

Determination of conjugated bile acids in human bile and duodenal fluid by reverse-phase high-performance liquid chromatography

Craig A. Bloch and John B. Watkins¹

Division of Gastroenterology and the Joint Program in Neonatology, Children's Hospital Medical Center and Boston Hospital for Women, Boston, MA 02115

Summary A simple method using reverse-phase liquid chromatography is presented for resolution and quantitation of the major conjugated bile acids of man, including the glycine and taurine conjugates of the dihydroxy bile acids, chenodeoxycholic and deoxycholic acid. Using modern, high-performance chromatographic equipment, analysis time is less than 30 minutes. The quantitative range of the method, with detection by refractive index, is 0.05 to 0.1 μmol of bile acid and the limit of detection for an injected sample is 0.01 μmol . This provides a sensitivity sufficient for analysis of dilute duodenal and gallbladder bile with minimal sample preparation.

Supplementary key words refractive index detection · reverse phase.

Bile acids in biological systems are usually present as either the glycine or taurine conjugates. This modification alters their physico-chemical properties (1) and, in some instances, may significantly influence their metabolism (2, 3). Previous methods for isolation and quantification of conjugated bile acids have involved multiple steps including extraction, purification, and group separation of the taurine and glycine conjugates with incomplete resolution of the dihydroxy conjugates (4). Quantification of conjugated bile acids is then performed either by enzymatic or gas-liquid chromatographic (GLC) analysis. The latter approach provides sensitive quantitative information; however, it has inherent disadvantages including the destruction of conjugates and the forma-

tion of artifacts during hydrolysis (5). Furthermore, both enzymatic and GLC methods are extremely time consuming.

Modern, high-performance liquid chromatography offers the possibility for rapid separation and precise quantification of bile acid conjugates. Detection by nondestructive means also permits recovery of the injected sample for further analysis. This report discusses a direct injection method for the separation and quantification of bile acid conjugates in bile and duodenal fluid, with complete resolution of the conjugated dihydroxy isomers of chenodeoxycholic acid and deoxycholic acid.

Experimental

Instrumentation. HPLC analysis was performed with a Waters Associates (Milford, MA) model M-6000 reciprocating pump and a model U6K septumless, loop injector. A Microbondapak C₁₈ reverse-phase column (Waters Associates) was used for all separations. Detection was achieved using a Waters Associates R401 differential refractometer or a Varian Associates (Palo Alto, CA) refractive index detector unit, part no. 00-430-00. Detector response was recorded with a Hewlett Packard (Lexington, MA) model 3380 integrator or a strip chart recorder. Areas were calculated by integration or by triangulation. The column and detector were maintained at ambient temperature with good stability.

Preparation of standards and samples. Conjugated bile acid standards were purchased as sodium salts from Calbiochem, (San Diego, CA) or Steraloids (Wilton, NH) and were found to be 98% pure in two thin-layer systems: *a*) chloroform-methanol-acetic acid-water 65:24:15:9 (v/v) (6) and *b*) butanol-0.01 M Tris-propionic acid 100:18.5:15 (7). [1-¹⁴C]Glycine-labeled glycocholic acid was purchased as the sodium salt from Amersham (U.K.) and used as radiolabeled internal standard; radiochemical purity was 97%. Gallbladder bile samples were prepared for analysis by dilution of 1.00 ml of bile with 1.86 ml of methanol, to yield methanol bile dilutions of 65:35 (v/v). For more dilute duodenal fluid, 1.00-ml samples were evaporated to dryness under nitrogen and redissolved in 0.5 ml of mobile phase. All samples were finally vortex-mixed, centrifuged at 1000 *g* for 5 min, and then injected onto the column; standards were all dissolved in methanol-water 65:35 (v/v) before injection.

Mobile phase. The mobile phase was prepared by diluting 350 ml of doubly distilled water with 650 ml of analytical grade methanol (Mallinckrodt #3024). Exactly 32.7 ml (0.6 mol) of analytical grade glacial acetic acid (Mallinckrodt #2504) was then added and the solution was titrated with 10 N sodium hydroxide

Abbreviations: TLC, thin-layer chromatography; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; LCA, lithocholic, 3 α -hydroxy-5 β -cholic acid; CDA, chenodeoxycholic, 3 α ,7 α -dihydroxy-5 β -cholic acid; DCA, deoxycholic, 3 α ,12 α -dihydroxy-5 β -cholic acid; UDA, ursodeoxycholic, 3 α ,7 β -dihydroxy-5 β -cholic acid; CA, cholic, 3 α ,7 α ,12 α -trihydroxy-5 β -cholic acid. The prefixes glyco (G) and tauro (T) are used for bile acids having glycine or taurine in amide linkage at C-24.

¹ Requests for reprints should be addressed to John B. Watkins, M.D., Children's Hospital Medical Center, Department of Medicine, Division of Gastroenterology, 300 Longwood Ave., Boston, MA 02115.

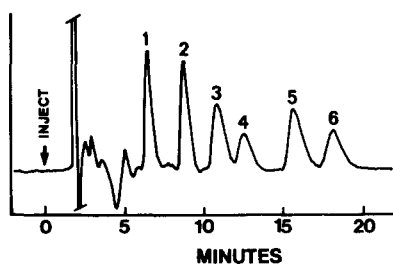


Fig. 1. Chromatogram of human gallbladder bile. Sample was prepared as stated in the experimental section; injection volume 30 μ l. 1, Taurocholic acid; 2, glycocholic acid; 3, taurochenodeoxycholic acid; 4, taurodeoxycholic acid; 5, glycochenodeoxycholic acid; 6, glycodeoxycholic acid. Flow rate, 2.0 ml/min; isobaric pressure, 2200 psi.

(approximately 7.0 ml) to an apparent pH of 4.7 with a glass electrode (Corning #476050, Medfield, MA). The mobile phase was filtered and degassed through a 0.45 μ m filter, type HA, Cat. #WPO4700 (Millipore Corp., Bedford, MA).

Results

The chromatographic separation of conjugated bile acids from a sample of human bile is shown in **Fig. 1**. There was complete resolution of the four conjugated dihydroxy bile acids, taurochenodeoxycholic acid, taurodeoxycholic acid, glycochenodeoxycholic acid, and glycodeoxycholic acid. Separation of the glycine and taurine conjugates of each bile acid is governed by both the pH and the ionic strength of the mobile phase. Lowering the ionic strength, adding a counter-ion (8, 9) for ion-paired chromatography (tetrabutylammonium⁺), or raising the pH resulted in decreased selectivity between glycine and taurine conjugates.

The influence of the number and/or orientation of the hydroxyl function is illustrated in **Fig. 2**. The elution order of conjugated bile acids with α -hydroxyl groups follows a progression from most polar to least polar, with the trihydroxy conjugated bile acids preceding the dihydroxy and monohydroxy conjugated bile acids. The 3 α ,7 β -dihydroxy conjugated bile acid, tauroursodeoxycholic acid, is minimally retained in comparison to its 3 α ,7 α -epimer.

The elution data from Figs. 1 and 2 were quantified in the form of normalized retention volumes (column 1, **Table 1**) in order to define the distribution ratio (10) and the selectivity of the system. The selectivity between the glycine and taurine conjugates (column 2) appears to be independent of the type of core bile acid. Similarly, the selectivity associated with a difference in the number and/or orientation of hydroxy groups (column 3) appears to be independent of the amino acid moiety. To determine re-

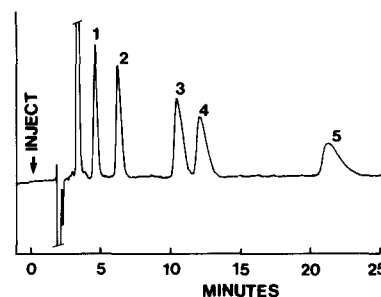


Fig. 2. Chromatogram of five taurine conjugated bile acids with differing numbers and/or orientations of hydroxyl groups. Bile acid concentrations, 3.3 mg/ml; injection volume, 30 μ l. 1, tauroursodeoxycholic acid; 2, taurocholic acid; 3, taurochenodeoxycholic acid; 4, taurodeoxycholic acid; 5, tauroolithocholic acid. Flow rate, 2.0 ml/min; isobaric pressure 2200 psi. Refractometer attenuation, 8.

covery and whether cross-contamination between eluted peaks occurred, [1-¹⁴C]glycine-labeled glycocholic acid was added to a sample of human duodenal bile, which was then prepared for analysis as described above. The linearity of the no. R401 refractive index detector response with varying quantities of five conjugated bile acids is demonstrated in **Fig. 3**. Although the slopes differed slightly in each case, the curves were linear over the range 0.05–0.40 μ mol (linear regression correlation coefficients ranged from 0.997 to 0.999). The lower limit of detection was 0.01 μ mol. In addition, though not shown in **Fig. 3**, linearity of response was maintained to 1 μ M, including that for glycocholic acid.

TABLE 1. Relative retention of conjugated bile acids

Normalized Retention Volume ^{a,b}	$\alpha \frac{\text{Glyco} -}{\text{Tauro} -}$ ^c	$\alpha \frac{\text{Tauro} -}{\text{Tauro} -}$ vs. $\alpha \frac{\text{Glyco} -}{\text{Glyco} -}$
TUDA - 1.95		
TCA - 3.21	$\frac{\text{CA}}{\text{CA}} - 1.52$	$1.95 - \frac{\text{CDA}}{\text{CA}} - 1.98$
GCA - 4.87		
TCDA - 6.26	$\frac{\text{CDA}}{\text{CDA}} - 1.54$	$2.31 - \frac{\text{DCA}}{\text{CA}} - 2.34$
TDCA - 7.40		
GCDA - 9.65	$\frac{\text{DCA}}{\text{DCA}} - 1.54$	$1.18 - \frac{\text{DCA}}{\text{CDA}} - 1.18$
GDCA - 11.38		
TLCA - 14.04		

^a The retention volume is normalized with respect to flow rate and clearance time by the equation $k' = (V_r - V_o)/V_o$, where k' is the normalized retention volume, V_r is the retention volume of the solute, and V_o is the retention volume of the unretained solvent front.

^b The data presented are derived from Figs. 1 and 2. Abbreviations for individual bile acids are as noted.

^c The selectivity factor, α , between two solutes is calculated according to the equation $\alpha = k_2/k_1$, where k_2 and k_1 are the normalized retention volumes of the more retained and less retained solutes, respectively.

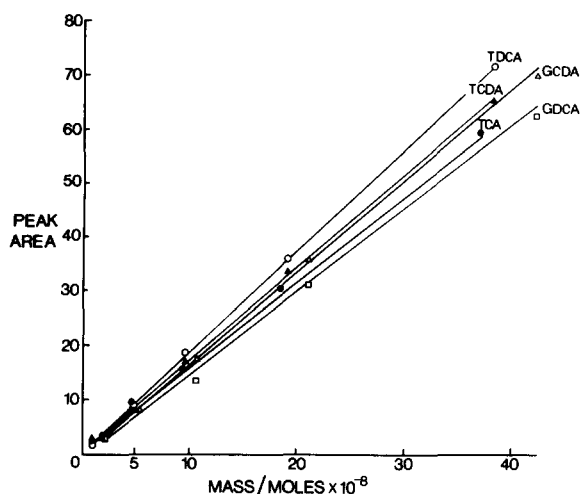


Fig. 3. Detector response vs. sample size of five conjugated bile acids. Correlation coefficients of linear regression ranged from 0.997 to 0.999.

Discussion

The conventionally used column and thin-layer systems for the separation of conjugated bile acids are complex, time-consuming, and generally fail to resolve the glycine and taurine conjugates of the dihydroxy bile acids. In the initial studies using HPLC, similar problems were encountered (11); however, it appeared that a reverse-phase system at a strongly acidic pH might provide the optimal chromatographic conditions for separation of bile acid conjugates (12–14). In our system, the selectivity between the taurine and glycine conjugates was optimized at an apparent pH of 4.7. This presumably is due to the differing degrees of dissociation of the glycine and taurine conjugates at this pH, and accounts for the loss of selectivity between the glycine and taurine conjugates at higher pH values (range 5.3–6.4) where the glycine conjugates approach complete dissociation. Peak symmetry and maintenance of retention volume independent of sample size were also dependent on the strong buffering capacity of the mobile phase, a characteristic of the chromatographic behavior of ionizable compounds (15).

The behavior of various conjugated bile acids in this system provides a sensitive probe for studying the retention mechanisms of ionic substances in reverse-phase chromatography. In particular, the importance of hydrophobic interactions (16, 17) may be illustrated by the diminished retention of tauroursodeoxycholic acid, the 7β epimer of taurochenodeoxycholic acid. The 7β -hydroxyl function would appear to hinder attachment to the stationary phase and thus promotes earlier elution. Additional study with conjugates containing equatorial hydroxyl groups is clearly

needed. Further information may be attained by characterizing elution behavior on columns with bonded ligands of differing chain lengths (18), function groups, and with different mobile phases.

At present, the sensitivity provided by the refractive index detector is less than that afforded by GLC (19) or fluorometric techniques (20); however, it is sufficient for quantification of conjugated bile acids in duodenal fluid and bile, and provides considerable advantages for quantitative separations and isolation during preparative procedures. The use of ultraviolet detectors equipped with the capacity to vary wave length may provide some increased sensitivity for the detection of bile acid conjugates. However, the maximum absorption for conjugated bile acids is in the range of 210 nm (12), where problems with excessive background absorption occur. Newer methods such as postcolumn derivatization (21) or a different means of detection could extend sensitivity to take greater advantage of the excellent chromatographic separation achieved by this method. □

The authors would like to thank Ann M. Tercyak for her interest and valuable contribution to this work, to Drs. Niels C. R. R ih  and H. William Taesch for their support, and to Ms. Sally Burke for secretarial assistance.

Manuscript received 1 August 1977; accepted 28 November 1977.

REFERENCES

- Carey, M. C., and D. M. Small. 1970. The characteristics of mixed micellar solutions with particular reference to bile. *Amer. J. Med.* **49**: 590–608.
- Brown, E. R., A. Eraklis, J. B. Watkins, and E. Goldstein. 1977. Bile acid sulfation mechanisms: Influence of conjugation. *Gastroenterology*. **72**: A-9/1032.
- Allan, R. N., J. L. Thistle, and A. F. Hofmann. 1976. Lithocholate metabolism during chemotherapy for gallstone dissolution: 2. Absorption and sulfation. *Gut*. **17**: 413–419.
- Eneroth, P., and J. Sj vall. 1971. Extraction, purification and chromatographic analysis of bile acids in biological materials. In *The Bile Acids: Chemistry, Physiology, and Metabolism*. Vol. 1: Chemistry. A. P. Nair and D. Kritchevsky, editors. Plenum Press, New York, N.Y. 121–171.
- Harris, W. S., L. Marai, J. J. Myher, and M. T. Ravi Subbiah. 1977. Bile acid ethyl esters: Their infrequent formation during routine bile acid analysis and identification by gas chromatography and mass spectrometry. *J. Chromatogr.* **131**: 437–441.
- Cass, O. W., A. E. Cowen, A. F. Hofmann, and S. B. Coffin. 1975. Thin-layer chromatographic separation of sulfated and nonsulfated lithocholic acids and their glycine and taurine conjugates. *J. Lipid Res.* **16**: 159–160.
- Palmer, R. H. 1971. Bile acid sulfates. II. Formation, metabolism, and excretion of lithocholic acid sulfates in the rat. *J. Lipid Res.* **12**: 680–687.

8. Fransson, B., and G. Schill. 1975. Isolation of acidic conjugates by ion pair extraction. I. Extraction of glucuronic, sulphuric and glycine conjugates. *Acta Pharm. Suec.* **12**: 107–118.
9. Hofmann, A. F. 1967. Efficient extraction of bile acid conjugates with tetraheptylammonium chloride, a liquid ion exchange. *J. Lipid Res.* **11**: 55–58.
10. Snyder, L. R., and J. J. Kirkland. 1974. Introduction to Modern Liquid Chromatography. John Wiley and Sons, Inc., New York, New York. 17–45.
11. Shaw, R., and W. H. Elliott. 1976. Bile acids: XLVIII. Separation of conjugated bile acids by high-pressure liquid chromatography. *Anal. Biochem.* **74**: 273–281.
12. Parris, N. A. 1977. Liquid chromatographic separation of bile acids. *J. Chromatogr.* **133**: 273–279.
13. Procházka, A. 1975. Steroids. In Liquid Column Chromatography Journal of Chromatography Library: vol. III. Z. Deyl, K. Macek, and J. Janak, editors. Elsevier Scientific Publishing Company, Amsterdam, Netherlands. 593–662.
14. Shaw, R., T. A. Smith, and W. H. Elliott. 1977. HPLC separation of nanomoles of conjugated bile acids. *Federation Proc.* **36**: 782.
15. Knox, J. H., and G. R. Laird. 1976. Soap chromatography—a new technique for separation of ionizable materials: dyestuff intermediates. *J. Chromatogr.* **122**: 17–34.
16. Karger, B. L., J. R. Gant, A. Hartkopf, and P. H. Weiner. 1976. Hydrophobic effects in reverse-phase liquid chromatography. *J. Chromatogr.* **128**: 65–78.
17. Horváth, C., W. Melander, and I. Molnár. 1977. Liquid chromatography of ionogenic substances with non-polar stationary phases. *Anal. Chem.* **49**: 142–154.
18. Hemetsberger, H., W. Maasfeld, H. Ricken. 1976. The effect of chain length of bonded organic phases in reversed phase high performance liquid chromatography. *Chromatographia.* **9**: 303–310.
19. Kuksis, A. 1976. Gas chromatography of bile acids. In Lipid Chromatographic Analysis. G. V. Marinetti, editor. Marcel Dekker, Inc., New York, New York. 479–610.
20. Schwarz, H. P., K. V. Bergman, and G. Paumgartner. 1974. A simple method for the estimation of bile acids in serum. *Clin. Chim. Acta.* **50**: 197–206.
21. Frei, R. W., L. Michel, and W. Santi. 1976. Post-column fluorescence derivatization of peptides: Problems and potential in high-performance liquid chromatography. *J. Chromatogr.* **126**: 665–677.