High Performance Liquid Chromatographic Analysis of Individual Bile Acids: Free, Glycine- and Taurine-conjugated Bile Acids

Sumihiko OKUYAMA,* Daisuke UEMURA,† and Yoshimasa HIRATA†

Nagoya University College of Medical Technology, Daiko-cho, Higashi-ku, Nagoya 461

†*Chemical Institute, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464*

(Received May 8, 1978)

High performance liquid chromatographic analyses of individual bile acids (cholic, chenodeoxycholic, deoxycholic, and lithocholic acids), free and conjugated with glycine and taurine, are described. The analyses of the free and glycine-conjugated bile acids are based on the esterification of the carboxyl group of bile acids with *O*-(*p*-nitrobenzyl)-*N,N'*-diisopropylisourea(PNBDI). The bile acids in the biological samples were extracted by an Amberlite XAD-2 column, and separated by DEAE-Sepharose CL-6B into free, glycine- and taurine-conjugated bile acids. After separation, the free and glycine-conjugated bile acids were directly esterified with PNBDI. Taurine-conjugated bile acids are unable to be esterified with PNBDI, the bile acids were hydrolyzed with NaOH to produce the free bile acids, and then esterified. The *p*-nitrobenzyl ester of bile acids has a characteristic ultraviolet absorption. Consequently the compounds were separated into the individual bile acids by high performance liquid chromatography, and detected by an UV-detector. An analysis of the individual bile acids in human bile is given for an example.

Despite the progress in gas-chromatographic analysis for the estimation of free bile acids,[1,2] individual conjugated bile acids can not be separated by this method. Moreover, the preparation of samples for gas-chromatography is complicated, incurring difficulties both in precision and accuracy. A method has been developed using high performance liquid chromatography[3] to give data on conjugated as well as free bile acids in clinical medicine.

The *p*-nitrobenzyl esters of glycine-conjugated and free bile acids possess characteristic ultraviolet absorption spectra, and based on this an attempt to apply this special quality for the analysis of these bile acids has been conducted. Taurine-conjugated bile acids have been separated from free and glycine-conjugated bile acids by anion exchange column-chromatography with DEAE-Sepharose CL-6B. After collection of the taurine-conjugated bile acids, hydrolysis with NaOH at 120 °C, esterification was conducted with PNBDI as well as the free and glycine-conjugated bile acids.

Experimental

Apparatus. A Varian LC 8500 liquid chromatograph was used throughout this work and fitted with an UV detector (JASCO UVIDEC 100), and a gradient elution accessory. A μBondapak C_{18} column, commercially available from Waters Associates, was used for the analysis of the *p*-nitrobenzyl esters of bile acids.

Materials. The bile acids were obtained from Sigma, Calbiochem, and Katayama Kagaku Kogyo. PNBDI was obtained from Regis Chem. Amberlite XAD-2 and Amberlyst A-15 from Rohm and Haas. Amberlite XAD-2 was washed with methanol, acetone, and water. Amberlyst A-15 was thoroughly washed with 2 M NaOH in 70% aqueous ethanol and followed by 70% ethanol until the elutant was neutral. It was subsequently washed with 2 M HCl in 70% ethanol and followed by 70% ethanol until the elutant was again neutral. DEAE-Sepharose CL-6B was made into the acetic form with acetic acid in 70% ethanol.

Procedure. Figure 1 outlines the analytical procedure. The mixture of 12 each individual bile acids (1 mg), free, glycine and taurine-conjugated, was dissolved in 0.1 M NaOH (10 ml) in saline and the solution agitated ultrasonically for 15 min. The solution was then applied to the column of Amberlite XAD-2[4] in water (1.0 g). The sample was allowed to flow through the column (100 mm/5 mm i.d.) at a rate of about 0.2 ml/min. The column was then washed with water until neutal and the bile acids eluted with methanol (30 ml). The elutant was then evaporated to dryness *in vacuo* at 50 °C, and the residue dissolved in 72% ethanol (30 ml). This was filtered through a column of Amberlyst A-15 in the H^+ form which removed interfering cations. The effluent from the column was passed through a column of DEAE-Sepharose CL-6B in the acetate form. Free bile acids passed directly through this column and were recovered in the first fraction (A). Glycine-conjugated and a small amount of the free bile acids were eluted with 0.1 M acetic acid in 72% ethanol (B). The taurine-conjugated bile acids were eluted with 0.15 M ammonium acetate (pH 6.6) in 72% ethanol (C). The separation was quantitative with minimal overlap between groups. The fractions were taken to near dryness on a rotary evaporator.

Fractions A and B were dissolved in 0.1 M NaOH (10 ml) in saline. The mixture of fraction A and B was allowed to flow through the column XAD-2 and after elution with methanol (30 ml), the elutant was evaporated to dryness. The residue was dissolved in water (10 ml), and the solution passed over a column of CM-Cellulose (1.0 g) in order to obtain the free acid from sodium salt. The flask used in the evaporation was washed subsequently with water (10 ml), ethanol (2 ml) and water (10 ml), and then each fraction passed through the column. The final volume of collected elutant was 30 ml. The solution was evaporated to dryness *in vacuo*.

Fraction C was dissolved in 7.5% NaOH (15 ml), and then hydrolyzed in a sealed tube at 120 °C for 4 h, since taurine-conjugated bile acids are unable to be esterified with PNBDI. After the hydrolysis, the hydrolysate was directly applied to the column of XAD-2, and the bile acids extracted with methanol (30 ml). After evaporation of the extract, the residue was dissolved in water (10 ml), and passed through a column of CM-Cellulose. The elutant was collected and evaporated to dryness. The bile acids in the mixture of fraction A and B, and the bile acids in fraction C were esterified with PNBDI in *t*-butyl alcohol (1 ml) for 24 h at room-temperature, as shown in Fig. 2.

The bile acid derivatives were treated with 0.1 M HCl

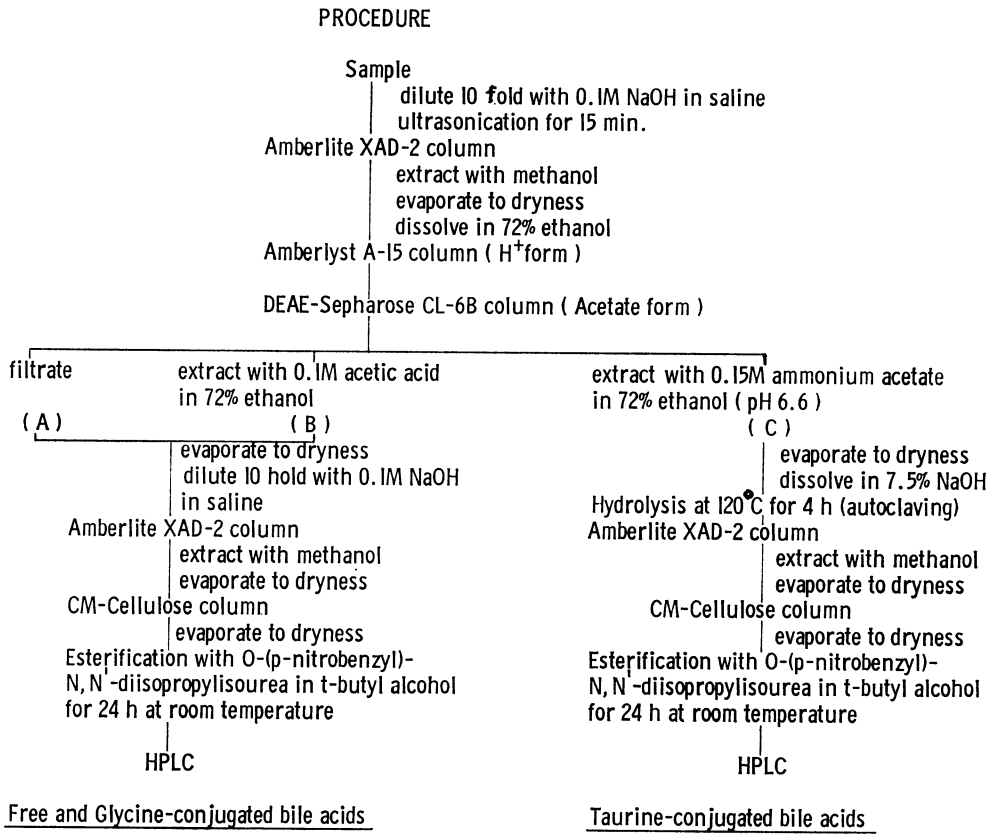

Fig. 1. The outline of analytical procedure of bile acids.

and water, and then analyzed by high performance liquid chromatography.

Operating Conditions. The conditions are shown in Table 1. The sample was injected with a microsyringe.

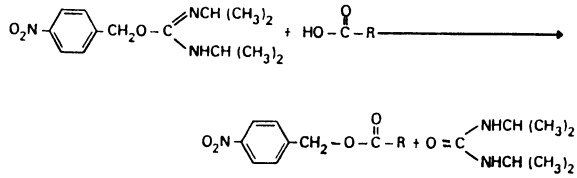

Fig. 2. The esterification reaction of bile acids with PNBDI.

Table 1. Operating conditions of high performance liquid chromatography for the determination of bile acids

Instrument	Varian LC 8500
Column	μBondapak C_{18} 30 cm × 3.9 mm i.d.
Mobile phase	Solvent A KH_2PO_4 (0.01 M): Methanol 1:1
	Solvent B KH_2PO_4 (0.01 M): Methanol 1:4
Flow rate	60 ml/h
Temperature	ambient
Detector	JASCO UV1DEC 100 UV 0.02 254 nm
Pressure	1250 psi
Recorder	10 mV 2.5 mm/min
Initial b%	65% 0—80 min
Gradient b%	0.4%/min 80 min—final

Results and Discussion

High Performance Liquid Chromatographic Analysis of Bile Acids. The high performance liquid chromatographic separation of the *p*-nitrobenzyl esters of the individual free and glycine-conjugated bile acids is shown in Fig. 3. The quantity of each bile acid was

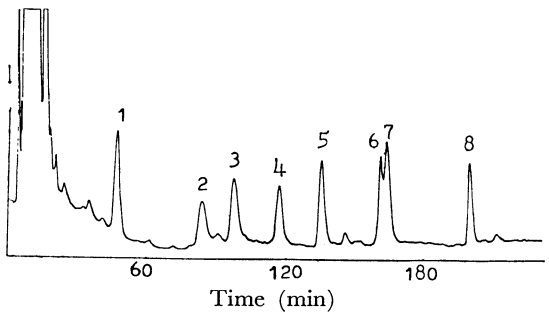

Fig. 3. High performance liquid-chromatographic separation of individual free and glycine-conjugated bile acids.

Bile acids

1.	glycocholic acid	GC
2.	glycochenodeoxycholic acid	GCDC
3.	glycodeoxycholic acid	GDC
4.	cholic acid	C
5.	glycolithocholic acid	GLC
6.	chenodeoxycholic acid	CDC
7.	deoxycholic acid	DC
8.	lithocholic acid	LC

6 μg. Each peak was identified by the addition of the *p*-nitrobenzyl ester of each authentic bile acid respectively. The separation between deoxycholic acid and chenodeoxycholic acid however was not sufficient.

High Performance Liquid Chromatographic Analysis of Bile Acids in Human Bile. Bile (1 ml), obtained by autopsy from a patient with obstructive jaundice due to cancer of pancreas head, was used in this experiment. Figure 4 shows the chromatogram of the free and glycine-conjugated bile acids in bile. The peaks were identified by the retention times. Figure 5 shows the chromatogram of taurine-conjugated bile acids in human bile. Since taurine-conjugated bile acids were hydrolyzed, each peak of the taurine-conjugated bile acid appears as the peak of the corresponding free bile acid. The concentration of individual bile acids in human bile is shown in Table 2. The total bile acid concentration in bile was 14.64 μM/ml, free 3.74 μM/ml, glycine-conjugated 8.08 μM/ml, taurine-conjugated 2.82 μM/ml. Accordingly glycine- and taurine-conjugated bile acids occupied 74% of the total bile acid in this human bile. The ratio of glycine-conjugated bile acid to taurine-conjugated bile acid (G/T) was 2.78.

The abbreviations of individual taurine-conjugated bile acids are expressed as follows:

taurcholic acid (TC)
taurochenodeoxycholic acid (TCDC)
taurodeoxycholic acid (TDC)
taurolithocholic acid (TLC)

$$\frac{C+GC+TC}{CDC+GCDC+TCDC} = 1.46$$

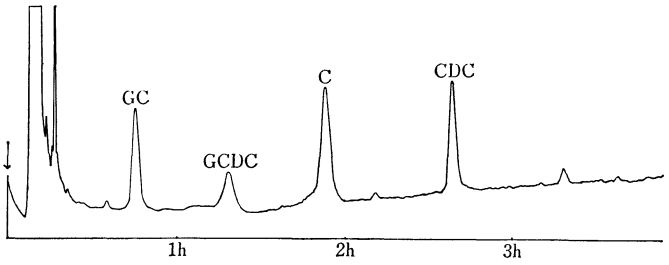

Fig. 4. High performance liquid-chromatographic analysis of free and glycine-conjugated bile acids in human bile.

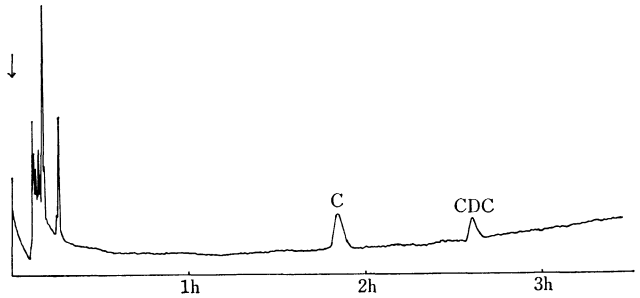

Fig. 5. High performance liquid-chromatographic analysis of taurine-conjugated bile acid in human bile.

Table 2. Bile acid in bile of a patient with obstructive jaundice due to pancreas head cancer

	Concentration in bile μM/ml
GC	4.93
GCDC	3.15
	8.08
TC	1.89
TCDC	0.93
	2.82
C	1.86
CDC	1.88
	3.74
Total	14.64

$\frac{C}{CDC}$	0.99	$\frac{GC}{GCDC}$	1.57	$\frac{TC}{TCDC}$	2.03
$\frac{GC+TC}{GCDC+TCDC}$	1.67	$\frac{C+GC+TC}{CDC+GCDC+TCDC}$			1.46
$\frac{\text{Conjugated}}{\text{Total}}$	0.74	$\frac{G}{T}$	2.78		

Significantly it means in obstructive jaundice that

$$\frac{C+GC+TC}{CDC+GCDC+TCDC}$$

is greater than 1.0.

Conclusion

The procedures described here make possible the individual separation of the conjugated and free bile acids. However, in order to complete the separation between DC and CDC in free bile acids, it is necessary to examine the conditions of the mobile phase and column. The investigation for the micro-determination of individual bile acids is in progress.

The development of the quantitative analysis of individual conjugated and free bile acids by high performance liquid chromatography may extend the value of bile acid studies in the clinical investigation of hepatobiliary disease.

References

1) D. H. Sandberg, J. Sjövall, K. Sjövall, and D. A. Turner, *J. Lipid Res.*, **6**, 182 (1965).
2) J. Roovers, E. Evrard, and H. Vanderhaeghe, *Clin. Chim. Acta*, **19**, 449 (1968).
3) S. Okuyama, D. Uemura, and Y. Hirata, *Chem. Lett.*, **1976**, 679.
4) I. Makino and J. Sjövall, *Anal. Lett.*, **5**, 341 (1972).