A simple method for the determination of lipid composition of human bile

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Summary The Entero-Test®, a device for easy sampling of gastrointestinal contents, including bile, has been used for determination of biliary lipid composition. The device consists of a weighted gelatine capsule containing 140 cm of a highly absorbant nylon line. The capsule is swallowed while one end of the string is taped to the face. After 3.5 h, when the line has reached the duodenum, gallbladder contraction is stimulated by intramuscular administration of ceruletide. The line is pulled out, and the last 15 cm are eluted four times in methanol. Total bile acids (by 3α-hydroxysteroid-dehydrogenase assay), individual bile acids (by high performance liquid chromatography), phospholipids (by assay of lipid-soluble phosphorus), and cholesterol (by gas-liquid chromatography) are determined in the eluate. Tests in vitro demonstrated no preferential binding and a good recovery of biliary lipids from the thread. Similar values of biliary cholesterol saturation were obtained by means of duodenal intubation and of the Entero-Test in a series of 12 subjects (r = 0.952). In 5 subjects, indvidual bile acids were also measured and were found to be similar with both techniques (r = 0.948). When the test was repeated over 3 days in a series of 7 subjects, biliary cholesterol saturation was found to be remarkably reproducible (CV = 7.6%). Thus, the Entero-Test is a convenient technique for the determination of biliary lipid composition, which can be particularly useful in longitudinal studies.—**Muraca, M., M. T. Vilei, L. Miconi, P. Petrin, M. Antoniutti, and S. Pedrazzoli.** A simple method for the determination of lipid composition of human bile. *J. Lipid Res.* 1991. **32:** 371-374.

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Analysis of biliary constituents for clinical or research purpose is usually carried out on bile obtained by duodenal intubation. This technique is, however, cumbersome and uncomfortable for the patient. A simpler method for bile collection has recently been proposed (1-3). The method is based on administration to the patient of the Entero-Test®, a commercially available device for sampling gastrointestinal contents. In the present work, a procedure has been validated for simultaneous determination of total and individual bile acids, cholesterol, and phospholipids in the bile collected with the Entero-Test.

METHODS

Subjects

Nineteen individuals volunteered in this study. Informed consent was obtained from each subject and the experimental protocol was approved by the Ethical Committee of the University Hospital. In order to compare the Entero-Test with the duodenal intubation technique, the capsule was administered to nine patients with stones in a functioning gallbladder and in three healthy individuals. After 1 to 3 days, all individuals underwent duodenal intubation under fluoroscopic guidance, and gallbladder bile was collected after intramuscular administration of ceruletide (0.4 μg per kg of body weight; Farmitalia Carlo Erba, Milan, Italy). The reproducibility of the procedure was assessed by administering the Entero-Test twice over a 3-day period to seven additional subjects (three gallstones patients with a functioning gallbladder and four healthy individuals). During this period the subjects did not indicate any change in their diet nor did they consume any drug.

Collection of bile

The Entero-Test (HDC corporation, Mountain View, CA) is a weighted gelatine capsule containing 140 cm of a highly absorbant nylon line. The device is administered after an overnight fast. The first 10 cm of the nylon line are pulled out from the capsule by the protruding loop. The capsule is then swallowed with water while the loop is held outside the mouth, and then taped to the face, to secure the line. The patient is encouraged to drink three glasses of water during the following 2 h. After 3.5 h, when the thread has moved into the duodenum, ceruletide (0.4 μg per kg of body weight) is administered intramuscularly to stimulate gallbladder contraction. Thirty minutes after the injection of ceruletide, the string is gently pulled out. The distal end of the line, which appears yellow with bile, is checked for pH with the colorimetric kit supplied with the Entero-Test. The finding of an alkaline pH confirms that the thread has reached the duodenum. The last alkaline-reacting 10-15 cm are then cut out and weighed in a test tube on an electronic balance. The volume of the duodenal fluid adsorbed to the thread is calculated by subtracting the dry weight of the segment. After addition of 5 ml of methanol, the tube is stored at −20°C for up to 1 week before analysis.

Elution of bile

The tube containing the line in methanol was brought to room temperature and put into an ultrasound bath for 5 min. The eluate was transferred into a 15-ml graduated test tube and put under nitrogen flow at 37°C. The elution procedure was repeated three times with 5 ml each of methanol. The eluate was evaporated to less than 10 ml and then brought to 10 ml with methanol.

Analysis of total and individual bile acids

Three ml of the eluate was transferred into a 15-ml test tube and evaporated under nitrogen. The residue was dissolved with 0.3 ml methanol in an ultrasound bath for 5

min. Total bile acids were determined by the 3α-hydroxysteroid dehydrogenase assay (Sterognost 3α Pho, Nycomed AS, Oslo, Norway) using 0.02 ml of sample. The remaining eluate was diluted with 13 ml of a mixture consisting of methanol–0.15 M NaOH 3:7 (v/v) and extracted with a Sep-Pak C18 cartridge (Waters Inc., Milford, MA) as previously described (4). The extract was analyzed by high-performance liquid chromatography (4) with a Perkin-Elmer series 3B liquid chromatograph.

Analysis of cholesterol

Cholesterol was extracted from the eluate as described by Abell et al.(5). Four ml of the eluate was transferred into a 20-ml test tube. One ml of methanol, 5 ml of water, and 10 ml of petroleum ether (bp 40°–70°C) were added in sequence, and the mixture was vigorously shaken for 2 min. Eight ml of the upper petroleum ether phase was transferred into a dry test tube and evaporated under nitrogen at 60°C. The residue was dissolved with 1 ml of chloroform containing 60 μg of 5α-cholestane as internal standard. The chloroform solution was evaporated to about 0.2 ml and 1 μl was injected into a gas chromatograph equipped with a flame ionization detector (Perkin-Elmer mod. 3920B). The column used was a DB-17, 15 m long, 1.0 μm film thickness (J & W Scientific, Folsom, CA) and chromatographic conditions were as follows: column temperature, 240°C; helium flow, 25 ml/min; air pressure, 2.5 bar; hydrogen pressure, 1.3 bar.

Analysis of phospholipids

Two ml of the eluate was transferred into a 10-ml test tube and extracted with 4 ml of chloroform and 1.25 ml of 0.73% NaCl (6). After two washings with 1 ml of saline upper phase, as described by Folch, Lees, and Sloane Stanley (6), 2 ml of the chloroform phase was transferred to a 20-ml test tube and evaporated to dryness. Inorganic phosphorus was determined in the residue as described by Fiske and SubbaRow (7) with some modifications aimed at enhancing the sensitivity of the assay. After addition of 0.8 ml perchloric acid, the tube was heated at 200°C until a clear, colorless solution was obtained. The sample was then cooled to room temperature, and 3 ml of water, 0.25 ml of 5% ammonium molybdate, 0.4 ml of the Fiske-SubbaRow aminonaphthol sulfonic acid reagent (7), and 1 ml of water were added in sequence and vortex-mixed at each step. The tube was left at 150°C for 7 min, cooled to room temperature, and absorbance was determined at 820 nm.

Only samples in which total lipid concentration in the bile-rich duodenal fluid was greater than 2 g/dl were considered acceptable (8). Biliary cholesterol saturation was calculated with the method of Carey (9), using a microcomputer program (10) and assuming a total lipid concentration of 10 g/dl.

RESULTS

Tests in vitro

Studies in vitro were performed in order to evaluate possible preferential binding and to check recovery of compounds after elution from the thread. Ten-cm segments of the thread were incubated for 30 min at 37°C with 13 different bile samples adjusted to pH 7.0 (total lipid concentration range: 2.4–6.5 g/dl). The threads were weighed, eluted, and analyses were performed as described in the Methods section. A 0.3-ml aliquot of each bile sample was diluted with 20 ml of methanol. The solution was evaporated, adjusted to 10 ml, and analyzed with the same methods described for the Entero-Test. The correlations were calculated between the absolute concentrations obtained by the analysis with the Entero-Test and by the direct analysis of bile. The correlation coefficients were 0.906 for total bile acids, 0.987 for phospholipids, 0.860 for cholesterol, and 0.897 for total lipids.

Stability studies were performed as follows. Ten-cm segments of the thread were incubated for 30 min at 37°C

TABLE 1. Biliary lipid composition in duodenal fluid

Subject	Procedure	BA	PL	CH	Total Lipids
			molar %		*g/dl*
1	DI	83.08	10.37	6.55	3.61
	ET	82.48	10.26	7.26	2.55
2	DI	80.07	16.89	3.05	2.44
	ET	83.78	12.97	3.26	3.36
3	DI	65.40	21.67	12.94	3.51
	ET	74.26	14.70	11.04	3.74
4	DI	76.48	20.19	3.33	2.90
	ET	85.38	12.55	2.07	2.51
5	DI	69.68	22.85	7.48	3.58
	ET	76.90	17.63	5.48	2.96
6	DI	78.79	15.48	5.72	4.10
	ET	84.45	10.63	4.92	4.40
7	DI	76.71	15.30	7.99	3.12
	ET	77.90	15.08	7.02	2.37
8	DI	69.13	18.93	11.95	3.23
	ET	75.33	15.24	9.43	3.71
9	DI	76.60	18.46	4.94	3.15
	ET	83.92	11.87	4.21	2.55
10	DI	84.81	11.64	3.63	2.67
	ET	82.97	13.40	3.64	2.76
11	DI	79.55	17.45	3.01	5.31
	ET	84.90	12.07	3.04	2.07
12	DI	80.95	14.01	5.46	3.99
	ET	86.34	10.42	3.24	2.68

Comparison of biliary lipid composition in four healthy subjects (2, 4, 11, 12) and in eight patients with gallstones by means of duodenal intubation (DI) and by the Entero-Test (ET); BA, bile acids, PL, phospholipids; CH, cholesterol.

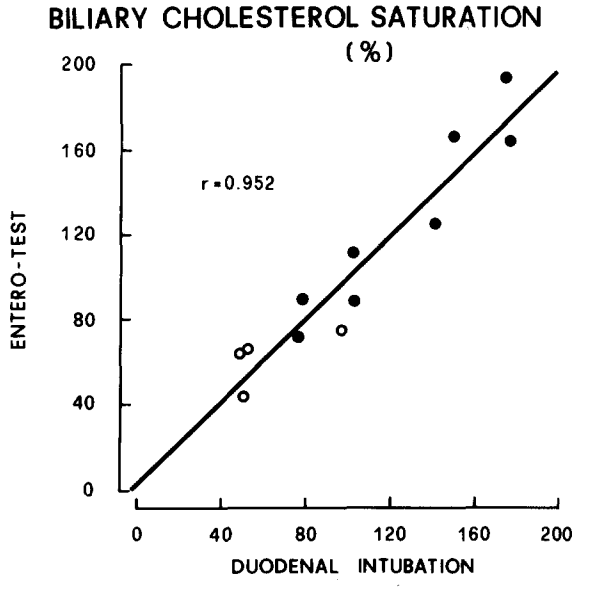

Fig. 1. Comparison of biliary cholesterol saturation in four healthy subjects (O) and in eight patients with gallstones (●) by means of duodenal intubation and of the Entero-Test.

TABLE 2. Day-to-day variation in biliary lipid composition

Subject	Day	BA	PL	CH	CSI
			molar %		%
1	1	74.91	18.21	6.87	108
	2	75.13	17.54	7.32	118
2	1	82.96	12.99	4.04	82
	2	79.82	16.05	4.13	72
3	1	81.52	15.04	3.28	60
	2	85.82	11.19	2.99	67
4	1	80.29	16.05	3.64	64
	2	77.33	18.32	4.35	70
5	1	83.97	10.94	5.09	114
	2	85.88	9.17	4.95	123
6	1	82.10	13.04	4.49	89
	2	87.75	9.12	3.13	79
7	1	80.68	11.74	7.58	160
	3	85.81	8.63	5.56	143

Day-to-day variation in biliary lipid composition and cholesterol saturation in four healthy subjects (2, 3, 4, 5) and in three patients with gallstones (1, 6, 7). Administration of the Entero-Test was repeated on the second or on the third day; BA, bile acids, PL, phospholipids; CH, cholesterol; CSI, cholesterol saturation index.

in several aliquots of two different bile samples, and analyses were carried out in duplicate both immediately and after storage at −20°C for up to 3 weeks. The concentrations of total bile acids and cholesterol remained constant up to 3 weeks, but the concentration of phospholipids was stable only up to 1 week.

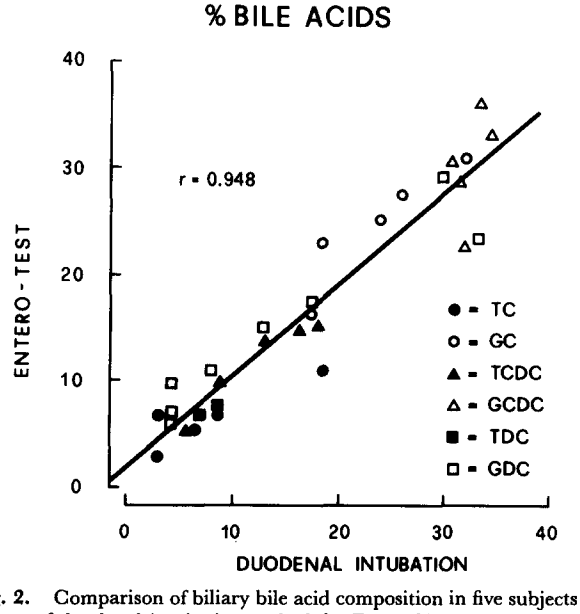

Fig. 2. Comparison of biliary bile acid composition in five subjects by means of duodenal intubation and of the Entero-Test.

Tests in vivo

The procedure was well accepted by all of the volunteers. A few subjects reported a vague abdominal discomfort after the injection of ceruletide. The volume of yellow-stained duodenal fluid bound to the thread varied between 190 and 360 μl. The results obtained with the Entero-Test were compared with the duodenal intubation technique of sampling bile. Significant correlations were obtained between the molar percent of individual lipids obtained with both procedures (**Table 1**). Correlation coefficients were 0.807 for total bile acids, 0.633 for phospholipids, and 0.955 for cholesterol. Similar results for cholesterol saturation index were obtained with both techniques (r = 0.952; **Fig. 1**). In five randomly selected patients (no. 3, 5, 7, 8, 11), individual bile acids were analyzed by high-performance liquid chromatography both in the duodenal aspirate and in the eluate from the Entero-Test. The correlation coefficient between the individual bile acid analysis of both tests was 0.948 (**Fig. 2**).

The day-to-day variations of the molar percent of the different biliary lipids and of cholesterol saturation index are reported in **Table 2**. The calculated coefficient of variation for biliary cholesterol saturation was 7.6%.

DISCUSSION

Since duodenal intubation is a cumbersome technique, the possibility of sampling bile with a simple device such

as the Entero-Test is appealing. This method has already been used for determination of bile acid profile and for quantification of bilirubin in human bile (1-3). Analysis of biliary lipids by means of the Entero-Test has also been reported, but methodological details have not been described (11). The methodology described in the present study has been adapted and validated for the determination of cholesterol saturation index on the small amounts of bile that can be collected with the thread, yielding results that are superimposable to those obtained with duodenal intubation (r = 0.952). In addition, the procedure allows the determination of individual bile acids in the methanol eluate from the thread. A correlation coefficient of 0.948 was found when comparing the composition of biliary bile acids by means of duodenal intubation and of the Entero-Test. Similar results have been obtained by Vonk et al. (2) in two healthy volunteers, by eluting the thread with phosphate buffer. The test is well tolerated and the results show good reproducibility. Indeed, biliary cholesterol saturation index was remarkably constant over a 2- to 3-day period (CV = 7.6%). Such characteristics are particularly important for longitudinal studies. Furthermore, the test can be used in an outpatient setting, since there is no need for radiological facilities. Thus, the present procedure should be a useful tool for determination of biliary lipid composition both for clinical and experimental studies.

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