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# The Occurrence of 7β-Hydroxylated Bile Acids in Human Bile

## Bile Acids and Steroids 76

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Ursodeoxycholic acid has been crystallized from human duodenal contents and identified by means of chromatographic properties, melting point, sulphuric acid spectra and infrared spectra. Column and paper chromatographic analyses and sulphuric acid spectra have indicated the presence of 3α,7β,12α-trihydroxycholanic acid in some human bile samples.

During a study of the quantitative relationships between bile acids in human bile, it was found that, besides the glycine and taurine conjugates of cholic, chenodeoxycholic and deoxycholic acids, some bile samples contained other compounds which behaved like bile acids on paper chromatograms [1]. The present paper gives some results regarding the nature of these compounds.

## EXPERIMENTAL

The bile acid analyses were done either with gallbladder bile (collected during abdominal operations) or with duodenal contents (collected through a polyvinyl tubing introduced through the nose).

Paper chromatography of bile, duodenal contents and purified bile acids was carried out according to Sjövall [2].

Reversed-phase partition chromatography according to Sjövall [3] and Norman [4] was used for the purification of the bile acids present in the biological material.

The following phase systems were used:

| Phase system | Moving phase (ml) | Stationary phase (ml) |
|---|---|---|
| F 1 | Methanol/water 165/135 | Chloroform/heptane 45/5 |
| C 1 | Methanol/water 150/150 | Chloroform/*iso*octanol 15/15 |
| C 2 | Methanol/water 144/156 | Chloroform/*iso*octanol 15/15 |

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When the conjugated bile acids were to be separated, about twenty volumes of ethanol were added to the bile or duodenal contents, respectively, and after filtration, the clear ethanol solution was evaporated to dryness *in vacuo*. The residue was dissolved in 1 N sodium hydroxide and, after acidification, the bile acids were extracted with butanol [5]. When the free bile acids were to be analyzed, the bile or the duodenal contents was hydrolyzed at 120°C for 6 h in 2 N sodium hydroxide. After acidification, the bile acids were extracted with ether.

Sulphuric acid spectra were recorded with a self-recording spectrophotometer (Perkin-Elmer, Spectracord 4 000) after dissolving the bile acid samples in 65 % sulphuric acid (20—40 μg per ml) and heating them for 15 min at 60°C.

## RESULTS

*A. 3α,7β-Dihydroxycholanic acid (ursodeoxycholic acid).* Descending paper chromatography of bile and duodenal contents, with ethylene chloride/heptane 50/50 as moving phase and 70 % acetic acid as stationary phase, reveals the presence of glycocholic (GC), glycochenodeoxycholic (GCD) and glycodeoxycholic (GD) acids in human bile. In several samples, a fourth spot which is weaker than the other ones can be detected between the spots of GC and GCD. In order to purify the compound causing this spot, the conjugated bile acids from about 10 ml of gallbladder bile (patient N.A.) were subjected to reversed-phase partition chromatography with phase system C 1. The effluent from the column was collected by means of a fraction collector and titration of the fractions with 0.02 N sodium hydroxide gave titration peaks at the places of taurine conjugated bile acids, GC and GCD. The fractions containing the latter two acids were combined and paper chromatography revealed only spots of GC and GCD, respectively. The fractions between these two acids from the column chromatogram were combined and evaporated to dryness. After butanol extraction, rechromatography with phase system C 1, on a column 1/5 as large as the first column, showed a titration peak corresponding to about 3 mg of bile acid. Paper chromatography of this material produced one spot at the place of the unknown compound. The residue was then hydrolyzed and after acidification extracted with ether.

Descending paper chromatography of the hydrolyzate with ethylene chloride/heptane 20/80 as moving phase and 70 % acetic acid as stationary phase gave one spot which appeared between the spots of cholic and chenodeoxycholic acids, run simultaneously as reference compounds. The position of the spot suggested that the compound (if a bile acid) might be a dihydroxy bile acid with two equatorial hydroxyl groups which caused the relatively slow rate of movement. Therefore, ursodeoxycholic acid (3α, 7β-dihydroxycholanic acid) and hyodeoxycholic acid (3α, 6α-dihydroxycholanic acid) were used as reference compounds and the unknown substance was found to move like the acid mentioned first. The sulphuric acid spectrum of the compound, eluted from the paper chromatograms [2], was identical with that of ursodeoxycholic acid.

The taurine conjugated bile acids from the bile of patient N.A. were hydrolyzed and subjected to chromatography with phase system F 1. Titration peaks were found at places of cholic (C), ursodeoxycholic (UD) and chenodeoxycholic (CD) acids (Fig. 1). Hyodeoxycholic acid is known to be eluted at approxim-

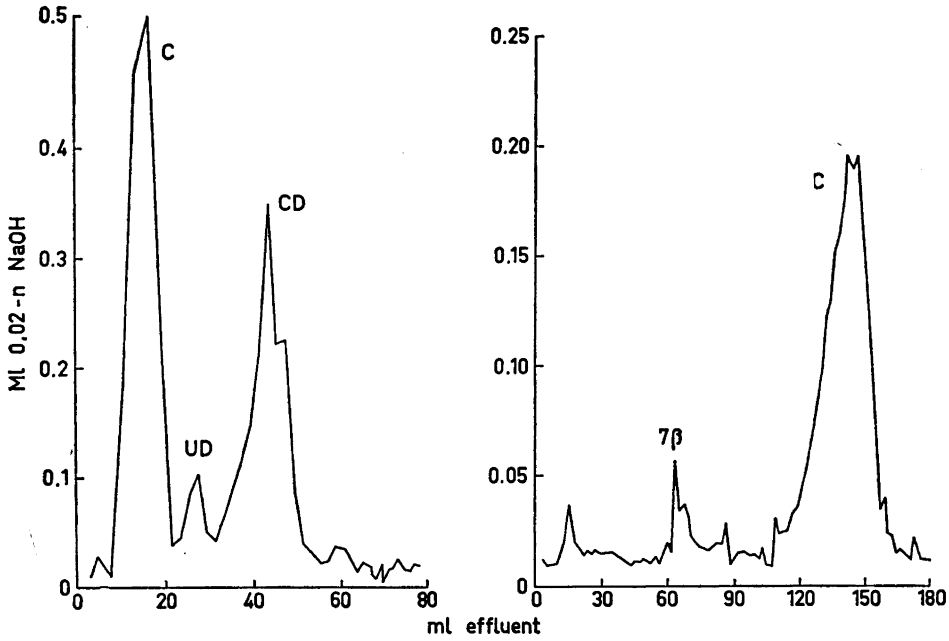


*Fig. 1.* Chromatography of hydrolyzed taurine conjugated bile acids from patient N.A. The front band from the chromatography with phase system F 1 (C, left curve) was rerun with phase system C 2 (right curve).

ately the same place as ursodeoxycholic acid, but paper chromatography of the peak at this place showed that the substance moved as UD.

The two samples of the compound, moving as ursodeoxycholic acid, isolated from the bile of patient N.A., were combined and weighed about 5 mg. A slow crystallization from ethyl acetate light petroleum took place, but, due to the small amount of material, only one recrystallization could be carried out resulting in crystals which had a melting point of 195—197°. The sulphuric acid spectrum was identical with that of UD.

The largest amount of the compound causing the spot between GC and GCD on the paper chromatograms was found in the duodenal contents of a one-year-old child (patient R.A.). About 10 ml of duodenal contents were hydrolyzed and the free bile acids separated with phase system F 1. Titration peaks were found at the places of C, UD and CD. The material in the fractions corresponding to UD was collected and extracted with ether from an acidified water solution. The residue, weighing about 10 mg, was dissolved in ethyl acetate and light petroleum was added. Crystals slowly appeared, were recrystallized twice, and gave about three mg of crystals which had a melting point of 201—202° not depressed by authentic ursodeoxycholic acid. Paper chromatographic behavior and sulphuric acid spectrum were identical with those of UD as was also the IR spectrum shown in Fig. 2.

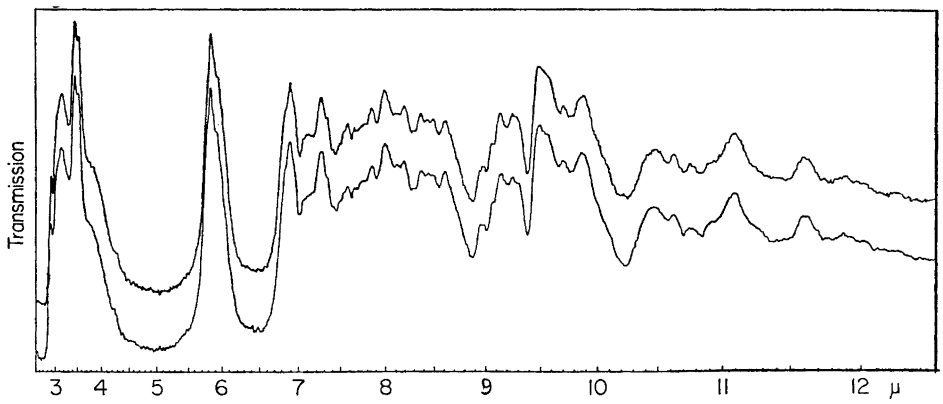


*Fig. 2.* Infrared spectra of authentic ursodeoxycholic acid (upper curve) and ursodeoxycholic acid isolated from the duodenal contents of patient R.A. (lower curve).

*B. 3α,7β,12α-Trihydroxycholanic acid.* This acid has been prepared recently by Samuelsson [6]; its presence in rat bile and feces has been demonstrated [7]. In chromatograms with phase system F 1, the acid appears together with cholic acid. In order to separate these acids, the "cholic acid bands"

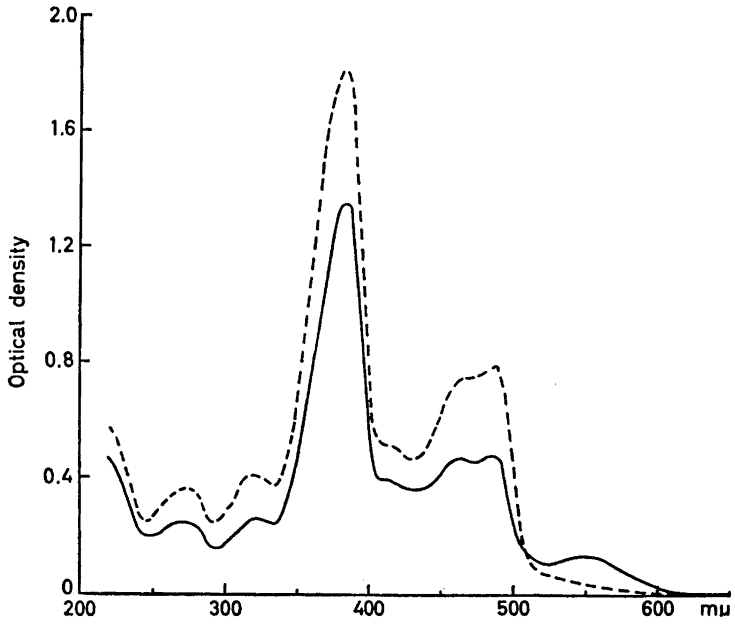


*Fig. 3.* Absorption spectra of 3α,7β,12α-trihydroxycholanic acid (– – – –) and the compound with identical chromatographic behaviour as this acid, isolated from the bile of patient N.A. (———). The samples were heated for 15 min at 60°C in 65 % sulphuric acid.

from patient N.A. (C, Fig. 1, left curve) and patient R.A. were subjected to chromatography with phase system C 2. The chromatogram of the band from patient N.A. is shown in Fig. 1 (right curve). A large titration peak, corresponding to cholic acid, is present (C), and there is also a minor peak at the place where 3α,7β,12α-trihydroxycholanic acid is known to be eluted [6]. Ascending paper chromatography of the material contained in these fractions, with ethylene chloride/heptane 50/50 as moving phase and 70 % acetic acid as stationary phase, showed the presence of a compound moving with the same rate as 3α,7β,12α-trihydroxycholanic acid. The sulphuric acid spectrum of this compound eluted from the chromatogram was very similar to that of 3α,7β,12α-trihydroxycholanic acid, kindly supplied by Dr. Samuelsson (Fig. 3). The low absorption maximum at 555 mμ of the compound isolated from bile, is due to the presence of small amounts of bromothymol blue used as indicator in the titration of the fractions from the column chromatography. Due to the small amounts of acid obtained from the bile (about 0.8 mg), further purification and identification could not be carried out.

The chromatogram of the "cholic acid band" from patient R.A. showed no titration peak at the place of 3α,7β,12α-trihydroxycholanic acid. However, paper chromatograms of the fractions where this acid could be expected to be eluted clearly showed a spot that had moved with the same rate as the reference acid 3α,7β,12α-trihydroxycholanic acid. If this compound was identical with the reference acid, it was present in amounts less than 1 % of the total bile acids of patient R.A.

## DISCUSSION

The non-ketonic bile acids in human bile hitherto isolated are cholic, chenodeoxycholic, deoxycholic and lithocholic acids. These acids all contain hydroxyl groups in one or several of the positions 3α, 7α and 12α. Bile acids with a 7β hydroxyl group have been isolated, *e. g.* from the bile of a certain bear species and from the coypu and the rat [7-9]. The present investigation has shown the presence of ursodeoxycholic, 3α,7β-dihydroxycholanic acid, in samples of human bile and suggests the presence of 3α,7β,12α-trihydroxycholanic acid. Except in the duodenal contents of a one year old child, the ursodeoxycholic acid accounted only for a few per cent of the total bile acids as judged from the spots on the paper chromatograms. In most samples, no spot could be detected at all at the place of ursodeoxycholic acid when the amount of bile used for routine analysis of bile acids was run (a total bile acid amount of about 50 μg).

The chromatographic analysis of bile from patient N.A. showed that ursodeoxycholic acid occurred among the taurine as well as the glycine conjugated bile acids. It is therefore reasonable to assume that it was conjugated with taurine and glycine. The ratio between these conjugates was roughly equal to that of the other bile acids.

Further work must be done for the final identification of the compound behaving like 3α,7β,12α-trihydroxycholanic acid. In the rat, this acid has been shown to be formed from cholic acid, probably through the action of microorganisms in cecum [7]. Several strains of *E. coli* have been shown to oxidize

the hydroxyl group of cholic and chenodeoxycholic acids at C—7, giving a 7-keto group [10]. The 3α-hydroxy-7-ketocholanic acid, that might thus be formed from chenodeoxycholic acid in the rat cecum, is known to be converted to ursodeoxycholic acid in the rat liver [9–11]. Bile acids are known to be absorbed from the cecum [7] and ursodeoxycholic acid could thus be formed from chenodeoxycholic acid (see also Ref.[9]). Whether the 7β-hydroxylated bile acids discussed in this paper are formed in the same way, remains to be investigated. The general importance of the intestinal flora for the composition of the biliary bile acids is, however, clearly seen in the case of deoxycholic acid, which has been shown to be formed by microbial action on cholic acid in man as well as in animals [12–13].

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