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NATURALLY OCCURRING CONJUGATED BILE ACIDS, MEASURED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, IN HUMAN, DOG, AND RABBIT BILE

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SUMMARY

The aim of this study was to determine the biliary pattern of conjugated bile acids after stimulation of their enterohepatic circulation.

Conjugated bile acids were separated by reversed-phase ion-pair chromatography without prior derivatization. A MicroPak SP-C18-IP-4 column was used as non-polar matrix, and an ionic alkyl compound, tetrabutylammonium phosphate, was added to the mobile phase, which was a mixture of acetonitrile and water. Quantification was made by UV absorption at 210 nm with external standardization. In fourteen human patients with external biliary drainage after papillotomy there was preferential glycine conjugation. The mean values were 36.5% for glycocholic acid, 33% for glycochenodeoxycholic acid, and 10.0% for glycodeoxycholic acid. Only 15.2% of the biliary bile acids were taurine metabolites. Conjugates of ursodeoxycholic acid were below 2.1%. In most cases, conjugated lithocholic acid was not detected. Within 4 h after ingestion of a standardized meal there were no significant changes in the biliary bile acid pattern.

In four dogs (beagles), glycine-conjugated bile acids were lacking. The mean values were 74.3% for taurocholic acid, 14.9% for taurodeoxycholic acid, and 5.3% for taurochenodeoxycholic acid. In six rabbits, 87.4% of biliary bile acids was identified as glycodeoxycholic acid and 5.3% as glycocholic acid. In conscious dogs, as well as in rabbits, the stimulation of biliary secretion by cholecystokinin and/or secretin had no effect on the biliary bile acid spectrum.

Evidently, there is a difference in the biliary composition of conjugated bile acids between humans, dogs, and rabbits. Because of the different physicochemical behaviour of glycine- and taurine-conjugated bile salts, it seems difficult to compare the therapeutic effect of gallstone dissolution in various species.

INTRODUCTION

Various investigations have shown that the bile acids, which are mostly conjugated with glycine or taurine, are discharged from the liver, the conjugates of cholic and chenodeoxycholic acids predominating in the pattern of human bile acids[1,2]. The investigations described here were undertaken in order to find out whether the bile

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acid pattern in the bile remains stable or whether there are temporary changes in different species.

EXPERIMENTAL

Instrumentation

The apparatus used was a Varian liquid chromatography system (Model LC 5500, autosampler 8085, UV-detector 200), equipped with a Vista 402 Datasystem (Varian, Walnut Creek, CA, U.S.A.).

Materials

The eluent was prepared from chromatography-grade water and acetonitrile (Merck, Darmstadt, F.R.G.). Tetrabutylammonium phosphate (Pic A) was from Waters Assoc. (Milford, MA, U.S.A.). Sodium salts or free acids of glycocholic acid, taurocholic acid, glycochenodeoxycholic acid, glycodeoxycholic acid, taurodeoxycholic acid, and taurolithocholic acid were from Supelco (Crans, Switzerland), glycolithocholic acid, glycoursodeoxycholic acid, and tauroursodeoxycholic acid from Calbiochem-Behring (Giessen, F.R.G.), and taurochenodeoxycholic acid from P.L. Biochemicals (Milwaukee, WI, U.S.A.).

Chromatography

The bile acids were determined according to the method described by Wildgrube *et al.*[3]. As stationary phase, a MicroPak SP-C18-IP-4 column (150 × 4.6 mm I.D.) (Varian) was used. Acetonitrile–water (54:46) with the addition of 0.5 mol/l tetrabutylammonium phosphate was used as the mobile phase for isocratic elution at a flow-rate of 1.0 ml/min. Samples of 10 μl were injected, and quantitated with reference to bile acid standards by UV absorption at 210 nm.

Samples

For the analysis, pure bile salts were diluted in methanol. Bile (1 ml) was diluted in 2 ml of methanol and centrifuged, and the supernatant liquid was filtered through a Millex filter (0.22 μm, Millipore, Molsheim, France).

A nasobiliary drainage was implanted in fourteen human patients after papillotomy because of a gallstone disease. Bile samples were taken from the patients via the permanent probe before breakfeast, immediately after a standard test meal, and at 30-min intervals for a total of 4 h, and were analysed immediately. Six rabbits, which had an average weight of 3500 g, were implanted with a catheter in the common bile duct under nembutal anesthesia, after ligature of the cystic duct in order to collect the bile continuously. After a basal period, the rabbits received cholecystokinin in various concentrations (0.5–2.0 U/kg body weight) intravenously to stimulate the biliary flow. In the case of four conscious dogs (beagles), which had a Thomas canula in the duodenum, the biliary secretion was examined under the influence of secretin and cholecystokinin for 2 h.

RESULTS

Methods

The lowest detectable concentration for glycocholic acid in standards and bile samples was found to be 1.0 nmol/ml, and that for glycolithocholic acid was 1.5 nmol/ml (signal-to-noise ratio 2:1). There was a linear relation between peak area and concentration up to 500 nmol/ml. The intraassay (n = 14) coefficient of variation for glycocholic acid at a concentration of 5 nmol/ml was 2.7%, and at 15 nmol/ml it was 1.9%. For glycolithocholic acid the coefficient of variation at a concentration of 5 nmol/ml was3.4%, and at 15 nmol/ml it was 2.8%. The interassay (n = 18) coefficient of variation for glycocholic acid at concentrations of 5 and 15 nmol/ml was 5.1% and 4.7%, and for glycolithocholic acid at 5 and 15 nmol/ml it was 6.4% and 6.0%, respectively. The recovery, measured by addition of pure bile salts to bile, was 97% (S.D. 3%).

The MicroPak SP-C18-IP-4 column gave a better resolution of the bile acid mixtures than the Ultrasphere I.P. column. Thus, we were able to separate the glycine and taurine conjugates of ursodeoxycholic acid as well. Both bile salts were eluted earlier than the corresponding conjugates of cholic acid (Fig. 1). Furthermore, the resolution between taurocholic acid and glycochenodeoxycholic acid was improved.

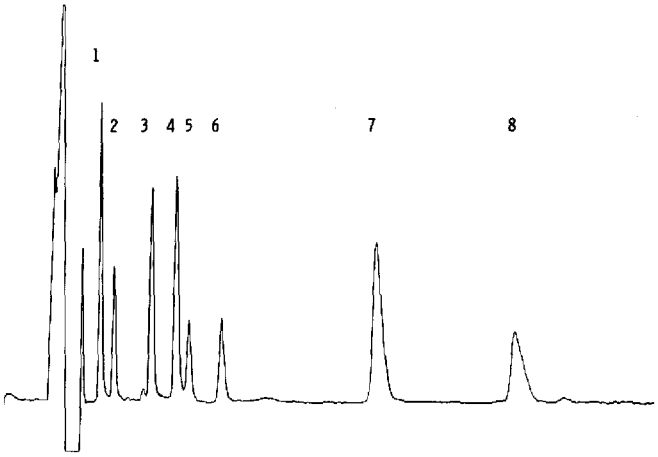

Fig. 1. Separation of a syunthetic mixture of glycine and taurine conjugates of bile acids. Conditions as in Experimental. For abbreviations see Table I. Peaks: 1 = GUDC; 2 = GC; 3 = TUDC; 4 = TC; 5 = GCDC; 6 = TCDC; 7 = GDC; 8 = TDC.

With the UV detector, the peak size was found to be pH dependent. Acidification of the mobile phase with phosphoric acid changed the behaviour of the bile salts. When the absorption at pH 8 was set to 100%, a change in the molar extinction coefficient was found, which was different for each bile acid conjugate (Fig. 2).

Bile acid patterns

The spectrum of conjugated bile acids in human subjects showed considerable intra-individual variability with the glycine conjugates of cholic acid (mean 36.5%, range 24.6–52.4%) and chenodeoxycholic acid (mean 3.0%, range 15.8–49.5%) dom-

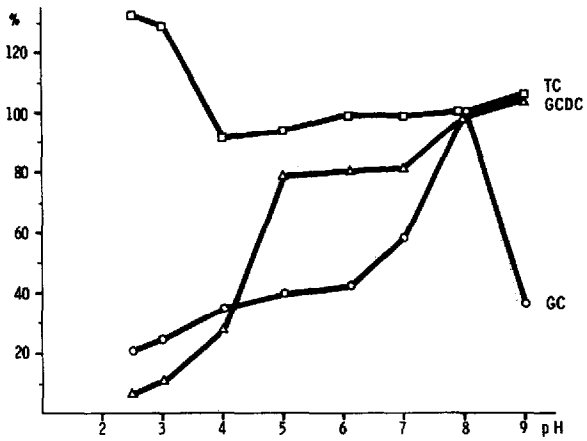

Fig. 2. The peak size of conjugated bile acids depends on the pH of the mobile phase. Conditions as in Experimental. For abbreviations see Table I.

inating (Table I). The percentage of taurocholic acid and taurochenodeoxycholic acid varied between 2.0% and 20.0%. Glycodeoxycholic acid, which is a bacterial product of glycocholic acid, was found in a mean concentration of 10.0% (range 2.5–16.1%). The concentrations of both conjugates of ursodeoxycholic acid were below 4.6% (mean 2.1%).

In dogs no glycine conjugated bile acids were found. Taurocholic acid (74.3%) and its bacterial degradation product, taurodeoxycholic acid (14.9%), dominated. The percentage of taurochenodeoxycholic acid was low (5.3%) (Table I).

In rabbits glycodeoxycholic acid (87.4%, S.D. 5.9%) dominated; glycocholic acid was found in 5.3% concentration (S.D. 3.8%). Taurine-conjugated bile salts were lacking (Table I).

TABLE I

BILE ACID PATTERN IN BILE (PERCENTAGE OF TOTAL BILE ACID CONCENTRATION)

Bile acid	*Human*	*Dog*	*Rabbit*
Glycocholic acid (GC)	36.5 (7.5)	–	5.3 (3.8)
Taurocholic acid (TC)	6.8 (3.4)	74.3 (4.6)	–
Glycochenodeoxycholic acid (GCDC)	33.0 (9.4)	–	–
Taurochenodeoxycholic acid (TCDC)	6.7 (3.2)	5.3 (2.1)	–
Glycodeoxycholic acid (GDC)	10.0 (5.6)	–	87.4 (5.9)
Taurodeoxycholic acid (TDC)	1.9 (2.3)	14.9 (3.8)	–
Glycoursodeoxycholic acid (GUDC)	1.4 (2.0)	–	–
Tauroursodeoxycholic acid (TUDC)	0.7 (1.5)	–	–

Biliary drainage

In humans no significant changes in the bile acid spectrum could be observed after a test meal. With regard to glycocholic acid there was a mean increase of 3.0% after 2 h. These results were confirmed by the investigations in dogs and rabbits. Regardless of changes in the bile acid output, secretin and/or cholecystokinin had no significant effect on the bile acid pattern (Table II).

DISCUSSION

The analysis of bile acids has decisively improved in both qualitative and quantitative aspects with the introduction of high-performance liquid chromatography (HPLC). After the initial reports on the separation of bile acids by means of HPLC, in which mainly derivatized metabolites were differentiated, various researchers aimed at a further optimization of this method[4]. Parris[5] was the first to quantify bile acids by their UV absorption, and thus stimulated further experiments intended to improve efficiency, sensitivity, and speed of analysis. Most authors have used reversed-phase columns (RP-18) and methanolic mobile phases[6–13], but studies very soon showed that these methods did not meet with the requirements of routine analyses[3]. On the basis of the specific physicochemical behaviour of bile acids we have elaborated an ion-pair chromatographic separation, which improved the efficiency of the HPLC method. Schill[14] and Persson and Schill[15] found that strong acids or bases changed their behaviour when a complementary ionized long-chain alkyl compound was added, and thus tetrabutylammonium phosphate (TBAP) improved the separation of acids on non-polar reversed-phase columns. Free and conjugated bile acids were regarded as amphipathic molecules, having both polar and non-polar as well as hydrophilic and lipophilic structural elements. While the degree of ionization of free bile acids is low (p*K ca.* 6) it is clearly enhanced by the carboxyl group of glycine (p*K ca.* 4) or by the sulphonate group of taurine (p*K ca.* 2). Glycine and taurine conjugates, therefore, require different concentrations of the counter-ion. After the addition of TBAP, the elution sequence changes so that the bile acids conjugated with glycine are eluted ahead of the taurine conjugates. Furthermore, conjugated trihydroxycholanic acids are eluted ahead of the conjugated di- and monohydroxycholanic acids (Fig. 1) when a column of octadecylsilane, bonded to 4-μm spherical silica particles (MicroPak SP-C18-IP-4) is used. Both glyco- and tauro-ursodeoxycholic acid are eluted earlier than the corresponding cholic acid conjugates.

Our investigations have also revealed another important function of the ion-pairing reagent (TBAP). After addition of TBAP, the pH of the mobile phase remains constant at pH 8, regardless of the amount of the added synthetic mixture of bile acids or bile. This is the reason for the reproducibility of the quantification of the various bile acids. The absorption at 210 nm was found to be highly dependent on pH (Fig. 2). In contrast to the absorption values at pH 8, acidification with phosphoric acid or further alkalinization with sodium hydroxide produced considerable changes in absorption for different metabolites. With detection at 210 nm, peak size was related to both the concentration and the molar extinction coefficient of each component. Our data confirm the well-known dependence of the extinction coefficient on the functional group in the bile acid molecule. Therefore, the quantification of bile acids may be impaired if the pH changes of the mobile phase are not controlled.

The determination of the bile acid spectrum of the fourteen patients clearly showed preferential glycine conjugation. The average percentage of cholic acid conjugates was only slightly larger than that of chenodeoxycholic acid, but there were differences in the bile acid spectrum among the patients. Glycocholic acid and glycochenodeoxycholic acid comprised more than two-thirds of all the bile acids; gly-

TABLE II

BILE ACID PATTERN IN BILE. TOTAL BILE ACID CONCENTRATION (TBA, μmol/ml). DEVIATION IN COMPARISON TO THE FASTING LEVEL

Conditions as in Experimental. Dogs: (a) secretin stimulation; (b) cholecystokinin stimulation. Rabbits: (a) secretin 0.5 IE/kg; (b) secretin 2.0 IE/kg. For abbreviations see Table I.

	Human			*Dog*			*Rabbit*		
	TBA (μmol/ml)	*Mean (%)*	*Deviation (%)*	*TBA (μmol/ml)*	*Mean (%)*	*Deviation (%)*	*TBA (μmol/ml)*	*Mean (%)*	*Deviation (%)*
Total bile acid	33.7			(a) 74.6 (b) 69.4			(a) 39.1 (b) 21.1		
GC		36.5	3.1		—			(a) 5.0 (b) 5.5	1.4 1.6
TC		6.8	1.1		(a) 74.6 (b) 74.0	6.9 5.1		—	
GCDC		33.0	3.3		—			—	
TCDC		6.7	1.2		(a) 5.5 (b) 5.2	1.1 0.9		—	
GDC		10.0	2.4		—			(a) 87.8 (b) 87.1	9.3 8.5
TDC		1.9	2.6		(a) 14.6 (b) 15.3	3.1 2.5		—	

codeoxycholic acid and taurocholic acid together accounted for 18% of the bile acids. Stimulation of the enterohepatic circulation by a meal did not significantly change the biliary pattern of any patient.

Our results show that rabbits, studied for their bile acid output, and dogs, studied after stimulation with secretin and/or cholecystokinin, experience no change in the bile acid pattern under physiological and pharmacological conditions (Table II). The bile acid pattern in humans seems to be determined mainly by glycine conjugates. The only quantitatively important differences were found in the ratio of glycocholic acid to glycochenodeoxycholic acid. As far as the bile acid conjugates are concerned, none of the laboratory animals that we have chosen for experimentally induced gallstones has a bile acid spectrum comparable with that of human subjects. Because of the differnt physicochemical behaviour of bile acid conjugates[16], it seems difficult to determine the efficiency of drugs for gallstone dissolution on the basis of experiments in rabbits or dogs.

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