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DETERMINATION OF RBC MEMBRANE BOUND LIPID IN ATORVASTATIN TREATED HYPERLIPIDAEMIA PATIENTS BY THIN-LAYER CHROMATOGRAPHY

Biochemistry

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ABSTRACT

Statin medication is essential to treat hypercholesterolemia as well as for primary and secondary prevention of cardiovascular disease. Thin-layer chromatography (TLC) is a technique that has been routinely used for the separation and identification of lipids like cholesterol, Triglycerides, free fatty acids, phospholipids and free cholesterol. In this study, 40 mg of atorvastatin treated hyperlipidaemia patients showed a significantly decreased lipids levels. But, atorvastatin treatment has some adverse events like myalgia, myopathy and rhabdomyolysis and these adverse effects can be ameliorated by co-administration of coenzyme Q_{10} (CoQ_{10}). The present study highlights the aspects of TLC-based lipid separations on RBC membrance.

KEYWORDS

Hypercholesterolemia, Coronary artery disease, Atherosclerosis, Obesity, Statin, coenzyme Q_{10}.

INTRODUCTION

Hypercholesterolemia is a primary risk factor for coronary artery disease, the major cause of premature death in developed countries. Lowering serum cholesterol levels has proved to be highly effective for cardiovascular risk reduction. Since cholesterol is synthesized by the mevalonate pathway. Statins or 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor is now the most prescribed class of drugs worldwide (1) (2). Statin therapy is regarded as well tolerated. Moreover, besides lowering the level of serum cholesterol, it has other positive effects such as those involved in improving the endothelial function, enhancing the stability of atherosclerotic plaques, decreasing oxidative stress and inflammation, and inhibiting the thrombogenic response (3).

Statins are the most efficient drugs for reducing plasma cholesterol level and generally well-tolerated (4). The most common adverse effects of statins are liver and muscle damage including elevated liver enzyme levels in serum, myopathy, myositis and rhabdomyolysis (5). As a result of a common biosynthesis pathway, both cholesterol and Coenzyme Q_{10} (CoQ_{10}) biosynthesis are decreased by statin treatment (6). CoQ_{10} (also known as Ubiquinone) is a water insoluble component of virtually all cell membranes and has multiple metabolic functions (7). Therefore, CoQ_{10} deficiency resulting from statin treatment may impair cellular energy metabolism and contribute to the development of myopathy and muscle symptoms, as described in patients treated with statins (8).

Thin-Layer Chromatography is more rapid and generally more sensitive than the other methods and has the added advantage of eliminating errors associated with recovery of the sample from chromatoplates (9). Thin layer chromotography (TLC) has been widely applied to the qualitative analysis of complex biologic lipid mixtures (10), as well as to the quantitation of selected biochemical entities.

MATERIALS AND METHODS

Reagents & Apparatus

Methanol, hexane, diethyl ether, glacial acetic acid, chromatography chamber, ITLC-SA (J.T. Baker®) (5×20&20×20 cm), developing chamber with cover, drying oven, heating blocks, centrifuge tubes, conical screw cap.

Study protocol

All participants in this study are of Indian origin. Apollo Hospitals Ethics committees approved the study protocols and all participants gave written informed consent. About 200 healthy normal subjects were taken as controls and 200 hyperlipidaemia subjects. From the hyperlipidaemia subjects, 100 who were 40 mgs atorvastatin treated hyperlipidaemia patients, 60 were atorvastatin treated hyperlipidaemic myalgia patients. Out of 100 atorvastatin treated hyperlipidaemic patients, 50 patients who were atorvastatin treated hyperlipidaemia without CoQ10 supplementation and 50 patients were atorvastatin treated hyperlipidaemia with CoQ10 supplementation. From atorvastatin treated hyperlipidaemic myalgia patients, 30 patients were treated without CoQ10 supplementation, 30 patients were treated with CoQ10 supplementation and are considered for this study groups.

Sample preparation

The 5ml blood samples were collected in purple colored EDTA tube, centrifuged at 1000*g* for 10 min and the supernatant (plasma) was discarded. Normal saline (2 mL) was added to the tubes, mixed and centrifuged at 5000*g* for 10 minutes followed by supernatant was decanted and this procedure was repeated twice. Tris HCl buffer (pH 7.4) 0.1mL was added (isotonic) and centrifuged for 5 min. This procedure was repeated thrice. The clear cells obtained were suspended in a hypotonic buffer and stored at 4 °C for 4 h. Then, cells were washed again with hypotonic buffer and then centrifuged at 10,000*g* for 30 minutes. This procedure was continued until the solution became colorless. Finally, isotonic buffer 2 drops were added to the solution and it was homogenized to get the red cell membrane. This was used for lipid extraction (11).

Extraction of lipids

The red cell membrane collected was again centrifuged for 10 min at 15,000*g*. The supernatant was decanted and the pellet obtained was suspended in 1 ml of methanol and homogenized. It was made up to 5 ml with methanol and centrifuged for 15 min at 15000 *g*. The supernatant was decanted, added 14 ml of chloroform to the tube and transferred in to a flat bottom flask. The content of this flask was evaporated in a fume hood. To the evaporated content, added 5 ml of chloroform- methanol (3; 1) mixture to dissolve the solid and the resulting solution is poured into a labelled centrifuge tube and covered with paraffin. These tubes are centrifuged at 6000 *g* and two differed layers of fluid were visible. The upper aqueous layer was aspirated and discarded while the lower layer was retained for lipid testing. Aliquots of this layer were stored at 4°C, used for the estimation of cholesterol, phospholipids and Triglycerides (12) (13).

Procedure

Lipids extract 500µl was dried in a glass centrifuge tube at 60°C under N2, dried lipid was dissolved in 10 µl chloroform (13). This volume was applied on ITLC-SA sheet, chloroform 1 ml was added and this aliquot was spotted. This procedure was repeated until approximately 25µl were applied. The applied samples were air dried, the chromatography solvent was prepared (enough to bring the solvent

level to a depth of 2 cm in the chamber). The sheet was placed in the covered chromatography chamber containing the solvent mixture (hexane: diethyl ether: glacial acetic acid, 100:8:2, v/v /v) and removed after exactly 20 minutes of migration. It was immediately dried in an 80 to 100°C oven to prevent diffusion of the spots. Each lipid fraction was seen within 5 min, after keeping the sheet in iodine chamber (Figure 1). The strip was removed from the chamber and the spots were quickly circled with a pencil. The iodine was allowed to volatilize at room temperature for 1 hr. Blank areas were also circled and carried through each assay. The circled areas were cut with scissors into strips approximately 4×6 mm. Esterified cholesterol, free cholesterol and phospholipid strips were placed in screw-cap test tubes to be used for each assay. The spots were handled with forceps to avoid contamination with skin lipids (14).

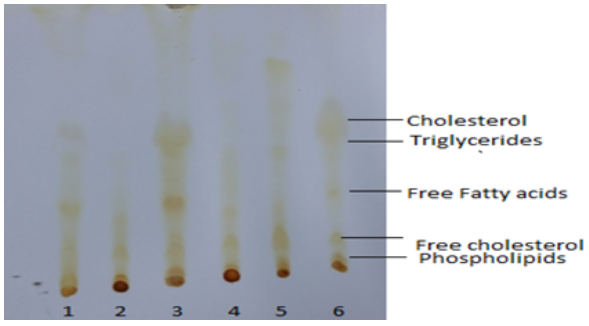

Figure1 Separation of lipid using thin layer chromatography (solvent: hexane: diethyl ether: glacial acetic acid, 100:8:2, v/v). 1.Control 2. Hyperlipidaemia subjects served as pretreatment group 3. Atorvastatin treated hyperlipidaemia patients without CoQ10 4. Atorvastatin treated hyperlipidaemia patients with CoQ10 5. Atorvastatin treated hyperlipidaemic myalgia patients' withoutCoQ10. 6. Atorvastatin treated hyperlipidaemic myalgia patients with CoQ10

RESULTS

Table 1 depicts the levels of lipid parameters among patients and control subjects. The mean value of total cholesterol, Triglycerides, free fatty acid and phospholipids of hyperlipidaemia patients were significantly high ($p<0.001$) when compared to control subjects.

Atorvastatin treated hyperlipidaemia patients without CoQ10 and atorvastatin treated hyperlipidaemic myalgia patients without CoQ10 showed a significant decrease in total cholesterol, triglycerides, free fatty acid and phospholipids when compared to hyperlipidaemia patients. Similarly, atorvastatin treated hyperlipidaemia patients with CoQ10 and atorvastatin treated hyperlipidaemic myalgia patients with CoQ10 showed a significant decrease in total cholesterol, TGs, free fatty acid and phospholipids, when compared to atorvastatin hyperlipidaemia patients without CoQ10 and atorvastatin treated hyperlipidaemic myalgia patients without CoQ10 (Table.1).

In this technique, Cholesterol is clearly separated from diglyceride and mono-glyceride from phospholipid, which remains at the origin; cholesterol ester and triglyceride migrate together near the solvent front, and the technique is sensitive, rapid and widespread analysis of the major lipid components. The separation achieved in solvent system is shown (Fig.1). It is routinely used in our laboratory for the determination of individual lipoprotein lipids and for in depth studies of disease processes where all the lipids are to be measured**.**

Table 1-Levels of Lipid profile status in control, hyperlipidaemia, atorvastatin and CoQ10 treated hyperlipidaemic myalgia patients.

Parameters	Control (n= 200)	Hyperlipidaemia patents (n = 200)	Atorvastatin treated hyperlipidaemia patients (n = 100)		Atorvastatin treated hyperlipidaemic myalgia patients(n = 60)		*p* Value
			Without CoQ10 (n = 50)	With CoQ10 (n = 50)	Without CoQ10 (n = 30)	WithCoQ10 (n = 30)	
Total Cholesterol	165.08 ±29.70	285.08 ± 41.83	175.87 ± 62.24	171.82 ±59.41	169 ± 30.21	164.28.±29.14	<0.001[abd] 0.004[c] 0.016[c]
Triglycerides	104.70 ± 55.56	289.27 ± 109.28	191.30 ± 107.88	178.23±98.15	162.40 ±90.80	154.25±86.41	<0.001[abd] 0.003[cc]
Free fatty acid	0.44±0.12	0.49 ±0.20	0.47 ±0.16	0.46±0.21	0.46±0.14	0.45±0.18	<0.001[a] 0.020[bd] 0.050[cc]
Phospholipids	29.2 ± 0.80	35.7 ± 1.69	32.5 ±3.06	31.3 ± 1.50	33.4 ± 1.31	31.7± 1.49	<0.001[a] 0.008[b] 0.005[c] 0.003[d] 0.001[e]

DISCUSSIONS

Statins have been used to treat various lipid disorders, such as hypercholesterolemia, hypertriglyceridemia, mixed dyslipidemia and homozygous familial hyperlipidemia. Statins inhibit an enzyme called HMG-CoA reductase, which controls cholesterol production in the liver. Additional enzymes in the liver cell sense that cholesterol production has decreased and respond by creating a protein that leads to an increase in the production of LDL receptors. These receptors relocate to the liver cell membranes and bind to passing LDL and VLDL (very low density lipoprotein). The LDL and VLDL then enter the liver and are digested (15).

In this study, biochemical parameters all found to be significantly higher when compared to control. The mean values of cholesterol (285.08 ± 41.83) were noticeably decreased in atorvastatin treated hyperlipidaemia patients (175.87±62.24, $p<0.001$). Several clinical trials have also shown mean value cholesterol (253.2±26.7) to be markedly decreased (190.25±20.00, $p<0.001$) in total cholesterol level (16). CoQ10 supplemented groups significantly reduction in cholesterol levels in atorvastatin treated hyperlipidaemia and atorvastatin treated hyperlipidaemic myalgia patients.

Atorvastatin treatment showed a marked decrease in triglycerides (191.30 ± 107.88, $p<0.001$) level when compared to untreated hypercholesterolemia patients (289.27 ± 109.28). Similarly, earlier studies have shown that triglycerides (280.5±44.5) were significantly decreased in statin treatment (160.7±26.70, $p<0.001$). CoQ10 supplementation has significantly reduced TG in atorvastatin treated hyperlipidaemia and atorvastatin treated hyperlipidaemic myalgia patients.

Plasma free fatty acids (FFAs) are the main precursors for hepatic triglyceride synthesis and secretion. Because atorvastatin reduced plasma FFAs, it may be that hepatic triglyceride synthesis and secretion are attenuated (17). Free fatty acids (0.47±0.16) were decreased by atorvastatin when compared to untreated hypercholesterolemia patients value of fatty acids (0.49±0.20). CoQ10 supplementation of atorvastatin treated hyperlipidaemia and atorvastatin treated hyperlipidaemic myalgia patients also have reduced FFA level.

The atorvastatin binds to phospholipids on the surface of lipoproteins (ie. Fluvastatin and lovastatin bind to LDL phospholipids) preventing the diffusion towards the lipoprotein core of free radicals generated during oxidative stress. The potent anti-oxidative potential of the metabolites (ie. Atorvastatin and fluvastatin metabolites) also results in lipoproteins protection from oxidation (18). In this study, atorvastatin treated hyperlipidaemia and atorvastatin treated hyperlipidaemic myalgia patients showed a significant decrease in phospholipids levels. CoQ10 supplementation of atorvastatin treated Hyperlipidaemia and atorvastatin treated hyperlipidaemic myalgia patients protects phospholipids and mitochondrial membrane proteins from peroxidation and protects DNA against the oxidative damage that accompanies lipid peroxidation.

CONCLUSION

The present study shows that the use of atorvastatin reversed the structural and functional features of erythrocyte membranes in hyperlipidaemia subjects. Also, hypolipidemic therapy had a beneficial impact on a balance between oxidant and antioxidant systems. Therefore, the extended use of statin medication demands awareness, recognition and proper evaluation and treatment of myalgia and myopathy by healthcare providers. Supplementations of CoQ10 significantly reduce its severity of myopathy in statin treated patients.

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