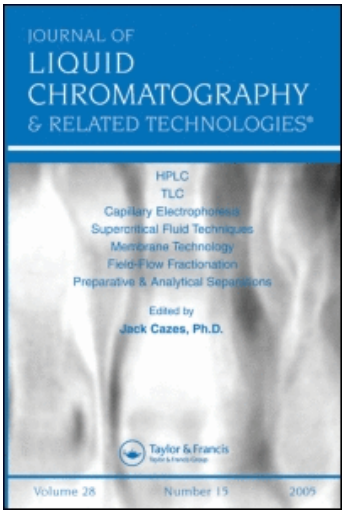

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A METHOD FOR QUICK DETERMINATION OF BILE ACIDS IN BILE OF PATIENTS WITH BILIARY LITHIASIS

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ABSTRACT

A single high-performance liquid chromatographic method is described for determining and measuring the major conjugated bile acids in human bile. Four different chromatographic conditions were applied using C-18 columns and an isocratic solvent system. An effective one-step purification with Sep-pak C-18 was adopted. The advantages of this method are an improved separation of the conjugated bile acids within a short period of time. The detection limit was 0.0125 μg/μl and linearity was up to 6

μg/μl. Recovery was always up to 96.6 %, this being sufficient for routine clinical application during bile acids evaluation in biliary lithiasis.

INTRODUCTION

Nowadays, biliary lithiasis presents an important health problem due to its high subject rate and economic repercussions.

Recent reports (1) have shown that bile acid secretion is altered in biliary lithiasis patients. Therefore it is necessary to develop an easy and rapid method to determine conjugated bile acids in the human bile and to evaluate its feasible alterations in hepatic and biliary diseases.

The classic methods that have been used to detect bile acids have many disadvantages: the gaseous-liquid chromatography requires a lot of time and the enzymatic kits only determine the total but not individual bile acids. The high-performance liquid chromatographic method (HPLC) is the suitable one for rapid and simultaneous bile acids determination (2,3,5,6). Previous to the bile acid separation by HPLC, purification of samples must be done in order to remove proteins, biliary pigments and other compounds that can interfere with it (8). Many methods have been used, the liquid-liquid extraction, Amberlite resins, etc., but the most recent method is that based on the use of discarded cartridges like octadecylsilane that show an improved recovery and a shorter analysis time.

The purpose of the present study is to evaluate a method to determine bile acids in human bile and to apply it to routine clinical evaluation in biliary lithiasis (2).

MATERIAL AND METHODS

Reagents: The sodium salts of taurocholic acid (TC), glycocholic acid (GC), taurochenodeoxycholic acid (TCDC), taurodeoxycholic (TDC), glycochenodeoxycholic acid (GCDC), glycodeoxicholic acid (GDC), taurolithocolic acid (TLC), glycolithocolic acid (GLC), and dexamethasone were supplied by SIGMA (St. Louis MO, USA). The water was purified with a Mili-Q water purification system purchased from MILLIPORE (Bedford MA, USA). Methanol and acetonitrile were HPLC-gradient from MERCK (Darmstard, FRG). All other chemicals were of analytical grade. The C-18 Sep-Pak cartridges were also purchased from MILLIPORE.

Apparatus: The HPLC system was made up of a L-6200 solvent delivery pump, a L-4250 detector and a D-2500 integrator by MERCK-HITACHI. The injector was a 7125 Reodyne with a 20 µl loop.

Operating conditions: The HPLC method was performed under isocratic conditions at room temperature. Analyses were performed on different reversed-phase C-18 columns: µBondapack C18 (10 µm particle size, 300 x 3.9 mm ID, WATERS); Spherisorb ODS 2 (5 µm, 150 x 3.9 mm, SUGELABOR); Lichrospher RP-18 (5 µm, 250 x 4 mm, MERCK); Lichrospher RP-18 (5 µm, 125 x 4 mm, MERCK).

The different mobile phases used were prepared daily with known quantities of phosphate or acetate buffer and with different percentages of methanol or acetonitrile. The pH was adjusted with phosphoric acid. All solvents were filtered through a 0.45 μm filter (type HA, MILLIPORE). Details of the chromatographic conditions applied in the HPLC systems are shown in Table 1. The flow in most of them was 1 ml/min. and the detection was performed at 205 nm and 0.04 aufs.

The chromatographic parameters: capacity factor (K') and selectivity (alpha) were determined as defined by Snyder and Kirkland (4). Peak areas were quantified by the integrator and it was calibrated by conjugated bile acids standards. To prepare a mixture of the eight conjugated bile acids, we dissolved each bile acid individually in methanol at a concentration of 1 mg/ml; 100 μl of each solution was then combined, evaporated under nitrogen stream and redissolved in 1 ml of the adequate mobile phase.

Sample preparation: Human bile was obtained by gallbladder punction during surgery in patients with symptomatic biliary lithiasis (n=10). This bile was immediately frozen. 500 μl of the human bile sample was centrifuged for 5 min. at 10.000 g without loss of bile acids in the precipitate. After that, an aliquot of 100 μl of the supernatant was diluted with 5 ml of 70 mM phosphate buffer at pH 7.0 and with 100 μg of dexamethasone added as an internal standard. The solution was mixed with a vortex mixer for 1 minute, after which the mixture was passed through a C-18 Sep-pak cartridge

Table 1-. Chromatographic systems.

SYSTEMS	COLUMN	MOBILE PHASE	%	FLOW
SYSTEM 1	COLUMN uBONDAPAK	acetonitrile/phospate buffer 10 mM pH 4.5	(30 / 70)	1
SYSTEM 2	COLUMN MERCK 125	methanol/acetate buffer 50 mM pH 4.3	(70 / 30)	0.8
SYSTEM 3	COLUMN MERCK 250	methanol/acetate buffer 50 mM pH 4.3	(70 / 30)	1
SYSTEM 4	COLUMN SPHERISORB	methanol/ acetate buffer 50 mM pH 4.3	(70 / 30)	1

conditioned previously with 5 ml of methanol, 10 ml of water and 5 ml of phosphate buffer 70 mM pH 7. The mixture was eluted successively with 10 ml of water, 3 ml of acetone 10%, 10 ml of water and 5 ml of methanol. The last fraction which contained the conjugated bile acids was evaporated to dryness under a nitrogen stream at 40ºC, and the residue was redissolved in 500 µl of the mobile phase and filtered through a 0.45 µm filter (MILLIPORE, type HV) before being injected into the HPLC (20 µl).

RESULTS

We applied different chromatographic columns in order to determine conjugated bile acids in human bile and obtained the best results with the Lichrospher RP-18 (5 µ m, 250 x 4 mm, MERCK) column. Chromatographic parameters are shown in Table 2 and chromatograms in Figure 2and in Figure 3. The elution order of the bile acids depends on the number of OH groups: trihydroxylated bile acids are eluted first, secondly dihydroxylated and finally monohydroxylated.

Table 2-. Chromatographic systems parameters. A=alpha; ND = non detected.

	TR	TR'	K	A
SYSTEM 1				
TC	8.56	6.12	2.50	
GC	13.16	10.72	4.39	1.75
TCDC	16.75	14.31	5.86	1.33
TDC	19.80	17.36	7.11	1.21
GCDC	35.84	33.40	13.68	1.92
GDC	41.71	39.27	16.09	1.17
TLC	N.D			
GLC	N.D			
SYSTEM 2				
	TR	**TR'**	**K**	**A**
TC	3.00	2.36	1.90	
GC	4.63	3.39	2.73	1.43
TCDC	5.90	4.66	3.75	1.37
TDC	6.80	5.56	4.48	1.19
GCDC	7.93	6.72	5.41	1.20
GDC	9.28	8.04	6.48	1.19
TLC	11.80	10.56	8.51	1.31
GLC	16.44	15.20	12.25	1.43
SYSTEM 3				
	TR	**TR'**	**K**	**A**
TC	5.71	3.78	1.95	
GC	6.98	5.05	2.61	1.33
TCDC	9.10	7.17	3.71	1.42
TDC	10.48	8.55	4.43	1.19
GCDC	11.66	9.73	5.04	1.13
GDC	13.34	11.41	5.91	1.17
TLC	17.58	15.65	8.10	1.37
GLC	23.36	21.43	11.10	1.37
SYSTEM 4				
	TR	**TR'**	**K**	**A**
TC	4.99	2.85	1.33	
GC	6.79	4.65	2.17	1.63
TCDC	7.62	5.48	2.56	1.17
TDC	8.58	6.44	3.00	1.17
GCDC	11.11	8.97	4.19	1.39
GDC	12.70	10.56	4.93	1.17
TLC	17.12	14.98	7.00	1.41
GLC	22.20	20.06	9.37	1.33

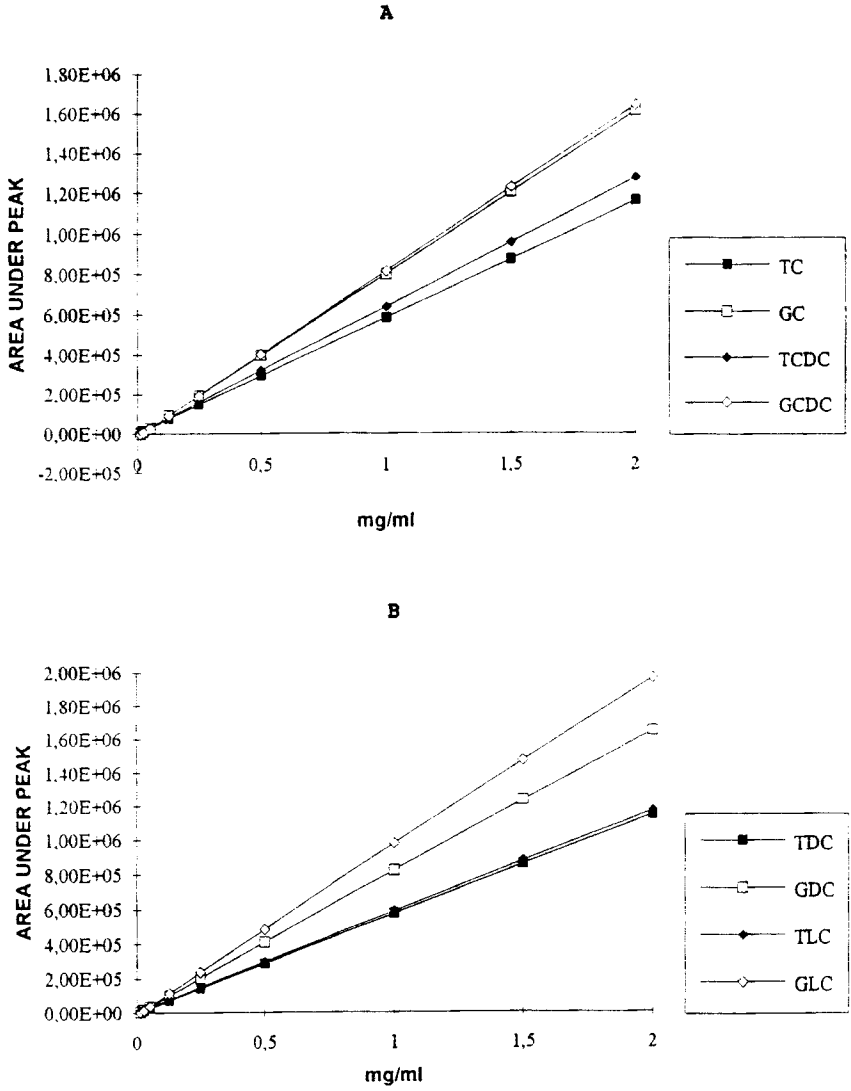

Fig 1-. Calibration curves. Correlation factors are: rTC: 0.996; rGC: 0.996; rTCDC: 0.998; rTDC: 0.997; rGCDC: 0.998; rGDC: 0.998; rTLC: 0.998; rGLC: 0.997.

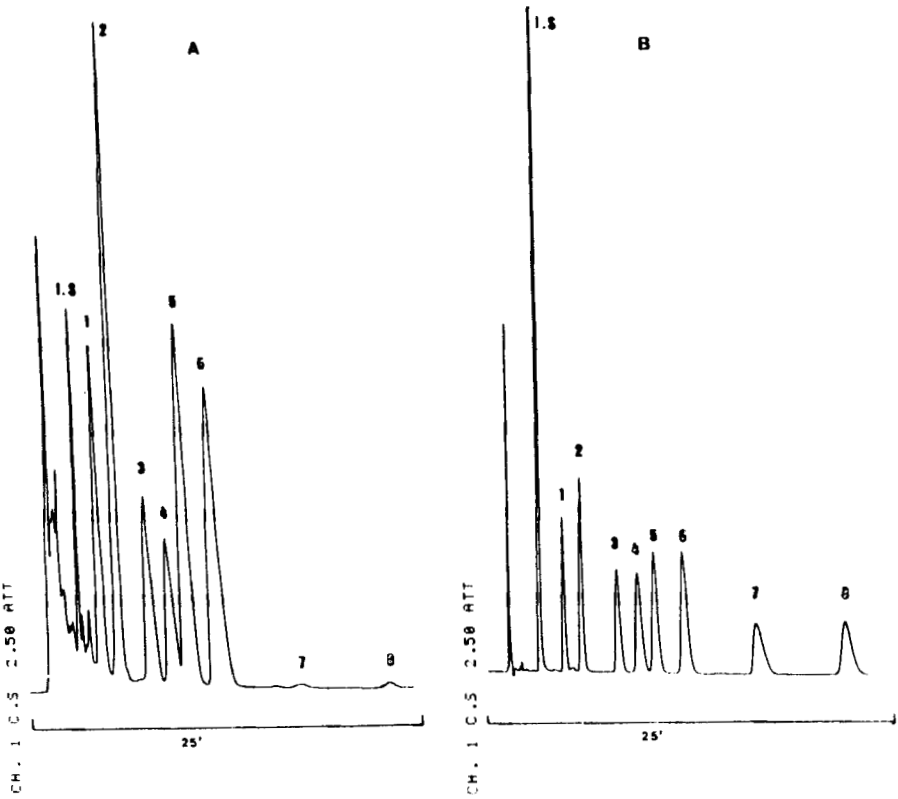

Fig 2- .A) Pathological Human bile chromatogram.

.B) Chromatogram of eight conjugated bile acids standards: 1. TC; 2. GC; 3. TCDC; 4. TDC; 5. GCDC; 6. GDC; 7. TLC; 8. GLC. I.S.= internal standard: dexamethasone.

Calibration: Known amounts of standards solutions of each bile acid were analysed in order to graph the calibration curves, which are shown in Figure 1. Correlation factors ranged from 0.996 to 0.999. The detection limits found were in the range of 2.5 ng/l, and the linearity was observed up to 6 µg/l.

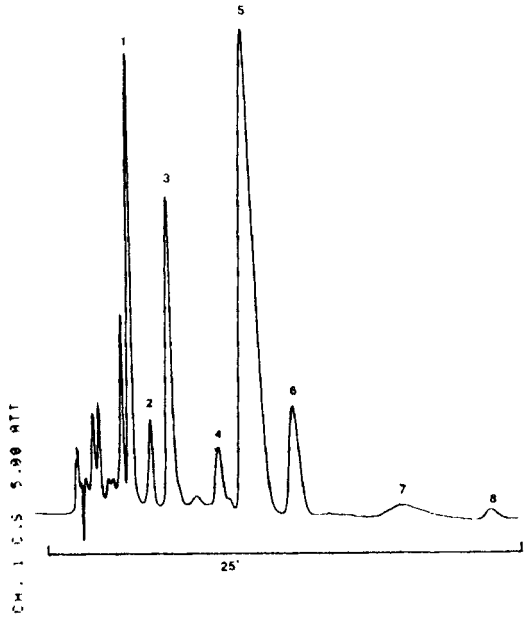

Fig 3-"Normal" Human Bile chromatogram.

Table 3-. Reproduction of human bile acids, C.V.= coefficient of variation

	TR	AREAS
n=5	CV %	CV %
TC	0.13	0.51
GC	0.10	0.79
TCDC	0.15	1.86
TDC	0.17	1.67
GCDC	0.16	0.95
GDC	0.20	1.99
TLC	0.25	5.94
GLC	0.26	1.71

Reproducibility: Reproducibility was tested by analysing 20 µl taken from the same stock bile sample on 5 different days. Results are shown in Table 3.

In order to determine the recovery, the bile samples were prepared by adding known amounts of each bile acid. The samples were subjected successively to purification by Sep-pak C 18 and determined by HPLC as described above. The recovery was always over 96.6 %, this being sufficient quantity for a routine clinical analysis. The results are shown in Table 4.

DISCUSSION

At present, high-performance liquid chromatographic methods are widely used for the assay of the conjugated bile acids by other authors (2, 5-14), but we have studied several chromatographic parameters in an effort to optimise the separation of the bile acids in human bile.

The reversed-phase is the appropriate system to separate non-polar and non-ionized substances; however, ionized substances like bile acids are separated on reversed-phase columns by different modifications: suppression ionic or the addition of a paired ionic chromatography (PIC) reagent (Waters). We do not consider the PIC to be adequate due to the low pH it needs. The acidification of the mobile phase entails a lot of undesirable problems: the functional groups of the column gel may be partially covered by these acid groups, reducing the separation efficiency and also means a shorter column time life. We used the ion suppression mode

Table 4-. Recovery (%) of bile acids from bile samples treated with Sep-pak, C.V.= coefficient of variation.

n=4	BILE mg/ml	ADDED mg/ml	EXPECTED mg/ml	FOUND mg/ml	RECOVERY %	CV %	R MEAN %	CV %
TC	0.8265	0.4	1.226	1.232	100.5	4.919	100.795	3.434
	0.9016	0.2	1.101	1.114	101.09	1.95		
GC	2.289	0.4	2.689	2.718	101.07	4.208	100.195	3.250
	2.437	0.2	2.637	2.619	99.32	2.293		
TCDC	0.9154	0.4	1.416	1.163	107.66	5.461	103.925	3.559
	1.020	0.2	1.222	1.222	100.9	1.658		
TDC	0.616	0.4	1.078	1.078	106.1	5.385	107.22	3.909
	0.646	0.2	0.9167	0.9167	108.34	2.433		
GCDC	1.892	0.4	2.336	2.336	101.9	3.906	101.075	3.296
	2.082	0.2	2.288	2.288	100.25	2.687		
GDC	1.450	0.4	1.933	1.933	103.78	3.54	102.45	3.076
	1.557	0.2	1.775	1.775	101.12	2.612		
TLC	0.041	0.4	0.5024	0.5024	113.78	4.331	116.14	5.065
	0.038	0.2	0.2827	0.2827	118.49	5.799		
GLC	0.072	0.4	0.4651	0.4651	98.42	4.512	97.62	4.570
	0.0795	0.2	0.2706	0.2706	96.82	4.629		

in which the pH of the mobile phase is adjusted according to the pK of the substances to be analysed. The conjugated bile acids show low pK values (pK taurine=2 and pK glycine=4.5) with the purpose of increasing the solubility at the intestinal pH. We adjusted the pH of the mobile phase at 4.3 because that pH confers improved separation and selectivity to the chromatographic system and an adequate control of the retention time.

The effect of the particle size was also studied. By using Lichrospher RP 18 (250 x 4 mm, MERCK), we could see that the 5 μm particle size fit better to the bile acid size than when another particle size (10 μm) is used, with a consequent loss of peak symmetry (system 1).

The effect of different buffers (phosphate and acetate) was also evaluated; although most of the authors think phosphate is better than acetate (6, 8, 11, 14), we have obtained an improved baseline and separation with acetate buffer (5).

Loss of resolution was observed with the use of acetonitrile instead of methanol. We think methanol is the most adequate solvent, although it has an inherently ultraviolet (UV) cut-off at 205 nm where conjugated bile acids are optimally detected; However acetonitrile as a solvent produces longer retention times for TLC and GLC, and also problems with the solubility of these previous bile acids (system 1).

The use of a shorter column (system 2) makes it possible to shorten the analysis time but with a loss of selectivity. We think the suitable column length is 250 mm (systems 3 and 4) and that system 1 shows an excessive length with excessive retention times.

The purification of the bile acids extract before HPLC analysis is achieved by Sep-pak C 18 cartridges (14). We consider that the precondition of the cartridge with the system methanol, water and phosphate buffer is a crucial step for successful purification. Also the use of phosphate buffer as a solvent of bile sample improves purification and removes interfering peaks, although the pH should never be over 7, because the solubility of cartridge silica gel increases dramatically over pH 7.

In conclusion, an isocratic HPLC method allows for the rapid and simultaneous determination of eight conjugated bile acids in pathological biles and makes it possible to evaluate the feasible involvement in biliary lithiasis. We think the method is not sensitive enough to determine bile acids in serum, because they are present in quantity hundreds of time less. The only disadvantage of this technique is that it does not determine unconjugated bile acids, although they are present in a very low percent in human bile.

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