreover, the use of naturally occurring bile acids such as ursodeoxycholic acid (UDCA) to dissolve cholesterol gallstones, has stimulated medicinal, chemical, and biochemical studies aimed at identifying the mechanism of action of these drugs and has aided in the design of new synthetic analogs with enhanced biological activity (8). New BA analogs have been developed by introducing into the steroid molecule substituents able to prevent some metabolic pathways and hence increase their bioavailiability and biological halflife (9–16).

Abbreviations: BA, bile acids; CHOL, cholesterol; UDCA, ursodeoxycholic acid; EHC, enterohepatic circulation; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LTA, lead tetraacetate; CD, circular dichroism; CMC, critical micellar concentration; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography.

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In recent years our interest has been directed toward the study of the BA side chain in order to identify the relationship between structure and biliary secretion and conservation in the enterohepatic circulation. In previous studies we reported the synthesis, physicochemical and biological properties of 3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic acid (9–12) and 3α,7β-dihydroxy-23-methyl-5β-cholan-24-oic acid (13). These studies disclosed some structural requirements for the biliary secretion of unconjugated BA and elucidated the role of conjugating enzymes on liver uptake and biliary secretion (13, 17, 18).

For many naturally occurring BA the amidation step is a prerequisite for efficient biliary secretion. All unconjugated steroid dihydroxy BA, being lipophilic, can be passively absorbed in the biliary tree, causing hypercholeresis and delayed biliary secretion (19, 20). This is a dose-dependent phenomenon, a function of substrate specificity for conjugating enzymes (CoA derivative formation) and relies on the available hepatic glycine and taurine pool. BA that cannot be conjugated, such as two diastereoisomers of 22,23-methylene-3α,7β-dihydroxy-5β-cholan-24-oic acid and 3α,7β-dihydroxy-23-methyl-5β-cholan-24-oic acid or nor-bile acids (13, 14, 19, 20), are not secreted as such into bile and alternative conjugating processes such as glucuronidation or sulfation take place. Thus any substituents able to increase the polarity or the ionization of the BA molecules could facilitate their biliary secretion. However, if the molecule is too hydrophilic it cannot be passively or actively absorbed by the intestine, given the poor molecular specificity of the ileal transport system, resulting in a loss from the enterohepatic circulation. These alternative metabolic pathways act as an excretory function and allow us to identify in the intestinal uptake and transport the major determinants in the conservation of a BA molecule in the enterohepatic circulation. The development of a bile acid with efficient ileal transport and poor metabolism could be an ideal strategy for improving the bioavailability of bile acids. The rationale of the present study was to identify a BA that behaves like an conjugated BA in terms of physicochemical properties in aqueous solutions, and one that is sparingly metabolized by the liver and still efficiently transported by the ileum.

As a further study of the role of BA side chain substituents in biliary lipid secretion and conservation in the EHC, we here report the physicochemical and biological properties of new side chain hydroxylated BA: the 22-hydroxy derivative of chenodeoxycholic acid (CDCA), the 23-hydroxy derivatives of chenodeoxycholic acid and deoxycholic acid (DCA) (R and S epimers), and 3α,7β-trihydroxy-5β-cholan-24-oic acid. The synthesis of R and S iosmers and their separation and structure assignment have been described previously (21).

The (22S) 3α,7α,22-trihydroxy-5β-cholan-24-oic acid (haemulcholic acid), (23R) 3α,7α,23-trihydroxy-5β-cholan-24-oic acid (phocaecholic acid), and (23R) 3α,12α,23-trihydroxy-5β-cholan-24-oic acid (bitocholic acid) are natural BA previously extracted from animal bile (22, 23). Haslewood (7) in 1961 isolated C-22 and C-23 hydroxylated bile acids from different animal species and postulated that these bile acids (major costituents of bile) are synthesized by the liver, which presumably contains a side chain-hydroxylating enzyme system.

In the present study the major physicochemical properties in aqueous solution were evaluated and compared with analogs with a similar ring structure but lacking the side chain hydroxy group. From the physicochemical point of view, the presence of a hydroxy group on the side chain in α or β position to the C-24 carboxylic moiety has a withdrawing effect which increases the degree of ionization of the carboxy group. Moreover, the nature of the side chain is altered in terms of charge and geometry so that the interaction of these bile acids with membranes, enzymes, and receptors with respect to natural bile acids will differ. Their metabolism and effect on biliary lipid secretion was investigated by infusing these bile acids intravenously in bile fistula Sprague-Dawley rats, which do not contain these bile acids in bile and do not physiologically hydroxylate on the side chain.

MATERIALS AND METHODS

Bile acids

The side chain-hydroxylated BA that were synthesized and studied were: (22S) and (22R) 3α,7α,22-trihydroxy-5β-cholan-24-oic acid, (23S) and (23R) 3α,7α,23-trihydroxy-5β-cholan-24-oic acid, (23S) and (23R) 3α, 7β-23 trihydroxy-5β-cholan-24-oic acid (**Fig. 1**). These BA were synthesized and the absolute configurations of the side chain were assigned as previously reported (21). Chenodeoxycholic acid, ursodeoxycholic acid, deoxycholic acid, and cholic acid were obtained from Calbiochem-Boehring, San Diego, CA.

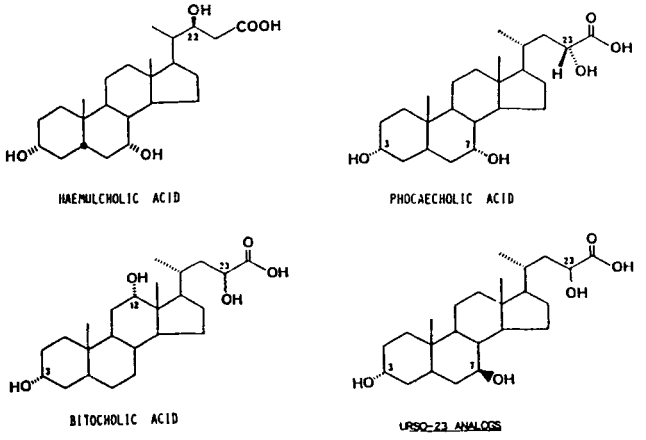

Fig. 1. Structure of side chain-hydroxylated bile acids; C-22 or C-23 R and S epimers were also studied. (see text).

Chemistry

The preparation of (23R) 3α,7α,23-trihydroxy-5β-cholan-24-oic acid {4a, phocaecholic acid}, (23R)-3α,12α,23-trihydroxy-5β-cholan-24-oic acid {4b, bitocholic acid}, and their (23S) epimers {5a} and {5b}, respectively, was achieved by means of a new efficient route to 23-hydroxylated bile acids involving α-oxygenation of the corresponding trimethyl silyl ketene acetals. Among several procedures investigated, the lead tetra acetate (LTA) oxidation of the ketene silyl acetal was found to be the method of choice for the preparation of the above compounds (**Fig. 2**). The conversion of chenodeoxycholic acid {1a} into phocaecholic acid {4a} and its 23S epimer {5a} exemplifies the sequence. Treatment of chenodeoxycholic acid methyl ester {1b} with lithium-diisopropylamide followed by addition of trimethyl silyl chloride quantitatively afforded the ketene silyl acetal {2} which by sequential treatment with LTA and triethylammonium fluoride was converted into the corresponding α-acetoxy ester {3} in 91% yield as a nearly equal inseparable mixture of the two C-23 epimers. The key step in the separation of these two epimers was the selective hydrolysis of the 23-acetoxy derivative {3}. Thus, ultrasonic irradiation of a solution of {3} in potassium carbonate-saturated methanol afforded a mixture of (23R)- and (23S)-monocathylates, {4c} and {5c}, respectively, which could be separated by medium pressure chromatography. Alkaline hydrolysis of the two monocathylates then afforded phocaecholic acid {4a} and its (23S) epimer {5a} (Fig. 2).

The same synthetic route was also applied to the conversion of deoxycholic acid {1c} into bitocholic acid {4b}. By submitting the two C-23 epimers, (23R. 3α,12α,23-trihydroxy-5β-cholan-24-oic acid {4b} and (23S) 3α,12α,23-trihydroxy-5β-cholan-24-oic acid {5b} to circular dichroism (CD) and ^{13}C NMR analyses, the configuration at C-23 of the naturally occurring bitocholic acid {4b} was conclusively established as R.

In particular, CD spectra showed that the methyl ester of bitocholic acid had a large negative Cotton effect, indicating a 23R (α_F) configuration for this bile acid, while the 23S (β_F)-epimer exhibited a curve opposite in sign, in agreement with the known behavior of analogous α-hydroxycarboxylic acids.

Furthermore, the (23R) carbon atoms of phocaecholic acid methyl ester {4d} and bitocholic acid methyl ester {4e} exhibited a more shielded chemical shift (68.40 and 68.43 ppm, respectively) than the (23S) carbon atoms of the corresponding epimers {5d} and {5e} (69.90 and 70.14 ppm, respectively). This difference, which can be attributed to a parallel 1,3 interaction of the 23S-hydroxy group with C-17, with a consequent decrease in the rotameric population in the S-epimers, provides a useful, general method for the correct attribution of the absolute configuration of the 23-CH group in the BA side chain. The preparation of (23R) and (23S) 3α,7β,23-trihydroxy-5β-cholan-24-oic acids was performed in the same way. Selected physicochemical properties of the four 23-hydroxy bile acids studied are reported in **Table 1**.

(22S) and (22R) 3α,7α,22-trihydroxy-5β-cholan-24-oic

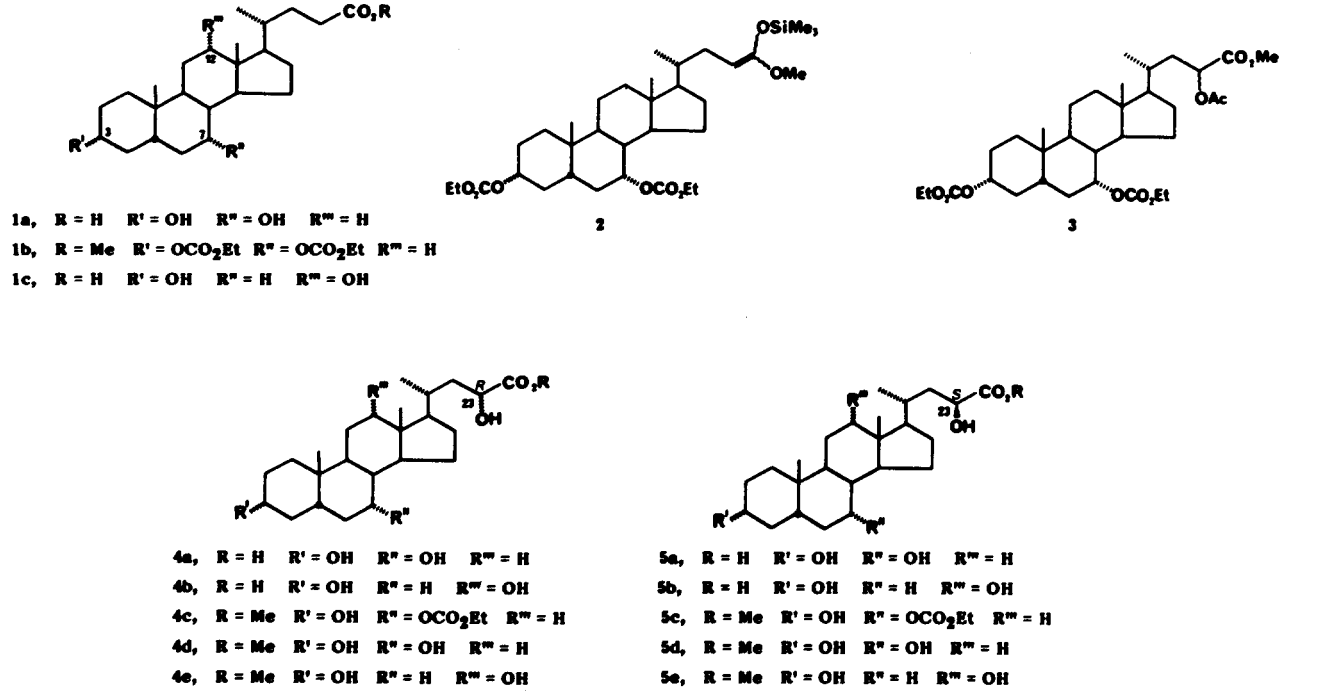

Fig. 2. Schematic representation of the bile acid derivatives used for the preparation of the side chain-hydroxylated bile acids.

TABLE 1. Selected physicochemical properties of the four 23-hydroxy bile acids

Bile Acid	OH Position	Melting Point °C	$[\alpha]_D^{20}$ (EtOH)	Polarity on SiO_2	Sign on CD
4a	3α,7α,23R	225	+ 21 (c = 1.06)	less polar	–
5a	3α,7α,23S	215	+ 15 (c = 1.00)	more polar	+
4b	3α,12α,23R	230	+ 48 (c = 0.85)	less polar	–
5b	3α,12α,23S	225	+ 38 (c = 1.80)	more polar	+

acids were synthesized by two convergent syntheses: the former was related to the reduction of α-diazo-β-hydroxy ester prepared by condensation of the bis-nor aldehyde, derived from chenodeoxycholic acid, with ethyl (lithio) diazoacetate. The key step in the latter involved the rhodium acetate dimer conversion of the same α-diazo-β-hydroxy ester into the corresponding β-keto ester.

Experimental design

The experimental design of the study was similar to that previously described for other natural and side chain-modified BA (9, 11, 14). This includes evaluation of the physicochemical properties in aqueous solution (water solubility, critical micellar concentration, acidity and hydrophylicity). Subsequently these BA were intravenously administered to bile fistula rats and their hepatic metabolism and the effect on biliary lipid secretion were evaluated. Finally in vitro studies were performed to assess the stability of the BA in the presence of intestinal bacteria containing 7-dehydroxylase and deconjugating enzymes (intestinal metabolism). These data were compared with those obtained with BA analogs lacking the hydroxy group on the side chain (see Table 2) with similar steroid structure and with BA with three hydroxy groups on the steroid ring.

Physicochemical properties

Critical micellar concentration. The CMCs were measured in water by the dye solubilization method with Orange OT as a water-insoluble dye (24). Different bile salt solutions were prepared in a concentration ranging from 0.1 to 100 mmol/l, and the pH was adjusted to 8 with sodium hydroxide. A sample of the final solution was analyzed for bile acid content by an enzymatic method using 3α-hydroxysteroid dehydrogenase (25). The sodium content was measured by atomic absorption spectroscopy. Bile salt solutions were rotated for 1 day at room temperature with an excess of crystalline dyes. The solutions were then filtered through a 0.22 μm Millipore filter, and absorbance was recorded at 490 nm and plotted against bile salt concentration.

Water solubility. BA (0.2 mmol) were suspended in 100 ml of 0.01 M $HClO_4$ (26). The solutions were refluxed for 24 h in order to achieve equilibrium. Two-ml aliquots of the saturated solutions were transferred to a thermostatted water bath 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 3α-hydroxysteroid dehydrogenase (25).

Hydrophilicity. The hydrophilicity of bile salts was measured by reverse phase high performance liquid chromatography (HPLC) using a Waters Inc. (Milford, MA) Liquid Chromatograph. A C-18 reverse phase column, 5 μm pore size and 10 cm length, was used. The analyses were carried out under isocratic conditions. As a mobile phase a mixture of 2-propanol–water 8:17 (v/v), pH 7, was used to ensure ionization of all BA. A retention factor (K') was calculated from the relative mobilities of the separated bile acids (27) using the formula:

$$K' = \frac{t_v - t_0}{t_0} \qquad \textit{Eq. 1}$$

where t_0 = retention of the solvent from and t_v = retention time of bile salts.

Acidity. Acidicity 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 (28). All the measurements were carried out at 25 ± 0.1°C.

In vivo studies

Hepatic metabolism and biliary secretion. BA were administered to male Sprague-Dawley rats (300–330 g). The rats 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 at a dose of 2 μmol/min per kg (five rats in each group) over 1 h, and then bile was collected for 2 h at 15-min intervals.

The bile flow was determined gravimetrically and the concentrations of bile acids, cholesterol, and phospholipids in the samples were determined by enzymatic methods

(25, 29, 30). BA compositions were determined by an HPLC method (31) and by TLC on silica gel G plates, 250 μm thickness. In particular, a known amount of bile (2–5 μl) was spotted onto TLC plates and after development, the plate was immersed in a 5% phosphomolybdic acid in ethanol solution, removed, and heated at 120°C for 10 min. BA were then quantified by densitometry using a CAMAG TLC-HPTLC 76500 scanner. The method is accurate and sensitive, and the precision was satisfactory (CV less than 8%).

The conjugated BA (glycine/taurine) were separated from the corresponding unconjugated BA by TLC using a solvent system composed of propionic acid–isoamyl acetate–water–1-propanol 15:20:5:1 (v/v/v/v). Biliary lipid secretion, calculated from the volume of secreted bile and from the biliary lipid concentration, was expressed as μmol/min per kg.

In vitro studies

Bacterial 7-dehydroxylase: substrate specificity. Fresh human stools were homogenized with saline–water 1:1 (v/v) under a nitrogen stream, and 500 ml was transferred into sterile vials to which 5 ml of sterilized chopped meat–glucose medium (Scott Lab., Fiskeville, RI) was added. BA were then added to this medium 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 Dicson Co., Orangeburg, NY). The incubations were carried out at 37°C and 1,2,4,8 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, and 2 ml of the supernatant was transferred into a tube to which 8 ml of 0.1 M NaOH was added.

The solutions were applied to a C-18 Bond-Elut cartridge which was eluted at a flow rate of 1 ml/min and washed with 10 ml of water; the BA were subsequently 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 then separated by using TLC and HPLC. 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). The analysis was carried out under isocratic conditions at a flow rate of 0.3 ml/min using a UV detector at 200 nm.

Expression of the data

The secretion rates of bile (SVo, μl/min per kg) and the secretion of BA (SBA), cholesterol (SCHOL), and phospholipids (SPL) expressed as μmol/min per kg were calculated. The effect of the intravenously administered BA on these parameters was expressed as maximum secretion rates:

$$S = \frac{S_{15} + S_{30} + S_{45} + S_{60}}{4} \qquad Eq.\ 2$$

where S_{15}–S_{60} are the secretion rates at different times during 60 min of intravenous infusion. S was calculated for bile (SVo), bile acids (SBA), cholesterol (SCHOL), and phospholipids (SPL).

RESULTS

Physicochemical properties

Table 2 shows the physicochemical properties in water for the C-22 and C-23 hydroxylated bile acids in comparison with unconjugated bile acids with similar steroid structure. The presence of a hydroxy group close to the C-24 carboxylic moiety greatly affects the dissociation constant of the molecule. The thermodynamic pKa of the 23-hydroxy bile acid is significantly lower than that of normal side chain bile acids, 3.8 for both phocaecholic acid and bitocholic acid and 5.07 and 5.02, respectively, for the parent compounds chenodeoxycholic acid and deoxycholic acid. The withdrawing effect of a C-22 hydroxy group, in the β position to the carboxy group is lower, but a significant reduction of the pKa of haemulcholic acid (4.2) was found. There were no differences between the R and S isomers for the side chain-hydroxylated BA that were studied. As far as the hydrophilicity is concerned, the presence of one more hydroxy group on the molecule generally increased its hydrophilic character with respect to the parent compound lacking the hydroxy group on the side chain and with similar steroid structure.

If we consider, for example, chenodeoxycholic acid, the increased hydrophilicity of haemulcholic and phocaecholic acid was similar to that due to the introduction of one more hydroxy group on the steroid nucleus (see cholic acid) geometrically oriented toward the α-face of the molecule. The hydrophilicity was slightly higher when the hydroxy group was in C-23 position compared to the C-22 position.

The C-22 and C-23 hydroxylated BA had CMC values only slightly higher than those shown by trihydroxylated BA and self-aggregated to form micelles at a concentration similar to that of other physiological BA. Moreover, the CMC was lower with respect to other trihydroxylated bile acids such as 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid but similar to 3α,7α,12α-trihydroxy or 3α,6α,7α-trihydroxy bile acids.

Finally, the water solubility of the protonated species was in the range of other steroid hydroxylated bile acids and 5–10 times higher than the corresponding dihydroxylated BA. No significant differences between the R and S isomers were found (Table 2).

TABLE 2. Physicochemical properties of side chain-hydroxylated bile acids in comparison with ring-hydroxylated bile acids

Bile Acid					
Position of Substituents	Trivial Name	S	CMC	K'	pKa
		μM	μM		
Dihydroxylated					
3α,7α	chenodeoxycholic	27	9	2.05	5.07
3α,7β	ursodeoxycholic	9	19	0.95	5.08
3α,12α	deoxycholic	28	10	2.8	5.02
Trihydroxylated: nucleus					
3α,6α,7α	hyocholic	45	17	1.1	5.03
3α,7α,12α	cholic	235	13	1.08	5.08
3α,7β,12α	ursocholic	1670	60	0.26	5.06
Trihydroxylated: nucleus and side chain					
3α,7α,22S	haemulcholic	79	14	1.17	4.2
3α,7α,22R		138	12	1.04	4.2
3α,7α,23R	phocaecholic	250	12	0.95	3.8
3α,7α,23S		180	13	0.91	3.8
3α,12α,23R	bitocholic	201	10	1.14	3.8
3α,12α,23S		90	12	1.08	3.8
3α,7β,23R/S	ursophocaecholic (synthetic)	20	48	0.32	3.8

Physicochemical data for common naturally occurring bile acids have been reported previously (see references 3, 14, 24, 26, and 28). Abbreviations: S, solubility; CMC, critical micellar concentration; K', hydrophilicity (C-18 HPLC).

Biliary lipid secretion

At the doses administered, the BA were efficiently taken up by the liver (the single pass hepatic extraction was similar for unconjugated and glycine-conjugated BA; A. Roda et al., unpublished data) and promptly secreted into bile. The biliary recovery and hepatic metabolism of these BA differed and **Fig. 3** shows the chemical forms recovered in bile during the experiments. While chenodeoxycholic acid, cholic acid, and ursodeoxycholic acid at the administered dose were recovered 70–80% conjugated with taurine and/or glycine, C-23 hydroxylated bile acids were mainly secreted into bile as such without extensive conjugation with glycine or taurine (less than 20%). They were efficiently recovered in bile; a few minutes after infusion they appeared in bile and rapidly disappeared once the infusion was stopped (**Fig. 4**). On the other hand, C-22 hydroxylated bile acids were secreted more than 60% conjugated with taurine and only 40% unconjugated (Fig. 3).

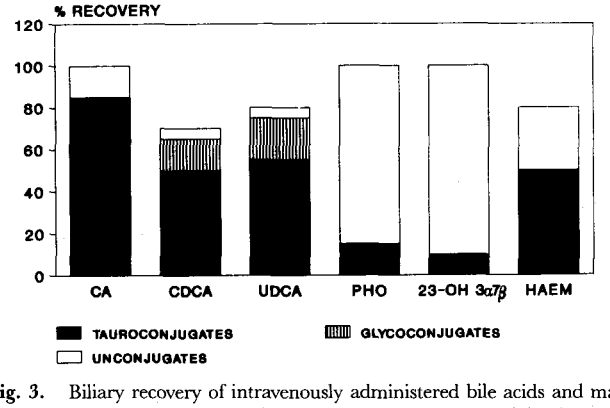

Fig. 3. Biliary recovery of intravenously administered bile acids and major metabolites recovered over 3 h expressed as a percentage of the dose administered; PHO, phocaecholic acid; 23-OH 3α,7β, 3α,7β-23 R/S trihydroxy-5β-cholan-24-oic acid.

Fig. 5 shows the effect of the intravenous infusion of phocaecholic and 3α,7β-23 R/S trihydroxy-5β-cholan-24-oic acids on bile flow in comparison with cholic and ursodeoxycholic acids. All BA increased bile flow as a function of their recovery in bile and the choleresis was higher for 3α,7β-23 R/S derivatives with respect to that of cholic, ursodeoxycholic, and phocaecholic acids. **Table 3** summarizes the secretion parameters of the BA expressed as SVo, SBA, SCHOL, and SPL (see equation 2). The mean maximum secretion rate of bile was significantly higher for 3α,7β-23 R/S trihydroxy-5β-cholan-24-oic derivative while no statistically significant differences existed among side chain-hydroxylated BA and other BA. Only CDCA and to a lesser extent phocaecholic acid affected (reduced) cholesterol secretion. There were no significant differences in maximum secretion rates of phospholipids.

In vitro studies

The C-23 and C-22 hydroxylated BA were 7-dehydroxylated with kinetics similar to those of chenodeoxy-

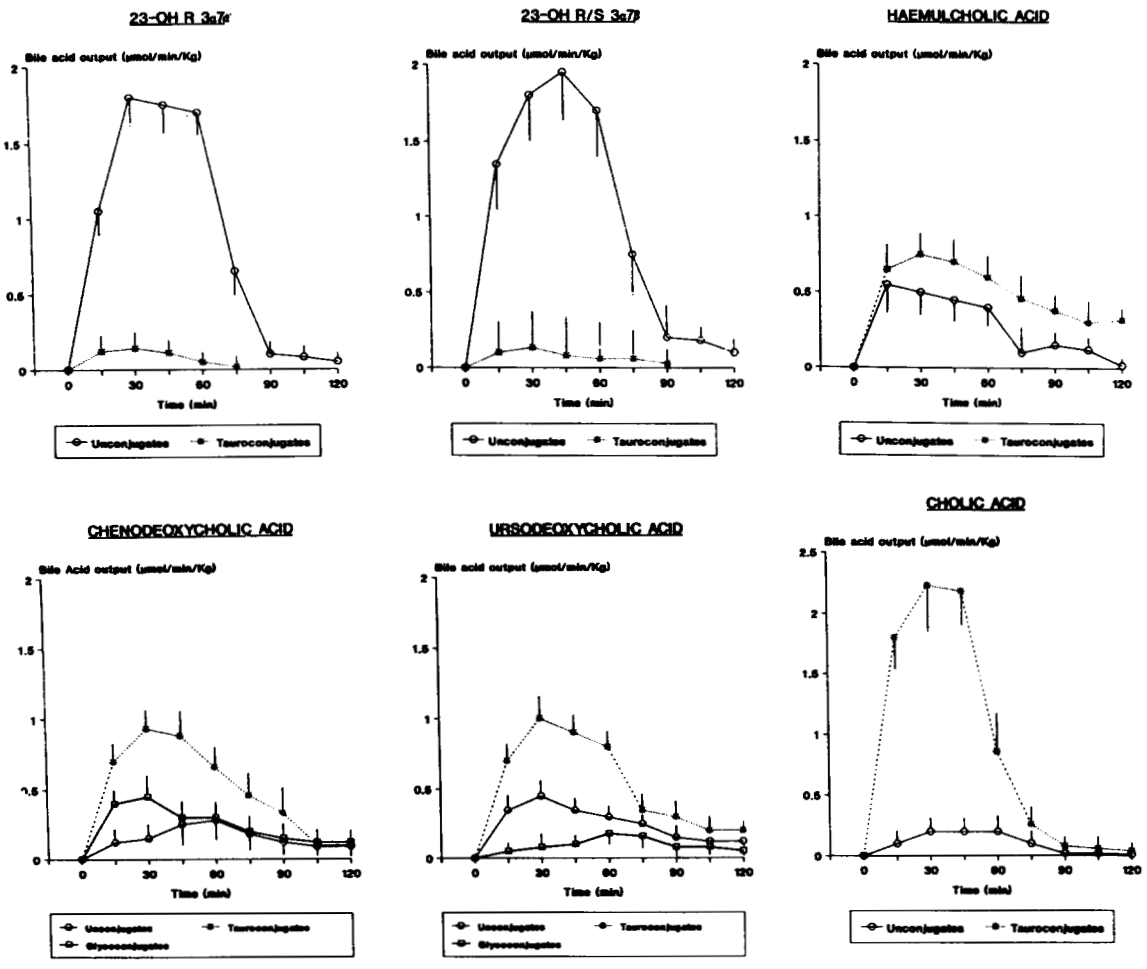

Fig. 4. Chemical forms recovered in bile after intravenous administration (2 μmol/min per kg over a period of 1 h) of side chain-hydroxylated bile acids and common bile acids in rat.

cholic acid and cholic acid and no major metabolites other than the corresponding 7-dehydroxylated BA were present. The 3α,12α analogs were quite stable and major metabolites such as side chain-dehydroxylated BA did not form, at least using our analytical tools (**Fig. 6**).

DISCUSSION

Physicochemical properties in aqueous solution

The experimental data suggest that the introduction of a hydroxy group on the side chain slightly modifies the properties of the BA with respect to other naturally occurring BA lacking a hydroxy group on the side chain. The detergent-like properties are quite similar; they still self-aggregate to form micelles at CMC values similar to trihydroxylated BA such as cholic acid. Moreover, the experimental data suggest that their structure is favorable for good self aggregation. The introduction of a hydroxy group on the side chain is less perturbing in terms of hydrophilic/hydrophobic balance than ring hydroxylation, in which a hydroxy group is oriented toward the β-face side of the molecule such as 3α,7β, or 3α,6α, in which the 7β and 6α hydroxyl is equatorial and inserted on the hydrophobic back of the molecule.

The most significant difference existing between these side chain-hydroxylated BA and the parent unconjugated compounds is the acidic character. The presence of a hydroxy group close to the carboxylic moiety plays a major role in determining the pKa of the BA. The electron withdrawing character of the hydroxy group considerably increases ionization and lowers the pKa by about 1.5 units. Acidity decreases with the distances of the hydroxy group from the carboxy group, being 4.2 for C-22 hydroxylated BA and 3.8 for the C-23 hydroxylated BA and 5 for common BA.

An interesting observation is that the acidity of the C-23 hydroxylated BA is similar to glycine-amidated BA (3.8) and this agrees with many biological properties observed (see below). The water solubility of the protonated

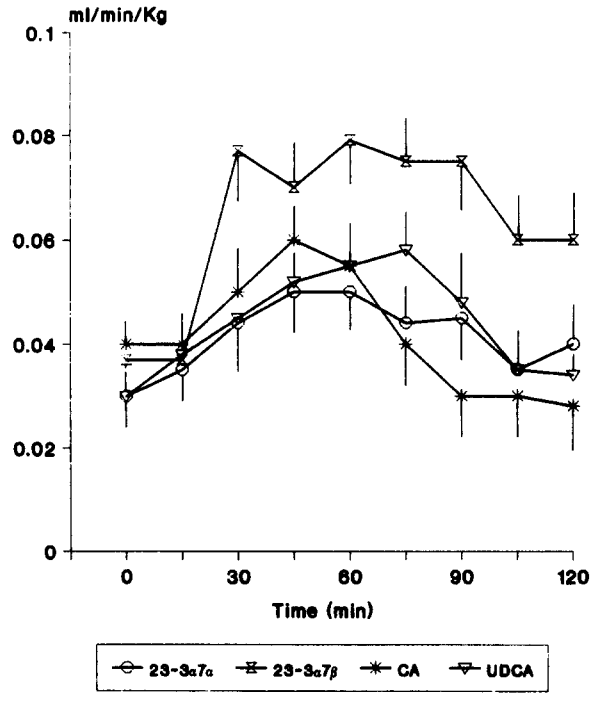

Fig. 5. Effect of side chain-hydroxylated bile acids on bile flow in comparison to cholic acid ursodeoxycholic acid; 23-3α,7α,phocaecholic acid; 23-3α,7β, 3α,7β-23 R/S trihydroxy-5β-cholan-24-oic acid.

form and lipophilicity agree with the expected values and are in line with other bile acids. Usually trihydroxylated BA with one hydroxy group on the side chain do not reach the hydrophilicity observed by some trihydroxylated BA such as 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid. This is due to the fact that the hydroxy group on the side chain does not modify the hydrophobic area of the steroid ring.

Biliary secretion

The unexpected efficient recovery in bile of C-23 and, to a lesser extent, C-22 BA seems directly related to the physicochemical characteristics, particularly acidity, which determine the extent of ionization at a given pH. The C-23 BA is recovered more than 80% unmodified and conjugated with taurine only to a small degree. On the other hand, the C-22 BA is recovered 40% unmodified and 60% conjugated with taurine and glycine. The analogs without the hydroxy group on the side chain were recovered in bile more than 90% conjugated with taurine and glycine and only 10% unconjugated. Thus the presence of a C-23 hydroxy group increases ionization of the molecule at the hepatic pH with respect to common unconjugated BA with a rise in the molecule's polarity. According to previously reported data for nor-bile acids and their corresponding amidated forms, this may account for a possible poor passive absorption in the biliary tree and consequently for efficient secretion into bile (13-17, 19).

It is noteworthy that a hydroxy group at the C-23 position mimics the amidation process at least with glycine, pKa values being quite similar. The mechanism of their biliary secretion may resemble that of glycine-conjugated BA, following the same pathway, even if other mechanisms cannot be excluded. On the other hand, C-22 BA behave slightly differently, the conjugation with glycine and taurine being a prerequisite for efficient secretion into bile; the pKa is somewhat higher (4.2), reaching values similar to common unconjugated BA such as UDCA or CDCA, which require amidation to be secreted. Thus, the polarity of such molecules, particularly 23-hydroxy BA, is quite similar to glycine-conjugated BA, but differences exist in the detergency; C-23 have less detergent properties than the glycine-conjugated natural analogs: 3α,7α,23-hydroxy versus glycochenodeoxycholic acid and 3α,12α,23-hydroxy versus glycodeoxycholic acid. When the glycine-conjugated BA were administered to rats at the same dose, the animals died, due to the high toxicity of these two BA in the rat (14). These BA are 7-dehydroxylated by human intestinal bacteria similarly to common and poorly side chain-dehydroxylated BA.

TABLE 3. Effect of 23-hydroxylated bile acids on biliary lipid secretion in comparison to ring-hydroxylated bile acids[a]

Bile Acid	SVO	SBA	SPL	SCHOL
	μl/min/kg		*μmol/min/kg*	
Phocaecholic R	42 ± 5	1.51 ± 0.2	0.17 ± 0.01	0.012 ± 0.002
3α,7α,23-OH S	43 ± 6	1.37 ± 0.3	0.24 ± 0.03	0.018 ± 0.001
3α,7β,23-OH R/S	80 ± 9	1.87 ± 0.2	0.27 ± 0.04	0.022 ± 0.002
CDCA	40 ± 3	1.40 ± 0.1	0.19 ± 0.01	0.009 ± 0.004
UDCA	54 ± 7	1.93 ± 0.2	0.30 ± 0.04	0.020 ± 0.002
CA	58 ± 6	2.98 ± 0.4	0.42 ± 0.05	0.034 ± 0.003

Abbreviations: S = mean maximum secretion of: VO, bile; BA, bile acids; PL, phospholipids; CHOL, cholesterol.
[a]Bile acids were administered at a dose of 2 μmol/min · kg^{-1}; values are given as mean ± 2 SD.

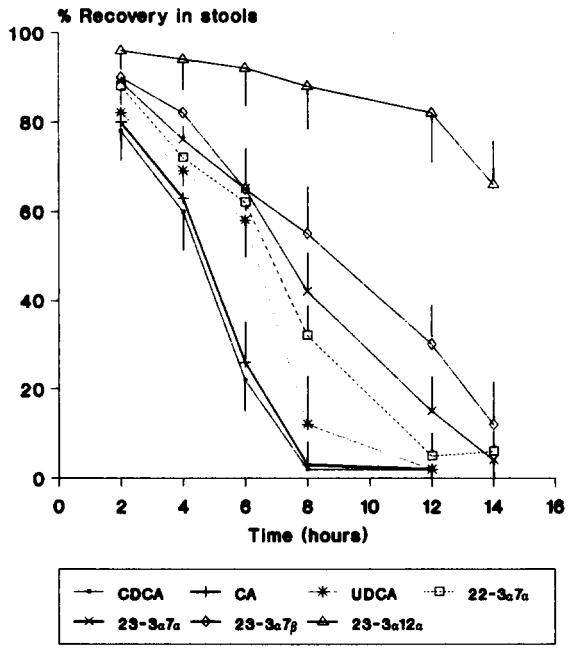

Fig. 6. Kinetics of in vitro metabolism of the C-22 and C-23 hydroxylated BA in human stools under anaerobic conditions. The results are expressed as the percentage of unmetabolized compound recovered in stools and are the mean of five sets of experiments ± SD. The major metabolite of the 7-hydroxy BA is the corresponding 7-dehydroxylated derivative; 22-3α,7α, 3α,7α-22 R/S trihydroxy-5β-cholan-24-oic acid; 23-3α,7α, 3α,7α-23 R/S trihydroxy-5β-cholan-24-oic acid; 23-3α,7β, 3α,7β-23 R/S trihydroxy-5β-cholan-24-oic acid; 23-3α,12α, 3α,12α-23 R/S trihydroxy-5β-cholan-24-oic acid.

The above data suggest interesting properties for these compounds from the point of view of medicinal chemistry. The C-23 hydroxylated synthetic analog of UDCA offers the following potential advantages over natural compounds. *a*) It is more hydrophilic and less detergent than UDCA and glycine-conjugated UDCA, which are the major metabolites that accumulate in bile after chronic UDCA feeding. The final result is that the toxicity of the bile acid pool in the presence of an appreciable amount of these BA could be reduced. *b*) The C-23 hydroxylated BA, when administered orally, does not require conjugation in order to be secreted into bile and, consequently, does not modify the endogenous pool of glycine as occurs when UDCA is chronically adminstered to human subjects. The final result will be that the glycine forms over the taurine one do not increase as observed during UDCA feeding from 3/1 to 10/1. With respect to the first series of studies on side chain UDCA analogs, which included BA with steric hindrance (23-methyl analogs) or steric plus electronic hindrance (22-23 cyclopropane analogs), the side chain-hydroxylated analogs are a further improvement. Many of the first analogs studied were poorly secreted into bile and were metabolized to polar compounds that were lost from the enterohepatic circulation. On the other hand, the 23-hydroxylated analogs were efficiently secreted into bile unmodified.

More extensive chronic studies are underway to demonstrate the extent to which these BA must accumulate in bile to act as a cholelitholytic drug or to reduce the "detergency" of the BA pool.

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