Studies on Bile Acids

THE MICROQUANTITATIVE SEPARATION OF CELLULAR BILE ACIDS BY GAS-LIQUID CHROMATOGRAPHY

By TATSUO OKISHIO, PADMANABHAN P. NAIR AND MAUREEN GORDON

*Biochemistry Research Division, Department of Medicine,
Sinai Hospital of Baltimore Inc., Baltimore, Md. 21 215, U.S.A.*

(Received 23 August 1966)

1. A method is described for the quantitative isolation of bile acids from cellular material. Homogenates of rat liver are freeze-dried and extracted exhaustively with 95% (v/v) ethanol containing 0.1% (v/v) of aq. ammonia (sp.gr. 0.88) and purified by anion-exchange chromatography on Amberlyst A-26. 2. The extracted bile acid conjugates are subjected to either of two hydrolytic procedures, one involving chemical and the other enzymic agents. A unique feature in this study is the introduction of an enzyme, a clostridial peptide-bond hydrolase, for the rapid cleavage of bile acid conjugates, replacing the classical drastic chemical hydrolysis with strong alkali. 3. After hydrolysis, free bile acids are methylated and converted into their trifluoroacetates for final determination by gas-liquid chromatography on a triple component column, FS-1265-SE 30-NGS. 4. For the purpose of identification of peaks, bile acid methyl esters are converted into their trimethylsilyl ethers by allowing the methyl esters to react with a new and potent silyl donor, bis(trimethylsilyl)acetamide. 5. The technique affords us a means of studying the metabolism of bile acids at the cellular and subcellular levels in tissues.

Cholic acid and chenodeoxycholic acid are the principal end products of cholesterol metabolism and constitute the primary bile acids in most mammals, giving rise in turn to the secondary bile acids, almost all of which retain the dominant 5 β -cholanolic acid structure (Haslewood, 1964). Our present knowledge on the biogenesis and enterohepatic metabolism of these substituted cholanolic acids has been limited to studies on bile, blood and intestinal contents (faeces) (Sjövall, 1964a) as the direct result of inadequate techniques for their isolation and determination from cellular material.

The present paper is an extension of previous work on gas-liquid chromatography of constituent bile acids from normal serum (Sandberg, Sjövall, Sjövall & Turner, 1965) and deals with the determination of bile acids from tissue homogenates and subcellular fractions. Advances in the methodology of the bile acids are now reported, including the enzymic cleavage of naturally occurring bile acids with a peptide-bond hydrolase derived from a strain of *Clostridium perfringens* (A.T.C.C. 19574), isolated by Nair, Gordon, Gordon, Reback & Mendeloff (1965). In contrast, drastic alkaline hydrolytic procedures are time-consuming and result in some degradative losses (Irwin, Johnston

& Kopala, 1944; Levin, Irwin & Johnston, 1961). Improvements have also been made in the final gas-liquid-chromatographic technique by using a highly reproducible triple component column (Okishio & Nair, 1966a). Bile acid methyl esters are converted into the corresponding trimethylsilyl ethers by allowing them to react with a new and potent silyl donor, bis(trimethylsilyl)acetamide. Our techniques have been applied to a study of major substituted cholanolic acids in subcellular fractions from rat liver (Okishio & Nair, 1966b). A preliminary report of this work has appeared (Okishio & Nair, 1965).

EXPERIMENTAL

Nomenclature of bile acids. Bile acids are referred to in this paper by the following trivial names: lithocholic acid, 3 α -hydroxy-5 β -cholanolic acid; hyodeoxycholic acid, 3 α ,6 α -dihydroxy-5 β -cholanolic acid; chenodeoxycholic acid, 3 α ,7 α -dihydroxy-5 β -cholanolic acid; deoxycholic acid, 3 α ,12 α -dihydroxy-5 β -cholanolic acid; cholic acid, 3 α ,7 α ,12 α -trihydroxy-5 β -cholanolic acid.

Materials. Standard free bile acids (cholic acid, deoxycholic acid, chenodeoxycholic acid, lithocholic acid and hyodeoxycholic acid) were obtained from commercial sources (Nutritional Biochemicals Corp., Cleveland, Ohio,

Scheme 1. Flow sheet indicating steps of isolation and quantitative separation of tissue bile acids.

U.S.A., and California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.). Taurocholic acid and glycocholic acid were prepared by the mixed anhydride method of Norman (1955).

Amberlyst A-26 (formerly XN-1006) anion-exchange resin (Rohm and Haas Co., Philadelphia, Pa., U.S.A.), neutral alumina, activity grade I (Bio-Rad Laboratories, Richmond, Calif., U.S.A.), and all solvents (Certified Reagents; Fisher Scientific Co., Pittsburgh, Pa., U.S.A.) were purified and stored as described by Sandberg *et al.* (1965).

Bis(trimethylsilyl)acetamide, a silyl donor, was prepared for us by Dr J. F. Klebe, Polymer Reactions Group, General Electric Co., Schenectady, N.Y., U.S.A.

An E.I.R. (Electronic Instruments for Research) analytical gas chromatograph (model AU-8) equipped with an argon ionization detector (20 mc of ^{90}Sr) was used. The following conditions were generally employed for gas-liquid chromatography: inlet temperature, 240°; column temperature, 210°; detector temperature, 230°; outlet flow rate, 75 ml./min. The electrometer gain was set at 1 m μ A to give a full-scale deflection on a 10 mv recorder at a detector voltage of 1000.

A triple-component column consisting of a mixture of FS-1265 (fluoroalkyl polysiloxane, QF-1; Dow Corning Corp., Midland, Mich., U.S.A.), NGS* and SE 30 (methyl polysiloxane; General Electric Co., Schenectady, N.Y., U.S.A.) phases was used for quantitative gas-liquid chromatography (Okishio & Nair, 1966a) of the methyl ester trifluoroacetates. The shifts in relative retention times observed on conversion of free bile acid methyl esters into the corresponding trimethylsilyl ethers aided in identification of the peaks.

Preparation of derivatives. The bile acids were methylated with diazomethane, prepared as described by Schlenk & Gellerman (1960). The extract containing bile acids was dissolved in 10.0 ml. of ether and 1.0 ml. of methanol. The solution was then allowed to react with excess of diazomethane (denoted by a persistent yellow colour) for 15 min., at the end of which the solvent was removed under partial vacuum.

Bile acid methyl esters were converted into their trifluoroacetates as described by Sandberg *et al.* (1965). Trimethylsilyl ethers were prepared by the addition of

* Abbreviation: NGS, neopentyl glycol succinate.

0.2–0.3 ml. of bis(trimethylsilyl)acetamide to bile acid methyl esters and the reaction mixture was maintained at 55° for 3 hr. The residue was dissolved in acetonitrile for gas-liquid chromatography.

Isolation procedure. Tissue homogenates of subcellular fractions were freeze-dried and then refluxed for 30 min. with 100 ml. of 95% (v/v) ethanol containing 0.1% (v/v) of aq. NH₃ (sp.gr. 0.88). The residue was re-extracted with the same volume of fresh solvent and filtered. The material left on the filter paper was washed with 50 ml. of fresh extractant. The extracts were pooled and evaporated to dryness under reduced pressure.

Bile acids in the residue were dissolved in 3.0–7.0 ml. of 0.1 N-NaOH (pH 11) and transferred to an Amberlyst A-26 anion-exchange resin column with the addition of 35 ml. of water. All subsequent steps in purification except enzymic hydrolysis and chromatography on alumina were performed as described by Sandberg *et al.* (1965) (Scheme 1).

Enzymic hydrolysis of bile acid conjugates. A strain of *Clostridium perfringens* that yielded an enzyme capable of cleaving the carbon-nitrogen bond of bile acid conjugates was isolated by Nair *et al.* (1965) from human urine, with trypticase-soya-blood agar (A.T.C.C. 19574). An alternative procedure for the cleavage of conjugated bile acids involved the use of a cell-free extract from this organism for the enzymic hydrolysis of the peptide bond. The enzyme was prepared as described by Nair *et al.* (1965). A sample of biological material (eluted from the Amberlyst column) containing approx. 100 µg. of total conjugated

bile acids was dried under reduced pressure and the residue redissolved in about 10 ml. of 0.01 M-phosphate buffer, pH 5.8. EDTA (disodium salt) (5 µmoles) was then added, followed by an excess of clostridial extract equivalent to 50 times the required amount. The mixture was incubated in a Dubnoff shaker for 5 min. at 37°, and diluted with 20 times its volume of water. Free bile acids released were recovered by extracting the solution three times with equal volumes of ether after acidification to pH 1 with 6 N-HCl.

Table 2. *Recovery of added free bile acids from rat-liver homogenate*

Free bile acid (40 µg.) was added to 20.0 ml. of 10% rat-liver homogenate and extracted as usual. A control was processed without added bile acids to determine the amount of endogenous bile acids.

Bile acid	Bile acid recovered (µg.)	Recovery (%)
Lithocholic acid	36.8	92.0
Deoxycholic acid	33.5	83.8
Chenodeoxycholic acid	32.9	82.3
Hyodeoxycholic acid	33.5	83.8
Cholic acid	32.7	81.8

Table 1. *Recovery of added glycocholic acid and taurocholic acid from rat-liver homogenates*

After extraction from a 10% homogenate, the conjugates were cleaved by the classical alkaline hydrolysis procedure.

Bile acid	Vol. of homogenate (ml.)	Bile acid added (µg.)	Bile acid* recovered (µg.)	Recovery (%)
Taurocholic acid	5.0	200	160	80.0
	10.0	500	395	79.0
	10.0	500	424	84.8
Glycocholic acid	10.0	200	157	78.5
	10.0	200	154	77.0

* Determined as free bile acids and calculated as conjugated bile acids. A control was processed in each case to determine the amount of endogenous bile acids.

Table 3. *Comparison of recoveries from chemical and enzymic hydrolysis of added bile acid conjugates*

Bile acids were added to a purified (Amberlyst-column) extract of bile acids from rat liver. Values are the means of several replicate analyses. A control was processed in each case to determine the amount of endogenous bile acids. The enzyme control did not contribute any peaks on gas-liquid chromatography.

Bile acid	Chemical hydrolysis				Enzymic hydrolysis			
	Bile acid added (µg.)	Cholic acid in standard (µg.)		Recovery (%)	Bile acid added (µg.)	Cholic acid in standard (µg.)		Recovery (%)
		Calc.	Found			Calc.	Found	
Glycocholic acid	200	175.6	140.8	80.2	100	87.8	78.7	89.6
Taurocholic acid	500	396.0	347.7	87.8	100	79.2	68.9	87.0

Alumina chromatography. Since we had not previously studied the isolation of lithocholic acid in detail, we re-examined this aspect and obtained quantitative recoveries by modifying the alumina chromatographic procedure. Bile acid methyl esters were transferred to the alumina column with about 10 ml. of benzene-hexane (1:9, v/v) and washed with about 40 ml. of the same solvent mixture. Lithocholic acid methyl ester was eluted with 70 ml. of benzene and all the other bile acid methyl esters with 40 ml. of methanol-acetone (1:9, v/v).

RESULTS

The isolation of both free and conjugated bile acids from tissue homogenates is represented in Scheme 1. Of several solvents examined, 95% (v/v) ethanol containing 0.1% (v/v) of aq. ammonia (sp.gr. 0.88) gave the best results. Since all other stages of purification and determination except enzymic hydrolysis of conjugates and alumina chromatography are essentially the same as those used in the gas-liquid chromatography of serum bile acids (Sandberg *et al.* 1965), we examined only the overall efficiency of the procedure.

The overall recovery of glycocholic acid and taurocholic acid added to a 10% homogenate of rat liver is presented in Table 1, where the conjugates were cleaved by alkaline hydrolysis. The recovery of unconjugated bile acids added to rat-liver homogenates shown in Table 2 consistently yielded over 80%. A comparison of the chemical and enzymic hydrolytic procedures was made to determine the relative merits of the two techniques (Table 3). The recovery of added glycocholic acid by enzymic cleavage was higher than that by chemical hydrolysis. There was no difference in the recovery of taurocholic acid by the two methods. Several homogenates of rat liver were extracted and the conjugates were subjected to both methods of cleavage. In each enzymic procedure, a control without any biological extract was maintained to exclude the possibility of interfering peaks contributed by the enzyme (Fig. 1). Within the range 0.2–1.0 μ g. a linear relationship exists between peak areas and amounts of trifluoroacetyl bile acid methyl esters injected into the column (Fig. 2).

In previous studies (Sandberg *et al.* 1965) a column with FS-1265 alone as the stationary phase was used. Since this column lacked reproducibility as well as consistency in response, we developed a highly reproducible triple-component column (FS-1265-SE30-NGS). Standard relative retention times for the trifluoroacetyl methyl esters for a series of 20 different substituted cholanic acids have been described (Okishio & Nair, 1966a). In the present study additional data are presented to characterize this column. Bile acid methyl esters were converted into their corresponding trimethylsilyl ethers before gas-liquid chromatography, and

Fig. 1. Gas-liquid chromatography of rat-liver bile acids in the form of methyl ester trifluoroacetates: comparison of enzymic and chemical hydrolytic procedures for conjugated bile acids. (a) Enzyme control with no extract of rat-liver homogenate; (b) enzymic cleavage; (c) alkaline hydrolysis in autoclave. 1, Deoxycholic acid; 2, hyodeoxycholic acid; 3, cholic acid. The conditions employed for gas-liquid chromatography were as described in the Experimental section.

retention times were determined relative to methyl deoxycholate (Table 4).

DISCUSSION

Of several types of biological material, bile, blood and faeces are the most widely studied for their bile acid composition (Sjövall, 1964a). In contrast, our knowledge of bile acids and their function at the cellular and subcellular levels has remained unexplored, largely because of the lack of suitable

methods of isolation and determination. The extraction of the substituted cholanic acids (bile acids) from cells (animal and bacterial) presents special problems since they are relatively more polar than other sterols and are often firmly bound to protein in the particulate material (Gustafsson & Norman, 1962).

To extract bile acids from faeces quantitatively, several solvent systems have been proposed, e.g. hot ethanol, chloroform-methanol (1:1, v/v) and methylal-methanol (4:1, v/v) (Sjövall, 1964a; Antonis & Bersohn, 1962). Our method for the extraction of bile acids from tissue homogenates is essentially an adaptation of the hot-ethanol system, except for the added ammonia, which assists in dissociation of protein-bound bile acids without cleavage of the conjugated forms. Preliminary purification on Amberlyst A-26 anion-exchange resin, as described by Sandberg *et al.* (1965), gave both free and conjugated bile acids.

The hydrolytic cleavage of bile acid conjugates presents a difficult problem in the analytical methodology of bile acids. Since alkaline hydrolysis usually results in some loss of bile acids, a new enzymic procedure has been developed. Cell-free extracts from *Clostridium perfringens* (A.T.C.C. 19574) rapidly cleave the naturally occurring conjugates of the bile acids under milder conditions than those employed for their chemical hydrolysis (Nair *et al.* 1965). Tissue extracts subjected to this hydrolytic procedure inhibit the enzyme (peptide-bond hydrolase) to a small extent, but this could be partially prevented by the addition of a small

Fig. 2. Linear relationship between amounts of trifluoro-acetylated methyl esters of bile acids and peak areas obtained from gas-liquid chromatography. ○, Lithocholic acid; ●, deoxycholic acid; □, chenodeoxycholic acid; ■, hydoxycholic acid; ×, cholic acid.

Table 4. Gas-liquid chromatography of trimethylsilyl ether derivatives of substituted methyl cholانات on FS-1265 and FS-1265-SE 30-NGS columns

Results are given for the compounds with free hydroxyl groups (OH) and for the trimethylsilyl ethers (TMSi). Values in parentheses were obtained at a column temperature of 220° (methyl deoxycholate retention time 23 min.). The outlet flow rate was 75 ml./min.

Stationary phase	Retention time relative to that of methyl deoxycholate			
	FS-1265*		FS-1265-SE 30-NGS	
	OH	TMSi	OH	TMSi
Substituted methyl cholانات				
3α-Hydroxy	0.50	0.30	0.34	0.20
3α,12α-Dihydroxy	1.00†	0.32, 0.60	1.00‡	0.22, 0.45
3α,7α-Dihydroxy	1.13	0.35, 0.80	1.10	0.22, 0.50
3α,7β-Dihydroxy	1.25	0.34, 0.62	1.23	0.28, 0.54
3α,6α-Dihydroxy	1.48	0.36	1.58	0.26
3α,6β-Dihydroxy	1.19	0.35	1.28	0.24, 0.56
3α,7α,12α-Trihydroxy	2.34	0.36, 0.60, 1.25	(3.31)	0.23, 0.47, 1.34
3α,6α,7α-Trihydroxy	2.63	0.45, 0.61	(3.86)	0.31, 0.47
3α,6α,7β-Trihydroxy	2.40	0.67	(3.34)	0.51

* 1.2-g. dissolved in 150 ml. of acetone for 15 g. of support.

† Methyl deoxycholate retention time 22 min. at column temperature of 210°.

‡ Methyl deoxycholate retention time 36 min. at column temperature of 210°.

amount of EDTA (disodium salt). Whereas enzymic hydrolysis gave a higher recovery for glycocholic acid (Table 3), no difference was noticed with taurocholic acid. This is probably attributable to the lower rate at which the latter is cleaved (Nair *et al.* 1965). The enzyme itself does not contribute any peaks to the final gas-liquid chromatography (Fig. 1) and has the added advantages of rapidity and lower temperature for hydrolysis.

The bile acids are usually converted into their methyl ester trifluoroacetates and chromatographed on two types of columns, one with FS-1265 as the only liquid phase and the other a mixture of phases consisting of FS-1265-SE 30-NGS. Identification of peaks is possible from standard relative retention data (Sjövall, 1964a; Okishio & Nair, 1966a). Under the specified reaction conditions, substituted cholic acids are known to provide the methyl esters and trifluoroacetates quantitatively. In contrast, not all of the trimethylsilyl ether derivatives of bile acid methyl esters are readily formed; the axial hydroxyl groups are less readily converted into the silyl ethers than the corresponding equatorial hydroxyl groups (Sjövall, 1964b; Briggs & Lipsky, 1965) (Table 4). The product obtained on conversion of all hydroxyl groups of a substituted cholic acid usually exhibits the shortest retention time compared with that of a partially converted one. No attempt has been made to identify the secondary peaks.

Another unique feature in this study is the introduction of a new silyl donor, bis(trimethylsilyl)acetamide, for the preparation of trimethylsilyl ether derivatives. This method has advantages over the classical method utilizing hexamethyldisilazane in that the reagent itself could serve as a solvent and that there are no precipitates, which require elimination. Bis(trimethylsilyl)acetamide readily transfers one of its silyl groups, forming the more stable monotrimethylsilylacetamide (J. F. Klebe, personal communication). This reagent has

been successfully used for the preparation of trimethylsilyl ether derivatives of vitamin D and the substituted tococls (Nair & deLeon, 1966; Nair, Sarlos & Machiz, 1966).

This work was supported by Grants AM-01417 and AM-02131 from the U.S. Public Health Service, National Institutes of Health, General Research Support Grant 5SO-1FR-05478-04 and Training Grant HE-5399 from the National Heart Institute. T.O. was a trainee of the National Heart Institute.

REFERENCES

- Antonis, A. & Bersohn, I. (1962). *Amer. J. clin. Nutr.* **11**, 142.
- Briggs, T. & Lipsky, S. R. (1965). *Biochim. biophys. Acta*, **97**, 579.
- Gustafsson, B. E. & Norman, A. (1962). *Proc. soc. exp. Biol., N.Y.*, **110**, 387.
- Haslewood, G. A. D. (1964). *Biol. Rev.* **39**, 540.
- Irwin, J. L., Johnston, C. G. & Kopala, J. (1944). *J. biol. Chem.* **153**, 439.
- Levin, S. J., Irwin, J. L. & Johnston, C. G. (1961). *Analyt. Chem.* **33**, 856.
- Nair, P. P. & deLeon, S. (1966). In *Progress in Biochemical Pharmacology*, vol. 2. Ed. by Paoletti, R. Basle: S. Karger A.-G. (in the Press).
- Nair, P. P., Gordon, M., Gordon, S., Reback, J. & Mendeloff, A. I. (1965). *Life Sci.*, **4**, 1887.
- Nair, P. P., Sarlos, I. & Machiz, J. (1966). *Arch. Biochem. Biophys.* **114**, 488.
- Norman, A. (1955). *Ark. Kemi*, **8**, 331.
- Okishio, T. & Nair, P. P. (1965). *Fed. Proc.* **24**, 564.
- Okishio, T. & Nair, P. P. (1966a). *Analyt. Biochem.* **15**, 360.
- Okishio, T. & Nair, P. P. (1966b). *Biochemistry*, **5**, 3662.
- Sandberg, D. H., Sjövall, J., Sjövall, K. & Turner, D. A. (1965). *J. Lipid Res.* **6**, 182.
- Schlenk, H. & Gellerman, J. L. (1960). *Analyt. Chem.* **30**, 1412.
- Sjövall, J. (1964a). *Meth. biochem. Anal.* **12**, 118.
- Sjövall, J. (1964b). In *New Biochemical Separations*, p. 65. Ed. by James, A. T. & Morris, L. J. London: D. Van Nostrand Co. Ltd.