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NEW AND VERSATILE METHOD FOR THE DETERMINATION OF FAECAL BILE ACIDS BY THIN-LAYER CHROMATOGRAPHY WITH DIRECT SCANNING FLUORIMETRY

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SUMMARY

A sensitive, versatile and precise method for quantitative analysis of individual faecal bile acids in humans by thin-layer chromatography with direct scanning fluorimetry is described. The method enables convenient quantitative measurements of faecal bile acids in larger series of samples for routine applications in gastroenterology. The overall coefficient of variation (including stool preparation and extraction) for the five predominant bile acids of human stool specimens (cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid and ursodeoxycholic acid) was 3.4–4.9%. Recoveries of free bile acids added to the faeces ranged from 91% to 106%. An excellent and linear correlation between this method and fused-silica column gas chromatography with temperature programming was established ($r = 0.91$–0.99). In clinical practice this thin-layer chromatographic method constitutes a reliable, simple and time-saving alternative to gas chromatography.

INTRODUCTION

Quantitative analysis of individual faecal bile acids is both of theoretical and clinical interest. Until recently, only gas chromatography (GC) [1–5] and – since 1987 – also high-performance liquid chromatography [6] have been considered selective and sensitive enough for this purpose. Both these elaborate techniques, however, cannot be used for routine application in larger analytical series.

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A versatile technique for the quantitative determination of individual unconjugated faecal bile acids in the faeces of rats by means of thin-layer chromatography with direct scanning fluorimetry (TLC–DSF) has been described by Friemann [7]. This method is applicable for clinical purposes since the larger portion (93%) of faecal bile acids is in the unconjugated form [5,8–11]. It offers the practical advantage over GC that larger series of samples can be analysed, adequate for clinical application, within short periods of time and with less expenditure than GC.

In this paper we report on the validity of a modified TLC–DSF method for the quantitative determination of individual unconjugated faecal bile acids in humans and compare TLC–DSF with fused-silica column GC.

EXPERIMENTAL

Chemicals

All chemicals used were of analytical grade. Bile acids were obtained commercially: cholic acid (C) from Sigma (Taufkirchen, F.R.G.), chenodeoxycholic acid (CDC) and ursodeoxycholic acid (UDC) from Calbiochem (Giessen, F.R.G.). Deoxycholic acid (DC), lithocholic acid (LC), hyodeoxycholic acid (HDC), 3,12-di-keto-cholanic acid, Amberlite XAD-2 (mesh 100–200 μm), hexamethyldisilazane and trimethylchlorosilane were obtained from Serva Labs. (Heidelberg, F.R.G.). Thin-layer plates (20 cm $\times$ 20 cm, silica gel 60 with concentration zone, layer thickness 0.25 mm), isooctane, ethyl acetate and 2-propanol (all LiChrosolv), and 2,7-dichlorofluorescein were purchased from Merck (Darmstadt, F.R.G.). 1-Methyl-3-nitro-1-nitrosoguanidine was obtained from EGA Chemie (Steinheim, F.R.G.).

Apparatus

TLC fluorescence detection was carried out with a Camag TLC scanner monochromator ($\lambda = 366$ nm) (Camag, Berlin, F.R.G.). Fluorimetry peaks of the individual bile acids were recorded by a Perkin-Elmer Sigma 10 chromatography data station with interface (Perkin-Elmer, Überlingen, F.R.G.). For GC a Varian Model 3700 (Varian, Darmstadt, F.R.G.) equipped with a flame ionization detector (270°C) and fused-silica column (Durabond 1, 0.25 μm layer thickness, 25 m $\times$ 0.25 mm I.D., ICT Handelsgesellschaft, Frankfurt, F.R.G.) was used. The injection port temperature was 270°C, with splitless injection. The carrier gas was hydrogen at a flow-rate of 2 ml/min. Peaks were recorded on a W&W Recorder 600 Tarkan (Kontron, Munich, F.R.G.). Gas chromatography–mass spectrometry (GC–MS) was carried out on a Varian MAT 311 A apparatus coupled to a Varian 3700 gas chromatograph (25 m fused-silica column, Durabond 5, programme 150–310°C at 4°C/min, carrier gas helium at 5 ml/min, open coupling) coupled to the computer Spectrosystem MAT 188. The ionization energy was 70 eV.

Collection and preparation of stool specimen

The fresh stool specimen were weighed and homogenized immediately after collection and stored at −20°C. Ethanol (40 ml) and the internal standard (2 mg of HDC) were added to a stool portion of 4 g. After homogenization, ethanol (20 ml) was added and the mixture was refluxed for 2 h in a Soxhlet apparatus prior to centrifugation at 10 000 *g* (15 min). The supernatant was decanted and collected. The residue was shaken with 70% ethanol (10 ml) and centrifuged again for 15 min at 10 000 *g*. The supernatant was added to the first portion.

Extraction of bile acids

Water (50 ml) and 1 *M* NaOH (1 ml) were added to the collected supernatants, which were eluted three times with light petroleum (boiling range 40–60°C) to remove the neutral steroids. Then water (40 ml) and 1 *M* HCl (3.5 ml) were added, and the bile acids were extracted three times into chloroform (60, 40 and 40 ml). Bile acids were further purified by addition of 0.05 *M* NaOH (40 ml) and 5 *M* HCl (2 ml), and again triple extraction with chloroform (40 ml) was carried out. The chloroform phase was evaporated. The dry residue was resuspended in 0.1 *M* NaOH (1.5 ml) and titrated to pH 7 with HCl. The resulting solution was passed through an XAD-2 column (5 ml) and washed with water (20 ml). The bile acids were eluted with methanol (2×10 ml). The methanol content of the first 10-ml portion was evaporated with nitrogen at 35°C for 2 h. The residue was freeze-dried after the addition of water (3 ml). The dry sample was redissolved in the second portion of the methanol eluate. The solution was then evaporated. The purified bile acids were dissolved in methanol (0.7 ml). Sample preparation for the GC analysis is described below.

Thin-layer chromatography

The ethanol solution of the standard contained 1 mg/ml each of C, CDC, DC, LC, UDC, HDC (internal standard) and 3,12-di-keto-cholanic acid. In order to calibrate the system, 10 μl of the standard solution were applied to the plate. Optimal conditions (that is linear detector response) were obtained for the measurement when no more than 20 μg of the bile acids (2–30 μl of purified faecal bile acid solution) were distributed on the plate. The plates were chromatographed with isooctane–2-propanol–acetic acid (30:10:1, v/v) for 40 min, dried and chromatographed again with isooctane–ethyl acetate–acetic acid (10:10:2, v/v) for 65 min.

Fluorescence detection

For quantitative analysis of bile acids the plates were vertically dipped (for 2 s) into a 0.2% 2,7-dichlorofluorescein ethanol solution. Bile acid fluorescence was measured with a TLC scanner and on-line plotter; results were calculated by the peak-height method.

Gas chromatography

Derivatization. Bile acids were esterified (by treating the evaporated sample) with diazomethane reagent (1 ml) followed by evaporation with nitrogen to dryness. Trimethylsilyl (TMS) ether derivatives of bile acid methyl esters were obtained by incubating the evaporated sample with pyridine–hexamethyldisilazane–trimethylchlorosilane (3:2:1, v/v) for 20 min followed by evaporation under a stream of nitrogen. The derivatives were redissolved in *n*-hexane [11].

Measurement. The solutions of bile acid derivatives (0.1–1.0 μl) in *n*-hexane were injected. The column temperature was raised at 4°C/min from 150 to 270°C and held at 270°C for 10 min. Bile acid amounts were calculated by the peak-height method [11]. Retention indices, expressed as methylene units, were calculated by coinjection of the sample and a mixture of straight-chain hydrocarbons from $C_{18}H_{38}$ to $C_{38}H_{78}$ [12]. All peaks obtained from the GC separation of the faecal specimen were controlled and identified by GC–MS.

RESULTS

Analysis of bile acids by TLC-DSF

The predominant faecal bile acids (C, CDC, DC, LC and UDC) were well separated and unequivocally defined by TLC–DSF (Fig. 1). The respective retention indices are listed in Table I. A representative TLC–DSF chromatogram of a standard mixture of bile acids and faecal bile acids is shown in Fig. 2.

The linearity of the fluorescence signal for all five faecal bile acids was excellent between 2 and 20 μg ($r=0.99$) (Fig. 3). The coefficients of variation (C.V.) for repeated ($n=18$) determinations of these five bile acids were found to vary between 0.8% (CDC) and 5.4% (LC).

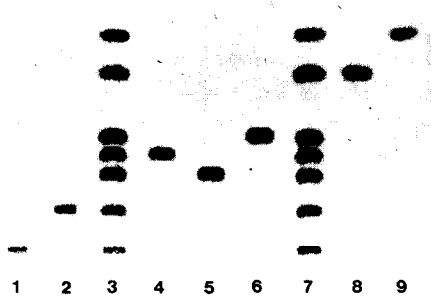

Fig. 1. TLC of free bile acids (anisaldehyde dye). Lanes: 1 = cholic acid; 2 = hyodeoxycholic acid (internal standard); 3 = standard bile acid mixture; 4 = chenodeoxycholic acid; 5 = ursodeoxycholic acid; 6 = deoxycholic acid; 7 = standard bile acid mixture; 8 = 3,12-di-keto-cholanic acid; 9 = lithocholic acid.

TABLE I

RETENTION INDICES OF BILE ACIDS

Retention indices of bile acids (1 mg/ml in ethanol) measured by TLC as described in Experimental. Values represent the means ± S.D. of ten experiments.

Bile acid	Abbreviation	Retention index
Cholic acid	C	0.55 ± 0.04
Hyodeoxycholic acid	HDC	1.00
Ursodeoxycholic acid	UDC	1.36 ± 0.04
Chenodeoxycholic acid	CDC	1.52 ± 0.06
Deoxycholic acid	DC	1.67 ± 0.07
Lithocholic acid	LC	2.63 ± 0.18

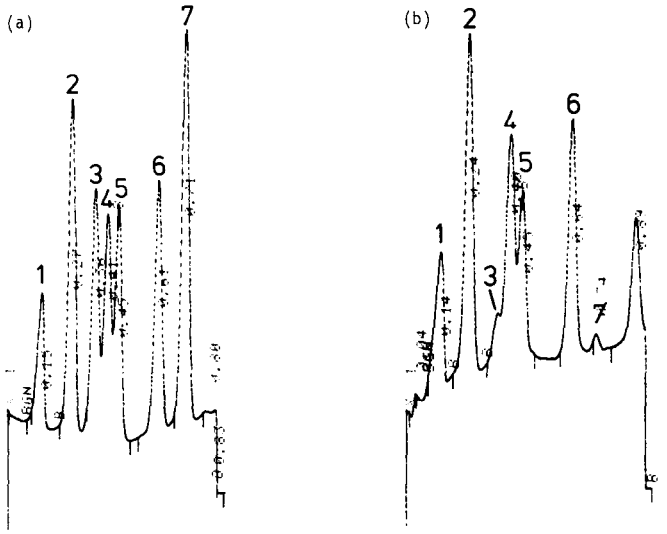

Fig. 2. Fluorescence detection of (a) free bile acids (5 μg per spot) in a standard solution and (b) faecal bile acids in a stool specimen from a patient with bile acid malabsorption, both separated by TLC. Peaks: 1 = cholic acid; 2 = hyodeoxycholic acid (internal standard); 3 = ursodeoxycholic acid; 4 = chenodeoxycholic acid; 5 = deoxycholic acid; 6 = 3,12-di-keto-cholanic acid; 7 = lithocholic acid.

The C.V. were distinctly higher when 3,12-di-keto-cholanic acid was the internal standard (4.4–11.4%). Furthermore, 3,12-di-keto-cholanic acid could not be derivatized for GC. Therefore, HDC was used as the internal standard for both methods.

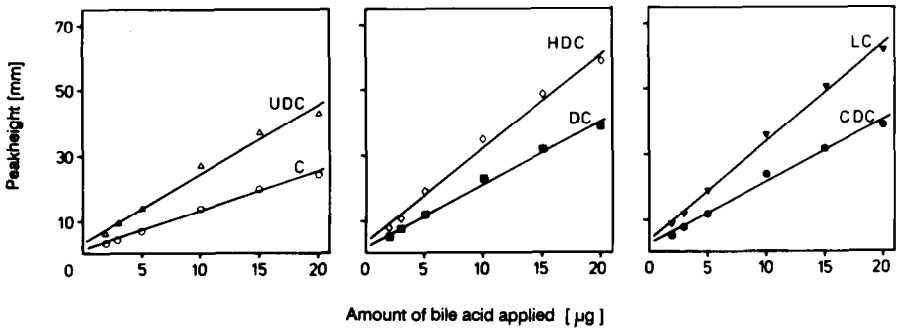

Fig. 3. Linearity of the fluorescence signal of a standard bile acid mixture (1 mg of each bile acid per ml ethanol) in the range 2–20 µg. For abbreviations see Table I.

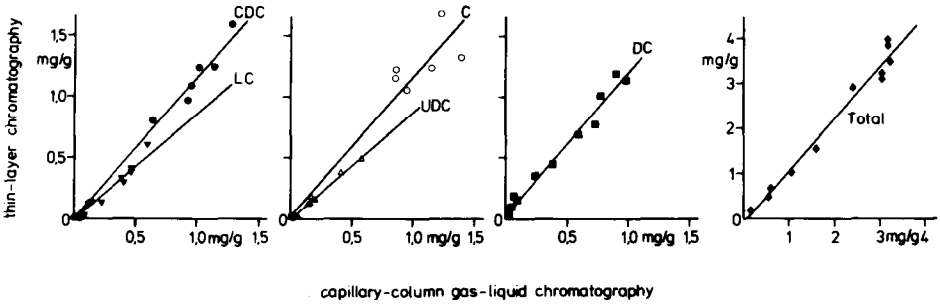

Fig. 4. Correlation between fused-silica column GC and TLC–DSF measurements of human faecal bile acids ($n=11$). Specimens were obtained both from persons with normal bile acid excretion and from persons with bile acid malabsorption. LC, $r=0.91$; CDC, $r=0.99$; UDC, $r=0.99$; C, $r=0.98$; DC, $r=0.95$; total faecal bile acids, $r=0.99$. For abbreviations see Table I.

The excellent reproducibility of the TLC–DSF method, including stool preparation and extraction (four-fold analysis), is characterized by a C.V. of 3.4–4.9% for the different bile acids. The recovery of a bile acid mixture added to the stool (2 mg of each bile acid per 1 g of wet faeces) was 95–106%.

Comparison of TLC and GC

TLC was compared with GC in the analysis of eleven stool specimens of human origin with both normal and abnormal faecal bile acid patterns (known bile acid malabsorption with increased excretion of C, CDC and UDC). The results are summarized in Fig. 4. A linear correlation between the two methods was found, with correlation coefficients for the respective faecal bile acids between 0.91 and 0.99.

DISCUSSION

The method described for the preparation and purification of faecal bile acids and their analysis by TLC–DSF proved to be very suitable for the quantitative analysis of free faecal bile acids. Conjugated bile acids were not taken into account since they constitute only a minor portion of faecal bile acids [5,8–11]. Experiments in our laboratory [7] with ^{14}C-labelled CDC revealed a recovery of 89% and are thus in accordance with the results of other authors [6,13]. Our recoveries of 95–106% of bile acid standard solutions added to human stool specimens at a clinically relevant concentration are within the range of previously published data obtained with GC methods [1,2,9]. The reproducibility of the analytical method described (C.V.=3.4–4.9%) is comparable with that of GC methods (4.7–9.4%) [11]. The use of HDC instead of 3,12-di-keto-cholanic acid (described in ref. 7) as the internal standard improved the C.V. of direct scanning fluorimetry, resembling those described by Van den Ende et al. [14].

Compared with previous data on the correlation of enzymic [9,15] and TLC methods with GC [16], a better linear correlation (r=0.99) for the quantification of *total* bile acids was obtained with TLC–DSF and GC as practised here. Moreover, an excellent correlation was found for the individual faecal bile acids (r=0.91–0.99). No comparable data have so far been published in the literature on the amount of *individual* faecal bile acids measured by GC in comparison with either enzymic or TLC methods.

For clinical analytical purposes, TLC–DSF represents a versatile, sensitive and precise method that is suitable for routine purposes. In contrast to GC, derivatization of bile acids is not necessary, thereby eliminating one potential source of variation. The results can be achieved at lower expense of equipment and time. Eight samples can be chromatographed on one plate simultaneously. Compared with other TLC methods [14,17–19], the use of 2,7-dichlorofluorescein offers the advantage of direct scanning fluorimetry on the original plate without additional elution procedures or heating, which again may be prone to imprecise quantification. In clinical practice these advantages outweight the theoretical disadvantage of discerning only five (the most important for gastroenterologists) bile acids in humans. Other bile acids contribute only a minor part to the total amount of human bile acids [11] and can be ignored with respect to the diagnosis of bile acid malabsorption and chologenic diarrhoea. Therefore, the described method is a practicable alternative to GC in the clinical setting, e.g. the identification of bile acid malabsorption.

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