Journal of Chromatography, 400 (1987) 149–161
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1116

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC–FLUORESCENCE DETERMINATION OF HUMAN FAECAL BILE ACIDS

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SUMMARY

A high-performance liquid chromatographic method with fluorescence detection has been developed for the determination of human faecal bile acids, especially free faecal bile acids. Faecal bile acids were extracted using an Amberlite XAD-2 column and then fractionated into four groups (free, glycine-conjugated, taurine-conjugated and sulphated bile acids) on a piperidinohydroxypropyl Sephadex LH-20 column. The free bile acid fraction and free bile acids obtained after enzymatic hydrolysis and/or solvolysis of the three other fractions were derivatized with 1-bromoacetylpyrene and dicyclohexyl-18-crown-6-ether. The derivatized bile acids were separated stepwise on a Shim-pack CLC-ODS column using acetonitrile–methanol–water (100:50:30) (A), (100:50:20) (B), and (100:50:0) (C) as mobile phases with changing automatically from A to C using a solvent changer. Calibration curves of bile acid standards were linear in the range between 20 and 400 pmole, when monitored at 370 nm (excitation) and 440 nm (emission). Percent recoveries of bile acids from human faecal samples were between 80 to 95%. The method is applicable to clinical use and is sensitive, reliable, and useful for the detailed determination of human faecal bile acids, especially of free faecal bile acids.

INTRODUCTION

In recent years, there has been a renewed interest in the measurement of bile acids in faeces. This is due to the possible effect of cholesterol and bile acid metabolism on diseases, such as atherosclerosis, gallstones, colorectal cancer, and disorders associated with a changed intestinal microflora. Due to such diseases, the qualitative and quantitative composition of bile acids in the enterohepatic circulation may change. For the understanding and diagnosis of the diseases it is valuable to elucidate the metabolic profiles of bile acids and related compounds in faeces in detail.

A variety of methods has been described for the determination of faecal bile acids and related compounds. Enzymatic and fluorometric methods[1–4], gas–liquid

0021-9673/87/$03.50

chromatography (GLC)[5–7], and GLC–mass spectrometry (MS)[8,9] have been used. Recently, high-performance liquid chromatographic (HPLC) methods have been developed for the determination of faecal bile acids[10,11], but these methods seem to be inadequate to determine faecal bile acids, which include considerable amounts of ketonic bile acids and 3β-hydroxy bile acids. Therefore, we employed HPLC with fluorescence detection using 1-bromoacetylpyrene as a derivatizing reagent[12]. In this paper we describe a sensitive and reliable HPLC method for the detailed determination of human faecal bile acids including ketonic and β-hydroxy bile acids. The method is especially suitable for the determination of free faecal bile acids.

EXPERIMENTAL

Reference bile acids

The bile acids were derived from cholic acid (Wako, Osaka, Japan). The remaining reference samples of bile acids and their derivatives were prepared according to the literature and their physical properties were in agreement with those previously reported[13–18].

The purity of the samples was better than 97%, as checked by thin-layer chromatography (TLC), GLC and/or GLC–MS.

All reference bile acids were derivatives of 5β-cholan-24-oic acid; 3α,7α,12α-trihydroxycholanic acid (cholic acid), 3α,7β,12α-trihydroxycholanic acid, 3β,7α,12α-trihydroxycholanic acid, 3α,6α,7α-trihydroxycholanic acid (hyocholic acid), 3α,6β,7α-trihydroxycholanic acid (α-muricholic acid), 3α,6β,7β-trihydroxycholanic acid (β-muricholic acid), 3α,7α-dihydroxy-12-ketocholanic acid, 3α,12α-dihydroxy-7-ketocholanic acid, 7α,12α-dihydroxy-3-ketocholanic acid, 3α,6α-dihydroxycholanic acid (hyodeoxycholic acid), 3α,7α-dihydroxycholanic acid (chenodeoxycholic acid), 3α,7β-dihydroxycholanic acid (ursodeoxycholic acid), 3β,7β-dihydroxycholanic acid, 3α,12α-dihydroxycholanic acid (deoxycholic acid), 3β,12α-dihydroxycholanic acid, 7α,12α-dihydroxycholanic acid, 7β,12α-dihydroxycholanic acid, 3α-hydroxy-7-ketocholanic acid, 3α-hydroxy-12-ketocholanic acid, 7α-hydroxy-3-ketocholanic acid, 12α-hydroxy-3-ketocholanic acid, 3α-hydroxy-7,12-diketocholanic acid, 7α-hydroxy-3,12-diketocholanic acid, 12α-hydroxy-3,7-diketocholanic acid, 3α-hydroxycholanic acid (lithocholic acid), 3β-hydroxycholanic acid (isolithocholic acid), 7α-hydroxycholanic acid, 12α-hydroxycholanic acid, 3,7,12-triketocholanic acid (dehydrocholic acid), 3,7-diketocholanic acid, 3,12-diketocholanic acid, 3-ketocholanic acid, cholanic acid, glycocholic acid, glycochenodeoxycholic acid, glycoursodeoxycholic acid, glycodeoxycholic acid, glycolithocholic acid, taurocholic acid, taurochenodeoxycholic acid, tauroursodeoxycholic acid, taurodeoxycholic acid, chenodeoxycholic acid 3-sulphate and glycolithocholic acid 3-sulphate, except for 3α-hydroxy-5α-cholan-24-oic acid (allolithocholic acid), 24-nor-3α,7α,12α-trihydroxy-5β-cholanic acid (norcholic acid), 24-nor-3α,12α-dihydroxy-5β-cholanic acid (nordeoxycholic acid), 23,24-bisnor-3α,7α,12α-trihydroxy-5β-cholanic acid (bisnorcholic acid) and 3β-hydroxychol-5-en-24-oic-acid (3β-hydroxychol-5-enic acid).

Other materials

Lauric acid, myristic acid, palmitic acid, and stearic acid were purchased from Wako. Dicyclohexyl-18-crown-6-ether was obtained from Nakarai (Kyoto, Japan),

acetonitrile and methanol for HPLC from Wako and cholylglycine hydrolase (E.C. 3.5.1.24) from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical grade, and solvents were purified by distillation prior to use.

Amberlite XAD-2 was purchased from Rohm and Haas (Philadelphia, PA, U.S.A.) and piperidinohydroxypropyl-Sephadex LH-20 (PHP-LH-20), Shim-pack CLC-ODS and CLC-TMS from Shimadzu (Kyoto, Japan). Sep-Pak C_{18} cartridges were obtained from Waters (Milford, MA, U.S.A.).

HPLC and chromatographic conditions

A Shimadzu Model LC-6A HPLC system, equipped with a system controller (SCL-6A), an automatic sample injector (SIL-6A), and a reversed-phase column (Shim-pack CLC-ODS, 150 × 6 mm I.D.; particle size 5 μm, Shimadzu) was used, and a Hitachi (Tokyo, Japan) Model F 1000 spectrofluorophotometer, equipped with a 40-μl micro flow-cell was used as a monitor at an excitation wavelength of 370 nm and an emission wavelength of 440 nm.

Quantitative analysis of bile acids was carried out using a Chromatopac C-R3A recorder (Shimadzu) with 7α,12α-dihydroxycholanic acid as an internal standard. The derivatization procedure for fluorescence detection of bile acids was performed according to the method of Kamada *et al.*[12]. The data were analyzed statistically using the Student *t*-test.

After derivatization with 1-bromoacetylpyrene, the separation of derivatives of bile acids was carried out at 25°C by stepwise elution with three acetonitrile–methanol–water mixtures [100:50:30 (A), 100:50:20 (B), and 100:50:0 (C)] as mobile phases at a flow-rate of 2 ml/min. The mobile phase was automatically changed by a solvent changer from A to B 20 min after injection of the sample solution from B to C after 60 min, and from C to A after 80 min.

Collection and homogenization of stools

Stools were collected for 3 consecutive days from healthy men ($n = 5$) and women ($n = 5$) of ages between 25 and 40 years. Specimens were collected directly into preweighted polythene bags and stored at −20°C until analysis. The daily samples were combined and allowed to thaw at 4°C and subsequently thoroughly mixed in the bag. A portion of 20 g of the stool was homogenized with four volumes of water, and a quarter of the homogenate was freeze-dried, the lyophilized faeces was mixed well and powdered.

Analytical procedures

Each freeze-dried faecal specimen (250 mg) was placed into a 100-ml round-bottomed flask containing 3 ml of 1 *M* sodium hydroxide and 27 ml of ethanol. To the mixture 40 μg of 7α,12α-dihydroxycholanic acid in 100 μl of ethanol was added as an internal standard.

The sample was refluxed for 1 h. After cooling, the supernatant was transferred through a glass fiber filter paper (GF/C; Whatman, U.K.) to a 50-ml tube with a stopper. The residue on the paper was washed with a small volume of ethanol to minimize any losses. The resulting extract was evaporated to dryness under a stream of air on a water bath at 65°C.

The dried extract was dissolved in 20 ml of 1 *M* sodium hydroxides, 4 ml of

methanol were added and the solution was mixed well. To the sample solution 20 ml of *n*-hexane was added, it was mixed well and then centrifuged at 700 *g* for 10 min. The *n*-hexane extraction was performed three times in the same way to remove neutral steroids from the sample.

The methanolic phase was condensed to half of the original volume at 65°C under a stream of air and was finally made up to 20 ml with distilled water. The resulting solution was passed through an Amberlite XAD-2 column, which was then washed with distilled water until the washes were neutral. Faecal bile acids were then eluted with 30 ml of 0.1% ammonium carbonate in 90% ethanol[19].

The bile acid fraction was evaporated to dryness with a rotary evaporator and one-tenth of the fraction was dissolved in 2 ml of 90% ethanol. This sample was applied slowly to a PHP-LH-20 column (40 × 6 mm, I.D.) at a flow-rate of 7–8 drops/min, and fractionated into four groups by a slight modification of the method of Goto *et al.*[20].

After elution with 90% ethanol (6 ml), free, glycine-conjugated, taurine-conjugated, and sulphated bile acids were eluted by stepwise elution with 0.1 *M* acetic acid in 90% ethanol (12 ml), 0.2 *M* formic acid in 90% ethanol (12 ml), 0.3 *M* acetic acid–potassium acetate in 90% ethanol (pH 6.5, 18 ml) and 1% ammonium carbonate in 70% ethanol (pH 7.8, 18 ml), respectively. Each fraction was evaporated to dryness with a rotary evaporator.

Hydrolysis of conjugated bile acids and solvolysis of sulphated bile acids

Glycine- or taurine-conjugated bile acids were subjected to enzymatic hydrolysis with cholylglycine hydrolase by the (slightly modified) method of Kamada *et al.*[12]. Sulphated bile acids were treated according to Parmentier and Eyssen[21] and Takikawa *et al.*[22].

Recovery test of faecal bile acids

The recovery of faecal bile acids was estimated, using a freeze-dried human faecal sample, divided into six 250-mg portions. Three of these samples were treated by the present procedure.

To the remaining three 40 μg each of cholic, deoxycholic, chenodeoxycholic, ursodeoxycholic, lithocholic, 3-ketocholanic acids, and 7α,12α-dihydroxycholanic acid as an internal standard were added. For recovery tests of the glycine- or taurine-conjugated and sulphated fractions, glycine or taurine conjugates of hydroxy bile acids, described above, and the sulphates of chenodeoxycholic and glycolithocholic acids were used, in addition to 7α,12α-dihydroxycholanic acid.

RESULTS

Separation of bile acids and fatty acids by the present HPLC method

Capacity ratios and relative retentions (relative to deoxycholic acid) of reference bile acids and fatty acids are listed in Table I. Generally, the bile acids were eluted in order of increasing polarity in this HPLC system. The bile acids with a β-hydroxyl group were eluted before those with an α-hydroxyl group on the corresponding parent bile acids.

A chromatogram of standard free bile acids in a mixture resembling the main human faecal bile acid pattern is shown in Fig. 1.

TABLE I

CAPACITY RATIOS (k') AND RELATIVE CAPACITY RATIOS (k'_{rel}) FOR BILE ACIDS AND FATTY ACIDS

For HPLC details see Experimental section. Values are means of three experiments, using stepwise elution with acetonitrile–methanol–water mixtures as mobile phases on a Shim-pack CLC-ODS column at a flow-rate of 2 ml/min.

*Standard***	k'	k'_{rel}*
C_{24} (free)		
TriOH		
3α,7β,12α-triOH	5.53	0.158
3α,12α,23-triOH (BA)	7.70	0.220
3α,6β,7α-triOH (α-MA)	8.60	0.246
3α,6β,7β-triOH (β-MA)	9.72	0.278
3α,6α,7α-triOH (HCA)	12.62	0.361
3α,7α,12α-triOH (CA)	15.80	0.452
DiOH-monoketo		
3α,12α-diOH-7-keto	8.52	0.244
3α,7α-diOH-12-keto	8.83	0.253
7α,12α-diOH-3-keto	12.53	0.259
MonoOH-diketo		
3β-OH-7,12-diketo (IsoRDHCA)	5.26	0.151
3α-OH-7,12-diketo (RDHCA)	5.30	0.152
12α-OH-3,7-diketo	6.79	0.194
7α-OH-3,12-diketo	7.19	0.206
Triketo		
3,7,12-triKeto (DHCA)	5.28	0.151
DiOH		
3α,7β-diOH (UDCA)	12.21	0.349
3β,7β-diOH	13.71	0.392
3α,6α-diOH (HDCA)	14.96	0.428
7α,12β-diOH	15.52	0.444
3α,12β-diOH	16.40	0.469
3β,12α-diOH	19.73	0.565
3α,7α-diOH (CDCA)	31.71	0.907
3α,12α-diOH (DCA)	34.95	1.000
7α,12α-diOH (I.S.)	38.72	1.108
MonoOH-monoketo		
3β-OH-12-Keto	14.80	0.423
3α-OH-12-Keto	18.28	0.523
7α-OH-3-Keto	27.54	0.788
12α-OH-3-Keto	30.08	0.861
MonoOH-mono-Ac		
3α-OH-7α-Ac	27.22	0.779
12α-OH-3α-Ac	44.20	1.265
Diketo		
3,7-diketo	15.65	0.448
3,12-diketo	17.30	0.495
MonoOH		
3β-OH-Δ^5	43.03	1.231
3β-OH	47.17	1.350
3α-OH (LCA)	49.65	1.421
3α-OH (AlloLCA)	53.13	1.520
7α-OH	70.36	2.013
12α-OH	72.41	2.072

(continued on p. 154)

TABLE I (*continued*)

*Standard***	k'	k'_{rel}*
Monoketo		
3-keto	52.71	1.508
Cholanic Acid	ND***	ND
C_{23} *(free)*		
TriOH		
norCA	12.88	0.369
DiOH		
norDCA	27.83	0.796
C_{22} *(free)*		
TriOH		
bisnor CA	12.16	0.348
Glycine conjugated		
TriOH		
G-CA	3.49	0.100
DiOH		
G-UDCA	5.86	0.168
G-CDCA	9.46	0.271
G-DCA	11.26	0.322
MonoOH		
G-LCA	18.59	0.532
Fatty acid		
Lauric acid	44.55	1.275
Myristic acid	71.33	2.041
Palmitic acid	121.96	3.490
Stearic acid	ND	ND

* Relative retention to 3α,12α-dihydroxy-5β-cholan-24-oic acid (DCA) (34.95). Reference bile acids are substituted 5β-cholan-24-oic acids, except for 3α-hydroxy-5α-cholan-24-oic acid.

** Greek letters denote configuration of hydroxy groups, C_{24}, C_{23} and C_{22} denote homologues with 24, 23 and 22 carbon atoms, respectively; Δ = a double bond; OH = hydroxy; Ac = acetoxy; G = glyco; T = tauro; BA = bithocholic acid; MA = muricholic acid; HCA = hyocholic acid; CA = cholic acid; RDHCA = reductodehydrocholic acid; DHCA = dehydrocholic acid; UDCA = ursodeoxycholic acid; HDCA = hyodeoxycholic acid; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid; LCA = lithocholic acid; I.S. = internal standard.

*** ND = Not detectable.

Reproducibility and calibration curves of bile acids in the present HPLC system

Reproducibility of retention times and peak areas were determined for five common free bile acids (Table II).

The calibration curves of all the reference bile acids were linear in the range between 20 and 400 pmole. The variations in retention times of the bile acids over a period of 3 months were negligible.

Recovery of bile acids from human faecal samples

The results of the recovery test showed a reproducibility of better than 10% standard deviation, with about 90% overall recovery for all the bile acids tested, although the less polar bile acids tended to give higher recovery. No extraneous peaks appeared with retention times close to 7α,12α-dihydroxycholanic acid, the internal standard.

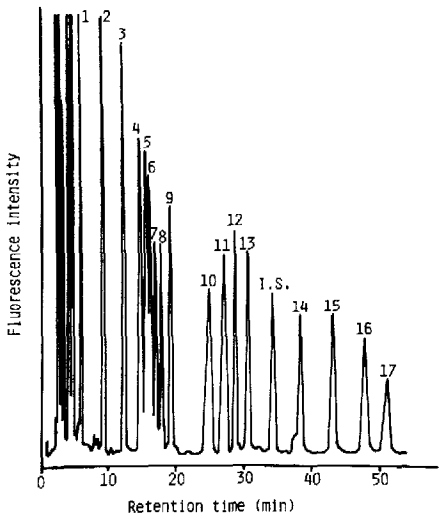

Fig 1. HPLC profiles with fluorescence detection of standard free bile acids in a mixture containing the main human faecal bile acids. Peaks: 1 = 3α,7β,12α-trihydroxycholanic acid; 2 = 3α,7α-dihydroxy-12-ketocholanic acid; 3 = 3α,7β-dihydroxycholanic acid; 4 = 3β-hydroxy-12-ketocholanic acid; 5 = 3α,7α,12α-trihydroxycholanic acid; 6 = 3α,12β-dihydroxycholanic acid; 7 = 3,12-diketocholanic acid; 8 = 3α-hydroxy-12-ketocholanic acid; 9 = 3β,12α-dihydroxycholanic acid; 10 = 7α-hydroxy-3-ketocholanic acid; 11 = 12α-hydroxy-3-ketocholanic acid; 12 = 3α,7α-dihydroxycholanic acid; 13 = 3α,12α-dihydroxycholanic acid; 14 = 3β-hydroxy-5-cholenic acid; 15 = 3β-hydroxycholanic acid; 16 = 3α-hydroxycholanic; 17 = 3-ketocholanic acid; I.S. (internal standard) = 7α,12α-dihydroxycholanic acid..

The efficiency of the present extraction procedure for endogenous bile acids in human faeces was not tested due to difficulties in obtaining faecal samples from subjects which had been given labelled cholic acid and chenodeoxycholic acid. Judging from a published method that was evaluated using a ^{14}C-labelled sample[7], the efficiency of the present extraction procedure may be appreciably lower for endogeneous faecal bile acids than for bile acids added to faecal samples.

TABLE II

COEFFICIENTS OF VARIATION (C.V.) OF RETENTION TIMES AND PEAK AREAS OF BILE ACID STANDARDS

For HPLC conditions see Experimental section. Values are mean ± standard deviation of five experiments for 200 pmole each of reference bile acids.

Bile acid	*Retention time (min)*		*Peak areas*	
	Mean ± S.D.	*C.V. (%)*	*Mean ± S.D.*	*C.V. (%)*
Ursodeoxycholic	12.02 ± 0.32	2.66	151313 ± 2264	1.50
Cholic	15.23 ± 0.49	3.22	146348 ± 1258	0.86
Chenodeoxycholic	28.00 ± 0.68	2.43	123649 ± 3839	2.10
Deoxycholic	29.82 ± 0.60	2.01	112612 ± 1169	1.04
Lithocholic	44.02 ± 0.98	2.32	115249 ± 1223	1.06

TABLE III

COMPOSITION OF FAECAL BILE ACIDS IN HEALTHY HUMAN SUBJECTS

Bile acid[*]	*Free bile acids*			
	Men		*Women*	
	μg/g dry faeces	*%*	*μg/g dry faeces*	*%*
3α,7β,12α-triOH	164.2 ± 124.8[**]	1.5 ± 0.6	440.6 ± 335.9[**]	5.0 ± 3.2
3α,7α-diOH-12-keto	477.7 ± 525.4	4.1 ± 4.1	392.1 ± 265.1	5.4 ± 3.8
3α,7β-diOH	86.5 ± 44.0	0.8 ± 0.1	128.4 ± 57.3	1.5 ± 0.8
3β-OH-12-keto	(144.4, 21.2, 268.5)[***]	–	165.0 ± 94.4	1.8 ± 0.6
3α,7α,12α-triOH	315.9 ± 193.7	3.2 ± 0.9	261.2 ± 126.4	2.8 ± 1.2
3α,12β-diOH	(19.3,249.0,2018.3)	–	216.8 ± 276.4	1.8 ± 1.6
3,12-diketo	(1187.6,238.6)	–	(393.0, 24.7, 118.2)	(5.5,0.5,1.5)
3α-OH-12-keto	(462.6,445.6)	–	443.6 ± 277.6	4.8 ± 3.2
3β,12α-diOH	771.4 ± 574.6	7.1 ± 3.4	654.5 ± 353.7	7.3 ± 1.7
7α-OH-3-keto	(199.3)	–	(19.9,227.7,152.8)	(0.1,2.8,1.6)
12α-OH-3-keto	505.4 ± 574.2	5.1 ± 1.4	626.3 ± 243.5	7.8 ± 3.0
3α,7α-diOH	613.9 ± 687.5	6.1 ± 5.2	442.8 ± 246.8	4.8 ± 1.2
3α,12α-diOH	2810.9 ± 993.5	29.7 ± 3.1	2262.5 ± 2057.7	22.4 ± 9.0
3β-OH-Δ^5	434.5 ± 291.6	4.0 ± 1.2	268.9 ± 191.2	4.2 ± 4.4
3β-OH	1192.3 ± 480.6	13.4 ± 5.5	924.5 ± 291.0	10.8 ± 1.8
3α-OH	1057.7 ± 506.7	10.8 ± 5.2	1149.1 ± 1026.1	11.0 ± 4.9
3-keto	359.0 ± 46.7	4.5 ± 2.4	526.8 ± 216.1	6.2 ± 2.5
Fraction				
Primary bile acids	929.8 ± 675.4	9.0 ± 4.3	704.0 ± 354.5	7.6 ± 2.2
Secondary bile acids	9065.7 ± 3814.5	90.9 ± 4.2	8386.3 ± 3955.0	92.4 ± 2.2
Total bile acids	9994.0 ± 4190.1		9090.2 ± 4275.5	
Total bile acids in men + women				
Amount (μg/g dry faeces)		9492.2 ± 3993.8		
Percent composition (%)		92.6 ± 2.4		

	Glycine-conjugated bile acids			
	Men		*Women*	
	μg/g dry faeces	*%*	*μg/g dry faeces*	*%*
3α,7β,12α-triOH	3.2 ± 2.5	0.9 ± 0.5	13.2 ± 13.4	3.1 ± 4.0
3α,7α-diOH-12-keto	1.9 ± 0.9	0.7 ± 0.3	4.0 ± 4.7	0.9 ± 1.3
3α,7β-diOH	11.3 ± 20.7	2.9 ± 4.8	6.6 ± 7.1	1.2 ± 0.7
3β,OH-12-keto	28.3 ± 12.4	9.9 ± 4.5	(24.5,24.5,9.9,5.3)	(1.8,9.0,3.1,1.3)
3α,7α,12α-triOH	13.7 ± 8.4	4.5 ± 2.4	51.1 ± 91.8	5.8 ± 5.8
3α,12β-diOH	14.9 ± 9.1	4.8 ± 2.5	(31.9,16.4,6.2,11.6)	(2.4,6.0,1.9,2.8)
3,12-diketo	(4.2,2.5)	(1.2,0.6)	–	–
3α,OH-12-keto	(6.6,2.1,3.8)	(1.7,0.6,0.9)	(27.7,5.2,0.9)	(2.1,1.9,0.3)
3β,12α,diOH	18.0 ± 12.5	5.2 ± 2.5	(11.3,14.1,4.1,8.2)	(0.8,5.2,1.3,2.0)
7α-OH-3-keto	35.2 ± 7.6	13.3 ± 7.9	73.1 ± 42.2	18.6 ± 14.5
12α-OH-3-keto	(13.2,47.1)	(8.3,11.2)	(5.6)	(2.1)
3α,7α-diOH	(17.2,18.6,1.6)	(4.3,5.4,1.0)	66.0 ± 112.2	8.0 ± 7.0
3α,12α-diOH	13.0 ± 7.2	4.3 ± 1.9	42.3 ± 68.4	5.5 ± 4.0
3β-OH-Δ^5	(49.7,8.5)	(14.4,3.7)	(8.4,7.6,1.6,2.0)	(0.6,2.8,0.5,0.5)
3β-OH	19.4 ± 13.3	6.1 ± 3.4	20.3 ± 20.9	4.4 ± 4.8
3α-OH	21.3 ± 17.9	6.4 ± 4.5	49.4 ± 54.9	8.5 ± 3.3
3-keto	90.8 ± 46.0	27.9 ± 14.2	146.2 ± 64.2	34.4 ± 9.7
Fraction				
Primary bile acids	21.2 ± 13.4	6.6 ± 3.3	117.0 ± 203.9	13.8 ± 12.6
Secondary bile acids	288.6 ± 102.2	93.4 ± 3.3	400.5 ± 259.5	86.2 ± 12.6
Total bile acids	309.7 ± 111.9		517.5 ± 461.6	
Total bile acids in men + women				
Amount (μg/g dry faeces)		441.8 ± 342.5		
Percent composition (%)		4.2 ± 1.6		

TABLE III (*continued*)

Bile acid[*]	*Taurine-conjugated bile acids*			
	Men		*Women*	
	μg/g dry faeces	*%*	*μg/g dry faeces*	*%*
3α,7β,12α-triOH	7.9 ± 5.5[**]	3.5 ± 2.0	2.5 ± 1.5[**]	1.2 ± 0.9
3α,7α-diOH-12-keto	3.2 ± 3.7	1.2 ± 1.0	1.6 ± 1.1	0.8 ± 0.6
3α,7β-diOH	(0.7,6.4,16.2)	(0.4,1.9,11.1)	2.9 ± 2.5	1.3 ± 0.9
3β-OH-12-keto	(32.9,15.4)	(9.5,10.6)	(21.1,46.3,23.9)	(11.3,25.4,9.6)
3α,7α,12α-triOH	19.8 ± 26.1	7.2 ± 7.1	18.5 ± 15.0	8.9 ± 7.1
3α,12β-diOH	(12.8,2.8)	(6.6,1.9)	(6.4,14.6,25.1,20.5)	(3.4,8.0,10.0,9.4)
3,12-diketo	(7.1)	(2.1)	–	–
3α-OH-12-keto	(15.6)	(4.5)	(0.8,5.7,0.6)	(0.4,3.1,0.3)
3β,12α-diOH	23.0 ± 10.3	11.2 ± 6.7	8.4 ± 4.8	4.2 ± 2.8
7α-OH-3-keto	26.5 ± 17.7	11.4 ± 5.8	33.3 ± 14.0	15.8 ± 6.0
12α-OH-3-keto	(12.1,7.6,10.5,24.7)	(7.1,3.9,3.0,17.0)	8.8 ± 7.0	4.3 ± 3.6
3α,7α-diOH	14.4 ± 13.2	5.0 ± 3.4	5.7 ± 3.6	2.8 ± 2.0
3α,12α-diOH	29.8 ± 23.0	13.9 ± 12.2	59.0 ± 38.4	26.8 ± 14.8
3β-OH-Δ[5]	(3.9)	(2.7)	(2.5,0.6)	(1.3,0.3)
3β-OH	24.2 ± 11.5	11.4 ± 6.9	14.9 ± 10.9	7.4 ± 5.9
3α-OH	25.6 ± 44.8	8.2 ± 12.5	4.5 ± 3.2	2.2 ± 1.5
3-keto	12.1 ± 11.0	4.9 ± 3.1	15.9 ± 6.7	7.7 ± 3.2
Fraction				
Primary bile acids	34.2 ± 32.8	12.3 ± 8.1	24.2 ± 17.0	11.7 ± 8.3
Secondary bile acids	205.9 ± 71.8	87.3 ± 8.1	185.4 ± 34.3	88.3 ± 8.3
Total bile acids	240.1 ± 97.9		209.6 ± 27.3	
Total bile acids in men + women				
Amount (μg/g dry faeces)			270.9 ± 91.4	
Percent composition (%)			2.4 ± 1.3	
	Sulphated bile acids			
	Men		*Women*	
	μg/g dry faeces	*%*	*μg/g dry faeces*	*%*
3α,7β,12α-triOH	4.4 ± 3.1	3.1 ± 2.7	3.4 ± 3.5	5.7 ± 6.8
3α,7α-diOH-12-keto	(1.3,2.8,1.6)	(1.0,3.2,2.2)	0.6 ± 0.2	0.8 ± 0.6
3α,7β-diOH	(52.7,88.6)	(6.4,24.3)	(0.4,0.6)	(0.3,0.6)
3β-OH-12-keto	16.0 ± 3.8	10.8 ± 8.1	(5.6,9.7,13.9,13.9)	(10.0,20.9,9.4,14.1)
3α,7α,12α-triOH	(2.5,2.4)	(2.9,3.3)	(0.6,7.7,6.2,4.7)	(1.1,5.2,5.2,4.8)
3α,12β-diOH	(2.4,8.4,15.6)	(2.8,11.6,4.3)	(1.7,0.7,1.5,3.1)	(3.0,1.5,1.3,3.1)
3,12-diketo	(12.6,5.4,4.4,3.9)	(9.9,6.2,6.1,1.1)	(2.7,3.3,3.3,1.8)	(5.8,2.2,2.8,1.8)
3α-OH-12-keto	(4.6)	(5.3)	(2.8)	(6.0)
3β,12α-diOH	31.0 ± 35.2	10.7 ± 11.3	15.6 ± 13.8	15.5 ± 13.2
7α-OH-3-keto	10.4 ± 6.4	9.7 ± 10.8	30.5 ± 27.0	28.1 ± 17.1
12α-OH-3-keto	(1.7,8.2,9.5)	(2.4,1.0,2.6)	2.2 ± 1.4	2.4 ± 1.1
3α,7α-diOH	26.3 ± 41.8	5.4 ± 4.1	(2.4,3.0,2.7,1.8)	(4.3,6.5,1.8,1.8)
3α,12α-diOH	(18.9,8.7,2.8)	(14.8,10.1,3.9)	6.2 ± 0.6	8.0 ± 4.0
3β-OH-Δ[5]	(3.0,0.6,158.5)	(2.3,0.7,19.2)	(1.8,1.5,1.4,3.8)	(3.2,1.0,1.2,3.9)
3β-OH	8.4 ± 6.9	4.9 ± 4.8	(1.7,6.8,1.0,1.6)	(3.0,4.6,0.8,1.6)
3α-OH	46.3 ± 95.1	8.3 ± 10.2	(6.8,4.3,4.7,14.8)	(12.2,9.2,3.2,12.5)
3-keto	5.6 ± 4.3	5.1 ± 5.8	6.1 ± 8.5	5.6 ± 5.1
Fraction				
Primary bile acids	29.0 ± 44.9	6.8 ± 3.7	5.8 ± 3.1	6.2 ± 0.8
Secondary bile acids	266.9 ± 276.1	93.2 ± 3.7	87.6 ± 39.5	93.8 ± 0.8
Total bile acids	295.9 ± 319.9		93.4 ± 42.4	
Total bile acids in men + women				
Amount (μg/g dry faeces)			124.3 ± 96.2	
Percent composition (%)			1.3 ± 1.0	

[*] For abbrevations see legend to Table I.

[**] Values are expressed as means ± S.D. for five healthy human faecal samples. Amounts of total faecal bile acids were 10269.5 ± 4235.8 μg/g dry faeces in healthy human subjects.

[***] Values in parenthesis are amounts found of the five samples and these values were included in the total.

Application to human faecal samples

A chromatogram of free bile acids from the faeces of a healthy human subject is shown in Fig. 2. The qualitative and quantitative profiles of faecal bile acids of healthy human subjects are summarized in Table III.

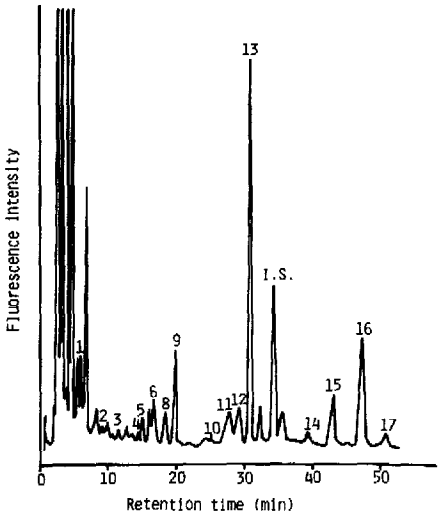

Fig. 2. HPLC profile with fluorescence detection of faecal free bile acids in a healthy human subject. Peaks as in Fig. 1.

Amounts of total faecal bile acids were 10818.1 ± 4205.8 μg/g dry faeces in men (ranging from 5921.4 to 15138.6), 9910.8 ± 4672.3 μg/g dry faeces in women (5198.7 to 17544.2), and 10269.5 ± 4235.8 μg/g dry faeces in all healthy human subjects.

Free bile acids account for 92.6 ± 2.4% of all bile acids in men. Of that, β-hydroxy acids and ketonic acids account for 35.3 ± 10.2% and 20.5 ± 3.4%, respectively. In women, these figures are 91.5 ± 2.4%, 35.3 ± 10.2%, and 20.5 ± 3.4%, respectively, and for all healthy human subjects 92.0 ± 2.3%, 33.1 ± 7.19% and 24.5 ± 7.4%, respectively. Sulphated bile acids in human faeces constituted 1.3 ± 1% of the total faecal bile acids. The average daily faecal excretion of bile acids in healthy Japanese subjects tested in this study was estimated to be about 480 mg/day.

The percentages of the major faecal free bile acids in men and women are shown in Fig. 3. The differences between the sexes are not significant.

DISCUSSION

Analytical procedures

In the present analytical procedures, the mild saponification did not give rise to appreciable artefacts from 3-keto bile acids except for dehydrocholic acid, and the

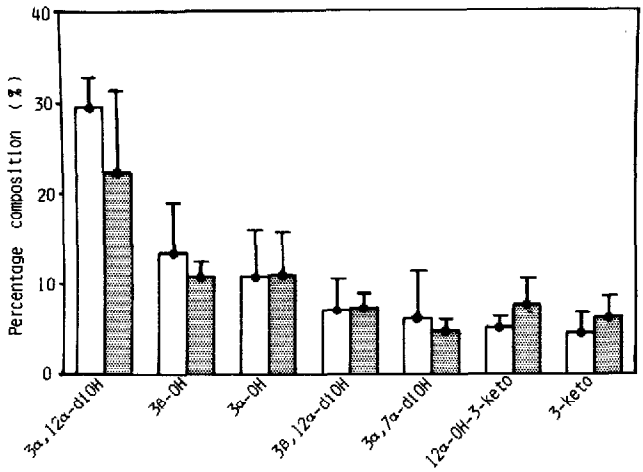

Fig. 3. Percentage composition of major faecal free bile acids in healthy human subjects. Symbols for the bile acids are as in Table I. Differences between men and women are statistically not significant. Values are expressed as mean ± standard deviation of five samples. Open bars, men: Shadowed bars, women.

recoveries were similar to those obtained with hydroxy bile acids added to faecal samples.

In recent years, highly sensitive HPLC methods have been developed using fluorescent derivatization reagents, such as 4-bromomethyl-7-methoxy coumarin[23], 9,10-diaminophenanthrene[24], and 1-bromoacetylpyrene[12]. These are derivatizatizing reagents for carboxylic acids and may be more useful than previous methods[1–6,10,11], particularly for the assay of ketonic and β-hydroxy bile acids at the C-3 position in human faeces. Phenacyl bromide-type reagents are favoured for the formation of carboxylic acid esters because of their high reactivity under mild conditions. Thus, we used 1-bromoacetylpyrene as a derivatizing reagent according to the method of Kamada *et al.*[12]. No appreciable amounts of artefacts of reference bile acids, including 3-keto bile acids, were formed in the derivatization procedure.

In this study, a stepwise elution system using three different mixtures (A, B and C) of acetonitrile, methanol and water mixtures was employed. The solvents were automatically changed using a solvent changer.

The chromatographic separation of the derivatized bile acids was carried out under various conditions. A good chromatogram of faecal bile acids was obtained using a Shim-pack CLC-ODS column and stepwise elution with the mixtures A and B. However, sometimes several small unknown peaks appeared with retention times between 60 and 180 min.

Among these peaks, the peaks with retention times of 65 and 110 min corresponded to myristic and palmitic acids, respectively. In order to avoid a time-consuming procedure for removing these peaks, solvent mixture C was employed. This mixture did not affect the capacity ratios and the detector response of the bile acids as obtained with mixtures A and B.

We have attempted to use a Shim-pack CLC-TMS column instead of Shim-pack CLC-ODS, but we failed to obtain chromatograms with satisfactory separation

of various faecal bile acids. The HPLC column was kept at 25°C because the retention times and the separation of bile acids were considerably affected by changes in the column temperature.

The identification of the HPLC peaks was based on the relative capacity ratios of the peaks for compounds with a free carboxyl group in chromatograms that were obtained after purification and group separation of human faecal bile acids. Previous knowledge about the composition of faecal bile acids was also used for the identification. However, the identifications are still tentative.

The extract of neutral steroids obtained by the present analytical procedure is useful for the analysis of neutral faecal steroids, particularly of faecal sterols, as reported previously[25]. The present method separated seventeen bile acids that are often found in human faeces[5,7,8] from each other.

Applicability of the method

It is clear from the results that the present method is applicable for the detailed analysis of bile acids in human faeces. The validity of the data obtained by this method is supported by data obtained by GLC using the current standard procedure[25]. In this study, there were significant differences in the qualitative and quantitative composition and in the recoveries of individual bile acids in human faeces. This indicates that the analysis of faecal bile acids is by no means as simple as many publications seem to imply.

Because of the complexity of the composition of faeces, it is impossible to develop a single universal method in which there is no overlap between classes of compounds. When a detailed analysis of all groups of faecal bile acids is required, then the present method may be too complex and time-consuming. However, it should be stressed that the inherent flexibility also renders the method suitable for routine applications, especially in the case of analyses restricted to the free bile acids, which constitutes the greatest fraction of the faecal bile acids.

Further studies on a large number of human faecal samples under pathophysiological conditions will be necessary to evaluate the present method. The method faciliates a much more detailed study of human faecal bile acids by HPLC and should be useful in investigations of the pathophysiology of the gastrointestinal tract.

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