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Spectrofluorimetric Determination of Individual Bile Acids in Biological Fluids: Duodenal Content and Bile

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A micromethod is described for the separation and quantitative determination of individual bile acids in duodenal content and bile. After a mild saponification procedure for splitting the taurine and glycine conjugates, the products are separated by thin-layer chromatography on silicagel layer with the solvent system *iso*-octane-ethyl acetate-acetic acid (60 ml + 35 ml + 5 ml) and determined by spectrofluorimetry. A series of control experiments has been performed on the validation of the procedure. Values obtained for human subjects or experimental applications of the method are presented.

Eine Mikromethode zur Trennung und quantitativen Bestimmung der einzelnen Gallensäure in Duodenal-Inhalt und Galle wird beschrieben. Nach milder Verseifung zur Spaltung der Taurin- und Glycinkonjugate erfolgt die Dünnschichtchromatographie auf Silicagel mit dem Laufmittel *iso*-Oktan/Äthylacetat/Eisessig 60 ml + 35 ml + 5 ml. Die nachfolgende Bestimmung wird spektrofluorimetrisch durchgeführt. Zur Bewertung des Verfahrens wurde eine Serie von Kontrollversuchen durchgeführt. Werte für den Menschen oder Laboratoriumstiere und Beispiele klinischer oder experimenteller Anwendung der Methode werden mitgeteilt.

Methods for the separation of individual bile acids from biological fluids include column and paper (1–4), thin-layer (5–13) or gas-liquid (14–17) chromatography. Quantitative measurements of the metabolites are made by an *in situ* semi-quantitative approach (6, 7) or densitometry without elution of zones from paper or layer, by colorimetry (1–4, 9), fluorimetry (13, 18, 19), enzymatic methods (10, 12) following elution of the steroids, or gas chromatography using internal standards (14–17). Recently, the introduction of spectrofluorimetry and gas chromatography resulted in a considerable increase in the sensitivity and specificity of the methods.

Determination of the individual free and conjugated bile acids in a large number of samples by gas chromatography appears to be rather laborious and the technique cannot be used for routine purposes. A procedure in which the individual conjugated bile acids are separated by thin-layer chromatography and quantitated by spectrofluorimetry overcomes this disadvantage.

Methods

Standards

Authentic cholic and deoxycholic acid were obtained from Fluka AG, chenodeoxycholic and lithocholic acid from Koch-Light Ltd., taurochenodeoxycholic, taurodeoxycholic, tauroolithocholic and glycolithocholic acid from Calbiochem Ltd., glycochenodeoxycholic and glycodeoxycholic acid were purchased from Sigma Chemical Corp. The purity of the standards was tested by thin-layer chromatography and phosphomolybdic acid reaction.

Chromatography procedures

Thin-layer chromatography was performed on Silicagel G Merck AG chromatoplates (20 × 20 cm and layer-thickness 0.2 mm). The plates were activated at 105°C for at least two hours and were not equilibrated in the chamber before development. The steroids were applied on strips of 1.5–2.0 cm.

Solvent systems:

- A. *iso*-Octane-ethyl acetate-acetic acid (50 ml + 40 ml + 10 ml)
- B. *iso*-Octane-ethyl acetate-acetic acid (50 ml + 30 ml + 20 ml)
- C. *iso*-Octane-ethyl acetate-acetic acid (60 ml + 35 ml + 5 ml)
- D. Chloroform-acetone-acetic acid (70 ml + 20 ml + 10 ml)
- E. Chloroform-acetone-acetic acid (80 ml + 15 ml + 5 ml)
- F. *n*-Butylalcohol-acetic acid-water (90 ml + 5 ml + 5 ml)
- G. *n*-Butylalcohol-acetic acid-water (95 ml + 3 ml + 2 ml)
- H. *n*-Propylalcohol-acetic acid-water (90 ml + 2 ml)
- I. *n*-Propylalcohol-acetic acid-water (95 ml + 4 ml + 1 ml)
- J. *n*-Butylalcohol-ethyl acetate-acetic acid-water (60 ml + 35 ml + 3 ml + 2 ml)
- K. *n*-Propylalcohol-ethyl acetate-acetic acid-water (40 ml + 55 ml + 3 ml + 2 ml)
- L. *n*-Propylalcohol-ethyl acetate-acetic acid-water (40 ml + 50 ml + 5 ml + 5 ml)
- M. *n*-Propylalcohol-ethyl acetate-acetic acid-water (30 ml + 65 ml + 4 ml + 1 ml).

Saponification

Authentic bile acid conjugates or the deproteinized biological material was dissolved in 5 ml 1.25 mol/l sodium hydroxide and the solution was saponified at 1.2 atm for 3 h in a pressure cooker. The mixture was cooled and acidified to pH 4.5 with about 3 ml 4.2 mol/l sulphuric acid.

This mild procedure was compared with two other methods. On splitting authentic bile acid conjugates by another mild procedure (16), the steroids were dissolved in 1 ml 200 g/l potassium hydroxide in ethylene glycol and hydrolysed at 220°C for 20 min. The mixture was cooled, acidified to pH 4.5 and processed as above. To compare the effectivity of methods, further, the bile acids were treated as in the first method with the exception that the conjugates were autoclaved at 2 atm (15 psi) for 3 h (14).

Spectrofluorimetry

Spectrofluorimetry of individual free bile acids was performed in a Hitachi-Perkin-Elmer Model 203 fluorescence spectrophotometer using a mercury light source. The wavelength of excitation was 436 nm and fluorescence was measured at 510 nm. The measurements were made on a micro scale in 0.6 ml cuvettes with a light path of 5 mm. A sensitivity setting around 1×10 (maximum 10×10) was used routinely on the fluorimeter. Spectro-

Tab. 1
The mobility of individual free and conjugated bile acids by thin-layer chromatography

Bile acids*	A**	B	C	D	Mobility ($R_F \times 100$) in solvent systems								
					E	F	G	H	I	J	K	L	M
C	20	59	2	22	4				72	85		85	
CD	69	91	21	82	57				93	94	95	95	98
D	78	94	23	82	56	93		98	93	95	95	96	98
L	91	98	67	96	88				98	96	96	98	98
TCD						40	50	70	63	32	31	56	5
TD						32	44	72	60	33	33	54	4
TL						47	56	72	68	39	47	59	9
GCD						75	68	82	78	70	84	84	85
GD						75	69	89	81	72	85	84	85
GL						78	76	89	84	81	89	91	91

* C = cholic, CD = chenodeoxycholic, D = deoxycholic, L = lithocholic, TCD = taurochenodeoxycholic, TD = taurodeoxycholic, TL = tauroolithocholic, GCD = glycochenodeoxycholic, GD = glycodeoxycholic, GL = glycolithocholic.

** For solvent systems see text.

fluorimetry of the conjugated bile acids in the duodenal content and bile was performed following saponification and thin-layer chromatography in the presence of Silicagel G adsorbent without elution of the steroids. For this reason, a comparison was made between the values of authentic cholic, chenodeoxycholic, deoxycholic and lithocholic acid in various concentrations measured directly in the presence of adsorbent following chromatography and those with no chromatographic treatment.

Method for duodenal content and bile

One ml of duodenal content or bile was deproteinized by adding 5 ml ethanol (960 ml/l) and boiling the mixture on a water-bath at 100°C for 5 min. The suspension was cooled, filtered through Whatman No 1 paper, the precipitate washed with ethanol, and the solution evaporated *in vacuo*. The residue was dissolved in 5 ml 1.25 mol/l sodium hydroxide, transferred into tubes, and the mixture was saponified at 1.2 atm for 3 h in a pressure cooker. The mixture was acidified to pH 5.4 with about 3 ml 4.2 mol/l sulphuric acid (wide-range indicator paper).

Free bile acids were extracted with 3 × 15 ml peroxide-free ether and the pooled extract was washed with 2 × 10 ml water, dried over anhydrous sodium sulphate and evaporated. The residue was dissolved in 10 ml ethanol (700 ml/l), extracted with 10 ml *n*-hexane, and the upper phase discarded. The ethanolic solution was evaporated *in vacuo*, and the residue dissolved in 5.0 ml absolute ethanol.

For the separation of metabolites, 0.05 or 0.1 ml of the ethanolic solution and 3, 5, 10 and 20 µg of chenodeoxycholic acid standard in ethanol were applied on 2 cm strips of the Silicagel G layer. Three further strips on the chromatoplate contained 5 µg cholic, chenodeoxycholic, deoxycholic and lithocholic acid each for reference. The chromatogram was developed without preliminary equilibration in the System C (two developments in the same direction). The distance run was 15 to 16 cm and one development lasted 40 to 50 min. The reference strips were sprayed separately with 50 g/l phosphomolybdic acid dissolved in ethanol-ether (50 ml + 50 ml), and the plate placed in an oven at 105°C for 5 to 10 min. The zones were marked accordingly and scraped into tubes. To each was added 2.0 ml 96.5 ml/l sulphuric acid. The adsorbent was suspended thoroughly and the suspension incubated at 60°C for 60 min in a water-bath. The tubes were centrifuged at 5000 rpm (3000 *g*) for 10 min at room temperature. Spectrofluorimetry of blank zones, standards and the extracts was carried out as described above. The conjugated bile acid concentration of the duodenal content and bile was calculated according to the calibration of the standards and correction factors validated by control experiments described below. When chenodeoxycholic or deoxycholic acid standards, the steroids with a medium fluorescence intensity, were used, the values for cholic were multiplied by a factor of 0.5 and those for lithocholic acid by a factor of 2 in routine analyses.

Results

Thin-layer chromatographic separation of free and conjugated bile acids

The mobility of individual free and conjugated bile acids is shown in Table 1. For the separation of free cholic, chenodeoxycholic, deoxycholic and lithocholic acid the solvent system A was found most suitable. However, in this system the mobility of lithocholic acid was so high as to make fluorimetric evaluation uncertain due to contaminations running near to the solvent front. For the resolution of the 4 major free metabolites the system C was found superior, following two developments in the same direction. Table 1 also gives the R_F values for some bile acid conjugates. For grouped separation of the free bile acids, taurine and glycine conjugates, the solvent systems containing *n*-butylalcohol or *n*-propylalcohol were used. System J invariably effected a complete separation of the 3 bile acid groups. It is noteworthy that by a minimum change in the proportion of the polar component in systems L and M, the mobility of taurine conjugates changed considerably while that of the glycine conjugates hardly altered.

Control of saponification

Table 2 shows values for the percent conversion of taurine and glycine conjugates of chenodeoxycholic, deoxycholic and lithocholic acid obtained by two mild and one "forcing" procedure. Saponification of the

Tab. 2
Efficiency of saponification procedures for bile acid conjugates

Bile acid conjugates	NaOH 1.2 atm	NaOH 2.0 atm	KOH Glycol
TCD*	99**	82	94
TD	99	82	95
TL	99	89	97
GCD	92	70	80
GD	96	72	80
GL	83	67	77

* For abbreviation of bile acid conjugates see Table 1.

** Percent conversion of 100 µg bile acid conjugate to the parent free steroid.

Fig. 1

Relative sulphuric acid fluorescence intensity of individual bile acids. C_A , CD_A , L_A in the presence of Silicagel G adsorbent. C , CD , D , L without adsorbent (individual estimates on straight lines not indicated)

conjugates with sodium hydroxide in an autoclave at 2 atm resulted in a significant loss of the bile acids, while it was found superior by the two "mild" procedures, especially with that performed at 1.2 atm.

Control of sulphuric acid spectrofluorimetry

The effect of adsorbent on fluorescence intensity of free bile acids was studied and the observations illustrated on Figure 1. The quantities of cholic, chenodeoxycholic, deoxycholic and lithocholic acid chosen were in the working range of determinations from biological material. As it can be seen from the calibration curves, the fluorescence intensity after the chromatography of bile acids on Silicagel G layer, measured in the presence of adsorbent, was found somewhat lower than that observed for the corresponding steroid without adsorbent, especially for the two dihydroxy steroids. On the other hand, the relative intensity of fluorescence increased with the number of hydroxyl groups, irrespective of the conditions of measurement.

To determine further the lower limit of quantitation, the silica-blank values were compared with those measured for the minimum amounts of bile acids chromatographed. Disregarding a bile acid fluorescence not exceeding twice that of the silica-blank fluorescence,

the lower limit of sensitivity was found to be 0.2 μg for cholic, chenodeoxycholic and deoxycholic and 0.4 μg for lithocholic acid.

In consequence of these observations, the individual bile acids originating from biological material were evaluated by spectrofluorimetry according to standards run simultaneously on chromatoplates. Since the relative fluorescence of the individual bile acids varied, chenodeoxycholic acid was used for calibration and corrections were made accordingly (Fig. 1) for the other metabolites. The sensitivity of the method permitted the determination of bile acids in a 1/100 volume of the duodenal fluid or bile processed using 3, 5, 10 and 20 μg of chenodeoxycholic acid for standardization.

Recovery

The recovery of free cholic, chenodeoxycholic, deoxycholic, lithocholic or taurochenodeoxycholic, glycochenodeoxycholic, taurodeoxycholic, glycodeoxycholic and glycolithocholic acids from water, duodenal content or bile was studied. Pooled specimens of the biological fluids were used and worked out as described above. Values of mean recovery and the standard error of the mean recovery are presented. The standard error was calculated as $s_{\bar{x}} = s/\sqrt{n}$, where s is the standard deviation and n the number of recovery experiments. Results are shown in Table 3. Since the values in respect of the free steroids or conjugates showed no essential differences, they are summarized. It can be seen from the figures that the recovery of bile acids was satisfactory and from the standard errors that it was not significantly different when free or conjugated bile acids were added to samples. Individual values for the recovery of large amounts showed an approximate 1/3 to 1/5 decrease of recovery both from water and bile.

Reliability

The reliability of the method was checked by parallel analyses of 7 duodenal and 5 bile samples originating

Tab. 3
Recovery of free and conjugated bile acids from water, duodenal content and bile

Bile acid*	Added mg/l	n	Recovery, % $\bar{x} \pm s_{\bar{x}}$
Water			
C, TCD, GCD, TD, GD, TL, GL	50—100	15	84 $\pm$ 3.5
C	1000	1	72
TCD	500	1	101
TD	500	1	90
Duodenal content and bile			
C, TCD, GCD, TD, GD, TL, GL	50—100	8	83 $\pm$ 3.3
C	1000	1	64
GCD	500	1	96
GD	200	1	83

* For abbreviations see Table 1.

Tab. 4
Results of parallel analyses

Bile acid	N*	$\bar{x} \pm SD$ [mg/l]
Duodenal content		
C**	2	1298 $\pm$ 184
	5	57 $\pm$ 8
CD	6	821 $\pm$ 91
D	6	739 $\pm$ 50
L	4	105 $\pm$ 24
Bile		
C	5	3390 $\pm$ 489
CD	4	1570 $\pm$ 289
D	4	78 $\pm$ 7
L	5	126 $\pm$ 41

* Number of duplicates.

** For abbreviations see Table 1.

from normal individuals or patients following surgery. Results are given in Table 4. The standard deviation of a single estimate was calculated as $SD = \sqrt{\sum d^2 / 2N}$, where d is the difference between duplicates and N the number of pairs. It is noteworthy from the figures that SD did not increase when low steroid levels were measured.

Specificity

There was an excellent separation of cholic, chenodeoxycholic + deoxycholic and lithocholic acid into 3 fractions and a mutual contamination of the two dihydroxy metabolites not exceeding 1/20 part of one component in the other by the system C after two developments; with the specific estimation of bile acids with spectrofluorimetry the specificity of method is thus assured. The degree of artefact formation during saponification was insignificant with the present mild procedure as shown by comparative examinations. Chromatograms originating from intestinal contents obtained by the solvent system of *iso*-octane-ethyl

acetate-acetic acid (60 ml + 35 ml + 5 ml) (system C, two developments) were in several occasions visualized *in toto* with the phosphomolybdic acid reaction. Only trace amounts of unspecific contaminants were detected, and the zones of the 4 main bile acid metabolites appeared to be homogenous.

Representative clinical and experimental observations

The cholic, chenodeoxycholic, deoxycholic and lithocholic acid concentration in the duodenal content of normal subjects and patients with gastrointestinal disease or in bile obtained from patients following surgery for cholelithiasis or choledochotomy was determined and values in illustrative cases are shown in Table 5. Values are also given for the major bile acid metabolites determined in the bile of dogs and guinea-pigs. These findings serve rather as an additional control of the method than as a clinical or experimental study. As can be seen, a preponderance and a great variation of the primary bile acids (cholic and chenodeoxycholic) were observed, with smaller amounts of the secondary acids, especially lithocholic. There was no correlation between the concentration or proportion of steroids and the clinical state of patients.

Discussion

The present method is based on mild saponification, thinlayer chromatographic separation and spectrofluorimetric determination of conjugated cholic, chenodeoxycholic, deoxycholic and lithocholic acid in duodenal content and bile. According to the control experiments, an effective saponification at moderate pressure and a reliable separation of the four major metabolites on Silicagel G by an ENEROTH's type (5) solvent system (*iso*-octane-ethyl acetate-acetic acid (60 ml

Tab. 5

Individual conjugated bile acids in duodenal content and bile of human subjects, dogs and guinea-pigs determined by thin-layer chromatographic separation* and spectrofluorimetry

Biological fluid	Sex	Age [years]	Diagnosis	Bile acids, [mg/l]				Total
				C	CD	D	L	
DC	♂	38	Normal	40	1480	1080	14	2614
DC	♂	38	Normal	100	1130	950	8	2188
DC	♂	35	Pancreas cc	440	1200	70	21	1731
DC	♂	74	Cholelithiasis, before surgery	3800	1650	100	100	5650
DC	♀	46	Prim. biliar. cirrh.	5000	1800	160	80	7040
B	♂	27	Choledocholithiasis, after surgery	500	840	120	200	1660
B	♂	67	Cholelithiasis, after surgery	4050	420	890	120	5480
B	♀	44	Choledocholithiasis, after surgery	400	1960	210	280	2850
B	♀	60	Choledocholithiasis, after surgery	2280	1300	780	100	4460
B	♂		Dog, normal**	5500	2800	3900	100	12300
B	♂		Dog, normal**	5000	800	1700	100	7600
B	♀		Dog, normal**	1200	1700	2200	200	5300
B	♂		Guinea-pig, normal***					10600
B	♂		Guinea-pig, normal***					7800
B	♂		Guinea-pig, normal***					5400

* The solvent system of *iso*-octane-ethyl acetate-acetic acid (60 ml + 35 ml + 5 ml) was used (two developments).

DC = Duodenal content, without stimulation of gall bladder, B = Gall bladder bile, following laparotomy.

** Weight between 12 and 14 kg.

*** Weight between 400 and 450 g

C = Cholic, CD = Chenodeoxycholic, D = Deoxycholic, L = Lithocholic acid.

+ 35 ml + 5 ml)) could be achieved. The proposed combination of solvent components and two developments in the same system and direction improved the resolution of the two dihydroxy bile acids. Determination of the individual bile acid concentrations by spectrofluorimetry appeared to be a method with high sensitivity, however, the accuracy of measurements considerably increased when the values were corrected due to the different fluorescence intensity of the various metabolites and when simultaneously chromatographed standards were employed for calibration.

LINDSTEDT (20) proposed a method for studying bile acid dynamics by the determination of the mass, radioactivity and proportion of individual bile acids in the duodenal fluid following oral administration of the bile acid isotope. Recent findings (21, 22) confirmed the validity of this approach. The present thin-layer chromatographic and fluorimetric procedure for the accurate determination of individual conjugated bile acids in intestinal contents also seems to be suitable on this purpose. Results on this respect will be published separately.

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