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Analysis of Human Bile Lipids by Gas-Liquid Chromatography

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The lipids of human gall-bladder bile have been fractionated by silicic acid chromatography and the fatty acid composition of the different lipid classes determined with gas-liquid chromatography.

Small amounts of cholesterol ester was found to be present in human bile. Lecithin is the predominating phospholipid.

Palmitic, stearic, oleic and linoleic acid were the major fatty acids in the bile lecithin indicating the presence of several different lecithins in human bile.

An asymmetric or specific positioning of the long chain fatty acids in the bile lecithin is described.

Relatively little is known about the composition of the fatty acids of human bile [1,2]. The major reason for this has been the lack of adequate methods for fatty acid analysis especially on a microscale. From preliminary studies with gas-liquid chromatography (GLC) of the total fatty acids of human bile, it appeared that the fatty acids constituted a complex mixture of several components. The studies were extended with separation of the total bile lipids of human gall-bladder on silicic acid and determination of the fatty acids of the different lipids by GLC.

Furthermore the lecithin of human gall-bladder bile was purified and the fatty acid distribution determined after enzymatic degradation. The results indicate the presence of several different lecithins in the human bile.

METHODS

Bile was collected by aspiration into methanol from the gall-bladder of patients without hepatobiliary disease and also from patients with gall-stones and biliary tract disease (to be published [3]). The bile was extracted with chloroform: methanol 2:1 according to Folch-Lee [4] as modified by Borgström [5]. The total lipids of the bile was subjected to chromatography on silicic acid in order to obtain a neutral fat nonesterified fatty acid fraction and a phospholipid fraction. Then the nonesterified fatty acids were removed from the neutral fat by extraction with alkaline 50 % ethanol. The neutral fat fraction (glycerides, cholesterol and cholesterol esters) were subjected to chromatography on silicic

Table 1. Composition of human gall-bladder bile lipids after separation on silicic acid. The bile was collected from subjects without known biliary disease.

Subject	Total Fat mg/100 ml	Cholesterol mg/100 ml		Fatty acids as percentage of total fatty acids			
		Free	Total	Cholesterol ester FA	Non-esterified FA	Glyceride FA	Phospholipid FA
1	700	110	116	0.7	3.3	4.0	92.0
2	852	120	128	0.4	5.1	3.1	91.4
3	902	125	135	0.9	6.1	7.0	86.0

acid for separation of cholesterol esters and a triglyceride-cholesterol fraction [6]. The phospholipids were fractionated by the procedure described by Hanahan *et al.*[5]

The progress of the chromatographic separation was followed by phosphorus assay and paper chromatography. Lecithin constituted the predominating phospholipid but small amounts of unidentified phospholipids were also found. The lecithins were rechromatographed on aluminium oxide [8]. Enzymatic degradation was performed with phospholipase A *(Crotalus adamanteus* [9]). The hydrolysis products were fractionated on silicic acid in order to separate free fatty acids and unchanged lecithin and lysolecithin. The fatty acids of the lysolecithin were obtained after hydrolysis, acidification and extraction with light petroleum.

GLC was carried out essentially as described by James and Martin [10] using an Argon Pye Chromatograph with an ionization chamber as detector. As stationary phase a polar polyester (LHC–R–296) [11] was used in the ratio Celite: stationary phase 4:1. For identification the samples were also chromatographed at 240°C with silicone oil (Dow Corning High vacuum grease) as stationary phase in the ratio Celite: stationary phase 4:1. The experimental procedures used otherwise have been described earlier [12].

Fatty acids are numbered as suggested by Insull *et al.*[13] The category 20–22? refers to a group of unsaturated fatty acids whose structure has yet to be determined.

RESULTS

The determination of the different lipid components of human gallbladder bile are summarized in Table 1. The phospholipid fatty acids constitute the major components of the total fatty acids of human bile. Small amounts of triglyceride and cholesterol ester fatty acids are also present, as well as small amounts of non-esterified fatty acids. Similar results have been obtained by Nakayama and Johnston [14].

Results of determinations of several components of human biliary fatty acids are summarized in Table 2. Three characteristic fatty acid patterns are found in the phospholipids, triglycerides and cholesterol esters of the human bile. Palmitic, oleic and linoleic acids are the major constituents in all of these lipid classes. Arachidonic acid is also found in significant amounts in the triglycerides, non-esterified fatty acids and the phospholipid fatty acids.

Palmitic acid represents approximately 13 % of the total fatty acids of bile glycerides, 57 % of the cholesterol ester fatty acids and 28 % of the phospholipid total fatty acids. Stearic acid occurs to the extent of 1.8, 9.8 and 3.0 % of the triglyceride, cholesterol ester and phospholipid fatty acids, respectively.

Table 2. The fatty acid composition of the different lipid classes of human gall-bladder bile. The fatty acids were determined with gas-liquid chromatography and the values given are the percentage of the fatty acid methyl esters. The component fatty acids are also designated by a dual symbol giving chain length and number of double bonds. The sum of each column is less than 100 % because of trace amounts of unusual fatty acids detected were omitted from the final tabulation.

Fatty acid		Tri-glyceride FA %	Non-esterified FA %	Cholesterol FA %	Phospho-lipid FA %
Saturated					
Lauric	12:0				1.0
Myristic	14:0	0.9	0.5	0.9	3.2
Pentadecanoic	15:0	0.7	0.7	1.0	1.4
Palmitic	16:0	12.8	22.3	37.2	28.1
Heptadecanoic	17:0	0.4	0.4	0.7	0.7
Stearic	18:0	1.8	8.3	9.8	18.3
Mono-unsaturated					
Palmitoleic	16:1	5.8	7.4	6.1	3.1
Oleic	18:1	6.9	18.6	26.3	14.1
Di-unsaturated					
Linoleic	18:2	39.9	26.8	15.1	12.7
Poly-unsaturated					
Linolenic	18:3	2.3	1.0		2.4
Arachidonic	20·4	14.4	6.2		2.4
	20—22 ?	9.5	5.3	0.7	8.0

The results of this study indicate that palmitic acid occurred as a predominating fatty acid component in the lysolecithin formed from bile lecithin by phospholipase A (Table 3). As with palmitic acid stearic acid was also found predominantly in the lysolecithin formed. Small amounts of lauric, myristic, pentadecanoic, palmitoleic, oleic and linoleic acid were also found in the lysolecithin, together with some unidentified fatty acids with 13 carbon atoms. In the fatty acids released by phospholipase A from the bile lecithin the polyunsaturated fatty acid predominated together with oleic acid. Palmitic acid was also present together with trace amounts of myristic and stearic acid. Linoleic acid was the major polyunsaturated fatty acid but linolenic and arachidonic acid were also found in significant amounts. There were two C_{20-22} fatty acids occurring on the gas-chromatogram of this fraction of the lecithin. The exact nature of these fatty acids has not yet been determined.

From the results of this investigation it is evident that there are several lecithins present in the human bile. Recently new evidence has been provided on the position of saturated and unsaturated fatty acid esters in purified egg lecithin [19]. For this reason a brief discussion on the distribution of the fatty acids of the bile lecithin will be given.

It has been known for several years [15] that snake venom phospholipase A liberates mainly unsaturated fatty acids from lecithins containing saturated and unsaturated fatty acids. In recent years it has also been shown that

Table 3. The fatty acid composition of human gall-bladder bile lecithin. The lecithin was purified by silicic acid column chromatography and hydrolysed with phospholipase A. The fatty acids released by the action of phospholipase A and the fatty acids of the lycolecithin were determined by gas-liquid chromatography.

Fatty acid		Percentage of total fatty acids in respective positions of human bile lecithin	
		Fatty acids freed from bile lecithin by phospholipase A	Fatty acids obtained after hydrolysis of lysolecithin
Saturated			
Lauric	12:0		0.9
	13: ?		5.3
Myristic	14:0	0.6	5.3
Pentadecanoic	15:0	0.7	5.4
Palmitic	16:0	17.1	53.8
Heptadecanoic	17:0	0.7	
Stearic	18:0	1.5	6.5
Mono-unsaturated			
Palmitoleic	16:1	6.4	7.9
Oleic	18:1	25.9	6.6
Di-unsaturated			
Linoleic	18:2	32.1	1.7
Poly-unsaturated			
Linolenic	18:3	1.5	
Arachidonic	20:4	5.1	
	20—22 ?	6.0	

both di-saturated and di-unsaturated lecithins are equally well hydrolysed to yield one mole of fatty acid and the corresponding lysolecithin [8]. It was therefore concluded [16] that the action of the enzyme was directed toward one particular ester group C-1 (α) or C-2 (β) in the lecithin molecule. Hanahan [17] using permanganate oxidation procedures, concluded that the snake venom phospholipase A specifically hydrolyzed the ester linkage in the C-1 (α) position. Several investigators [9] have confirmed this finding, but conflicting results [18] have also been obtained, suggesting the lack of specificity of phospholipase A. Tattrie [19] has with a new approach investigated the position of the fatty acids on lecithin. Purified egg lecithin was enzymatically hydrolysed to the corresponding mixture of α-β-diglycerides with lecithinase D. Myristic acid was incorporated into the α position of the diglyceride and the resulting triglycerides were hydrolysed with pancreatic lipase, which specifically cleaves fatty acids from the α and α' positions. Only saturated fatty acids were freed by the pancreatic lipase, proving that the saturated fatty acids occupy the α position of egg lecithin. Since snake venom phospholipase removes only unsaturated fatty acid from egg lecithin the site of hydrolysis must be at the β position of the lecithin according to Tattrie [19]. The specificity of phospholipase A therefore seems well established, although further work is necessary to definitely settle the question about the site of attack of the enzyme.

From the analysis shown in Table 3 it is apparent that there is an asymmetric distribution of the fatty acids in the human bile lecithin. According to the discussion above the predominant ester pattern should be saturated fatty acids in the C-1 (α) ester position and unsaturated fatty acids in the C-2 (β) ester position. However, both C-1 and C-2 position contained saturated and unsaturated fatty acids.

COMMENTS

Bile contains lecithins and small amounts of soaps to which an accessory function in emulsification has been ascribed [20]. The complex picture of the fatty acid composition of the different lipid classes of the bile and especially the complex composition of the fatty acids of the lecithins makes further studies on the metabolism of the biliary fatty acids necessary. There are very little information available on the influence of the dietary fat on the fatty acid pattern of the bile. Furthermore it will be of interest to investigate how the fatty acid pattern of bile might vary in biliary disease.

Recently Borgström [5] has analysed the fatty acid composition of human bile lecithin with the aid of paper chromatography. He found only oleic acid released form the lecithin by phospholipase A and palmitic acid in the lysolecithin. The difference in results has to be found in the different material and methods used for the analyses.

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REFERENCES

1. Sobotka, H. *Physiological Chemistry of the Bile*, Williams and Wilkins, Baltimore; Bailiere, Tindal and Cox, London, 1937.
2. Isaksson, B. *Acta Soc. Med. Upsaliensis* **56** (1951) 177.
3. Blomstrand, R. and Ekdahl, P. *Proc. Soc. Exp. Biol. and Med.* **104** (1960) 205.
4. Folch, J. and Lee, J. *J. Biol. Chem.* **191** (1951) 833.
5. Borgström, B. *Acta Chem. Scand.* **11** (1957) 749.
6. Borgström, B. *Acta Physiol. Scand.* **25** (1952) 101.
7. Hanahan, D. J., Dittmer, J. C. and Warashina, E. *J. Biol. Chem.* **228** (1957) 685.
8. Hanahan, D. J., Rodbell, M. and Turner, L. D. *J. Biol. Chem.* **206** (1954) 431.
9. Long, C. and Penney, I. F. *Biochem. J.* **68** (1957) 382.
10. James, A. T. and Martin, A. J. P. *Biochem. J.* **63** (1956) 144.
11. Orr, C. and Callen, J. E. *J. Am. Chem. Soc.* **80** (1958) 249.
12. Blomstrand, R. and Dahlbäck, O. *Acta Soc. Med. Upsaliensis* **114** (1959) 177.
13. Insull, W. Jr., James, A. T. and Ahrens, E. H. Jr. *J. Clin. Invest.* **38** (1959) 892.
14. Nakayama, F. and Johnston, c.q. Personal communication.
15. Wittcoff, H. *The Phosphatides*, Reinhold Publishing Corp, New York 1951, p. 105.
16. Zeller, E. A. *Federation Proc.* **11** (1952) 316.
17. Hanahan, D. J. *J. Biol. Chem.* **207** (1954), 879.
18. Marinetti, G. V., Erbland, J. and Stotz, E. *J. Am. Chem. Soc.* **81** (1959) 861.
19. Tattrie, N. H. *J. Lipid Research* **1** (1959) 60.
20. Fürth, O. and Minibeck, H. *Biochem. Z.* **237** (1931) 139.

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