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Short Communication

Evaluation of faecal bile acid profiles by HPLC after using disposable solid-phase columns*

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Introduction

A considerable number of epidemiological studies have suggested that bile acids are aetiologically important in the development of colorectal cancer [1–4]. However, it has not yet been determined how bile acids act in tumour promotion or initiation [4–6].

The possible link between cholecystectomy and large bowel cancer has been widely investigated but the results have been varied and often contradictory. These variations may, in some cases, depend on the different methodologies applied [4, 7].

It is a well known fact that the complexity of bile acid analysis in faeces is mainly due to a broad spectrum of polarities of bile acid derivatives and also due to the great amount of interfering endogenous compounds present in the matrix [8].

Therefore, it is necessary to obtain methodologies that are sensitive, accurate, precise and sufficiently validated to perform bile acid analysis in different biological fluids. High-performance liquid chromatography (HPLC) enables rapid and simple separation and also accurate determination of individual bile acids, essential for routine clinical evaluations of alterations of their pattern. It should be the preferred method since it has less requirements for sample preparation than GLC methods and consequently, hydrolysis and derivatization can be omitted.

Although the chromatographic methods have been optimized, sample preparation for accurate and reproducible results remains a challenge. Many procedures for sample preparation and group separation have been recommended [9–11] but reversed-solid phase extraction on disposable cartridges has given the best results during clean-up.

It was the aim of this work to achieve a simplified and efficient methodology that enabled extraction and dosage of main bile acids in faeces. It would be used to determine the faecal bile acid profile of human groups with high risk of developing colonic cancer such as cholecystectomized patients [12, 13]. These profiles could be proven useful for diagnosis in the daily routine. Previous work has shown some increase in the amount of secondary bile acids of such patients and it has been suggested that these acids play a role as co-carcinogens [4, 12, 14].

Experimental

Materials

Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic

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acid (LCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA) and taurolithocholic acid (TLCA) sodium salts were obtained from Sigma (St Louis, MO, USA). Ursodeoxycholic acid (UDCA), glycoursodeoxycholic acid (GUDCA) and tauroursodeoxycholic acid (TUDCA) sodium salts were from Tokyo Tanabe (Tokyo, Japan). The radiolabelled bile acids, [24–14C] CA (40.0 mCi $mmol^{-1}$), [24–14C] CDCA (50.0 mCi $mmol^{-1}$), [1–14C] GCA (44.6 mCi $mmol^{-1}$), [24–14C] TCA (51.0 mCi $mmol^{-1}$) were purchased from Du Pont, NEN (Boston, MA, USA). [24–14C] LCA (55 mCi $mmol^{-1}$) were obtained from Amersham International (Amersham, UK).

All chemicals used were of analytical grade and solvents were redistilled. Deionized double distilled water was used in all the experiments. Mega Bond Elut C_{18} cartridges (1 g) were obtained from Analytichem International (Harbor City, CA, USA). They were washed with methanol (6 ml) and distilled water (12 ml) prior to use. Bond Elut SI cartridges (500 mg) were purchased from Analytichem International. They were washed with 20 ml of ethanol–chloroform–water (20:80:1, v/v/v) before being used.

HPLC grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate was from J.T. Baker (Phillipsburg, NJ, USA). Eluents were filtered through a 0.2 μm Nylon membrane and degassed.

Instrumentation

HPLC study was carried out with a Varian Model 5020 liquid chromatograph equipped with a Varian UV-100 detector. Data were processed with a Varian 4270 integrator (Varian, Palo Alto, CA, USA). A Micropack MCH-5 column (300 × 4 mm i.d., 5 μm) (Varian) and a guard column silica C_{18} (40 × 4 mm i.d., 5 μm) (Varian) were employed. Liquid scintillation counting was carried out using a Wallak Model 1410 (Turku, Finland).

Collection and sample preparation

Faeces was collected for 3 days, frozen immediately and stored at −20°C until they were analysed. The stools were pooled and homogenized with cold distilled water in a stepwise manner [10] until the suspension was no longer particulate. After homogenization, an aliquot (equivalent to approximately 1 g of the original sample) was immediately transferred to a pre-weighed bottle and the sample was lyophilized at constant weight to determine the faecal dry weight. For analyses, bile acids were extracted from 5 ml of faecal homogenate by sequential alcoholic refluxes [10] consisting of 90% ethanol (2 h), 80% ethanol (2 h) and chloroform–methanol (1:1, v/v) (1 h). The organic extracts were pooled and evaporated to dryness on a rotary evaporator under nitrogen. The dried extract obtained was resuspended in 10 ml of sodium hydroxide (0.5 M) with 5% acetonitrile by sonication for 3 min. Later the non-polar neutral sterols were extracted with 3 × 10 ml of *n*-hexane which was discarded. The alkaline–acetonitrile phase was taken to neutrality with phosphoric acid and immediately applied to a Bond Elut C_{18} cartridge. After being washed with 6 ml of distilled water, bile acids were eluted with 4 ml of methanol. The methanolic extract was evaporated to dryness, redissolved in 3 × 1 ml of ethanol–chloroform–water (20:80:1, v/v/v) by sonication of each fraction for 3 min, cooled rapidly to 4°C and passed through a Bond Elut silica cartridge. The eluent was collected as part of the first fraction, then 3.5 ml of ethanol–chloroform–water–acetic acid (20:80:1:0.02, v/v/v/v) was applied to the column and this eluent was the rest of the first fraction which contained the free bile acids. Then, glyco and tauro derivatives of bile acids were eluted with 7 ml of ethanol–chloroform–water–acetic acid (60:40:3:5, v/v/v/v) and each fraction was taken to dryness. The former fraction was dissolved in 1 ml of methanol, and the latter in 500 μl of methanol. Both were filtered through a 0.2 μm Nylon membrane before being injected.

Standard solutions

Stock solutions of all conjugated bile acids were prepared in methanol at 3 mg ml^{-1} concentration and working solutions containing 6 μg ml^{-1} were obtained by suitable dilution in methanol. Stock solutions of the free bile acids were prepared in methanol in concentration of 3 mg ml^{-1}. Working standards solutions were obtained from stock solutions by dilution with methanol to a final concentration of 0.6 mg ml^{-1}.

Chromatographic conditions

Free bile acids were eluted using as mobile phase ammonium dihydrogen phosphate (pH 7.50; 0.3%)–acetonitrile (50:50), flow rate: 0.7 ml min^{-1}. Detection was performed at 208 nm and 0.05 aufs. Temperature: 30°C, injection volume: 10 µl. For conjugated bile acids the mobile phase consisted of two solvents: solvent A was ammonium dihydrogen phosphate (pH 6.8; 0.3%) and solvent B was acetonitrile, and the gradient elution profile was: solvent B increased from 24 to 35% during the first 19 min, to 37% during the next 11 min and then to 40% for the next 5 min. Flow rate: 0.7 ml min^{-1}, temperature: 30°C, injection volume: 50 µl detection was performed at 208 nm and 0.02 aufs.

Statistical analysis

The data were analysed using Kruskall–Wallis test for several group means (corrected for ties).

Results and Discussion

The aim of this work was to propose a simplified methodology that, by obtaining reliable results, could save time and consequently could be used for diagnosis in the clinical routine laboratory.

As correct sampling is known to be of great importance, Setchell's methodology was used instead of published alternatives because it allows a more representative sample of biological material [7, 10]. In the extraction step, sequential alcoholic refluxes [10] were used because a quantitative recovery of bile acids is obtained and artifacts are limited. The purpose of the subsequent hexane treatment was to eliminate neutral steroids [11]. During the next step of the sample clean-up a reversed-phase C_{18} was used [10] which offers advantages over the previous polimeric polistyrene materials as Amberlite XAD-2 resin [15].

Despite not using Amberlyst A-15 or SP-Sephadex indicated by other techniques in order to complete the purification process [10], no significant interferences were observed in the HPLC bile acid analysis.

In the proposed method, the separation of different bile acid fractions was carried out using a normal phase in silica cartridges recommended for fractioning of bile acids in serum [16].

Since the HPLC method proposed for bile acid analysis separates the glyco and tauro conjugates in one run, it only requires separation of two groups, free and conjugated compounds [17]. This last feature is an advantage of HPLC over GLC, which requires previous separation of the three fractions [16, 18–20]. Other HPLC techniques even need a previous division of glyco from tauro conjugates by PHP-Sephadex [21].

In the present method the validity of the results was evaluated by recovery of known amounts of radiolabelled bile acids added as internal standards to aliquots of the stool homogenate (Table 1). These compounds were chosen because of their very different polarities. The recovery of all bile acids was also estimated by adding unlabelled standards to faecal homogenates and the percentages obtained ranged from 60 to 100%. These values agreed with those reported by other authors. Low values (60 and 67%) found for GLCA and LCA, respectively, could be due to their lower solubility at neutral pH [21] and to the low overlapping of GLCA with the free fraction produced during the fractionation step through silica [16].

The reproducibility of the method was also tested and the values obtained are shown in Table 1.

Although glyco and tauro derivatives are present in low quantities, their differential conjugation and the glyco/tauro (G/T) ratio become important information in order to elucidate the pathophysiological behaviour of bile acids [22]. In this regard, Ferguson *et al.* have pointed out that the ability of bile acids to act as tumour promoter varies more due to conjugation or deconjugation rather than to primary or secondary forms [23]. Conjugation of bile acids with glycine and taurine modifies the physicochemical characteristics of such bile acids, which may be relevant for their bio-

Table 1
Recovery of radiolabelled bile acids added to faeces

Bile acid	Recovery (%) *mean ± SEM	RSD (%)
Cholic	97.4 ± 3.8	7.8
Chenodeoxycholic	71.2 ± 0.7	2.0
Lithocholic	67.1 ± 1.8	5.4
Taurocholic	101.1 ± 1.8	3.5
Glycocholic	87.2 ± 3.7	8.5

*Mean values from four analyses of each individual bile acid.

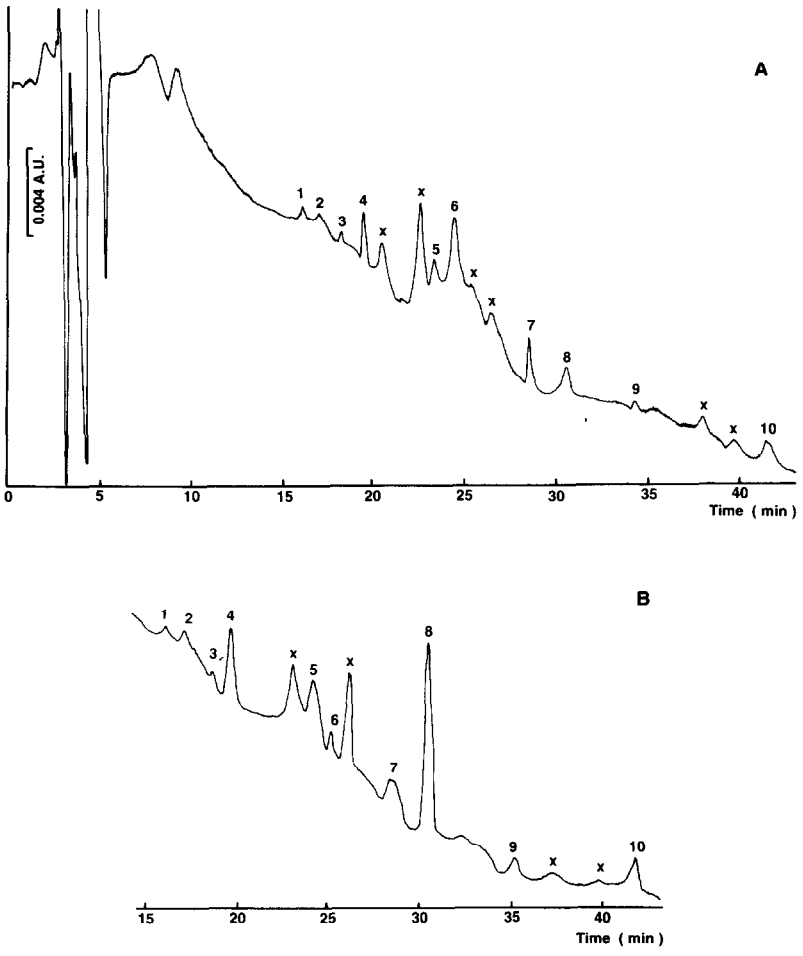

Figure 1
Conjugated bile acid profiles: (A) healthy subject; (B) cholecystectomized patient. 1, TUDCA; 2, TCA; 3, GUDCA; 4, GCA; 5, TCDCA; 6, TDCA; 7, GCDCA; 8, GDCA; 9, TLCA; 10, GLCA; X, unknown. Operating conditions as described in the text.

Table 2
Individual faecal bile acids in healthy subjects and cholecystectomized patients

	Free form		Glyco conjugates		Tauro conjugates	
			(mg/100 g dried faeces/day)			
Bile acid	H	C	H	C	H	C
UDCA	37.80 ± 4.37	25.00 ± 7.80	0.39 ± 0.27	1.03 ± 0.71	0.38 ± 0.33	0.45 ± 0.37
CA	42.66 ± 14.45	37.70 ± 11.00	1.95 ± 0.72	2.10 ± 1.42	0.29 ± 0.13	0.42 ± 0.24
CDCA	97.47 ± 27.18	48.20 ± 11.30	3.30 ± 1.52	5.37 ± 3.50	4.03 ± 2.03	1.61 ± 0.5
DCA	160.13 ± 33.83	279.50 ± 84.10	1.80 ± 1.26	13.70 ± 10.00	2.19 ± 0.72	1.67 ± 1.04
LCA	241.28 ± 77.60	217.90 ± 39.97	2.85 ± 1.45	2.99 ± 0.75	0.31 ± 0.24	0.47 ± 0.16

H = healthy subjects (n = 8); C = cholecystectomized patients (n = 8).
Results are expressed as means ± SEM.

logical activity [21]. However, the conjugation profile has not previously been considered by other authors [10, 12].

Figure 1 shows the conjugated bile acid profiles of both a healthy subject and a cholecystectomized patient. The results obtained from control subjects and cholecystectomized patients groups are given in Table 2. But it is not within the scope of this study to evaluate and discuss the main and slight variations among the different profiles obtained and their relative ratios. Just by taking into account the importance of the G/T ratio, a significant increase in the GDCA/TDCA ratio

($P < 0.05$) in the cholecystectomized patients can be seen compared to healthy subjects.

Although the sulphated bile acid fraction was separated using this technique and quantified by an enzymatic method, the results are not reported in this study.

Further studies in cholelitiasis and colonic cancer patients applying this methodology are in progress.

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