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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND QUANTIFICATION OF INDIVIDUAL HUMAN BILE ACIDS

G. MINGRONE* and A.V. GRECO

Istituto di Patologia Medica, Catholic University, Rome (Italy)

and

S. PASSI

Ospedale S. Gallicano, Rome (Italy)

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SUMMARY

The reversed-phase high-performance liquid chromatographic separation and quantification of individual bile acids is described.

Taurine- and glycine-conjugated bile acids were separated and detected directly by an ultraviolet absorbance detector operating at 200 nm. Simultaneous quantitation of at least 100 ng of each conjugated bile acid is possible.

Carboxylic (free and glycine-conjugated) bile acids were esterified with *p*-bromophenacylbromide. The reaction, using N,N-diisopropylethylamine as catalyst, yields quantitatively the strongly absorbing *p*-bromophenacyl esters which can be determined by absorbance measurement at 254 nm. Simultaneous quantitation of less than 20 ng of each bile acid is possible.

The present method is applied to the quantitation of individual bile acids in ten human gallbladder bile samples.

INTRODUCTION

Current methodology available for measurement of bile acids* shows

*Abbreviations used in the figures and tables: CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid. The prefixes glyco- (G) and tauro- (T) are used for bile acids having glycine or taurine in amide linkage at C-24.

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differences in the data produced by each procedure. Determination of individual bile acids in biological fluids by thin-layer chromatography (TLC) and fluorimetry has been reported [1]. TLC requires the use of time-consuming elution procedures; besides, the resolution of glycine and taurine conjugates is, in practice, not satisfactory. Total bile acids can be quantified by the use of 3α-hydroxysteroid-dehydrogenase. This method can not differentiate between the individual bile acids [2—4]. A specific radioimmunoassay method has been used to determine cholyl and chenyl conjugates [5—10]. Despite the improvement of gas—liquid chromatography (GLC) for the measurement of free bile acids [11, 12], it is not possible to separate individual conjugated bile acids by this method. The preparation of a sample for GLC shows inherent disadvantages, including the destruction of conjugates, formation of artefacts during vigorous alkaline hydrolysis, and incomplete hydrolysis [13]. However, a quantitative enzymatic hydrolysis of bile acid conjugates has been reported [14].

Modern high-performance liquid chromatography (HPLC) offers the possibility of separating and quantifying individual bile acids. Detection by non-destructive refractive index or ultraviolet (UV) detectors also permits recovery of the injected samples for further analysis. In this connection, numerous studies have been reported [15—21].

In the present work, a reversed-phase HPLC separation and quantification of individual bile acids is reported.

EXPERIMENTAL

Reagents

Acetonitrile Lichrosolv, methanol Lichrosolv, *p*-bromophenacylbromide, phosphoric acid, acetic acid and diisopropyl ether were all from Merck (Darmstadt, G.F.R.) and were used as received. N,N-Diisopropylethylamine, dioxane, chloroform, 2,2,4-trimethylpentane and isopropanol (all from Merck) were redistilled before use.

Silica gel (high purity grade) precoated plates without organic binder (Stratochrom SI-AP, Carlo Erba, Milan, Italy) were used for TLC.

Cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, glycocholic acid (sodium salt), glycochenodeoxycholic acid (sodium salt), glycolithocholic acid (sodium salt), taurodeocycholic acid (sodium salt) and taurolithocholic acid (sodium salt) were obtained from Calbiochem, Lucerne, Switzerland. Ursodeoxycholic acid was kindly supplied by Zambon Pharmaceuticals, Milan, Italy. Their purity was checked by TLC prior to use, as described by Cass et al. [22] for conjugated bile acids and by Panveliwalla et al. [1] for free bile acids. All were found to be 96—98% pure.

HPLC

Analyses were conducted using the 1084 B liquid chromatograph (Hewlett-Packard) equipped with either a single-wavelength (254 nm) UV detector or a scanning spectrophotometer with a wavelength range of 190—540 nm. The liquid chromatograph includes an integrator given areas and times for each peak in the chromatogram.

A reversed-phase Brownlee Labs column (Santa Clara, CA, U.S.A.), 25 cm × 4 mm I.D., RP-18, particle size 5 μm, was used for analysing all bile acids. Chromatographic column and solvents were operated at 40 ± 1°C.

HPLC analysis of standard taurine- and glycine-conjugated bile acids

The conditions were as follows. Mobile phase A was methanol—water (70:30). Phosphoric acid was used to adjust the pH of the mobile phase to 3.1. Flow-rate was 0.5 ml/min, the detector UV (200 nm). Sensitivity was a.u. from 8.0 to 128.0 × 10^{-4} /cm (depending on the amount of injected substances). Chart speed was 0.4 cm/min. Standards were all dissolved in mobile phase A before injection.

HPLC analysis of standard free and glycine-conjugated bile acids

*Derivatization procedure.*The reaction of free and glycine-conjugated bile acids with *p*-bromophenacylbromide, using N,N-diisopropylethylamine as catalyst, yields quantitatively strongly absorbing *p*-bromophenacyl esters that can be determined by HPLC with absorbance measured at 254 nm.

Briefly, 1 mg of bile acid is dissolved in 5 ml of anhydrous acetonitrile—methanol (9:1) containing 2.5 mg of *p*-bromophenacylbromide; 5 μl of N,N-diisopropylethylamine are added to catalyze the reaction which is complete in about 2 h at 25°C. Heated to 50—60°C, the reaction is complete in about 15 min. For 0.5—20 μg of bile acids, which are the amounts we used for calibration curves, a three-fold molar excess of *p*-bromophenacylbromide and a six-fold molar excess of N,N-diisopropylethylamine must be maintained. The *p*-bromophenacyl esters can be purified from excess reagents by TLC using benzene—dioxane (70:30) as eluent. The excess reagent and its degradation products migrate with the solvent front, *p*-bromophenacyl esters are revealed by a shortwave UV lamp (254 nm) and extracted three times with 5 ml of acetonitrile.

HPLC analysis. Mobile phase B was acetonitrile—water (70:30) at pH 3.10 (with phosphoric acid). The run was isocratic for 5 min, then gradient elution from 70% to 100% acetonitrile in 50 min. Detector: UV (254 nm). Flow-rate: 1 ml/min. Sensitivity: a.u. from 4.0 to 128.0 × 10^{-4} /cm (depending on the amount of injected substances). Chart speed: 0.25 cm/min.

With this mobile phase, if the excess derivatizing reagent and its degradation products are not eliminated by TLC (as is the case when derivatizing bile acids from biological fluids), they interfere with the peak of glycocholic *p*-bromophenacyl ester. Another mobile phase consisting of 100% acetonitrile (flow-rate 0.5 ml/min) is used for separation of glycocholic ester from the interfering derivatizing reagent excess.

Isolation of bile acids from human gallbladder bile samples

The samples were drawn during the removal of the gallbladders from subjects with cholesterin gallstones. Samples of the bile (0.2 ml) were deproteinized by treatment with 1 ml of ethanol (kept at —20°C overnight) and centrifuged. The residue was washed twice with ethanol and centrifuged. The supernatant and combined washes were divided into two parts. One half (for the investigation of conjugated bile acids) was evaporated to dryness and redissolved in 1 ml of mobile phase A and filtered through a Swinney filter prior to injection. The

remaining part (for the analysis of carboxylic bile acids) was acidified to pH 2.5—3.0 with 1 *N* HCl and evaporated to dryness. Nitrogen was blown through the vessel until the odour of HCl was gone. The residue was redissolved in chloroform—methanol (1:2) and purified by TLC with chloroform as solvent.

Bile acids remained at the origin, while the less polar lipids, including cholesterol, free fatty acids, triglycerides, cholesterol esters, etc., moved with or near the solvent front [1]. Areas corresponding to the origin were scraped off from the plates and extracted three times with chloroform—methanol (1:2). The combined chloroform—methanol extracts were dried under nitrogen and used for the derivatization procedure.

The esterification procedure was carried out as reported above. *p*-Bromophenacylbromide and N,N-diisopropylethylamine were added on the basis of the amounts of glycoconjugates present in the bile and determined directly at 200 nm with mobile phase A.

RESULTS

Fig. 1 shows the linear detection response to quantities of eight standard conjugated bile acids between 0.1 and 2.0 μg, with the use of a UV absorbance detector operating at 200 nm. The detection limit at a sensitivity of 8.0×10^{-4} a.u./cm is of the order of 10 ng. The detectable level is based on a

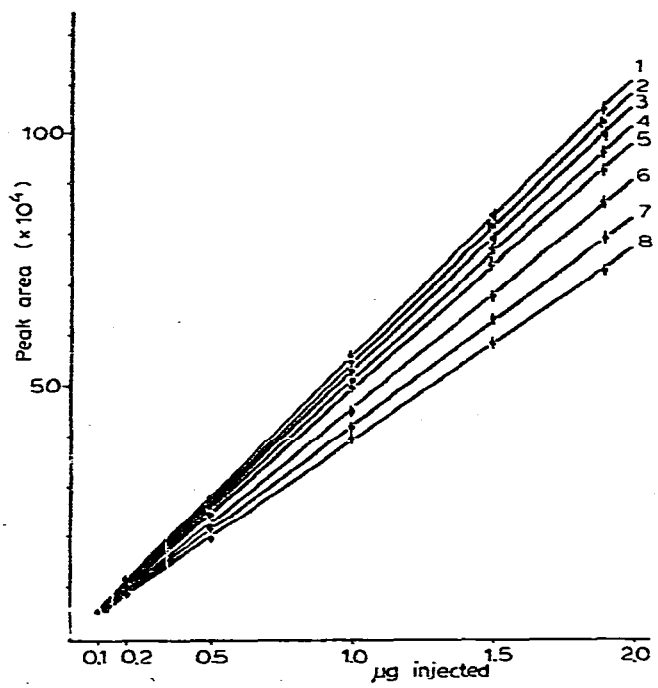

Fig. 1. UV (200 nm) detector response. Conditions are as described under Experimental. Each point is the mean ± S.D. of five determinations carried out for each conjugate dissolved in elution solvent mixture. 1, TCA; 2, TDCA; 3, TLCA; 4, TCDCA; 5, GCA; 6, GDCA; 7, GLCA; 8, GCDCA.

response of twice the noise level. At 210 nm, a response about three times lower is obtained.

Fig. 2 shows the linear detection response to quantities of carboxylic bile acids as *p*-bromophenacyl esters between 0.05 and 2.0 μg. Levels from 0.1 to 20 μg of standard free and glycine-conjugated bile acids were derivatized as

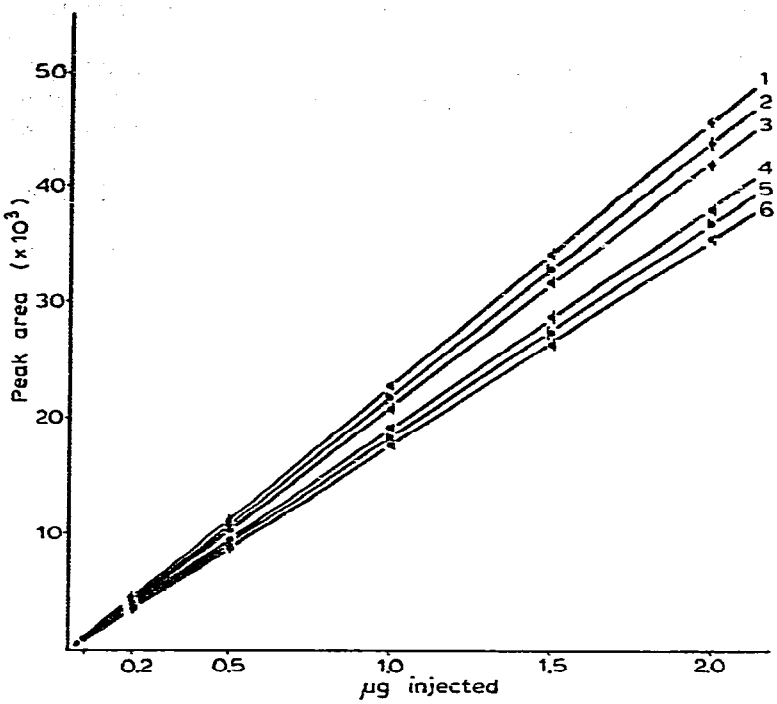

Fig. 2. UV (254 nm) detector response. Conditions are as described under Experimental. Each point is the mean ± S.D. of five determinations carried out for each *p*-bromophenacyl ester. 1, LCA; 2, DCA—CDCA; 3, CA; 4, GLCA; 5, GDCA; 6, GCA.

TABLE I

RECOVERY OF FREE AND GLYCINE-CONJUGATED BILE ACIDS AFTER TLC AND DERIVATIZATION

Each result represents the average of five experiments.

Bile acids	μg deposited on TLC plates	μg measured by HPLC after extraction from TLC plates and esterification procedures (mean ± S.D.)
CA	10	9.51 ± 0.36
DCA	10	9.55 ± 0.38
CDCA	10	9.60 ± 0.33
LCA	10	9.38 ± 0.44
UDCA	10	9.75 ± 0.29
GCA	10	9.48 ± 0.51
GDCA	10	9.50 ± 0.46
GCDCA	10	9.58 ± 0.41
GLCA	10	9.46 ± 0.39

described under Experimental. The reaction volume was always 1 ml and 100 µl were injected into the column. The *p*-bromophenacyl esters are strictly correlated to their molecular weights: the lower the molecular weight, the higher the peak area. λ_{max} for these esters is 255 nm with log ϵ values of about 4.5.

At the maximum detector sensitivity (1×10^{-4} a.u./cm), 1 ng represents the detection limit.

It was then ascertained that the carboxylic bile acids could be recovered from the plates (chloroform was used as eluent) and quantified. After extraction from the plates and esterification, the free and glycine-conjugated bile acids were measured by HPLC. Table I reports these results. Fig. 3 demonstrates the separation of a synthetic mixture of nine carboxylic bile acids as *p*-bromophenacyl esters.

Fig. 4 shows the separation of a synthetic mixture of conjugated bile acids.

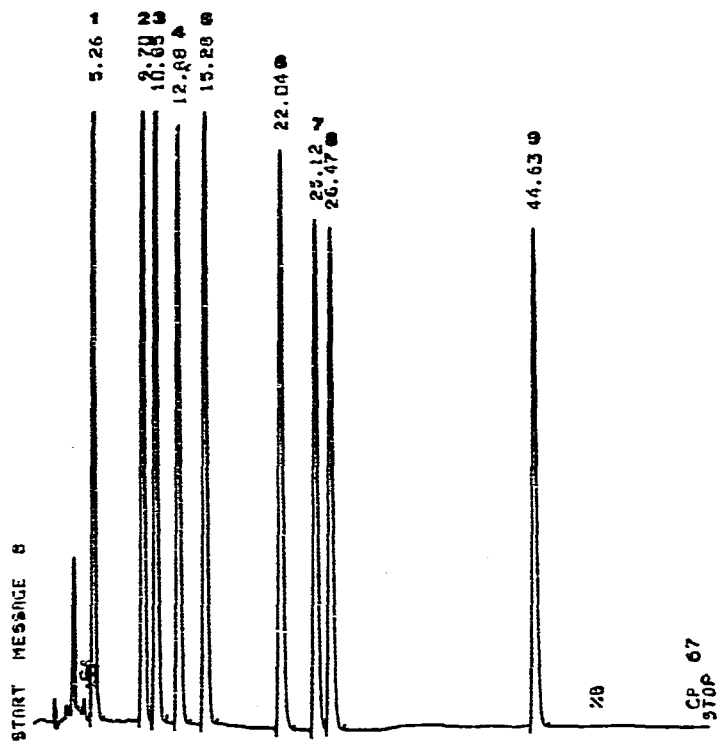

Fig. 3. Separation of standard free and glycine-conjugated bile acids as *p*-bromophenacyl esters, separated from excess derivatizing reagent by TLC. Conditions are as described under Experimental with a UV detector (254 nm). Amount injected was 1 µg of each standard. Peak identification: 1, GCA; 2, GCDCA; 3, GDCA; 4, CA; 5, UDCA; 6, GLCA; 7, CDCA; 8, DCA; 9, LCA.

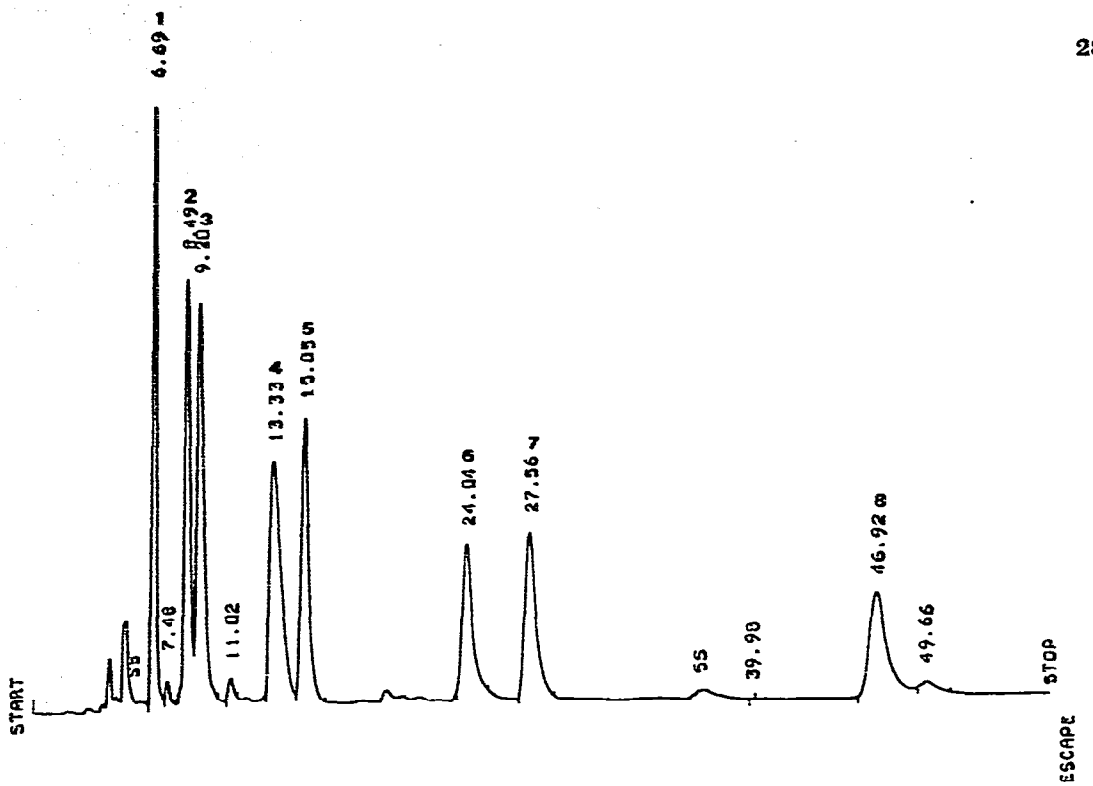

Fig. 4. Separation of a synthetic mixture of conjugated bile acids. Conditions are as described under Experimental, with a UV detector (200 nm). Amount injected was 2 μg of each standard. Peak identification: 1, TCA; 2, TCDCA; 3, TDCA; 4, TLCA; 5, GCA; 6, GCDCA; 7, GDCA; 8, GLCA. Unidentified peaks were observed. These were not investigated further.

TABLE II

AMOUNTS OF BILE ACIDS IN TEN HUMAN GALLBLADDER BILE SAMPLES

Values are given in μg/ml.

Sample	Cholate		Chenodeoxy-cholate		Deoxycholate		Lithocholate	
	T*	G**	T	G	T	G	T	G
I	1196	1724	2009	3501	311	1980	270	350
II	3960	2033	1850	1580	tr***	430	tr	tr
III	1820	1360	1205	1870	tr	520	tr	tr
IV	3850	6733	5012	6036	1125	2148	tr	tr
V	2146	1993	2630	1836	386	990	258	1036
VI	1285	963	865	830	89	225	tr	385
VII	2376	2467	2261	2625	318	750	tr	420
VIII	2040	2180	2520	1850	427	780	tr	tr
IX	1680	1640	2040	1320	tr	1215	tr	tr
X	2950	2350	3050	2010	217	853	tr	tr
Mean ± S.D.	2330 ± 974	2344 ± 1608	2344 ± 1138	2346 ± 1484	288 ± 337	298 ± 633	54 ± 111	220 ± 339

*T, taurine conjugates.
**G, glycine conjugates.
***traces (<0.5 μg/ml).

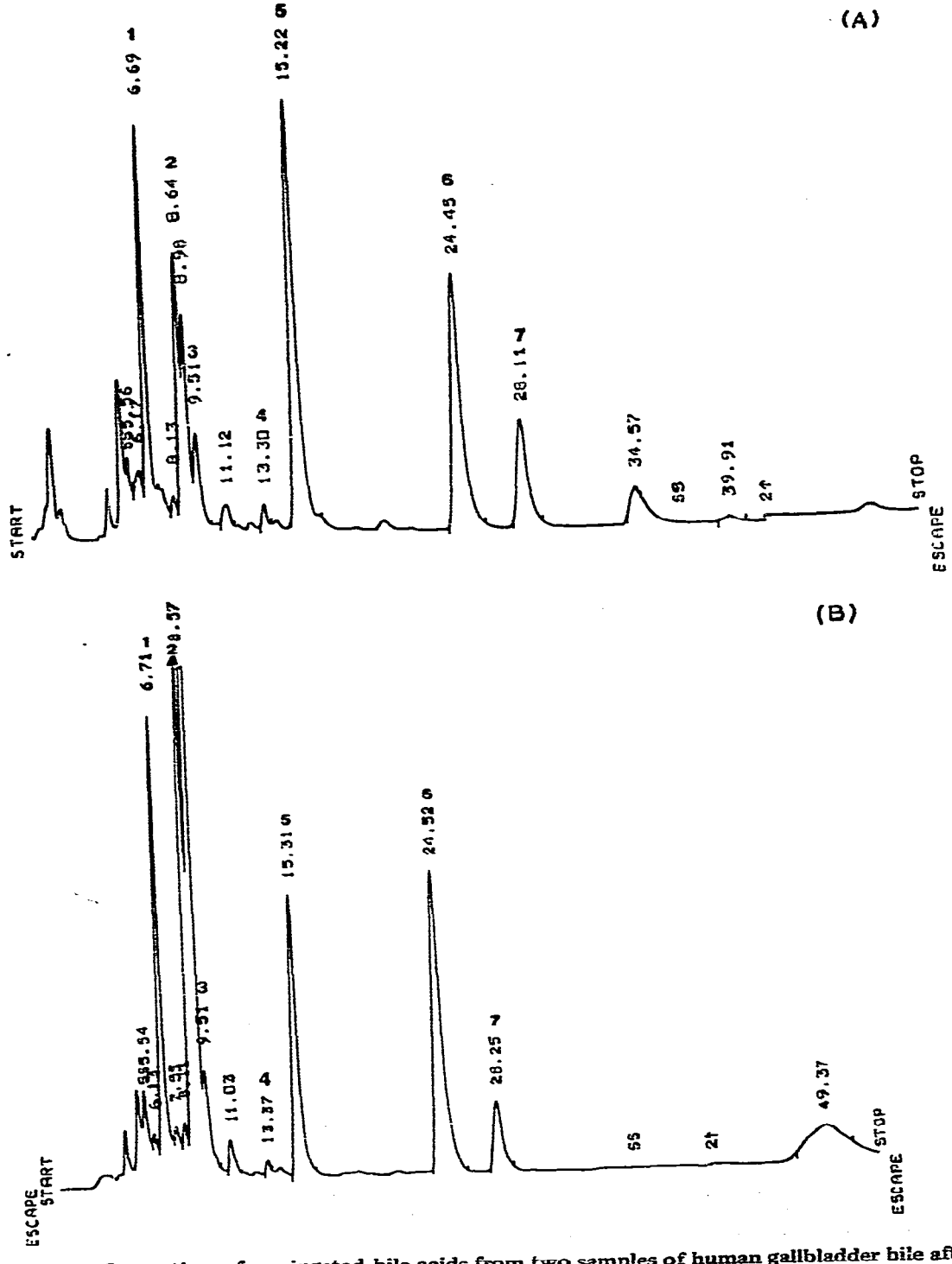

Fig. 5. Separation of conjugated bile acids from two samples of human gallbladder bile after deproteinization of the bile. For further details and peak identification see Fig. 4 and the text.

Only conjugated bile acids were found in ten samples studied, free bile acids were not found to be present.

Fig. 5A and B demonstrates the separation of conjugated bile acids from two samples of human gallbladder bile after deproteinization of the bile. Finally, Table II shows the amounts (μg/ml) of bile acids found in ten human gallbladder bile samples.

DISCUSSION

The recent use of reversed-phase HPLC coupled to a UV detector [16, 17, 21] or to a refraction index detector [18, 19, 21] for the analysis of conjugated bile acids prompted us to inquire into the possibility of improving the reversed-phase system and testing it on human bile samples. The pH of the mobile phase we used (methanol—water, 70:30) significantly affected the elution of conjugated bile acids and the shape of the eluting peaks. Acidification of the mobile phase at pH 3.1 improved both the separation of the acids and the shape of the eluting peaks by reducing tailing.

Because of our interest in measuring in the lower concentration range, the use of a UV detector provided a much greater sensitivity than that afforded by a refractive index detector. In our studies the operating wavelength of the UV detector was fixed at 200 nm. In fact, in the mobile phase A, 203 nm was the wavelength for maximal absorption of both taurocholate and glycocholate, with 200 nm or less for other conjugates, as determined directly by the scanning spectrophotometer connected to the liquid chromatograph. The sensitivity of the detection decreases progressively as the wavelength of the detector is increased. At 210 nm, about a three-times lower response is obtained; at 254 nm the response is minimal. This imposes the use of pure solvents in the preparation of the mobile phase in order to avoid high noise levels, above all when working at low attenuations. Linear relationships were found between peak areas and quantities of the conjugated acids between 0.1 and 2.0 μg, as shown in Fig. 1.

With the carboxylic bile acids, strongly UV-absorbing (254 nm) derivatives were prepared. The *p*-bromophenacyl esters proved to be particularly useful in this respect. The maximum for these esters is 255 nm with log ϵ values of about 4.5. This is more than sufficient to provide nanogram sensitivities.

Simultaneous determination of glycine- and taurine-conjugated bile acids were carried out on ten human gallbladder bile samples. No free bile acids were found in any of the ten samples. Appreciable differences were found among total bile acids and also among individual bile acids with each sample. Further studies on a large number of samples are necessary to elucidate this biological variability and its importance in relation to physiological and/or pathological events. In this connection, because of its sensitivity and reproducibility, the present method facilitates a much more accurate study of bile acids, allowing the investigation of a possible relationship between states of liver function and bile acid patterns.

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