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## FTIR-ATR spectroscopy as a diagnostic tool in the diagnosis of hypercholesterolemia induced by cholesterol-rich diet in rats

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### ABSTRACT

Clinical Diagnosis is the process that identifies a possible disease or disorder that can be diagnosed through analysing specific components with multiple techniques and procedures that identifies the internal physiological changes. FTIR-ATR is a non-invasive, reagent-free diagnostic tool which can be rapidly and simultaneously analyze several components in the biological fluids and organs and which can be employed in analyzing the biomolecules. Hypercholesterolemia was induced in Wistar rats by feeding the animals with the regular diet supplemented with 1% cholesterol and 0.5 % cholic acid saturated with coconut oil for 6 weeks. End of induction study, experimental animals were sacrificed for biochemical and FTIR- ATR spectral analysis. Experimental serum samples were analyzed immediately for spectral recordings in the Mid IR region of 4000-450 cm<sup>-1</sup>. The intensity of the olefinic =CH bond (3012 cm<sup>-1</sup>) can be used as an index of relative concentration of double bonds in the lipid structure from unsaturated lipids. The C=O stretching at 1740 cm<sup>-1</sup> monitor the glycerol backbone near the aqueous part of the lipids. Results on biochemical variations obtained with both these methods were compared. Based on results obtained the study suggest that the FTIR-ATR technique might be an additional technique to know more details at molecular levels in the diagnostic sector. Further, this technique could be standardised for routine utility in disease diagnosis and management in the medicinal field.



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### INTRODUCTION

Cholesterol (from ancient Greek chole -means "bile" and sterol - means solid followed by the chemical suffix -ol for an alcohol, a sterol essential structure component to maintain structural integrity and fluidity. Cholesterol reduces the permeability of the plasma membrane to neutral solutes, hydrogen, sodium ions etc., (Yeagle 1991). Within the cell membrane, cholesterol also functions in intracellular transport, cell signalling and nerve conductions. The rise of cholesterol in the body give a condition in which excess cholesterol is deposited

in artery walls called atherosclerosis and the condition that block blood to the vital organs which result in blood pressure or stroke. Based on the content of protein and fat portions differentiated as HDL also called good cholesterol since it removes cholesterol from the cell. LDL otherwise called lousy LDL cholesterol that cause plaque build on the walls of arteries leads to the risk of heart diseases.

Hypercholesterolemia is the presence of a high level of cholesterol in blood. It is not a disease, but a metabolic dearrangement that can be secondary to many diseases and can contribute too many forms of the disease, most notably cardiovascular disease. According to the lipid hypothesis, abnormal cholesterol level or high LDL particle and lower concentrations of functional HDL particles are strongly associated with cardiovascular disease. Cardiovascular diseases are increasing day by day due to overutilization of fats or due to genetic reasons. It is a leading cause of morbidity and mortality from infancy to old age. Cardiovascular diseases have remained one of the leading causes of death all over the world. (Goff D C 2006). The development of these diseases has been linked to several factors such as high-calorie diet intake, lack of exercise, smoking, age, alcohol consumption and genetic disposition (Mitchell B D 2006). These factors ultimately result in disorders of lipid and lipoprotein metabolism including lipoprotein over production and deficiency (Syed M 2000). Causes of hyperlipidemia could be primary or secondary. Primary (genetic) causes are single or multiple gene mutations that result in either over production or defective clearance of triglycerides and low-density lipoprotein cholesterol, or under production and excessive clearance of high-density lipoprotein cholesterol (Schaefer E J 1985). Secondary type causes other diseases such as diabetes mellitus, chronic liver disease, hypothyroidism and primary biliary cirrhosis. Hyperlipidemia has also been associated with enhanced oxidative stress related to increased lipid peroxidation (Visavadiya N P 2007).

Till date numerous physiological biomarkers based on serum lipid, glucose and hormone have been identified that are associated with increased cardiovascular risks. These markers display cellular lipid interactions and physiological functions of serum lipid bearing proteins and assist in clinical decision making and authenticated risk type. There are so many established cardiovascular risk markers based on confirmed clinical outcomes related to biomolecules structure, and functions. Many of these biomarkers, alone or in combination, can be incorporated into risk prediction models to determine whether their addition increases the model's predictive ability. Moreover, various

cardiovascular risk prediction models have been updated by incorporating traditional risk factors like molecular, immunological genetic, imaging, and biophysical factors for more authentic and reliable estimation of cardiovascular risk.

Administration of cholesterol suspension produced an elevation in serum level of total cholesterol, triglycerides, LDL-C, VLDL-C and decrease in serum HDL-C level. Diet rich in cholesterol and saturated fatty acids increases the availability of acetyl-CoA, a precursor for cholesterol biosynthesis. This, in turn, increases the activity of HMG-CoA reductase, the rate-determining enzyme in cholesterol biosynthesis thus increasing the synthesis of cholesterol in the body (Hillgartner F B 1995). Cholesterol and fatty acids in the diet cause down-regulation of LDL receptors either by changing hepatic LDL receptor activity or LDL-C production rate or both. This results in the elevation of serum LDL-C level (Hillgartner F B 1995).

The cholesterol ester transfer protein (CETP), a key enzyme in reverse cholesterol transport and HDL-C metabolism increases in the high-fat diet. This increases the transfer of cholesterol esters from HDL-C to triglyceride-rich particles in exchange for triglycerides. This leads to the increased serum concentration of triglycerides and a decrease in the serum concentration of HDL-C (Rang H P 2007). Lecithin cholesterol acyl transferase (LCAT) is an enzyme involved in esterification of cholesterol, maturation of HDL-C and flux of cholesterol from cell membranes into HDL-C, thus enhancing clearance of cholesterol from the body in a process called "reverse cholesterol transport" (Shepherd J 1994). The activity of LCAT is decreased in diet-induced hypercholesterolemia (Lacko A G 1996).

## MATERIALS AND METHODS

### *Experimental Design*

Experimental protocols and procedures used in this study were approved by the animal house of Research and Development, Saveetha Medical College and Hospital, Thandalam Chennai, India. All experiments were carried out according to the guidelines for care and use of experimental animals, and are approved by the Committee for Control and Supervision of Experiments on Animals (CPCSEA). The Institutional Animal Ethical Committee approved the study proposal. 6 (both sexes weighing between 120 and 150 g) Wistar rats per cage were housed in polypropylene cages (32.5 × 21 × 14) cm lined with raw husk which was renewed every 48 h. The animal house was maintained at an average temperature (24.0°C ± 2°C) and 30-70 % RH, with 12 hr. light-dark cycle (lights on from 8.00 a.m. to 8.00 p.m.). Animals received

human care and were fed with commercial pellet diet and the animals were acclimatised for one week before the start of the experiment.

Animals were randomly divided into 2 groups of 6 rats each. Group 1 served as the normal control and received distilled water. Animals in group 2 (hypercholesterolemic Wistar rat) induced feeding with 1% cholesterol and 0.5% cholic acid saturated with coconut oil in addition to administration of standard diet for 6 weeks.

### SAMPLING AND ANALYSIS

At the end of the 6-week induction treatments, rats were fasted overnight and then sacrificed by cervical dislocation. Blood was collected from the heart into EDTA tubes. Serum was obtained by centrifugation at 3000 rpm for 15 min. The blood serum properly stored for estimation of biochemical parameters including Albumin, Glucose, LDL concentration by CLIA and Total serum T4, T3 and TSH concentration were determined by ELISA (detection kits provided by Transasia, Zemun, SCG) in a reputed clinical laboratory in Chennai.

### FTIR-ATR Spectral Analysis

The serum samples of experimental animals were adequately preserved in ice bags and immediately transported to the wet lab for spectral studies. The studies carried out at Sophisticated Analytical Instrumentation Facility (SAIF-SPU), St. Peter's University, Avadi, Chennai-600 054, using Perkin Elmer Two Spectrophotometer. Experimental serum samples were analysed immediately for spectral recordings in the Mid IR region of 4000-450  $\text{cm}^{-1}$ . The Total Internal Reflection (TIR) is the principle/phenomenon forms the basis for the FTIR-ATR spectroscopy (Katon J E 1996, Baulsir C F 1996). FTIR spectral measurements were carried at room temperature, and each measurement was repeated to ensure the reproducibility of the spectra. These spectra were subtracted against the background of air spectrum. After every scan, the crystal is cleaned with isopropyl alcohol or methanol soaked tissue and background of new reference air were taken to ensure the crystal cleanliness.

### STATISTICAL ANALYSIS

All statistical analysis was performed using the Statistical Package for Social Science (SPSS, version 17) for Microsoft Windows. The data were not normally distributed and therefore non-parametric tests were performed. Descriptive statistics were presented as numbers and percentages. The data were expressed as Mean and SD.

A one-way analysis of variance (ANOVA) was used. The Independent sample student "t" test was used to compare continuous variables between the two

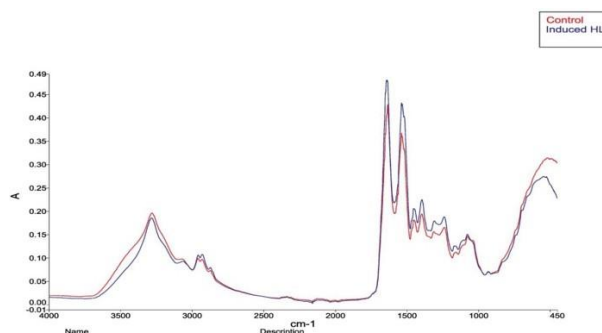
groups. A two-sided p-value < 0.05 was considered statistically significant.

## RESULTS

### Biochemical Evaluation of Blood serum in Wistar rat fed with high cholesterol to establish Hypercholesterolemia

At the end of experiments, blood samples analysed for biochemical parameters. The hyperlipidemia markers assayed include like cholesterol, Triglycerides, HDL cholesterol followed by T3, T4, TSH, etc., The other biochemical parameters were also analysed for clinical correlations include creatinine, urea, uric acid, calcium, phosphorous, total protein, albumin etc., Table 1 shows Various blood chemical parameters in blood employed for evaluation. Feeding cholesterol-rich diet on rats with body weight of  $180 \pm 10.0$  g led to a rapid progression of hypercholesterolemia resulting might cause atherosclerosis.

A vibration band assignment is done with the idea of the group frequencies of the various analytes present in the sample. The spectral pattern for control rat and hyperlipidemia caused by cholesterol-rich diet given in Figure 1.



**Figure 1: FTIR-ATR spectral overlaid pattern of blood serum of healthy control and hyperlipidemia induced cholesterol-rich diet in experimental Wistar rat**

### Internal standard parameter ratio

These spectra were used in internal ratio Parameter calculation and analysis requires spectra with a change in sensitive peaks and no change in sensitive peaks for control and hypercholesterolemic experimental groups.

The internal ratio parameter of protein, lipid and glycogen of control and hypercholesterolemic experimental animals are given in Table 3.

Internal ratio parameter is calculated to fortify the results obtained from the FTIR intensity of absorptions. Internal ratio Parameter ignores the difference in the amount of sample analyzed, it nullifies the contradiction in the quantity of the sample ( $I_{2961} / I_{1742}$ ,  $I_{1634} / I_{934}$ ,  $I_{1538} / I_{1165}$  and  $I_{1453} / I_{532}$ ). The peak ratio is calculated for (Protein)<sub>sym</sub> & <sub>asym</sub>

**Table 1: Changes in biochemical composition levels in Blood serum of control and Wistar rat fed high-fat diet**

Biochemical Composition in blood serum	Control	High-Fat Diet	Statistics
Urea (mg/dl)	40±7.10	41±6.40	NS
Creatinine (mg/dl)	0.88±0.4	0.91±0.5	NS
Total Protein (gm/dl)	7.1±2.89	7.81±2.11	NS
Albumin(gm/dl)	4.3±1.11	4.4±1.06	NS
Globulin (gm/dl)	2.8±0.77	3.1±0.42	NS
Plasma Glucose mg /dl	112±5.42	139±3.18*	P<0.05
Uric acid (mg /dl)	4.8 ±1.11	5.2±1.03	P<0.05
Calcium(mg /dl)	7.9±2.43	8.6±1.67	P<0.05
Total Cholesterol(mg /dl)	167±31.10	269±8.72	P<0.001
Triglyceride (mg /dl)	120±30.89	194±5.03	P<0.001
HDL Cholesterol (mg /dl)	47±9.80	39±3.09	P<0.01
T3(ng/dl)	161±15.87	218±8.19	P<0.01
T4(µ/dl)	5.9±1.20	15.7±1.00	P<0.01
TSH (mIU/dl)	4.8±1.33	6.91±3.11	P<0.01

**Table 2: FTIR-ATR Vibrational band assignment of biomolecules of healthy control serum of Wistar rat**

S.No	Wave Number (cm <sup>-1</sup> )	Vibrational Band assignment
1	3283	N-H stretch due to protein and urea
2	3071	Amide B band due to an overtone of Amide I band and olefinic group C-H stretch Lipids of Unsaturated fatty acid
3	2961	C-O-C Asymmetric / Symmetric stretch vibrations of the methyl group of Protein and C-H Lipids ( Fatty acids and TGL)
4	2931	Asymmetric stretching vibrations of methylene group of protein and lipids
5	2879	Symmetric stretching vibrations of methylene group of protein and lipids
6	1742	C=O group of cholesterol ester (HDL)
7	1634	Aryl substituted C=C Amide I band mainly due to C=O, C=N and N-H stretching
8	1538	Amide II band due to NH vibrations stretching coupled with C-N stretching vibrations in protein.
9	1453	Asymmetric bending vibrations of lipids, proteins of CH <sub>3</sub> groups.
10	1395	Free Amino Acid and Fatty acids;
11	1313	Amide III erythrocyte
12	1240	Amide III and Asymmetric PO <sub>4</sub> stretching vibration mode of Nucleic acid
13	1165	Ring vibrational mode of C-O-H and C-O-C bonds (CO-O-C) asymmetric cholesterol ester, Phosphoric acid
14	1115	Stretching vibration of glycogen
15	1076	C-O characterisation stretching of glucose
16	1040	Primary alcohol C-O stretch glucose mucopolysaccharide
17	934	Ribose, phospholipids
18	532	Polysulfidic S-S stretch in cystic acid

Via- (Lipids -FA-TGL) and HDL cholesterol ester ( $I_{2961}/I_{1742}$ ), Amide I and Ribose phospholipid ( $I_{1634}/I_{934}$ ), Amide II and (Chol.estr)<sub>asym</sub>-PO<sub>4</sub> ( $I_{1538}/I_{1165}$ ) and (Lipoprotein)<sub>asym.vib.</sub> and (Cystic acid)s-s -str. ( $I_{1453}/I_{532}$ ). The p values calculated were 0.0016, 0.0001, 0.0001 and 0.0006 respectively are highly significant among control and high cholesterol fed Wistar rat. As a result, it is suggested that all these absorbances ratios considered

as a biomarker in evaluating the Hypercholesterolemia status.

## DISCUSSION

Hypercholesterolemia (level of cholesterol in blood) is induced in rats by feeding the animals with a healthy diet supplemented with 1% cholesterol and 0.5% cholic acid saturated with coconut

**Table 3: The changes in the FTIR-ATR band internal peak ratio calculation for various molecules in the serum of control and Induced hypercholesterolemia experimental rats**

Peak ratio	Wave Number (cm <sup>-1</sup> )	Absorbance		P Value
		Control	HLL	
(Protein) <sub>sym&amp;asym</sub> Vib-(Lipids -FA-TGL) /HDL choletsreol ester	I <sub>2961/1742</sub>	4.2393±0.166	5.3366±0.178	0.0016
Amide I / Ribose -phospholipid	I <sub>1634/934</sub>	5.5893±0.151	6.7853±0.172	0.0001
Amide II/(Chol.estr) <sub>asym</sub> -PO <sub>4</sub>	I <sub>1538/1165</sub>	2.9338±0.180	3.4134±0.182	0.0001
(Lipoprotein) <sub>asym</sub> -vib./ (Cystic acid) <sub>s-s</sub> -str.	I <sub>1453/532</sub>	0.5768±0.177	0.7510±0.132	0.0006

oil for 6 weeks. The association between hypercholesterolemia and atherosclerosis has been demonstrated in many studies and trials (Lichtman A H 1999; Gordon T 1997). In this study, hypercholesterolemia was induced in rats by feeding cholesterol suspension with oil orally for 30 days and achieved hypercholesterolemia but achieved hypercholesterolemia with above 200mg/dl cholesterol oral feeding for 10 days (Reeves P G 1993). Feeding cholesterol-rich diet on rats with body weight of 180 ± 10.0g led to a rapid progression of hyperlipidemia resulting might cause atherosclerosis.

Hyperlipidemia may be manifested by elevation of total cholesterol, low-density lipoprotein and triglycerides concentrations and a reduction in high-density lipoprotein concentration. A significant increase in Total Cholesterol Triglyceride and a decrease in HDL on rats developed hypercholesterolemia (Table 1) observed in this study support earlier study and studies explained that the direct effect of plasma lipids and the effect of atherosclerosis. (Ferdinandy P 1997, Roach P D 1993). Hyperlipidemia is a major risk factor in the pathogenesis of atherosclerosis, a physiologic disorder that affects the coronary, cerebral and peripheral arterial circulation. (Gordon T 1997, Reeves P G 1993). A significant moderate elevation in T3, T4 and TSH was observed in our present results do not clarify the exact cellular mechanisms by which cholesterol diet leads to elevated thyroid hormones. The moderate increase in serum T3, T4 and TSH level in Wistar rats and no substantial functional atherosclerosis develop due to cholesterol diet. There was no significant difference in the other biochemical parameters (creatinine, urea, uric acid, calcium, phosphorous, total protein, albumin etc.,) in the experimental animals.

The cholesterol-rich diet fed to experimental rat for four weeks shows there was an increase in blood cholesterol level (269±8.72) and decrease in the serum HDL level (39±3.09) in the cholesterol-rich fed hyperlipidemia compare to control group (167±31.10 - cholesterol and 47±9.80 - HDL). Besides, the triglyceride and LDL level in Wistar rat fed cholesterol-rich diet was found to be higher (194±5.03 and 165± 5.10) than the control healthy

rats (120±30.89 and 96±5.11) and values obtained were statistically highly significant (P<0.001). These results obtained were agreed with the study of and they reported that rats fed with standard cholesterol diet (coconut oil/cholesterol diet) to develop hypercholesterolemia with an increase in TGL, and HDL cholesterol. (Augustin K T 2001). Diet rich in cholesterol and saturated fatty acids increases the availability of acetyl Co A, a precursor for cholesterol biosynthesis. This, in turn, increases the activity of HMG-CoA reductase the rate determining enzyme in cholesterol biosynthesis thus increases the synthesis of cholesterol in the body. Further, the amount of cholesterol returning to liver is increased and thus plasma HDL-cholesterol raises (Dietschy J M 1993). This increases the transfer of cholesteryl esters from HDL-cholesterol to triglyceride-rich particles in exchange for triglycerides. This leads to the increased serum concentration of triglycerides and a decrease in the serum concentration of HDL-cholesterol.<sup>8</sup> A significant moderate elevation in T3, T4 and TSH were observed (P<0.01) in this study do not clarify the exact cellular mechanisms by which cholesterol diet leads to an elevated thyroid hormone (Dietschy J M 1993).

The prominent absorption peak 3283 cm<sup>-1</sup> is due to the N-H stretching mode (amide A) of proteins. The spectral region 3072 cm<sup>-1</sup> comprises of C-H and O-H stretch of lipids of unsaturated fatty acids and N-H stretching vibrations of the Amide B band due to an overtone of amide I band. The symmetric /asymmetric stretching vibrations of the methyl group of protein and C-H lipids (fatty acids and triglycerides) are found to be present around 2930-2875 cm<sup>-1</sup>. The absorption peaks at 1743 cm<sup>-1</sup>. Corresponds to the C=O group of cholesterol ester (HDL). The strong absorption band at 1634 cm<sup>-1</sup> corresponds to aryl substituted C=C amide I band mainly due to C=O, C=N and N-H stretch, whereas the vibration at 1538 cm<sup>-1</sup> is attributed as amide II band due to NH vibrations stretching coupled with C-N stretching vibrations in protein. The absorption peaks in the region (1400-1300) cm<sup>-1</sup> arise due to the C-H deformation of methyl and methylene group of the proteins, lipids. The asymmetric and symmetric P-O stretching vibrations

are found to be around  $1245\text{ cm}^{-1}$  and symmetric P-O stretching of nucleic acid vibrations and ring vibration mode of C-O-H and C-O-C bonds (CO-O-C) asymmetric cholesterol ester, Phosphoric acid is found to be around  $1245\text{ cm}^{-1}$  and  $1165\text{ cm}^{-1}$  respectively. The spectral region 1115-1040 predominantly occupied by C-O characterisation and stretching of glucose and glycogen. The ribose and Phospholipids and polysulfidic S-S stretch in cystic acid vibrations are found to be at  $934\text{ cm}^{-1}$  and  $517\text{ cm}^{-1}$  respectively (Table 2).

### Summary

FTIR-ATR is a non-invasive, reagent-free diagnostic tool which can be rapidly and simultaneously analyze several components in the biological fluids and organs and which can be employed in analyzing the biomolecules. In this study, hypercholesterolemia was induced in experimental animals by feeding the animals with the normal diet supplemented with 1% cholesterol and 0.5 % cholic acid saturated with coconut oil for 6 weeks. The FTIR-ATR spectroscopic techniques employed to evaluate biomarkers by studying the variations on biomolecule composition in blood serum and organs of control and experimental animals. Experimental serum samples were analyzed immediately for spectral recordings in the Mid IR region of  $4000\text{--}450\text{ cm}^{-1}$ . The intensity of the olefinic =CH bond ( $3012\text{ cm}^{-1}$ ) can be used as an index of relative concentration of double bonds in the lipid structure from unsaturated lipids.

The C=O stretching at  $1740\text{ cm}^{-1}$  monitor the glycerol backbone near the aqueous part of the lipids. Results on biochemical variations obtained with both these methods were compared. The cholesterol-rich diet in the experimental animal shows increased blood cholesterol, triglyceride level and decreased serum HDL level in hyperlipidemic rat group compared to the control group. The calculated internal peak ratio absorbance for (protein)<sub>sym & asym</sub> vib- (lipids-FA-TGL)/ HDL cholesterol ester, Amide I / ribose -phospholipid, amide II/ (cholesterol ester)<sub>asym</sub>-PO<sub>4</sub> and (Lipoprotein)<sub>asym</sub>.vib/ (cystic acid) s-s- -str. were increased more in the hyperlipidemic rat as an atherogenic index for evaluating pathological conditions.

By this research study, it has been evident that, the FTIR-ATR can be an alternate as well as additional tool in clinical analysis compared with other techniques for understanding the biological nature of the disease for prognosis and management. Further, this technique could be standardised for routine utility in disease diagnosis and management in the medicinal field.

### CONCLUSION

The cholesterol-rich diet in the experimental animal shows increased blood cholesterol, triglyceride level and decreased serum HDL level in hyperlipidemic rat group compared to the control group. The calculated internal peak ratio absorbance for (protein)<sub>sym & asym</sub> vib- (lipids-FA-TGL)/ HDL cholesterol ester, Amide I / ribose -phospholipid, amide II/ (cholesterol ester)<sub>asym</sub>-PO<sub>4</sub> and (Lipoprotein)<sub>asym</sub>.vib/ (cystic acid) s-s- -str. Were increased more in the hyperlipidemic rat as an atherogenic index for evaluating pathological conditions. The FTIR-ATR spectral analysis for Wistar rat fed with high cholesterol diet obtained was comparable with biochemical changes obtained and thereby spectral analysis supported the biochemical analysis using existing routine methods. The standardisation of the FTIR-ATR spectral analysis for diseases diagnosis is needed for further studies.

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**Conflict of Interest:** None

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