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Note

Separation of bile acids on Chromarods: a new qualitative and semi-quantitative thin-layer chromatographic technique

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Most bile acids are present in bile and serum as their N-acyl glycine or taurine conjugates. The method described allows a rapid determination of the glycine/taurine ratio and the ratio of the di- to trihydroxy compounds. Previous methods are based on paper[1], thin-layer[2,3] and ion-exchange chromatography[4] and paper[5] and cellulose acetate electrophoresis[6]. In the method described here, bile acids are separated on Chromarods using two successive solvent mixtures. Quantitation is carried out by scanning the rods using a flame-ionization detector (FID) (scan time 1 min).

EXPERIMENTAL

Chemicals

Pure (> 98%) conjugated and unconjugated bile acids were obtained from Supelco (Bellafonte, PA, U.S.A.). Servachrom XAD type 2, 50–100 μm mesh, was obtained from Serva (Heidelberg, G.F.R.). Chromarods were supplied by Iatron Laboratories (Tokyo, Japan). All other chemicals were of analytical-reagent grade and were used without further purification.

Apparatus

The Iatroscan TG-10 Analyzer is produced by Iatron Laboratories. The apparatus consists mainly of a Chromarod holder frame, a scanning system and an FID (Fig. 1). The signal is simultaneously integrated with an Infotronics CRS-100 integrator and recorded on a Perkin-Elmer 56 recorder with a span of 10 mV and a paper speed of 6 cm/min. The Chromarods (Fig. 2) are thin rods of quartz-like material with a boundary sintered layer of silicic acid as the stationary phase. The samples are applied on the rods with a Terumo syringe (1 μl).

Sample preparation

Standard bile acid solutions are prepared by dissolving weighed amounts of standards in either water or methanol. Duodenal aspirate is collected by duodenal

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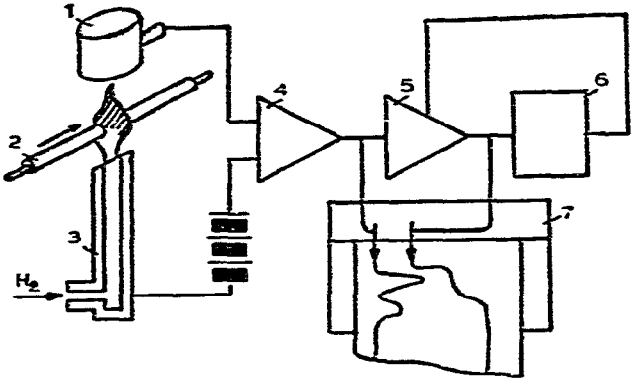


Fig. 1. Schematic diagram of the Iatroscan TG-10. 1 = Collector electrode; 2 = Chromarod; 3 = burner; 4 = current amplifier; 5 = integral amplifier; 6 = integral reset circuit; 7 = recorder.

intubation in patients after an overnight fast. All bile samples are collected in an ice-bath and frozen to −20 °C within 30 min.

Extraction of bile acids from duodenal fluid

A 0.1-ml volume of duodenal fluid is diluted with 9 ml of 0.1 *M* sodium hydroxide in saline and placed in an ultrasonic bath for 15 min. The sample is applied quantitatively on a column (28 cm × 0.8 cm I.D.) containing 0.5 g of Amberlite XAD-2 (flow-rate 6 drops/min) and washed with 10 ml of doubly distilled water to neutrality. Bile acids are eluted from the column with 5 ml of methanol. The eluate is evaporated under nitrogen and the residue redissolved in methanol to obtain a concentration of about 0.04 μmol/μl of bile acids.

Application and separation of bile acids on Chromarods

To obtain a straight baseline, the Chromarods are first passed through the flame for re-activation and clean-up. A 0.5-μl volume of the bile samples is then applied on the rods and developed in a saturated thin-layer chromatography (TLC) chamber at 4 °C. The upper phase of the first solvent mixture, toluene–acetic acid–water (10:9:1), is allowed to move to the top of the rods (development time *ca.* 1 h). After air-drying, the rods are developed with a second solvent, chloroform–methanol–

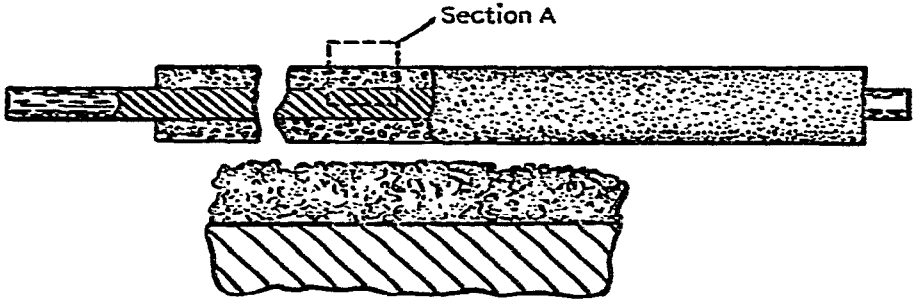


Fig. 2. Enlarged schematic view of a Chromarod showing the inner quartz rod and the outer sintered silica gel layer.

acetic acid–water (13 : 4 : 2 : 1) to a height of *ca.* 5.5 cm (development time *ca.* 10 min). The second eluent is evaporated at 115 °C for 20–25 min.

RESULTS AND DISCUSSION

Separation

Several solvent systems were investigated. The use of two successive solvent mixtures results in a efficient separation of the bile acids and their conjugates (Figs. 3 and 4). The R_F values of the fractions are given in Table I.

The R_F values are highest for monohydroxy acids and lowest for trihydroxy acids, according to the difference in polarity. The reproducibility of the R_F values is affected by room humidity (change of activity of the Chromarods) and room temperature (change of equilibrium of the solvent mixtures). Therefore, rigorous control of the chromatographic conditions is essential. Improved uniformity of the Chromarods is also needed in order to obtain a better reproducibility of the resolution between different rods.

Quantitative response

Mixtures of bile acid standards are applied in amounts of 0.2–2.5 μg per standard on Chromarods and developed as described above. Lithocholic acid (1 μg) is used as an internal standard. The calibration graph, obtained by plotting the surface ratio of bile acid standard to internal standard *versus* the amount of the bile acid, was linear up to a bile acid concentration of 1.5 μg per standard. For larger amounts a slightly different slope is found with all calibration graphs.

Relative molar response factors for the different bile acids and their conjugates are calculated in relation to glycodeoxy- and glycochenodeoxycholic acid (Table II).

The detection limits are determined by the sensivity ($<$ 100 ng) and by the separation capability ($>$ 3.0 μg) due to the overloading effect of the Chromarods.

Calculation

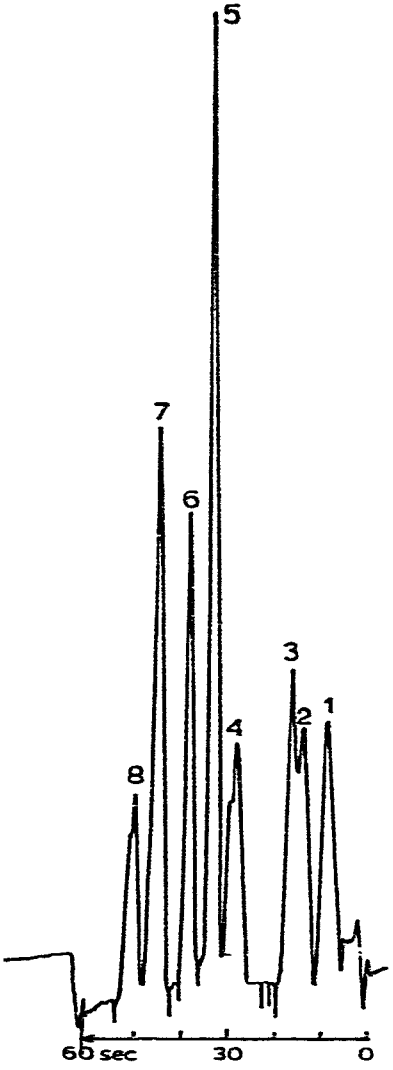
The use of an internal standard lowered the reproducibility of the results. For normal work the areas were divided by the corresponding relative molar response factors and the results expressed as a molar percentage.

Precision

The "within run" precision of the method is assessed by ten replicate analyses of a mixture of bile acid standards. A coefficient of variation of 5.0% on the molar percentage composition of the mixture was found in a concentration range of 0.8–2.0 μg per standard. This coefficient of variation was as high as 10% for lower concentrations but only 2% for higher concentrations. Five replicate bile acid analyses on one duodenal aspirate resulted in the same coefficient of variation (Table III). The "between run" precision (coefficient of variation = 5.0%) was obtained by re-analysis during 5 consecutive days of a duodenal aspirate (Table IV).

Validity of the method

The validity of the method was assessed by comparing the results obtained by the proposed method with the TLC method[3-7]. Using the Wilcoxon matched pairs



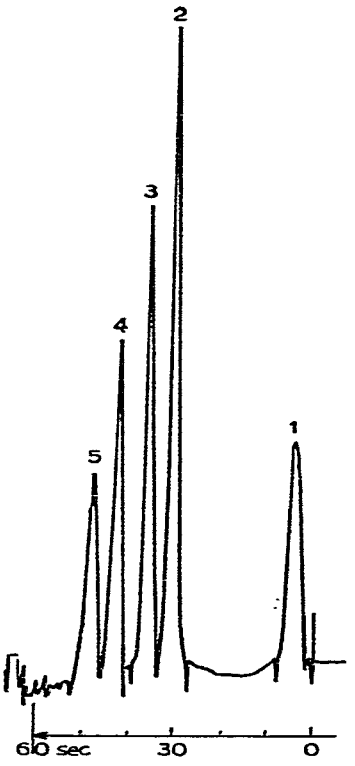


Fig. 3. Separation of standard mixture of bile acids: 1 = lithocholic; 2 = deoxycholic; 3 = chenodeoxycholic; 4 = cholic; 5 = glycodeoxycholic + glycochenodeoxycholic; 6 = glycocholic; 7 = taurodeoxycholic + taurochenodeoxycholic; 8 = taurocholic.

Fig. 4. Separation of bile acids from duodenal aspirate: 1 = cholesterol; 2 = glycodeoxycholic + glycochenodeoxycholic acid; 3 = glycocholic acid; 4 = taurodeoxycholic + taurochenodeoxycholic acid; 5 = taurocholic acid.

signed-ranks test, we found for $N = 47$ a $Z < 1.9599$ for $\alpha = 0.05$. Therefore, we concluded that it cannot be proved that there is a significant difference between the two methods at the $\alpha = 0.05$ level.

Results for bile samples

Eighty-two samples of duodenal fluid, obtained by intubation in 70 persons,

TABLE I

R_F VALUES OF BILE ACIDS FOR A CONCENTRATION OF 0.8 μg PER STANDARD

Bile acid	*Free acid*	*Glyco conjugate*	*Tauro conjugate*
Lithocholic acid	0.81 ± 0.02		
Deoxycholic acid	0.70 ± 0.02	0.47 ± 0.03	0.30 ± 0.02
Chenodeoxycholic acid			
Cholic acid	0.53 ± 0.02	0.41 ± 0.02	0.21 ± 0.02

were examined. Twenty-five of these samples could be considered as normal (absence of cholesterol hypersaturation, bacterial overgrowth, and disease in the gastrointestinal tract, liver, bile ducts and pancreas). This group showed a mean concentration of glycine conjugates of 67.9 ± 15.7 mol-%, tauro conjugates of 33.1 ± 0.6 mol-%, free bile acids of 1.7 ± 3.7 mol-% and summed trihydroxy bile acids of 39.9 ± 11.6 mol-%.

The 57 samples from pathological patients tend to show a slight but diagnostically insignificant lowering of the concentration of the glycine conjugates (56 ± 11.9 mol-%). Only two of the six patients with marked presence of free bile acids in serum showed an elevated concentration of free bile acids in their duodenal aspirate. This is explained by the fact that considerable deconjugation is taking place further on in the gastrointestinal tract.

Supersaturated bile does not display any specific pattern in this approach.

TABLE II

RELATIVE MOLAR RESPONSE FACTORS (R.M.R.F.) OF THE BILE ACIDS AND THEIR CONJUGATES

*Bile acid**	$F_i \cdot \frac{MG_i}{MG_{GDO,GCDC}} \pm$ *standard deviation* ($n = 5$)**
DO, CDC	0.58 ± 0.04
C	1.01 ± 0.07
GDC, GCDC	1.00
GC	0.78 ± 0.05
TDO, TCDC	0.70 ± 0.05
TC	0.59 ± 0.04

* DO = deoxycholic acid; CDC = chenodeoxycholic acid; C = cholic acid; GDO = glycodeoxycholic acid; GCDC = glycochenodeoxycholic acid; GC = glycocholic acid; TDO = taurodeoxycholic acid; TCDC = taurochenodeoxycholic acid; TC = taurocholic acid.

** F_i = relative response of bile acid; MG_i = molecular weight of bile acid; $MG_{GDO,GCDC}$ = molecular weight of GDO, GCDC.

CONCLUSION

The results indicate that separation and quantitation on a molar percentage basis in duodenal aspirates are easily achieved after extraction with Amberlite XAD-2 and rod-layer chromatography. The advantages of this method are its simplicity and its speed. Purity control of new standards is made very easy. The

TABLE III

"WITHIN RUN" PRECISION ON ONE DUODENAL ASPIRATE FOR $n = 5$

*Bile acids**	*Analysis 1 (%)*	*Analysis 2 (%)*	*Analysis 3 (%)*	*Analysis 4 (%)*	*Analysis 5 (%)*	*Mean ± S.D. (%)*	*Coefficient of variation (%)*
GDO, GCDC	42.2	43.4	44.2	44.1	44.5	43.7 ± 0.9	2.1
GC	18.8	16.5	17.2	17.4	17.8	17.5 ± 0.8	4.6
TDO, TCDC	27.6	28.0	17.9	27.9	27.5	27.9 ± 0.4	1.4
TC	11.3	11.4	10.6	10.5	10.2	10.8 ± 0.5	4.6

* For abbreviations, see Table II.

TABLE IV

"BETWEEN RUN" PRECISION ON ONE DUODENAL ASPIRATE DURING FIVE CONSECUTIVE DAYS

*Bile acids**	*First day (%)*	*Second day (%)*	*Third day (%)*	*Fourth day (%)*	*Fifth day (%)*	*Mean ± S.D. (%)*	*Coefficient of variation (%)*
GDO, GCDC	41.8	41.0	41.5	40.3	40.6	41.0 ± 0.6	1.5
GC	38.0	38.8	37.4	37.0	37.2	37.2 ± 0.7	1.9
TDO, TCDC	12.4	12.9	12.4	13.4	13.4	12.9 ± 0.5	3.9
TC	7.8	7.4	8.6	9.2	9.0	8.4 ± 0.8	9.5

* For abbreviations, see Table II.

advantages over high-performance liquid chromatography are the multi-sample system and the rapid quantitative detection with a universal FID detector.

Perhaps separations can be improved further by using longer and more standardized Chromarods.

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REFERENCES

1 J. Sjövall, *Clin. Chim. Acta*, 4 (1959) 652.
2 D. Panveliwalla, B. Lewis, I. D. P. Wootton and S. Tabaqchali, *J. Clin. Pathol.*, 23 (1970) 309.
3 R. Beke, G. A. De Weerdt, J. Parijs, W. Huybrechts and F. Barbier, *Clin. Chim. Acta*, 70 (1976) 197.
4 D. H. Sandberg, J. Sjövall, K. Sjövall and D. A. Turner, *J. Lipid Res.*, 6 (1965) 182.
5 N. Kaplowitz and N. B. Javitt, *J. Lipid Res.*, 14 (1973) 224.
6 R. Beke, G. A. De Weerdt, J. Parijs and F. Barbier, *Clin. Chim. Acta*, 71 (1976) 27.
7 A. Bruusgaard, *Clin. Chim. Acta*, 28 (1970) 495.