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HPLC-fluorescence determination of bile acids in pharmaceuticals and bile after derivatization with 2-bromoacetyl-6-methoxynaphthalene*

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Abstract: 2-Bromoacetyl-6-methoxynaphthalene was used as a pre-chromatographic fluorescent labelling reagent for the high-performance liquid chromatographic (HPLC) analysis of bile acids. The derivatization reaction was performed in an aqueous medium in the presence of tetrahexylammonium bromide by ultrasonication at 40°C to give fluorescent esters which were separated by reversed-phase HPLC and detected fluorimetrically (λ_{ex} = 300 nm, λ_{em} = 460 nm). Applications to the determination of ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) in their pharmaceutical formulations are described. The method was also applied to the determination of free and conjugated bile acids in human bile samples.

Keywords: *Bile acids; column liquid chromatography; fluorogenic pre-column derivatization; 2-bromoacetyl-6-methoxynaphthalene.*

Introduction

The determination of individual bile acids (BA) in biological fluids can be of value in the diagnosis of hepatobiliary diseases [1–3]. The analysis, however, presents problems because of the complex composition (free and conjugated form), low concentration and weak detectability of the compounds. Among various methods [1, 4], high-performance liquid chromatography (HPLC) involving pre-chromatographic labelling with a fluorophore appears to be promising with respect to sensitivity and resolution [5, 6]. The derivatization reaction can be carried out at the 3α-OH group (free, glycine- and taurine-conjugated BA) or at the carboxylic group (free and glyco-BA) on the side chain (Scheme 1). Fluorophores reactive toward the carboxylic function, such as the alkylating agents 4-bromomethyl-7-methoxycoumarin [7], bromoacetylpyrene [8, 9], *N*-(9-acridinyl)bromoacetamide [10] and 9-bromomethylacridine [11], have been successfully applied to the HPLC–fluorescence determination of bile acids in a variety of matrices. Recently, 2-bromoacetyl-6-methoxynaphthalene has been proposed as a useful fluorescent labelling reagent for the HPLC analysis of aliphatic carboxylic acids [12]. The derivatization reaction was peformed in acetonitrile in the presence of triethylamine.

Detailed studies by Van der Horst *et al.* [11, 13–15] have demonstrated that carboxylic acids can be derivatized directly in an aqueous matrix by using a micellar system. This approach is attractive because it allows a simpler pretreatment of the sample and avoids laborious extraction procedures. The aim of the present study was to investigate the optimum reaction conditions for the derivatization of free and glycine-conjugated bile acids with the fluorophore 2-bromoacetyl-6-methoxynaphthalene in an aqueous medium in the presence of the ion-pair reagent tetrahexylammonium bromide. Chromatographic (HPLC) separations of the derivatized BA were then accomplished on RP-18 columns using mixtures of acetonitrile–water as the mobile phase in the isocratic or gradient mode.

The method was applied to the HPLC (fluoroscence detection) analysis of bile acids in various matrices; it proved to be suitable for the determination of ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) in their dosage forms and for the analysis of the major bile acids in human bile.

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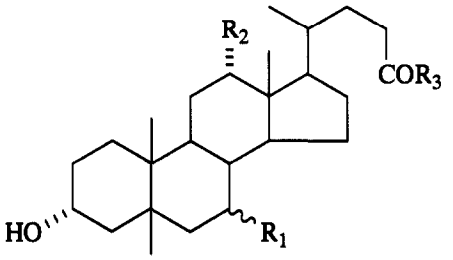

I R_1 =  , R_2 = OH F R_3 = OH

II R_1 =  , R_2 = H G R_3 = $NHCH_2COOH$

III R_1 = H , R_2 = OH T R_3 = $NHCH_2CH_2SO_3H$

IV R_1 =  , R_2 = H

V R_1 = R_2 = H

I: Cholic acid (CA); II: chenodeoxycholic acid (CDCA); III: deoxycholic acid (DCA); IV: ursodeoxycholic acid (UDCA); V: lithocholic acid (LCA)

Scheme 1
Structure of the major free (F) and conjugated [(G) and (T)] bile acids.

Experimental

Materials

Unconjugated bile acids were obtained from Aldrich Chimica (Milan, Italy) and the glyco-conjugated acids were from Sigma (St Louis, MO, USA). Tetrahexylammonium bromide (THxABr) and *n*-hexanoic (caproic) acid were from Fluka (Buchs, Switzerland) and all the other chemicals were from Farmitalia C. Erba (Milan, Italy). The reagent 2-bromoacetyl-6-methoxynaphthalene (Br-AMN) was prepared and purified as previously described [12].

Solid-phase extraction (SPE) was performed on Bond-Elut (BE) C-18 and SAX cartridges (500 mg) (Analytichem International, Harbor City, CA, USA).

Choloylglycine hydrolase [EC 3.5.1.24] was supplied by Sigma Chemical (St Louis, MO, USA).

Apparatus

The liquid chromatograph comprised a Varian 2010 pump and a Varian 2070 fluorescence spectrophotometer, operating at an emission wavelength at 460 nm with an excitation wavelength of 300 nm, connected to a personal computer IBM XT-PC. The JCL6000 chromatography data system was used. Manual injections were carried out using a Rheodyne model 7125 injector with a 50-μl sample loop.

The solvents were degassed on line with a degasser ERC-3312 Erma (Tokyo, Japan). IR spectra were recorded in a Nujol mull on a Perkin–Elmer 298 IR spectrophotometer. UV spectra were recorded on a Jasco Uvidec 610 double-beam spectrophotometer.

Sonorex Super RK 102 (35 kHz) (Bandelin, Berlin, Germany) equipment with thermostatically controlled heating (30–80°C) was used for ultrasonication.

Synthesis of 6-methoxynaphthacylester of n-hexanoic acid

The method previously reported [12] for the preparation of the corresponding ester of valproic acid was followed. The reaction product was purified by crystallization from methanol–water to give a white compound; a single spot was detected by TLC on silica gel with ethyl acetate–petroleum ether (7:3, v/v) as the solvent for development. UV detection at 254 and 366 nm. m.p.: 79–80°C; calculated for C_{19} H_{22} O_4, C 72.58, H 7.05%; found C

72.19, H 7.22. IR (cm^{-1}): 1735 (CO ester), 1690, 1620, 1265, 1170, 1155, 1025, 905, 870, 810. UV(ethanol): λ_{max} = 313 nm (ϵ = 1.325×10^4).

Solutions

Solutions of the reagent (Br-AMN) (2.1 mg ml^{-1} for the analysis of free BA or 21 mg ml^{-1} for glyco-conjugated BA) were prepared in acetone and were found to be stable for 2 weeks at 4°C. Tetrahexylammonium bromide (THxABr) solution (20 mM) was prepared in aqueous 100 mM phosphate buffer (pH 7.0). Stock solutions of free and glycoconjugated BA were prepared in methanol whereas stock solutions of their sodium salts were prepared in water; these solutions were then appropriately diluted to give standard solutions in water–methanol (9:1, v/v) of the desired concentration (see Calibration graphs). Solutions of the internal standards, the 6-methoxynaphthacylesters of *n*-hexanoic acid (40 μg ml^{-1}) and of valproic acid (43.6 μg ml^{-1}), were prepared in methanol–water (75:25, v/v).

Derivatization procedure

A 50 μl volume of 0.01% (w/v) KOH methanolic solution was evaporated to dryness in a 3-ml micro reaction vessel and then 0.2 ml of bile acid solution, 0.3 ml of 20 mM tetrahexylammonium bromide (THxABr) in phosphate buffer (pH 7.0) and 50 μl of the reagent solution (2.1 and 21 mg ml^{-1} for free and glyco BA) were added. The derivatization reaction was allowed to proceed for 10 min at 40°C, under ultrasonication. Then, 50 μl of the appropriate internal standard solution and 0.3 ml of acetonitrile were added; the reaction mixture was ultrasonicated at room temperature for 1 min and 50-μl aliquots of the resulting clear solution were injected into the chromatograph.

Chromatographic conditions

Routine HPLC analyses of UDCA and CDCA in commercial dosage forms were performed on a 250 × 4.6 mm i.d. column packed with 5-μm Hypersil RP-18 under isocratic conditions. UDCA was chromatographed using a mobile phase of acetonitrile–water (70:30, v/v) at a flow rate of 1.1 ml min^{-1}; for the simultaneous determination of UDCA and CDCA the HPLC separation was achieved using a mobile phase of acetonitrile–water (78:22, v/v) at flow rate of 1 ml min^{-1}.

Routine analysis of BA in bile samples were performed with gradient elution.

Free and glycine conjugated BA. A 250 × 4.6 mm i.d. column packed with 5-μm Hypersil RP-18 was used with a mobile phase of water (A)–acetonitrile (B) of varying composition (v/v). The gradient profile adopted was: t = 0, 60% B; t = 10 min, 60% B; t = 20 min, 80% B; t = 45 min, 80% B; and t = 50 min, 60% B at a flow rate of 1.0 ml min^{-1}.

Glycine-conjugated BA. A 250 × 4.6 mm i.d. column packed with 5-μm Ultracarb RP-18 was used with a mobile phase of water (A)–acetonitrile (B) of varying composition (v/v). The gradient profile adopted was: t = 0, 65% B; t = 10 min, 65% B; t = 15 min, 80% B; t = 25 min, 80% B; and t = 30 min, 65% B at a flow rate of 1.2 ml min^{-1}.

Calibration graphs

Bile acids in pharmaceutical formulations. Standard solutions of UDCA (6.23–37.5 μg ml^{-1}) and CDCA (10–37.5 μg ml^{-1}) were prepared in methanol–water (10:90, v/v). A 0.2 ml volume of each standard solution was subjected to the derivatization procedure and 0.1 ml of the internal standard solution (caproic acid ester for UDCA analysis and valproic acid ester for the CDCA and UDCA simultaneous analysis) was added. The peak-height ratio of bile acid ester to internal standard was plotted against the corresponding acid concentration to obtain the calibration graphs.

Bile acids in human bile. The procedure described above was applied to mixed standard solutions of CA, CDCA, DCA, LCA and UDCA (0.8–6.4 nmol ml^{-1}) in methanol–water (10:90, v/v). Valproic acid ester was used as the internal standard.

Similarly for the determination of glycine conjugated bile acids, calibration graphs were obtained from standard solutions of GCA, GCDCA, GDCA, GLCA and GUDCA (5–50 nmol ml^{-1}) in methanol–water (10:90, v/v), using valproic acid as the internal standard.

Analysis of pharmaceutical formulations

For the analysis of UDCA in Deursil capsules, the contents of five capsules were powdered and an amount, equivalent to about 25 mg of the drug, was treated with 100 ml of

methanol by magnetic stirring for 10 min. After filtration, an aliquot of the clear solution was diluted (1 to 10) with water to give a concentration of 25 μg ml^{-1}. For the simultaneous determination of UDCA and CDCA (magnesium salts) in Bilenor capsules, the contents of five capsules were powdered and an amount, equivalent to about 25 mg of the drug mixture was treated with 100 ml of water by magnetic stirring for 10 min. After filtration, the clear solution was further diluted to give a concentration of 25 μg ml^{-1} in methanol–water (10:90, v/v).

A 0.2-ml aliquot of the sample solution was subjected to the derivatization procedure and the drug content in each sample was determined by comparison with an appropriate standard solution.

Analysis of human bile

Duodenal human bile was collected through a nasoduodenal tube after gall-bladder stimulation with an intravenous injection of Cerulein (0.4 mg, Farmitalia, Milan, Italy). Bile was stored at −20°C until the analysis.

A bile sample (50 μl), diluted with 5 ml of 0.05 M phosphate buffer (pH 7.5), was subjected to solid-phase extraction using BE-C18 and BE-SAX cartridges in succession, according to a previously described method [16]. This procedure enabled the free, glycine- and taurine-conjugated fractions to be separated.

The taurine fraction was submitted to enzymatic hydrolysis using cholylglycine hydrolase following a previously described procedure [17] and was then subjected to SPE (C-18) clean-up procedure. After enzymatic hydrolysis, 500 μl of the aqueous solution was treated with 4 ml of 0.1 M NaOH and then applied to the SPE cartridge. The column was washed with 10 ml of water and the BA were eluted with 4 ml of methanol and dried under vacuum.

The final residue was dissolved in 2 ml of methanol at 40°C by ultrasonication for 3 min. After filtration through a 4-mm filter unit (0.2-μm Nylon), an 0.5-ml aliquot of the solution was treated with 50 μl of 0.001% w/v KOH solution in methanol and then evaporated to dryness. The residue was dissolved in 0.2 ml of methanol–water (10:90, v/v) at 40°C by ultrasonication for 3 min and then subjected to the derivatization procedure as described above. Bile acids were determined in each sample by comparison with an appropriate standard solution.

Results and Discussion

Derivatization reaction

In order to apply 2-bromoacetyl-6-methoxynaphthalene (Br-AMN) to the direct analysis of bile acids in an aqueous medium, the optimization of the derivatization system was examined. Tetrahexylammonium bromide (THxABr) was used as the ion-pair reagent suitable for carboxylic acids [11, 13, 14] and to provide an ionic micellar system in an aqueous medium at pH 7.0. To achieve optimum conditions the effects of temperature, reagent and ThxABr concentration, ultrasonication and organic solvent on the course of the reaction were investigated.

The course of the reaction on CDCA under different conditions is illustrated in Fig. 1. At ambient temperature, with ultrasonication, the derivatization reaction was found to be complete after 3 min; similar results were obtained for UDCA, DCA, LCA and their glyco-conjugated derivatives whereas a longer reaction time (10 min) was required for CA and GCA. At 40°C, with ultrasonication, the derivatization of CA and GCA was complete within 6 min. Higher temperatures did not offer significant advantages; in some cases (Fig. 1).reduced responses were observed. The effect of the quaternary ammonium salt concentration on the reaction course was also evaluated. The observed derivatization profile reached a maximum at 10 mM THxABr for the

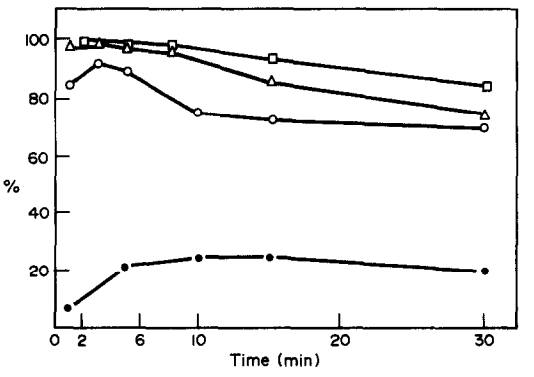

Figure 1
Influence of the temperature and ultrasonication on the derivatization of CDCA with Br-AMN (pH 7.0): (□) ambient temperature with ultrasonication for 3 min; (△) ambient temperature with continuous ultrasonication; (○) at 60°C with continuous ultrasonication; (●) ambient temperature without ultrasonication.

examined bile acids with the exception of CA and GCA; these compounds required 20 mM THxABr to obtain high derivatization rates. Under these conditions (40°C and 20 mM THxABr) the yields of the bile acid esters increased to reach a plateau at a molar ratio of reagent to bile acid of about 25 and a further reagent excess did not interfere.

Ultrasonication of the reaction mixture proved to be a useful device; when ultrasonication was omitted and the reaction was carried out by stirring, the yields of the bile acid esters were significantly lowered (Fig. 1). The important role played by ultrasonication in synthetic organic chemistry is well recognized [18]. With ultrasonication, the aqueous reaction mixture appeared turbid and this condition was found to be essential to achieve high yields of derivatized bile acids.

Addition of organic solvents (methanol, acetone, acetonitrile) prevented the formation of a turbid reaction mixture and this resulted in low derivatization rates; this was consistent with results observed for non-ionic micellar systems [14]. The effect of the methanol content in the reaction mixture on the derivatization of CDCA is shown in Fig. 2. Accordingly, 10% methanol was chosen as the solvent for the derivatization reaction. The negative effect of organic solvents and kinetic considerations [19] led to the use of a reduced volume (0.3 ml) of concentrated reagent solution in acetone. Under the described conditions, the derivatization reaction in studies on CDCA was found to be essentially quantitative by comparison with an authentic specimen of CDCA naphthacylester.

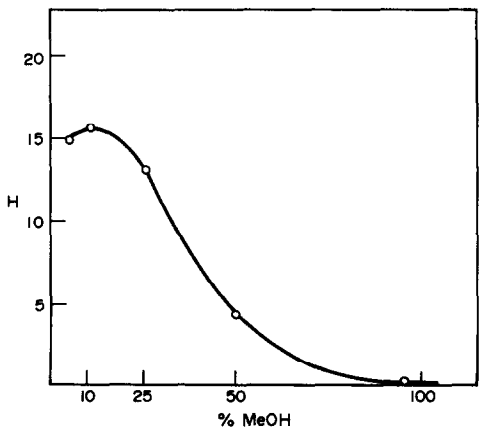

Figure 2
Influence of the methanol content in the reaction mixture on the derivatization of CDCA with Br-AMN at ambient temperature and pH 7.0 (reagent to analyte molar ratio = 30:1). H = peak height.

The turbid reaction mixture could be directly injected into the chromatograph but improved reproducibility was obtained when acetonitrile was added to the mixture and the resulting clear solution was injected.

Chromatography

As a result of previous experience [12], reversed-phase chromatography was chosen and binary mixtures of acetonitrile–water were used to achieve adequate separation of free and glycine conjugated bile acids derivatized with Br-AMN. Columns (250 × 4.6 mm i.d.) packed with two different 5 μm RP-18 materials (Ultracarb and Hypersil) were used. The Ultracarb column proved to be more retentive than the Hypersil column and enabled good separations to be achieved but a higher acetonitrile content in the mobile phase was required for the elution of the derivatized free bile acids. High contents of organic modifier are responsible for a significant quenching of the analyte fluorescence resulting in lower sensitivity [12]. Thus, the Ultracarb column was used for the HPLC analysis of glycine conjugated bile acids whereas the Hypersil column was found to be of general application.

The separations were carried out under isocratic or gradient conditions. Isocratic conditions were suitable for the HPLC determination of UDCA and CDCA in pharmaceutical formulations but were unable to provide adequate resolution of more complex samples in a reasonable analysis time. Thus gradient elution was used for the HPLC separation of all the principal bile acids of biological importance. A representative simultaneous separation of the fluorescent naphthacylesters of free and glycoconjugated bile acids is illustrated in Fig. 3. The peaks of the bile acids are fully resolved and separated from the reagent peaks.

Analysis of UDCA and CDCA in pharmaceutical formulations

Ursodeoxycholic acid (UDCA) is the 7β-epimer of chenodeoxycholic acid (CDCA); these compounds are both useful for the dissolution of cholesterol-rich gall-stones in patients with a functioning gall-bladder. The analytical solutions were subjected to the derivatization reaction with Br-AMN; the

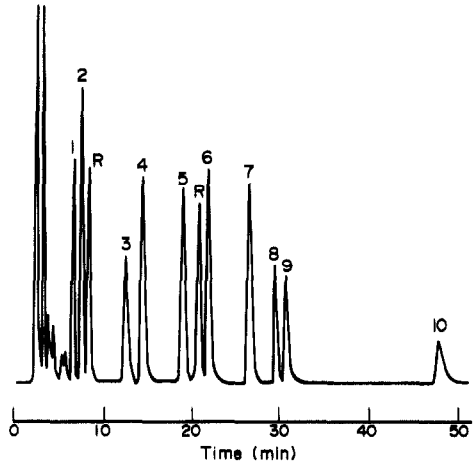

Figure 3
Representative HPLC separation at ambient temperature of a standard mixture of free and glyco-conjugated bile acids (30 μM) derivatized with Br-AMN: (1) GCA, (2) GUDCA, (3) GCDCA, (4) GDCA, (5) CA, (6) UDCA, (7) GLCA, (8) CDCA, (9) DCA, (10) LCA. R = Reagent peak. Column: 5-μm Hypersil RP-18 (250 × 4.6 mm i.d.). Gradient elution with mixtures of water (A)–acetonitrile (B) of varying composition (v/v). The gradient profile adopted was: t = 0, 60% B; t = 10 min, 60% B; t = 20 min, 80% B; t = 45 min, 80% B; and t = 50 min, 60% B at a flow rate of 1.0 ml min^{-1}. Fluorescence detection λ_{ex} = 300 nm; λ_{em} = 460 nm. Attenuation = 8–4.

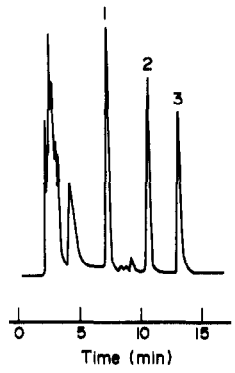

Figure 4
HPLC chromatogram obtained from a commercial formulation sample derivatized with Br-AMN: (1) UDCA, (2) valproic acid (the internal standard) and (3) CDCA. Column: as in Fig. 3. Mobile phase: acetonitrile–water (78:22, v/v) at a flow rate of 1.0 ml min^{-1}. Detection as in Fig. 3. Attenuation = 8.

fluorescent naphthacyl-esters obtained were then analysed by HPLC with fluorescence detection (λ_{ex} = 300 nm; λ_{em} = 460 nm). A typical chromatogram obtained (isocratic conditions) from a commercial sample containing both UDCA and CDCA is shown in Fig. 4. Under these conditions a linear relationship between the peak-height ratio (y) of drug ester to internal standard (valproic acid ester) and drug concentration (x; μg ml^{-1}) were obtained for UDCA ($y = 0.033x + 0.0065$; $r = 0.9980$, $n = 6$) and CDCA ($y = 0.0204x - 0.0052$; $r = 0.9980$, $n = 6$). Where the formulation contained only UDCA, caproic acid naphthacyl-ester was used as the internal standard and the calibration graph was: $y = 0.0556x + 0.010$ ($r = 0.9985$; $n = 6$) where y is the response ratio and x is the drug concentration (μg ml^{-1}). The satisfactory within-run precision of the method (derivatization and chromatography) was indicated by the relative standard deviation (RSD = 1.5–1.8%, $n = 8$) of the peak-height ratio (drug to I.S.) from replicate ($n = 8$) analyses of a single standard solution. The method was then applied to the HPLC determination of UDCA and CDCA in commercial dosage forms; the assay results (Table 1) were found to be in close agreement with the claimed content of drug. The accuracy of the method was verified by the analysis of commercial samples spiked with known amounts of the drugs; quantitative recovery was obtained in each instance.

Table 1
Assay results for the HPLC determination of UDCA and CDCA in commercial dosage forms (capsules). The results are the mean value of five determinations and are expressed as the percentage of the claimed content

Formulation	Drug	Found (%)	RSD (%)
I*	UDCA	99.40	1.24
II†	UDCA	102.00	1.43
	CDCA	100.80	2.10

*Other ingredients: starch, magnesium stearate and colloidal silica.
†Other ingredients: hydroxypropylmethylcellulose, magnesium stearate and starch.

Analysis of bile acids in human bile

Bile acids are present in biological fluids in the free (F) form (intestinal content and stools) and as glycine (G) and taurine (T) conjugates (bile and serum). In human bile the bile acids are mainly amidated with glycine and taurine and are present at the millimolar level. Only in the terminal ileum and in the colon are the primary bile acids (CA, CDCA) deconjugated and 7-dehydroxylated to form free secondary bile acids (DCA, LCA). Few attempts have been dedicated to the evaluation of unconjugated bile acids in human bile, where they have been found present at the micromolar level by gas-chromatography–mass spectrometry [20]. Their determination, however,

could be of diagnostic importance since the free fraction composition can be altered in the presence of hepatobiliary diseases [20]. Thus the HPLC determination of the major unconjugated C_{24} bile acids in human bile has been included in this work.

According to a previously published method [16], bile acids were first extracted from bile samples by solid-phase extraction (SPE) using C-18 sorbent and then separated in F, G and T groups using strong anion-exchange cartridges (Bond-Elut SAX). Subsequently, the resolution of each fraction into individual bile acids was performed by HPLC. In particular, unconjugated and glycine-conjugated bile acids were derivatized with Br-AMN and the resulting naphthacylesters were analysed by HPLC with fluorescence detection (λ_{ex} = 300 nm; λ_{em} = 460 nm); the taurine-conjugated bile acids were converted to the corresponding free forms by enzymatic hydrolysis prior to their derivatization. The recovery after the BE-SAX fractionation was previously reported [16] to be at least 87% for each bile acid and the results of further investigations carried out in the authors' laboratory confirmed these data. Linear relationships between peak-height ratio (analyte to I.S.) and analyte concentration (nmol ml^{-1}) were found for each bile acid (Table 2). The within-run precision of the method (derivatization and chromatography) was satisfactory as indicated by the RSD range (2.0–3.6%) of the peak-height ratios from replicate (n = 8) analyses of a single standard solution.

The method described above was applied to the analysis of human bile samples; representative HPLC chromatograms obtained for the unconjugated glyco- and tauro-conjugated fractions are reported in Figs 5, 6 and 7, respectively. The identity of the separated compounds was assigned by retention coincidence after analysing final analytical solutions reinforced with authentic bile acid standards.

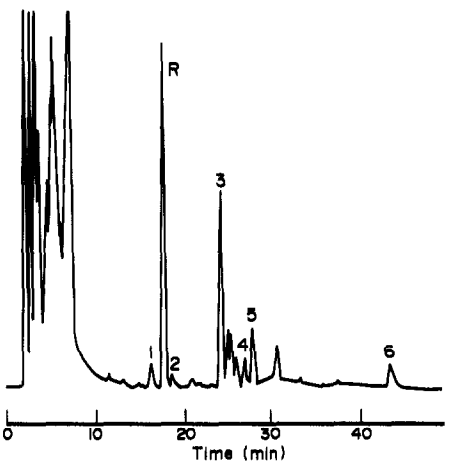

Figure 5
HPLC chromatogram of the derivatized unconjugated bile acid fraction obtained from a human bile sample from a patient with cholesterol gall-stones after UDCA treatment. (1) CA, (2) UDCA, (3) valproic acid (internal standard), (4) CDCA, (5) DCA and (6) LCA. R = Reagent peak. Chromatographic conditions and detection as in Fig. 3. Attenuation = 1.

Under the chromatographic conditions of Fig. 5, the peaks of free bile acids were fully resolved from the peaks of glyco-conjugates and use of this method should avoid errors in assignation due to imperfect group fractionation. However, the detection of free bile acids requires maximum instrumental sensitivity (attenuation = 1) and, under these conditions, the background interference can prevent the identification and quantitation of these compounds. In this application the need for purified analytical solutions and unambiguous peak identification constitute critical aspects of the

Table 2
Data for the calibration graphs (n = 6) obtained by the HPLC method for free and glyco-conjugated bile acids derivatized with Br-AMN

Compound	Slope	Intercept	Correlation coefficient	Conc. range (nmol ml^{-1})
CA	0.325	0.024	0.9975	0.8–6.4
UDCA	0.390	0.012	0.9970	0.8–6.4
CDCA	0.203	−0.002	0.9980	0.8–6.4
DCA	0.210	−0.006	0.9983	0.8–6.4
LCA	0.105	0.010	0.9980	0.8–6.4
G-CA	0.065	−0.006	0.9980	5–50
G-UDCA	0.091	0.007	0.9985	5–50
G-CDCA	0.051	−0.010	0.9999	5–50
G-DCA	0.065	−0.014	0.9999	5–50
G-LCA	0.023	−0.005	0.9997	5–50

Table 3
Biliary bile acid composition in three patients with cholesterol gall-stones before (B) and after (A) 1 month's treatment with 150 mg day^{-1} of ursodeoxycholic acid. Results* are expressed in mmol l^{-1} for the glycine (G) and taurine (T) fractions and as μmol l^{-1} for the unconjugated (F) fraction

		CA			CDCA			DCA			LCA			UDCA			Total	
Patient		G	T	F	G	T	F	G	T	F	G	T	F	G	T	F	Conj. (mmol l^{-1})	Free (μmol l^{-1})
1	B	5.10	3.56	6.0	5.84	2.54	7.0	4.57	2.54	83.0	0.5	0.50	47.0	0.20	0.05	†	25.40	143.0
	A	8.40	5.60	7.0	4.25	2.20	13.0	1.80	2.50	51.0	†	0.15	23.0	2.30	0.90	5.0	28.10	99.0
2	B	4.28	1.95	†	4.87	2.53	†	3.52	1.95	68.0	0.1	0.30	46.0	†	†	†	19.50	114.5
	A	4.40	0.55	5.0	4.23	0.52	†	2.70	0.28	47.0	†	0.09	41.0	4.23	0.10	7.0	20.12	100.0
3	B	6.12	2.04	†	4.48	2.05	9.0	3.05	1.43	54.0	0.6	0.20	77.0	0.20	0.20	†	20.37	140.0
	A	5.30	0.95	†	9.10	1.10	15.0	5.00	0.53	35.0	†	0.13	58.0	1.37	0.16	†	23.64	108.0

*RSD % (n = 3) was 2.5–4.3% for glycine- and taurine-conjugates and 4.5–12% for free bile acids.
†Not detectable.

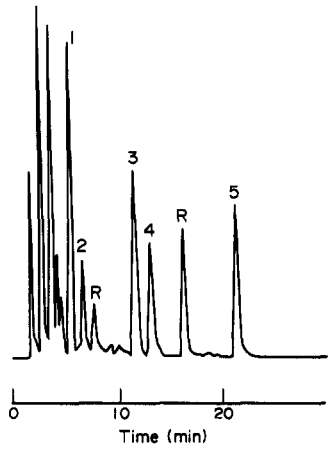

Figure 6
HPLC chromatogram of the derivatized glyco-conjugated bile acid fraction obtained from a human bile sample (as in Fig. 5). (1) GCA, (2) GUDCA, (3) GCDCA, (4) GDCA and (5) valproic acid (internal standard). R = Reagent peak. Column: 5-μm Ultracarb RP-18 (250 × 4.6 mm i.d.). Gradient elution with mixtures of water (A)–acetonitrile (B) of varying composition (v/v). The gradient profile adopted was: t = 0, 65% B; t = 10 min, 65% B; t = 15 min, 80% B; t = 25 min, 80% B; and t = 30 min, 65% B. Detection as in Fig. 3. Attenuation = 16.

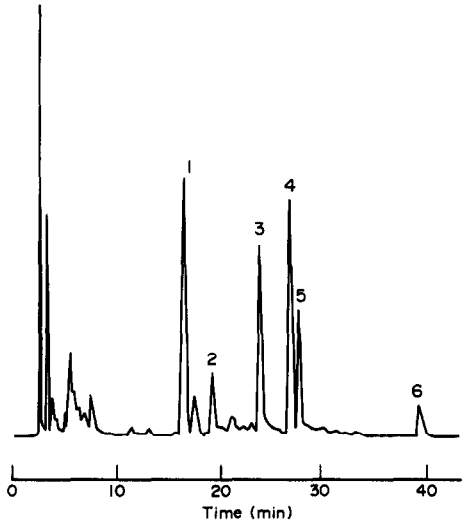

Figure 7
HPLC chromatogram of the derivatized tauro-conjugated bile acid fraction (subjected to enzymatic hydrolysis) obtained from a human bile sample (as in Fig. 5): (1) CA, (2) UDCA, (3) valproic acid (I.S.), (4) CDCA, (5) DCA and (6) LCA. Chromatographic conditions and detection as in Fig. 3. Attenuation was modified during the analysis (16–8–2).

HPLC method. As a measure of the sensitivity, the detection limit (S/N = 3 with attenuation = 1) was about 1–2 pmol of the injected naphthacyl-esters.

The results obtained are summarized in Table 3, where the bile acid composition in duodenal bile of three patients before and after treatment with UDCA at low dose (150 mg day^{-1}) is reported.

Before treatment the bile contained only trace amounts of UDCA and the main bile acids were cholic and chenodeoxycholic acids. The free function accounted for only 0.5–0.7% of the total BA and the main unconjugated BA were LCA and DCA. The main fraction was the glycine-amidated fraction with a glycine/taurine ratio usually of about 2:1.

After treatment for 1 month with UDCA the bile became partially enriched with the amidated form of this BA, particularly with GUDCA; free UDCA was present in trace amounts. The UDCA treatment drastically modified the glycine/taurine ratio which increased to about 10:1 with the exception of that for LCA, which was probably due to the poor solubility of GLCA. The unconjugated LCA and DCA were reduced after treatment but remained the main unconjugated BA.

The above data are in line with previous reports on biliary bile acid composition in patients with cholesterol gall-stones and the modifications induced by UDCA treatment [20, 21]. Preliminary exploratory information has been obtained on the unconjugated bile acid fraction which was found to be very low (0.3–0.7% of the total bile acids).

Conclusions

The present study confirmed the potential of HPLC with fluorescence detection for the analysis of the main bile acids in complex matrices such as human bile; 2-bromoacetyl-6-methoxynaphthalene proved to be a useful pre-chromatographic fluorescent labelling reagent. Further investigations are required in order to better define the applicability of the method for the evaluation of unconjugated bile acids in biological fluids and of their variation in relation to hepatobiliary diseases or pharmacological treatment. Studies are in progress on the analysis of serum bile acids.

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