

CHARACTERIZATION OF FREE AND BOUND FATTY ACIDS IN HUMAN GALLSTONES BY CAPILLARY GAS LIQUID CHROMATOGRAPHY

*N.A. CHANNA, F.D. KHAND¹, M.A. NOORANI² and M.I. BHANGER²

Institute of Biochemistry, University of Sindh, Jamshoro, Pakistan

¹Department of Biochemistry, Isra University, Hyderabad, Pakistan

²Center of Excellence in Analytical Chemistry, University of Sindh, Jamshoro, Pakistan

(Received June 16, 2003 and accepted in revised form August 22, 2003)

Forty-four human gallstone samples either of pure cholesterol or cholesterol and bilirubin were randomly selected and analyzed by capillary gas liquid chromatography for the relative percentage composition of free and total fatty acids. The results showed that bound fatty acids were present in higher amounts than the free fatty acids. Amongst the bound fatty acids the percentage occurrence for palmitic acid was highest followed by stearic, oleic, linoleic and myristic acids. Fatty acids myristic, palmitic and linoleic were present in higher amounts in cholesterol gallstones, whereas stearic acid in cholesterol and bilirubin gallstones. When compared, no significant difference ($p < 0.05$) in the levels of free and bound fatty acids were seen in gallstones of males and females. The results suggest that bound fatty acids have a role to play in the structure of gallstones.

Keywords : Gallstones, Free fatty acids, Bound fatty acids, Capillary gas liquid chromatography.

1. Introduction

Lipids, particularly cholesterol and free fatty acids, are present, at different levels in human gallbladder bile and mucosa [1]. According to Mingrone et al. [2 - 4] increased amounts of mucin and free fatty acids are found in the bile of patients with gallstone disease.

Free fatty acids are reported to stimulate the hypersecretion of mucin, a well-known nucleating factor in bile [5 - 7]. Further to clarify the role, if any, of free and bound fatty acids in the structure and/or in the formation mechanism of gallstones, present study was planned to measure the amounts of free- and total-fatty acids in gallstone samples. For this purpose forty four gallstone samples were randomly selected and analyzed for fatty acid contents by capillary gas liquid chromatography.

2. Materials and Methods

2.1. Experimental

Forty four gallstone samples (36 pure cholesterol and 8 cholesterol and bilirubin) surgically recovered from as many patients (36

females and 8 males) were analyzed for the amounts of free and bound fatty acids by capillary gas chromatography. The gas chromatography was performed on a Perkin Elmer Model 8700. Capillary gas chromatography equipped with flame ionisation detector and fused silica capillary column DB-1, 2.5m \times 0.25mm and 0.2 μ m film thickness. Other conditions were as follows: Oven temperature 170°C, increased 7°C per minute, Final temperature 270°C and iso time 4 minutes. Nitrogen was used as a carrier gas with a flow rate 3.5/min and split ratio 1:18. The injector temperature was 260°C and detector temperature was 270°C. A 1 μ l sample of methyl ester was injected each time. The fatty acids were identified by comparison of their retention time with those of standard samples obtained from Fluka, Germany. All chromatograms were recorded on an EPSON LX-300 printer.

For each human gallstone sample lipid extract was prepared and then the methyl esters of free and total fatty acids were prepared.

* Corresponding author : nachanna2000@usa.com

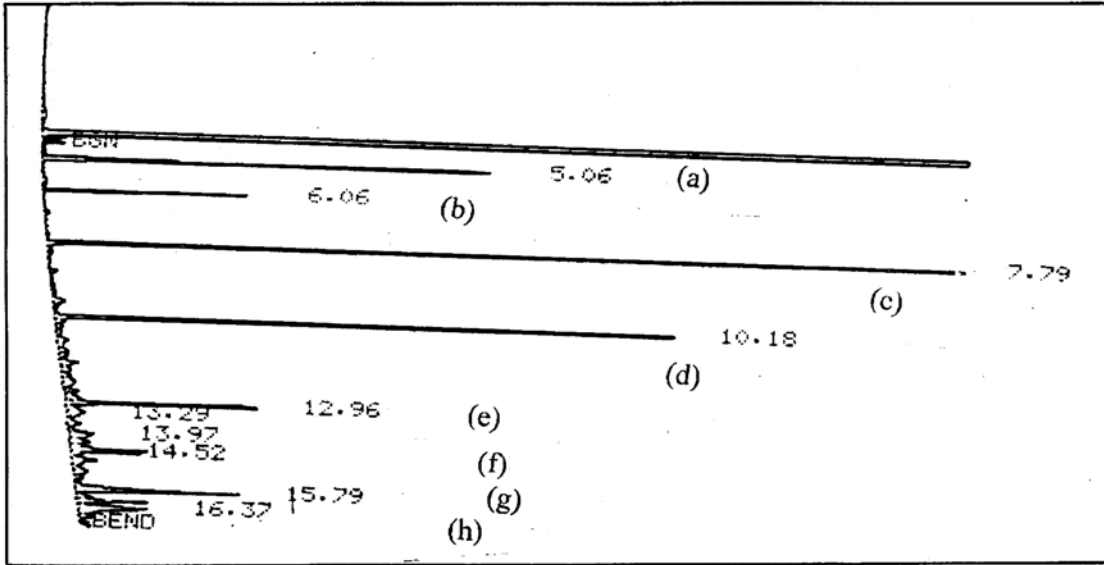


Figure 1. Capillary gas chromatogram of standard fatty acids; (a) C-8, (b) C-10, (c) C-12, (d) C - 14, (e) C - 16, (f), C -18:2, (g) C - 18: 1, (h) C - 18:0.

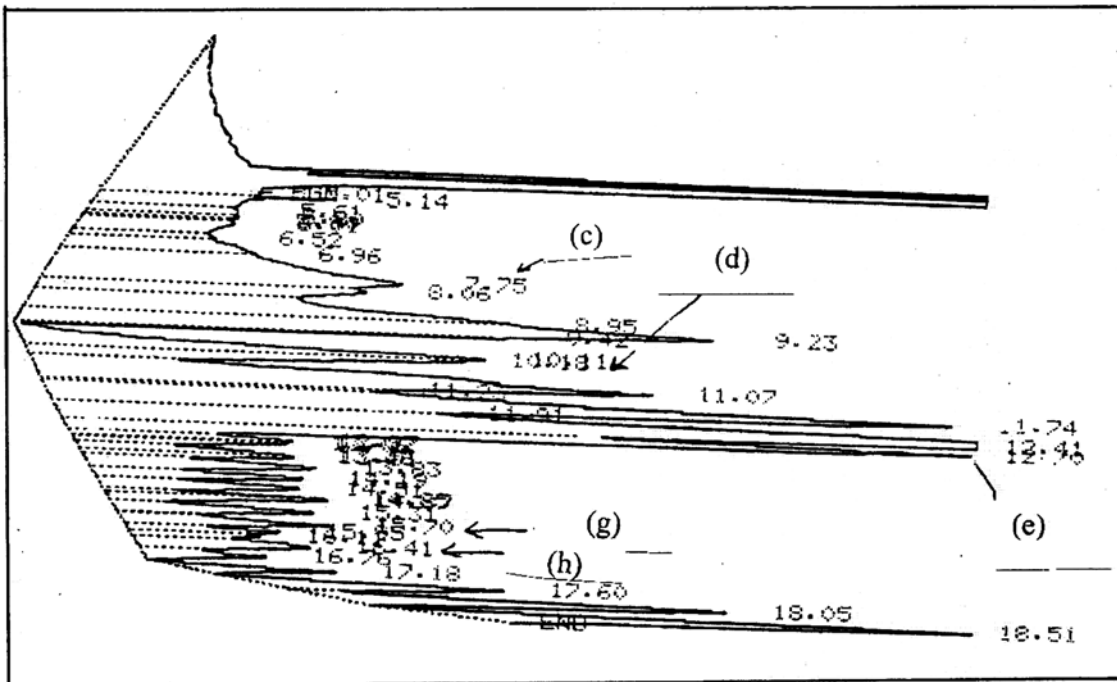


Figure 2. Capillary gas chromatogram of fatty acids in a human gallstone sample.

2.1.1. Preparation of lipid extract from gallstone sample:

Samples were prepared as reported by Folch et al. [8]. One gram of stone powder was dissolved in 20 ml of a mixture of chloroform-methanol, 2:1(v/v) and filtered. The filtrate after mixing with 200 ml 0.1N NaCl solution was shaken and left for few hours to separate into two layers. After discarding

the upper alcoholic phase by siphoning, the surface of the remainder was rinsed away by washing three times with small portions of a mixture of chloroform-methanol-water, 3:48:47 (by volume). The residual wash fluid and the lower phase were homogenized with methanol. After getting clear mixture the solvent was evaporated with N₂ gas at 50°C. Finally the mixture of chloroform-methanol, 50:50 (v/v) was added to

residual material to get clear lipid extract of human gallstone samples.

2.1.2. Sample preparation for the free fatty acids in gallstone samples

Samples for the free fatty acid measurements in gallstones were prepared with slight modification in the procedure as reported by Liebich et al. [9]. 3 ml lipid extract was concentrated by slow evaporation under nitrogen. This was then dissolved in 12 ml mixture of methanol-toluene, 4:1 (v/v). Added 1.2 ml acetyl chloride and the mixture after heating at 100°C for 60 min. on the heating stirring module, was neutralized by shaking with 30 ml 6% of potassium carbonate and centrifuged. The supernatants thus obtained were directly applied to capillary gas liquid chromatography for free fatty acid analysis.

2.1.3. Sample preparation for the total fatty acids in gallstone samples

Samples were prepared as described by Liebich et al. [9] with slight modification. 3 ml lipid extract concentrated by slow evaporation under nitrogen was dissolved in 30 ml methanol followed by the addition of 0.6 ml acetyl chloride and stirred at 25°C for 45 min. The contents after neutralization with 18 ml of 6% potassium carbonate were well mixed with 3 ml n-hexane, and centrifuged (1800 rpm for 10 min). The supernatants obtained were then analyzed by capillary gas liquid chromatography.

3. Results and Discussion

Figures 1 and 2 respectively represent the typical capillary gas chromatograms of standard fatty acids and of fatty acids in a human gallstone sample.

Percentage compositions of bound and free fatty acids are shown in Figures 3 and 4 respectively. Amongst the bound fatty acids the relative percentage composition for palmitic acid was highest followed by stearic, oleic, linoleic and myristic acids. Similarly, amongst the free fatty acids the relative percentage composition for oleic acid was the highest followed by palmitic, linoleic, stearic and myristic acids. The variations seen in the levels of both free and bound fatty acids in human gallstones might be owing to difference in the dietary habits and dietary content of fatty acids, as these have an effect on the fatty acid levels of bile and / or plasma [10 - 13]. The finding that the bound and free fatty acids palmitic and oleic acids respectively, occurred in highest amounts in human gallstones suggest that both the fatty acids have a role to play in the structure of and / or in the formation mechanism of gallstone.

Figure 5 shows comparison of bound and free fatty acids levels in human gallstones. The presence of higher amounts of bound than free fatty acids in human gallstones suggest that fatty acids especially palmitic acid is involved in the structure of gallstones.

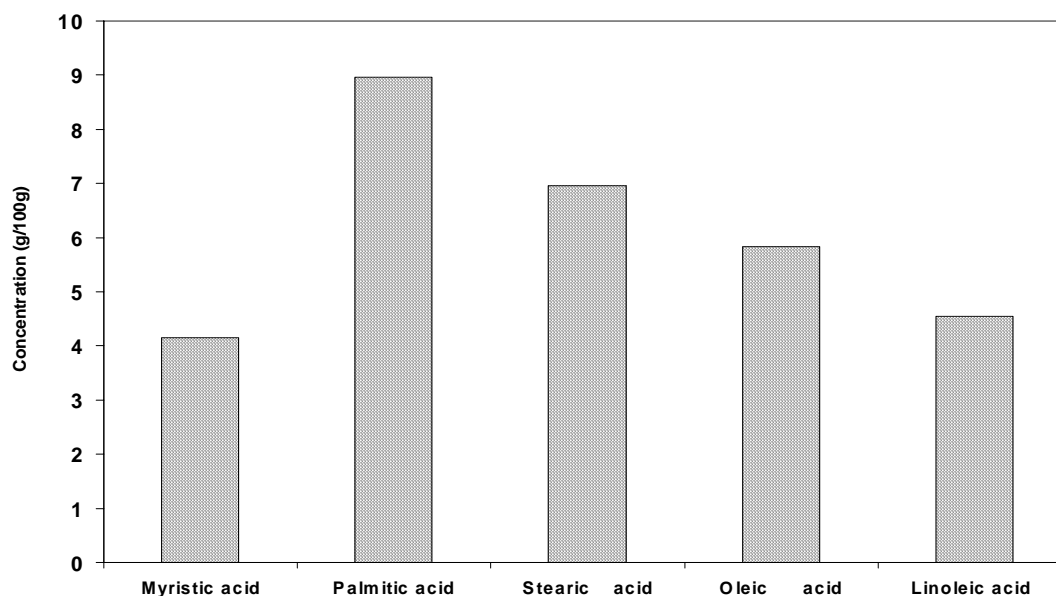


Figure 3. Percentage composition of bound fatty acids in human gallstone

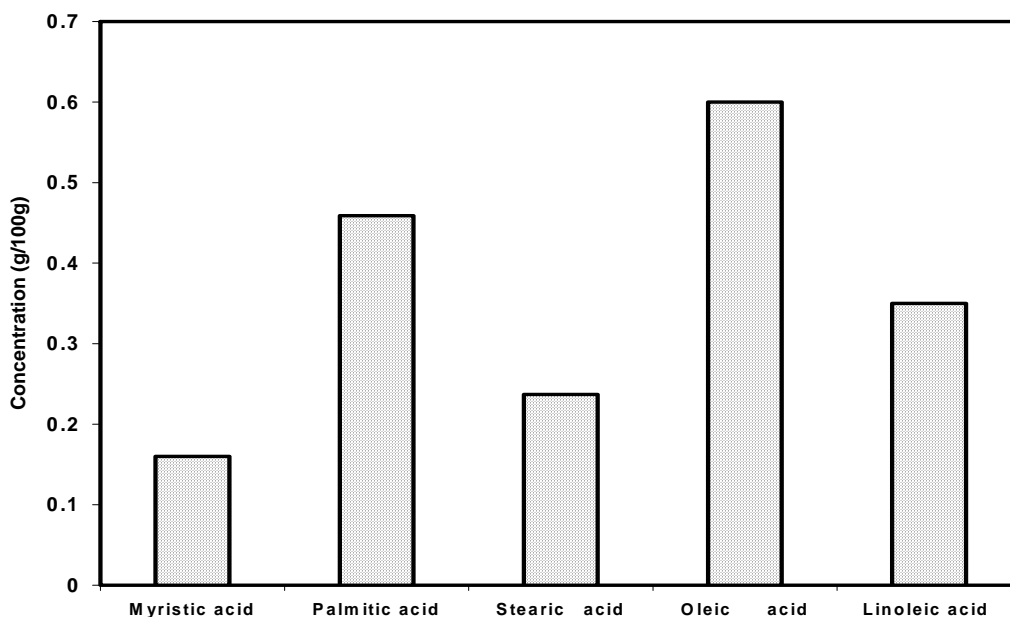


Figure 4. Percentage composition of free fatty acids in human gallstones.

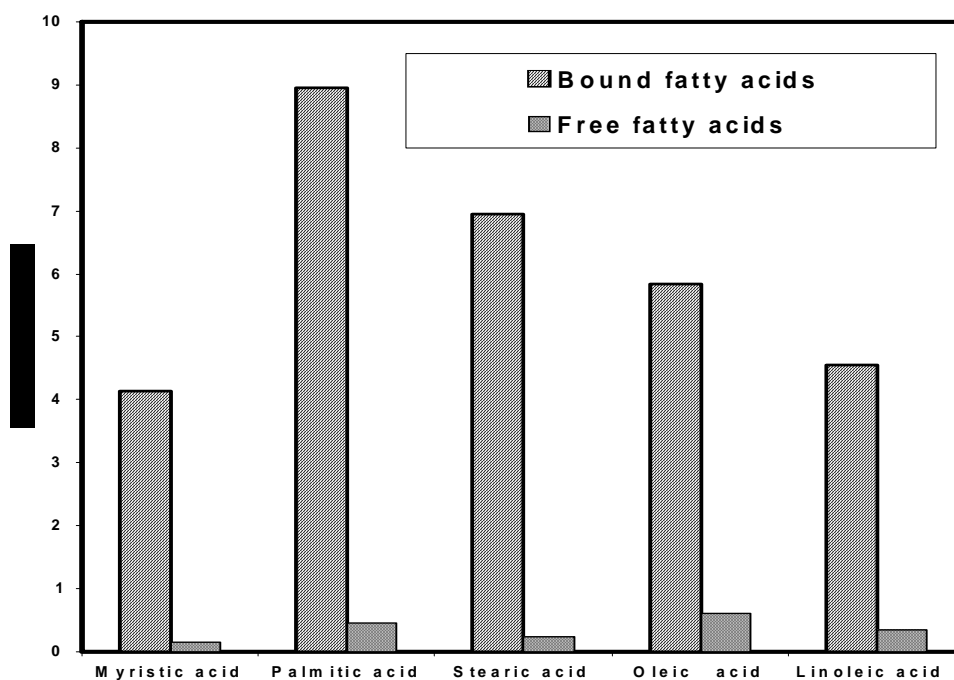


Figure 5. Comparison of free and bound fatty acids in human gallstones.

Table 1 shows that both free and bound forms of myristic, palmitic and linoleic acids were present in the highest amounts in cholesterol gallstones whereas stearic acid in cholesterol and bilirubin gallstones. This table further reveals that oleic acid in bound and free forms was more abundant in

pure cholesterol and cholesterol and bilirubin gallstones respectively. As palmitic acid is cholesterol raising fatty acid [14], its free and bound levels were higher in pure cholesterol than cholesterol and bilirubin gallstones. In contrast, levels for stearic acid were higher in cholesterol and bilirubin than in pure cholesterol stones.

Table 1. Statistical comparison of bound and free fatty acids in two types of gallstones.

Fatty Acids		Stone Type	p value
Myristic Acid	Free	Cholesterol > Cholesterol and Bilirubin	0.94
	Bound	Cholesterol > Cholesterol and Bilirubin	0.19
Palmitic Acid	Free	Cholesterol > Cholesterol and Bilirubin	0.13
	Bound	Cholesterol > Cholesterol and Bilirubin	0.75
Stearic Acid	Free	Cholesterol and Bilirubin > Cholesterol	0.24
	Bound	Cholesterol and Bilirubin >Cholesterol	0.54
Oleic Acid	Free	Cholesterol and Bilirubin >Cholesterol	0.58
	Bound	Cholesterol > Cholesterol and Bilirubin	0.51
Linoleic Acid	Free	Cholesterol > Cholesterol and Bilirubin	0.72
	Bound	Cholesterol > Cholesterol and Bilirubin	0.35

Table 2. Sex-wise comparison of fatty acids in human gallstones

Fatty Acids		Female (N = 36) Mean \pm SEM	Male (N = 8) Mean \pm SEM	p Value
Myristic Acid	Free	0.16 \pm 0.05	0.17 \pm 0.10	0.94
	Bound	4.53 \pm 1.10	3.30 \pm 2.00	0.62
Palmitic Acid	Free	0.47 \pm 0.15	0.18 \pm 0.10	0.13
	Bound	9.77 \pm 1.80	7.84 \pm 3.20	0.62
Stearic Acid	Free	0.32 \pm 0.10	0.14 \pm 0.10	0.24
	Bound	7.80 \pm 2.50	4.50 \pm 2.20	0.34
Oleic Acid	Free	0.65 \pm 0.34	0.04 \pm 0.26	0.51
	Bound	6.94 \pm 1.80	4.19 \pm 2.90	0.46
Linoleic Acid	Free	0.38 \pm 0.08	0.22 \pm 0.13	0.35
	Bound	5.08 \pm 0.97	4.08 \pm 2.40	0.72

Interestingly, oleic acid was the only fatty acid, which showed reverse trend in bound and in free forms. We offer no explanation at this stage as to why oleic acid in bound and free form occurred in highest amounts in pure cholesterol and in cholesterol and bilirubin gallstones respectively.

Comparison for both free and bound fatty acid levels found in pure cholesterol and cholesterol and bilirubin gallstones (Table 2) revealed no significant difference ($p > 0.05$) between the males and females. This indicates that gender has no effect on the amounts of free and bound fatty acids measured in human gallstones.

4. Conclusions

The results suggest that fatty acids namely palmitic, myristic and linoleic acids are involved in the structure of cholesterol gallstones, whereas stearic acid in cholesterol and bilirubin gallstones.

References

- [1] P. E. Ross, E. Kouroumlis, A. Clarke, D. Hopwood and I. A. D. Bouchier, *Clin. Chim. Acta.* **144** (1984)145.
- [2] G. Mingrone, A. V. Greco, S. Passi, *Biochim. Biophys. Acta.* **751** (1983) 138.
- [3] G. Mingrone, A. V. Greco, E. A. Mastromaci, *Clin. Sci.* **78** (1990) 175.

- [4] G. Mingrone, A. V. Greco, E. Finotti and S. Passi, *Biochim. Biophys. Acta.* **958** (1988) 52.
- [5] S. P. Lee, *J. Pathol.* **134** (1981) 199.
- [6] R. S. Pemsingh, B. R. MacPherson and G. W. Scott, *Hepatology.* **7** (1987) 1267.
- [7] M. L. Shiffman, B. F. Smith and J. T. LaMont, *Gastroenterology.* **87** (1993) 270.
- [8] J. Folch, M. Lees and G. H. Sloane Stanley, *J. Biol. Chem.* **266** (1957) 497.
- [9] H. M. Liebich, C. Wirth and B. Jakober, *J. Chromatogr.* **572** (1991) 1.
- [10] G. W. Melchior, H. B. Lofland and R. W. St Clair, *Metabolism.* **27** (1978) 1471.
- [11] E. A. Trautwein, A. K. Rau, J. Dietrich, S. Drusch and H. F. Erbersdobler, *Br. J. Nutr.* **77** (1977) 605.
- [12] S. S. Jonnalagadda, E. A. Trautwein and K. C. Hyes, *Lipids.* **30** (1995) 415.
- [13] E. A. Trautwein, *Z. Ernährungswiss.* **33** (1994) 2.
- [14] K. C. Hyes, *Can. J. Cardiol., Suppl G* **11** (1995) 39G.