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Note

High-performance liquid chromatographic analysis of bile acids in hamster bile

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During the course of studies on biliary lithiasis in the golden hamster, we analysed the pattern of conjugated bile acids present in the bile of these animals. The golden hamster has been used as an experimental model in this type of study [1, 2] and the pattern of conjugated bile acids found in the hamster was compared with that of human bile [3, 4]. Although the analysis of the bile acid composition of bile has been achieved with several analytical techniques, high-performance liquid chromatography (HPLC) is clearly the method of choice [5—13], especially since fluorometric detection methods have been introduced [14—18]. When gas chromatography was used for the analysis of the bile acid composition of hamsters the glyco and tauro derivatives could not be identified or determined, and the results obtained always refer to cholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and lithocholic acids.

In the present report we describe the separation of ten conjugated bile acids found in hamster bile which are identical to those found in human bile.

MATERIALS AND METHODS

Reagents

Methanol and acetonitrile were HPLC grade (E. Merck, Darmstadt, F.R.G.). Glass-bidistilled water was used in all the experiments. All other reagents used were HPLC grade (Fisher Scientific, New Jersey, U.S.A.). Solvents were filtered through a 0.45-μm Millipore membrane and degassed.

The sodium salts of taurocholic acid (TCA), taurochenodeoxycholic acid

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(TCDCA), taurodeoxycholic acid (TDCA), taurolithocholic acid (TLCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA) and glycolithocholic acid (GLCA) were obtained from Sigma (St. Louis, MO, U.S.A.). The sodium salts of tauroursodeoxycholic acid (TUDCA) and glycoursodeoxycholic acid (GUDCA) were a gift from Tokyo Tanabe (Tokyo, Japan). Dexamethasone (Sigma) was used as internal standard. Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were employed to purify the bile samples.

Animals

Golden hamsters (Centro Panamericano de Zoonosis, Buenos Aires, Argentina) were used. Six females and six males, each weighing 90 ± 10 g were kept in individual cages with water and standard roden Chow (Purina Labina, Buenos Aires) during 40 days. They were weighed twice a week. The room temperature was 24°C and the lights were turned off from 8 p.m. to 8 a.m. Hamsters were fasted for 16 h before being killed. They were in good health at the time of death.

Equipment

A liquid chromatograph Varian Model 5020 (Palo Alto, CA, U.S.A.) equipped with a spectrophotometric detector (Vari-chrom VUV 10 Varian), a recorder (Model 9176, Varian), and a data processor (CDS-111 L Chromatography Data System, Varian) were used. A Micropack MCH-5 column (300 mm × 4 mm I.D., particle size 5 μm) was employed.

Operating conditions

The mobile phase consisted of two solvents: solvent A was 0.3% ammonium dihydrogen phosphate, pH 7.5, and solvent B was acetonitrile. Gradient elution profile: solvent B increased from 28% to 35% during the first 19 min and then to 40% during the next 11 min. Flow-rate: 0.8 ml/min. Temperature: 32°C. Injection volume: 10 μl. Detection was performed at 210 nm and 0.05 a.u.f.s.

Sample preparation

The animals were killed by a blow on the head between 9 a.m. and 11 a.m., and the gallbladders were removed.

A normal saline solution (50 μl) was injected into each gallbladder and 130—230 μl of bile were aspirated. The bile samples were kept at —20°C and processed as soon as possible. A 100-μl volume of bile was diluted with 2 ml of 0.5 *M* phosphate buffer (pH 7.0) and this solution was passed through a Sep-Pak C_{18} cartridge (2 drops per sec) previously washed with 2 ml of methanol and 5 ml of water. The cartridges were then washed with 8 ml of water, 2 ml of 1.5% ethanol and finally with 4 ml of methanol which eluted the bile acids. The methanolic solution was evaporated to dryness in vacuo at 40°C. The residue was dissolved in 1 ml of methanol with 100 μg of internal standard (dexamethasone) and filtered through a 0.2-μm Sartorius membrane filter before being injected into the chromatograph.

RESULTS

Quantitative analysis of bile acids in hamster bile

A reference methanolic solution of ten standard conjugated bile acids containing 140—200 μg/ml of each one and 100 μg/ml of internal standard were chromatographed according to the operating conditions (Fig. 1). The

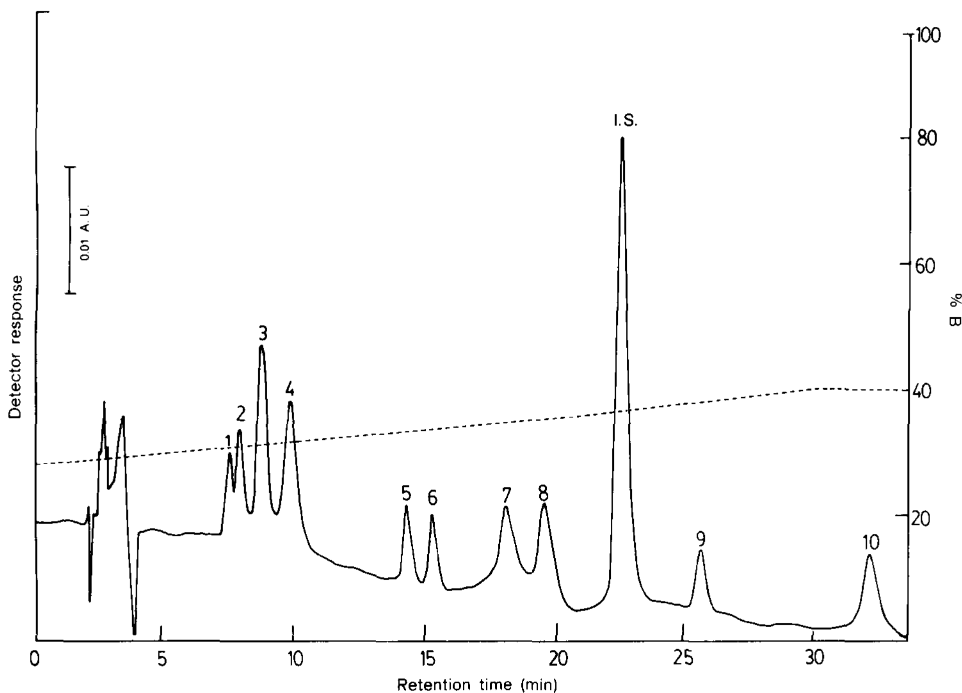

Fig. 1. Chromatogram of a reference solution of conjugated bile acids. Mobile phase: solvent A = 0.3% ammonium dihydrogen phosphate (pH 7.5), solvent B = acetonitrile. Peaks: 1 = TUDCA; 2 = TCA; 3 = GUDCA; 4 = GCA; 5 = TCDCA; 6 = TDCA; 7 = GCDCA; 8 = GDCA; 9 = TLCA; 10 = GLCA; I.S. = dexamethasone.

TABLE I

RELATIVE RETENTION TIMES OF CONJUGATED BILE ACIDS REFERRED TO TAURODEOXYCHOLIC ACID

Bile acid	Relative retention time
Tauroursodeoxycholic acid	0.49
Taurocholic acid	0.52
Glycoursodeoxycholic acid	0.57
Glycocholic acid	0.64
Taurochenodeoxycholic acid	0.94
Taurodeoxycholic acid	1.00
Glycochenodeoxycholic acid	1.19
Glycodeoxycholic acid	1.29
Taurolithocholic acid	1.68
Glycolithocholic acid	2.10
Dexamethasone (internal standard)	1.48

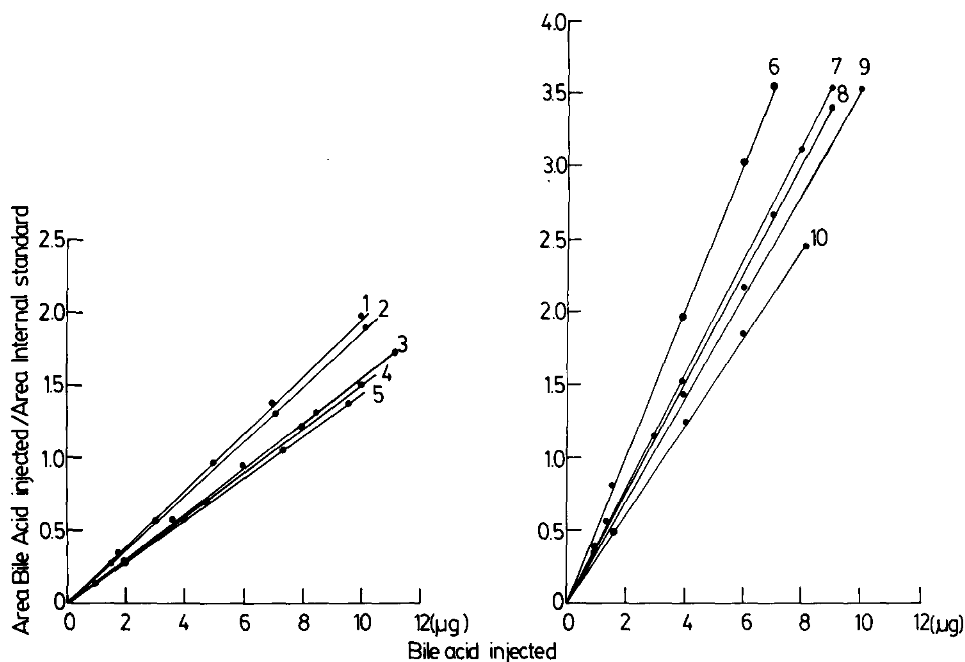

Fig. 2. Calibration curves for conjugated bile acids with dexamethasone as internal standard. Correlation coefficients of linear regression ranged between 0.996 and 0.999. 1, TUDCA; 2, TDCA; 3, TLCA; 4, TCA; 5, TCDCA; 6, GUDCA; 7, GLCA; 8, GCA; 9, GCDCA; 10, GDCA.

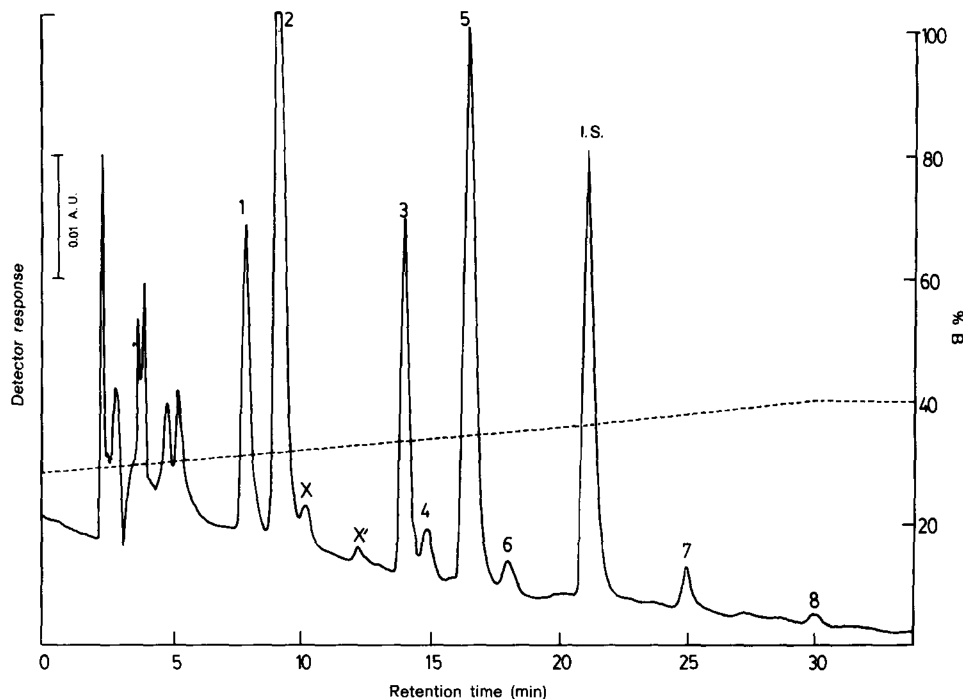

Fig. 3. Bile acid composition of hamster bile. Mobile phase: solvent A = 0.3% ammonium dihydrogen phosphate (pH 7.5), solvent B = acetonitrile. Peaks: 1 = TCA; 2 = GCA; 3 = TCDCA; 4 = TDCA; 5 = GCDCA; 6 = GDCA; 7 = TLCA; 8 = GLCA; X, X' = unknown; I.S. = dexamethasone.

TABLE II

BILE ACID COMPOSITION IN BILE OF GOLDEN HAMSTERS

Sample	Sex*	Percentage of total bile acids										TBA (mg/ml)
		TCA	GCA	TCDCA	GCDCA	TDCA	GDCA	TLCA	GLCA	TCBA	GCBA	
1	M	13.4	19.9	21.2	36.6	6.2	2.7	trace	—	40.8	59.2	9.7
2	M	28.9	16.1	24.2	22.9	5.2	0.6	1.3	0.8	59.6	40.4	21.1
3	M	18.7	22.9	25.5	26.2	3.2	1.0	2.5	trace	49.9	50.1	28.5
4	M	15.5	23.2	26.1	28.0	4.2	3.0	trace	—	45.8	54.2	27.0
5	M	10.3	10.5	35.7	36.0	2.6	2.1	trace	2.8	48.6	51.4	9.4
6	M	20.5	20.1	26.8	19.7	9.5	2.4	0.4	0.6	57.2	42.8	24.6
7	F	4.2	28.8	22.4	44.6	trace	trace	—	—	26.6	73.4	12.0
8	F	14.4	18.2	32.0	28.1	4.4	0.4	2.2	0.3	53.0	47.0	27.6
9	F	5.2	20.3	31.3	42.5	trace	0.7	trace	—	36.5	63.5	17.5
10	F	8.3	29.2	21.1	32.3	5.8	3.3	trace	—	35.2	64.8	14.4
11	F	6.3	28.7	33.7	30.4	trace	0.9	trace	—	40.0	60.0	11.8
12	F	20.3	16.8	41.6	19.9	trace	trace	1.4	trace	63.3	36.7	17.4

*M = male; F = female.

TABLE III

COMPARISON OF ANALYTICAL RESULTS FOR BILE ACIDS IN HAMSTER BILE OBTAINED BY HPLC WITH SEP-PAK C_{18} CARTRIDGES AND WITH ETHANOL IN THE CLEAN-UP OF THE SAMPLE

Sample		Concentration (mg/ml)							
		TCA	GCA	TCDCA	GCDCA	TDCA	GDCA	TLCA	GLCA
Bile A	Sep-Pak C_{18}	3.98	5.01	8.82	7.74	1.20	0.12	0.61	0.090
	Ethanol	4.12	5.25	8.87	7.92	1.24	0.14	0.60	0.088
Bile B	Sep-Pak C_{18}	1.20	4.20	3.04	4.64	0.85	0.47	Trace	—
	Ethanol	1.05	4.06	2.95	4.55	0.98	0.41	Trace	—

TABLE IV

REPRODUCIBILITY OF DETERMINATION OF BILE ACIDS IN HAMSTER BILE BY HPLC

$n = 5$.

Bile acid	Mean ± S.D. (mg/ml)
TCA	3.25 ± 0.11
GCA	4.63 ± 0.10
TCDCA	5.12 ± 0.09
GCDCA	6.01 ± 0.09
TDCA	1.12 ± 0.07
GDCA	0.63 ± 0.05
TLCA	1.05 ± 0.06

relative retention times are shown in Table I. A linear calibration response to each bile acid in the range of 0.3—10 μg is shown in Fig. 2. The detection limits found were in the range of 75 ng for GUDCA to 200 ng for GDCA and 250 ng for TUDCA to 350 ng for TCDCA. Twelve hamster bile samples were chromatographed according to the procedure previously described (Fig. 3). The results are summarized in Table II. Similar HPLC bile acid patterns were obtained when the bile samples were deproteinized with ethanol, centrifuged at 1000 *g* for 5 min, washed and dried in vacuo at 40°C. There was a good correlation between the results obtained with these samples and the samples analysed

directly as described above (Table III). In order to examine the recovery of bile acids known quantities of standard bile salts were added to a hamster bile sample, before the purification step with Sep-Pak C_{18} cartridge. The recovery values were 90% for GCA, 92% for GCDCA and 99% for TCA. The reproducibility of the analysis was tested on a hamster bile pool for $n = 5$ with standard deviations of 1.5—7.9% (Table IV).

DISCUSSION

Reversed-phase HPLC performed on individual samples of hamster bile allowed the separation of glyco and tauro conjugates of bile acids in 30 min using dexamethasone as internal standard. This separation could not be achieved with an isocratic system [13], but with the gradient system reported in this paper good reproducibility and recovery values were obtained. The use of the Sep-Pak C_{18} cartridge allowed direct clean-up of the sample without previous deproteinization. The reported HPLC separations of conjugated bile acids required longer elution times [8, 10—12], more elaboration [7, 9, 12, 19, 20], or had less sensitivity [5—7]. More elaborate techniques require a more complicated sample purification which increases analysis time and costs [14—18]. Our method is well adapted to the small amount of bile present in the hamster.

Loss of resolution of the column was observed with time of use, but the column could be regenerated by washing first with methanol—water (70:30), then with tetrahydrofuran, chloroform, tetrahydrofuran and finally with methanol—water (70:30).

The composition of hamster bile showed appreciable variations among the samples examined. The conjugation ratio with glycine and taurine showed great variability (Table II). TUDCA was not detected in the bile samples and GUDCA was identified in two samples in trace amounts. In hamster bile a major proportion of GCDCA and TCDCA was observed compared to GCA and TCA. TDCA and GDCA were present in low proportions. These results are similar to those reported in human bile by other authors [12, 19, 20] using HPLC.

Two unknowns were present in most of the chromatograms (Fig. 3). These peaks could be related to bile acid conjugated forms reported by Bergman et al. [21]. Work is in progress to identify them.

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