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Efficacy of an Ayurvedic Drug Thyronil over Thyroid Disorder using Blood Samples by FTIR-ATR technique

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Abstract : Fourier Transform Infrared - Attenuated Total Reflectance (FTIR-ATR) technique is a modern spectroscopic technique used for elemental analysis of biological samples. During the recent times the use of FTIR-ATR has received quite a lot of attention not only for understanding the biological nature of the disease, but also for diagnosing it. From a diagnostic and therapeutic point of view, it is fundamental to study the physical and chemical changes occurring in the tissues and cells due to diseases and disorders. In the present study, FTIR-ATR spectra of the pre and post treatment of the hypothyroid and hyperthyroid disorder patients along with the healthy subjects are represented to find the efficacy of the drug thyronil. The FTIR-ATR spectral analysis revealed the differences in some major metabolic components in blood viz., LDL, total cholesterol and triglycerides that clearly demarcated between control and thyroid disordered patients. Measurements were recorded on 150 blood samples belonging to 50 hypothyroid, 50 hyperthyroid and 50 healthy subjects. Spectral recordings were taken before treatment, 60 days after treatment, 90 days after treatment and compared with the healthy spectra. Hypothyroid patients are found with elevated TG levels associated with increased levels of LDL. Hypothyroid patients may also exhibit elevated levels of HDL mainly due to increased concentration of HDL particles. Hypothyroid patients spectra show a remarkable increase from the control persons in LDL, total cholesterol, triglycerides and the biomarkers decrease towards the healthy spectrum during the ayurvedic drug therapy whereas spectra of hyperthyroid patients show a remarkable decrease in values of LDL, total cholesterol and triglycerides and increase towards the healthy ones during the course of treatment. The efficacy of the ayurvedic drug is validated by calculating the values of the biomarkers that brings the difference in the values of the disorder and are calculated using the internal ratio parameters viz triglycerides/ glucose [$R_1 (I_{3060}/I_{1080})$], total cholesterol/ glucose [$R_2 (I_{2933}/I_{1080})$] and LDL/ glucose [$R_3 (I_{1460}/I_{1080})$]. These parameters could be used as a basis for deriving a spectral method for analyzing thyroid disordered blood samples. It is shown that Fourier Transform Infrared - Attenuated Total Reflectance spectroscopy (FTIR-ATR) could be a possible technique for the analysis of efficacy of the ayurvedic drug thyronil using blood in thyroid disorder patients.

Keywords : FTIR-ATR, hypothyroid, hyperthyroid, pregnancy, Ayurvedic drug thyronil, efficacy.

Introduction

The thyroid gland is one of the most important glands of the endocrine system and secretes mainly two hormones (triiodo-thyronine and thyroxine) that are required for many physio-logical processes such as homeostasis, normal growth and energy production [1]. The thyroid gland consists of a thin layer of epithelial cells (follicular cells) that produce the precursor of the thyroid hormones, thyroglobulin [2], which is stored in structures known as colloids until they are needed by the body. The gland can produce high amount of hormone (hyperthyroidism) [3] or low amount of hormone (hypothyroidism). Abnormalities in thyroid function can have an adverse effect on reproductive health and result in reduced rates of conception, increased miscarriage risk and adverse pregnancy [4] and neonatal outcome.

The thyroid disorder status has been assessed by measuring T_3 , T_4 and TSH in serum by ELISA method [5]. In addition to this, other biochemical components such as protein, lipids and glucose were also analyzed to correlate the thyroid disorder status. An approach to the ayurvedic treatment for thyroid is undertaken in the present study. The main benefit of the Ayurvedic treatment is that it has no side effects as it is prepared by using the essence of fruits, spices, vegetables and natural herbs. The drug thyronil, an Ayurvedic medicine is used in the treatment of thyroid disorder. The drug thyronil includes the alkaloid of *Commiphora mukul* (Kanchanar Guggulu) [6]. *Commiphora mukul* is responsible for treating thyroid disorder [7].

Current study focused on concept of economic and timely screening of the thyroid disorder condition adopting reliable method. FTIR-ATR spectroscopic technique is used as a diagnostic tool in detecting disease condition by analyzing the biomarker(s) of blood in thyroid disorder women patients. The spectral bands ($4000 - 450 \text{ cm}^{-1}$) obtained from blood was characterized for different biomarkers such as LDL, Total cholesterol and triglycerides to assess the diseases status which aid in complete screening to control and management the disease. The chemical bonding stretches reveals the nature of bio molecule levels in blood. The results obtained on control and experimental subjects were showed statistically more significant. The spectral study gives additional information about the molecular basis which help in the detection and severity of the disorder condition [8].

The main aim of this study is to find the efficacy of the ayurvedic drug “**thyronil**” over thyroid disorder using human blood by FTIR-ATR spectroscopic method. Healthy blood samples and those affected with abnormal values of protein, lipids and glucose due to thyroid disorder (hyperthyroid and hypothyroid) on various days of treatment (pre-treatment, 60 days, 90 days) are analyzed by employing FTIR-ATR spectroscopic technique. An attempt has been made to analyze the variation of light absorption characteristics of some specific absorption bands of protein, lipids and glucose in blood for healthy and thyroid disordered subjects [9].

Materials and Methods

Blood samples from 50 hyperthyroid and 50 hypothyroid women patients and 50 healthy persons (not affected by any disease or disorder and checked by the medical practitioner) of age group between 22 and 28 years were collected from a leading clinical laboratory in Chennai, India. The samples were collected from the hypothyroid and hyperthyroid patients before the treatment, 60 days after the treatment and 90 days after the treatment respectively. The ayurvedic drug thyronil was given to the thyroid disorder patients by the medical practitioner and care was taken that they took regular intake of the ayurvedic drug. The blood samples of the same patients were collected after 60 days of treatment and 90 days of treatment with the regular intake of the drug, thyronil. During a blood test, a small sample of blood is taken from the body. It is usually drawn from a vein in the arm using a needle and preserved in EDTA solution to prevent coagulation of blood until the sample reach the lab.

20 μL of the blood sample is pipetted using a micropipette and the blood sample was then subjected to FTIR-ATR spectral measurements. Blood samples were analyzed immediately for spectral recordings in the Mid IR region of $4000 - 450 \text{ cm}^{-1}$, in the absorption mode. As water is a good absorber of infrared radiation, it affects the actual spectral response of the test sample and dominate in the FTIR spectrum of blood sample and therefore it was placed on the IRE crystal and water content on the sample is removed by air drier. FTIR spectral measurements were carried out at room temperature. FTIR spectral measurements of blood samples

were carried out at Sophisticated Analytical Instrumentation Facility (SAIF), SPIHER, Avadi, Chennai – 600054, India, using Perkin Elmer Spectrum – Two FTIR spectrophotometer with attenuated total reflection accessory having highly reliable and single bounce diamond as its internal reflectance element (IRE) [10]. The FTIR-ATR spectroscopy is based on the phenomenon known as total internal reflection (TIR). This radiation strikes the interface between the IRE and the blood sample that has a lower refractive index than that of a diamond to enable total internal reflection. This internal reflectance creates an evanescent wave that extends beyond the surface of the crystal into the blood sample, held in good optical contact with the crystal. It can be easier to think of this evanescent wave as a bubble of infrared that sits on the surface of the crystal. This evanescent wave protrudes only a few microns of the order of 0.5 microns to 5 microns beyond the crystal surface and into the sample. The spectral readings were done at 16 scans of accumulation with resolution 4 per centimeter. Data were analyzed by using SPSS software package, a descriptive data that include mean, standard deviation, standard error, t-values and p-values were collected for pre and post treatment of each group and used for analysis.

FTIR-ATR Vibrational Analysis

The average FTIR-ATR absorption spectrum of human blood of healthy subjects is shown in Fig. 1. Spectral signatures identified with the idea of the vibrational frequencies of the bio-molecules present in the blood such as protein, lipids, nucleic acid, phospholipids, carbohydrates and glucose are given in Table 1. IR absorption spectra of blood provide information about these key biological components present in the blood tissue. In IR absorption spectra of blood, three major regions can be distinguished, i.e., lipids ($2800\text{--}3000\text{ cm}^{-1}$), protein ($1600\text{--}1700\text{ cm}^{-1}$, $1500\text{--}1560\text{ cm}^{-1}$) and nucleic acid ($1000\text{--}1250\text{ cm}^{-1}$) bands as well as additional bands typical of a specific group.

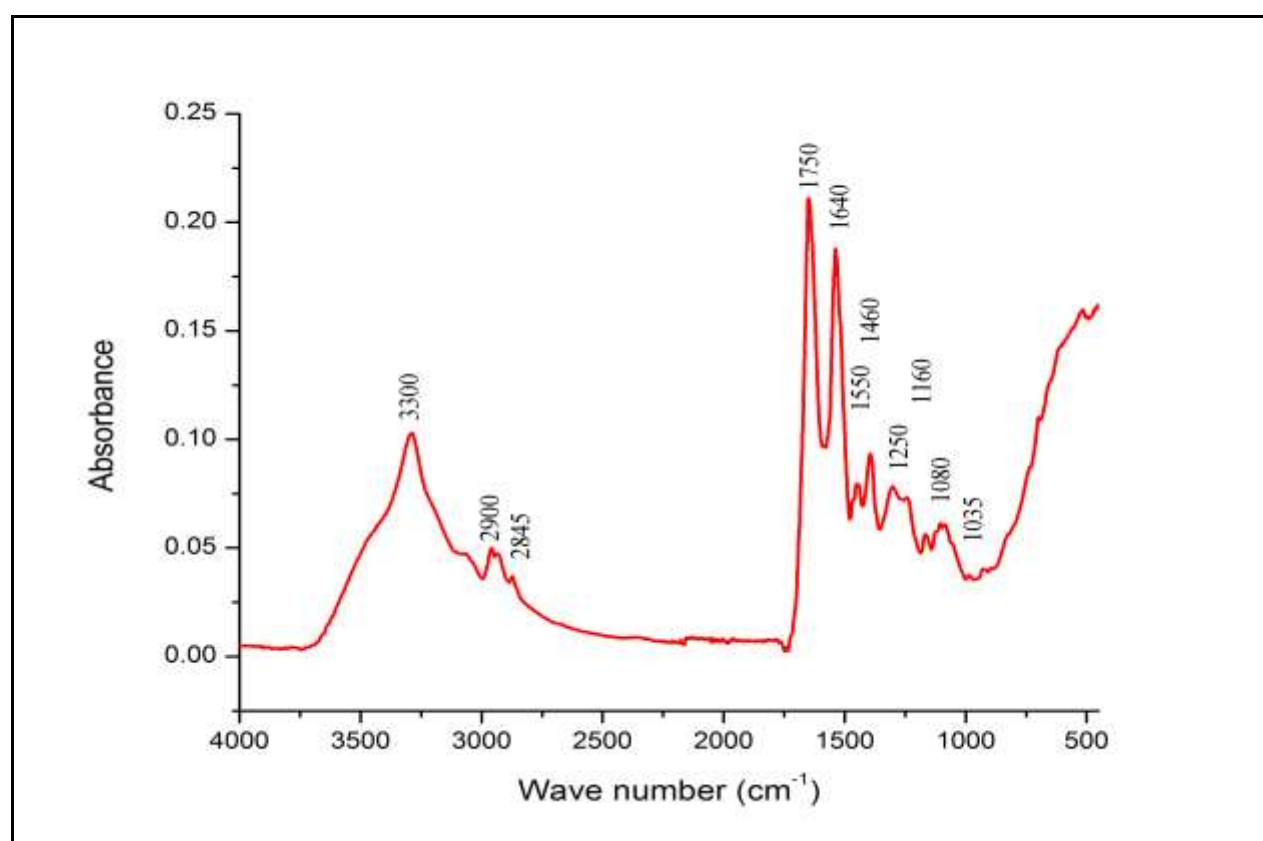


Fig. 1 Characteristic average FTIR-ATR spectrum of healthy human blood

Table 1 FTIR-ATR Spectral Vibrational Band Assignments of Human Blood

Vibrational Band (cm ⁻¹)	Component Identification	Vibrational Mode
3280	Amide A	Symmetric N-H Stretching
3060	Triglycerides (TGL)	Acyl CH ₃ stretching mode
2955	Methyl stretches of lipids in plasma	Asymmetric stretching of CH ₃
2933	Methyl stretches of lipids in plasma (Total Cholesterol, TC)	Symmetric stretching of CH ₃
2875	Methylene stretches of proteins and lipids	Asymmetric CH ₂ Stretching
2845	Methylene stretches of lipids in oral mucosa	Symmetric CH ₂ stretching
1735	High Density Lipoprotein (HDL)	C=O groups of cholesterol esters
1645	Amide I (α -helix)	C=O stretching
1550	Amide II	C=O stretching coupled with C-N stretching and bending deformation of N-H
1460	Methylene bending of lipids (LDL)	Bending vibration of CH ₂ groups
1440	Methyl bending of lipids (LDL) & proteins	Asymmetric bending of CH ₃
1375	Methyl bending of lipids & proteins	Symmetric CH ₃ bending
1250	Amide III and Lipid phosphates	C-N stretching and asymmetric PO ₂ stretching mode of nucleic acids
1180	Carbohydrates	C-OH groups of amino acids and C-O symmetric stretching of carbohydrate
1115	Glucose	Stretching of Glycogen
1080	Glucose	CO Symmetric stretching of glucose
1060	Phospholipids and cholesterol	COC asymmetric stretching vibration

The mid infrared spectra of blood can provide qualitative and quantitative analysis of protein, Low Density Lipids (LDL), Amide and Glucose. A band assignment is done with the idea of the group frequencies of the various bio-molecules present in the sample. The prominent absorption peak 3280 cm⁻¹ is due to the N-H stretching mode (Amide A) of protein. The acyl stretching mode of CH₃ at 3060 cm⁻¹ is due to the triglycerides (TGL). The methyl (CH₃) asymmetric and symmetric modes are observed at 2955 cm⁻¹ and 2933 cm⁻¹ is due to the lipids and total cholesterol (TC). The methylene (CH₂) asymmetric and symmetric modes at 2875 cm⁻¹ and 2850 cm⁻¹ are due to the lipids (11). In the IR spectra, very weak evidence of the protonated carboxyl group (COOH) exists, as reflected by the small band of the C=O stretching High Density Lipoprotein (HDL) at around 1735 cm⁻¹ (12). The broad and strong peak at 1640 cm⁻¹ is due to C=O stretching coupled with an in-plane bending of the N-H and C-N stretching modes (Amide I band) (13). While the amide II band centered at around 1550 cm⁻¹ is due to C=O stretching coupled with C-N stretching and bending deformation of N-H in the protein backbones (14). The bands are exemplified as medium, broad absorption at 1460 cm⁻¹ (LDL), while the band at 1375 cm⁻¹ is due to bending deformation of CH₃ vibration of amino acid (12). The absorption band at 1250 cm⁻¹ is due to contributions of amide III that occurs due to CN stretching and asymmetric stretching modes of nucleic acids and PO₂⁻ (15). The band observed at 1180 cm⁻¹ is due to the C-OH groups of amino acid and the C-O groups of carbohydrate (16). The spectral band at 1118 cm⁻¹ is due to the glycogen. The band around 1080 cm⁻¹ is due to the contribution of symmetric stretching of CO that corresponds to glucose. The band around 1060 cm⁻¹ is due to the COC asymmetric stretching that corresponds to phospholipids and cholesterol. The quality of the spectra has been fairly appreciated through the utilization of FTIR-ATR spectroscopy.

Results and Discussions

The mid infrared spectra of blood can provide qualitative and quantitative analysis of protein, Low Density Lipids (LDL), Amide and Glucose. Increased thyroid hormone levels stimulate fat mobilization, leading to increased concentrations of fatty acids in plasma. They also enhance oxidation of fatty acids in many tissues. Finally, plasma concentrations of cholesterol and triglycerides are inversely correlated with thyroid hormone levels - one diagnostic indication of hypothyroidism is increased blood cholesterol concentration [17]. Human cells, tissues and body fluids are generally composed of water, lipids, proteins, carbohydrates, glucose and nucleic acids. Thyroid hormones induce the 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase, which is the first step in cholesterol biosynthesis (18). Moreover, triiodothyronine (T_3) regulates LDL receptors by controlling the LDL receptor gene activation. This T_3 -mediated gene activation is done by the direct binding of T_3 to specific thyroid hormone responsive elements (TREs) (19). Furthermore, T_3 controls the sterol regulatory element-binding protein-2 (SREBP-2), which in turn regulates LDL receptor's gene expression (20). T_3 has also been associated with protecting LDL from oxidation (21). Thyroid hormones can influence HDL metabolism by increasing cholesteryl ester transfer protein (CETP) activity, which exchanges cholesteryl esters from HDL (high density lipoprotein) to the very low density lipoproteins (VLDL) and TGs (triglycerides) to the opposite direction (20). Beyond their effect on lipid profile, thyroid hormones can equally affect a number of other metabolic parameters related to cardiovascular disease risk. Indeed, thyroid function can influence adipocyte metabolism and the production of adipokines (22). Hyperthyroidism has been associated with increased levels of adiponectin, whereas hypothyroidism is not associated with significant changes in adiponectin (23). Although decreased thyroid function or hypothyroidism is accompanied by reduced activity of HMG-CoA reductase, TC (total cholesterol) and LDL (low density lipoprotein) levels are increased in patients with hypothyroidism (24). This is due to the decreased LDL-receptors' activity, resulting in decreased catabolism of LDL and HDL (25). Hypothyroid patients are found with elevated TG levels associated with increased levels of LDL. Hypothyroid patients may also exhibit elevated levels of HDL mainly due to increased concentration of HDL particles. Moreover, decreased activity of the CETP results in reduced transfer of cholesteryl esters from HDL to VLDL, thus increasing HDL levels (26). Hypothyroid patients have increased lipoprotein levels (27), which are associated with increased cardiovascular disease risk.

The incidence of hyperthyroidism is lower compared with hypothyroidism in the general population. Despite the increased activity of the HMG-CoA reductase, levels of TC, LDL tend to decrease in patients with hyperthyroidism. This is due to increased LDL receptor gene expression resulting in enhanced LDL receptor-mediated catabolism of LDL particles (28). A decrease in HDL level is also observed in hyperthyroidism, due to increased CETP-mediated transfer of cholesteryl esters from HDL to VLDL and increased catabolism of HDL (29). On the other hand, no changes in blood pressure (27), has been described in hyperthyroid patients. Therapy of clinical hyperthyroidism results in restoration of those alterations of lipid metabolism (28). Increased thyroid hormone levels stimulate fat mobilization, leading to increased concentrations of fatty acids in plasma. They also enhance oxidation of fatty acids in many tissues. Finally, plasma concentrations of cholesterol and triglycerides are inversely correlated with thyroid hormone levels - one diagnostic indication of hypothyroidism is increased blood cholesterol concentration (30).

Thyroid hormones stimulate almost all aspects of carbohydrate metabolism, including enhancement of insulin-dependent entry of glucose into cells and increased gluconeogenesis and glycogenolysis to generate free glucose (20). Insulin resistance is also correlated with thyroid function (31). TSH is positively associated with fasting and postprandial insulin concentration and negatively with insulin sensitivity (32) Hyperthyroidism is typically associated with decreasing blood glucose control and increased insulin requirements. The excessive thyroid hormone causes increased glucose production in the liver, rapid absorption of glucose through the intestines, and increased insulin resistance (a condition in which the body does not use insulin efficiently). It may be important to consider underlying thyroid disorder if a person has unexplained weight loss, deterioration in blood glucose control, or increased insulin requirements. Having diabetes increases a person's risk for heart disease, and many people with diabetes have a heart condition such as coronary heart disease or heart failure. Since hyperthyroidism causes rapid heart rate and increases the risk of abnormal heart rhythm, it may also bring on angina (chest pain), worsen heart failure or interfere with the treatment of heart failure, as well as further increase the risk of other heart problems. Hypothyroidism rarely causes significant changes in blood glucose control, although it can reduce the clearance of insulin from the bloodstream, so the dose of insulin may be reduced which increases the blood glucose control. Moreover, low normal FT4 levels are significantly

associated with increased insulin resistance. Protein levels are found to be increased in hypothyroidism whereas the protein levels are decreased in hyperthyroidism (33).

The assignments of bands for specific modes have been achieved by interpreting the FTIR spectra of human blood. Average overlaid spectra of 50 pre and post treatment of the hypothyroid and hyperthyroid disorder patients along with the 50 healthy subjects are represented in Fig. 2 and Fig. 3 respectively.

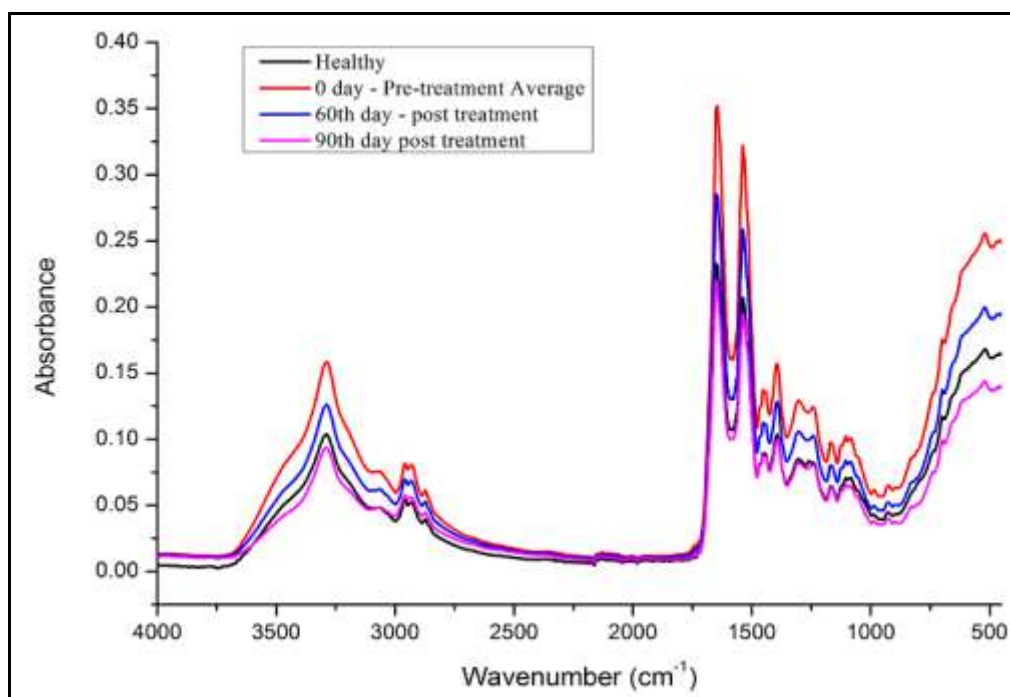


Fig. 2 Overlaid Average Spectra of Pre and Post Treatment of Hypothyroid Blood samples

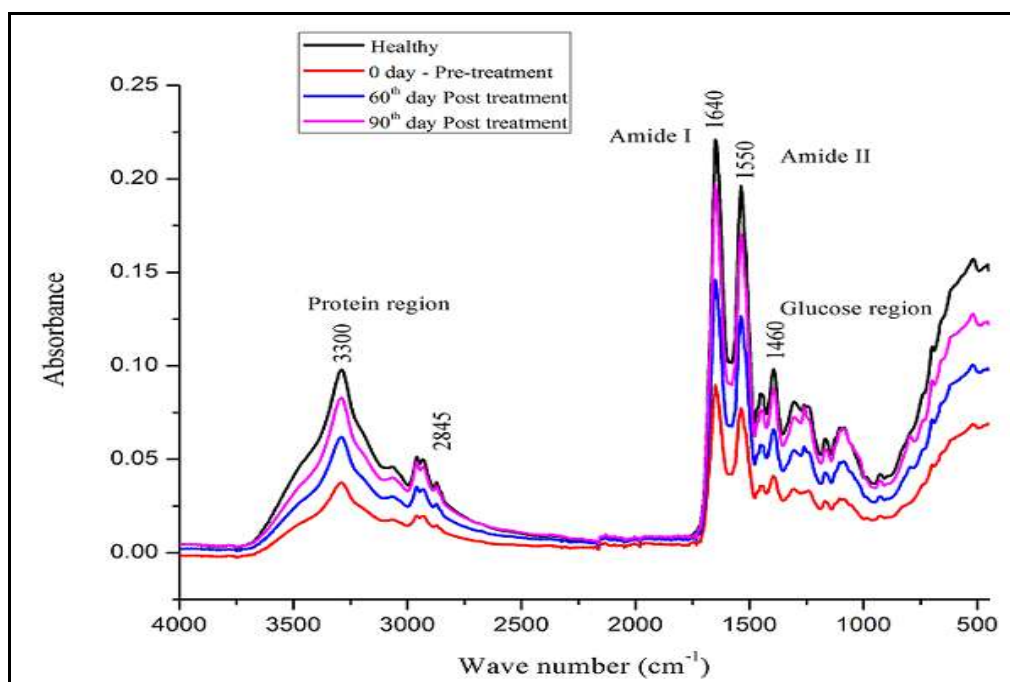


Fig. 3 Overlaid Average Spectra of Pre and Post Treatment of Hyperthyroid Blood samples with healthy blood samples

To quantify the spectral signatures in the blood samples of pre and post treatment, the absorbance values of the spectral peaks such as 3060, 2933, 1460, 1080, were noted and the intensity ratio parameters were calculated. The significance of the intensity ratio results is estimated using dependent 't' test statistical methods. For the statistical interpretation, the p value must be less than 0.05, then it is considered to be statistically significant. The percentage of efficacy of drug therapy can be calculated using the formula,

$$\text{Efficacy Percentage} = \frac{[IRP.(Pre) - IRP.(Post)]}{IRP.(Pre)} \times 100$$

Considerable spectral differences have been observed in the region of protein (1500 – 1700 cm^{-1}), LDL (2800 – 3400 cm^{-1}) and glucose (930 – 1250 cm^{-1}). Three intensity parameters viz. LDL/Glucose [$R_1(I_{1460}/I_{1080})$], Triglycerides/Glucose [$R_2(I_{3060}/I_{1080})$] and Total cholesterol/ Glucose [$R_3(I_{2933}/I_{1080})$] are calculated among the prominent absorption peaks due to the LDL, triglycerides, total cholesterol and glucose for the estimation of efficacy of thyronil in hypothyroid patients. Three intensity parameters viz. LDL/Glucose [$R_1(I_{1460}/I_{1080})$], Triglycerides/Glucose [$R_2(I_{3060}/I_{1080})$] and Total cholesterol/ Glucose [$R_3(I_{2933}/I_{1080})$] have been calculated to study the efficacy of thyronil in hyperthyroid patients. The sampling analysis of dependent 't' test was carried out for pre and post blood samples. The dependent 't' test is used to compare the means between two related groups on the same continuous dependent variables. The mean, standard deviation, standard error for pre and post treatment on hypothyroid and hyperthyroid patients blood samples were found from the three intensity parameters viz. LDL/Glucose [$R_1(I_{1460}/I_{1080})$], Triglycerides/Glucose [$R_2(I_{3060}/I_{1080})$] and Total cholesterol/ Glucose [$R_3(I_{2933}/I_{1080})$] are shown in Table 2 and Table 3 respectively. Fig. 4 and Fig. 5 show the histograms that show the comparison of mean intensity ratio parameters for healthy subjects, pre and post treatment of hypothyroid and hyperthyroid patients' blood samples respectively. The efficacy of the ayurvedic drug thyronil varies between 60 – 85 % in hypothyroid and hyperthyroid patients. There is a statistical significant difference in levels of LDL, total cholesterol and triglycerides for each of the internal ratio parameters. The low p value less than 0.05 from Table 2 and 3 indicate that there is a significant difference between the pre and post treatment blood samples.

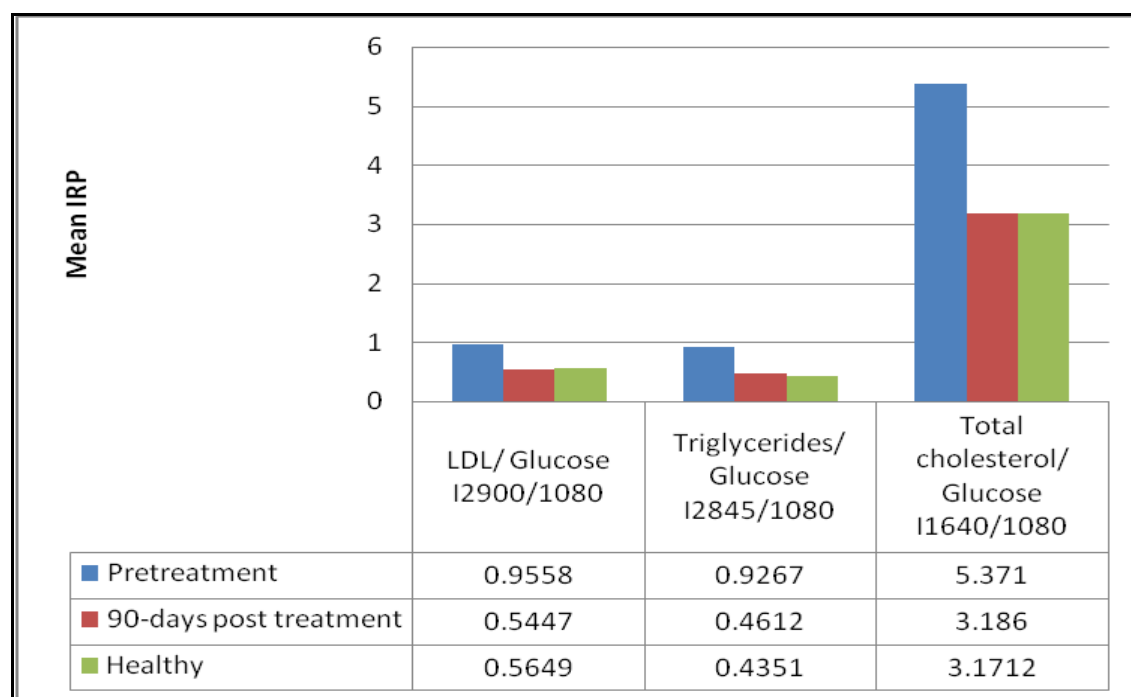


Fig. 4 Histogram – FTIR-ATR - Mean Intensity Ratio Parameter of the Efficacy of the drug thyronil in hypothyroid patients

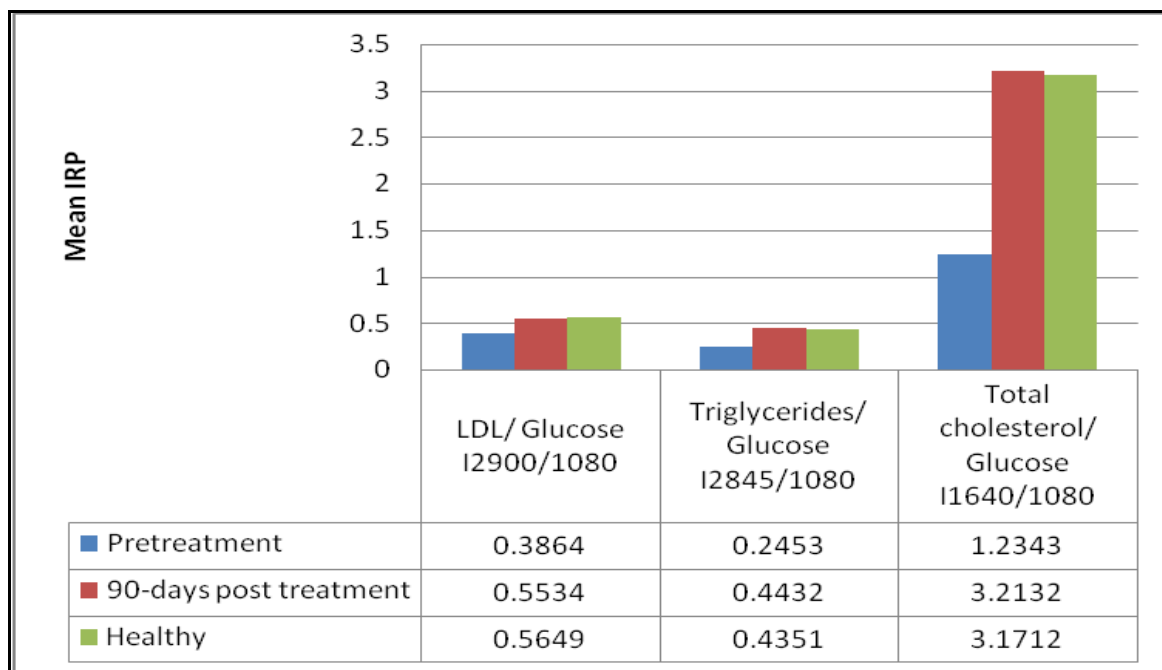


Fig. 5 Histogram – FTIR-ATR - Mean Intensity Ratio Parameter of the Efficacy of the drug thyronil in hyperthyroid patients

Table 2 Mean, standard deviation, t value and p value of intensity ratio parameters of hypothyroid blood samples

IRP Ratios	Pre treatment				Post-treatment				t-value	p-value
	N	Mean IRP	Standard Deviation	Standard Error	N	Mean IRP	Standard Deviation	Standard Error		
LDL/ Glucose I2900/1080	50	0.9558	0.0335	0.0075	50	0.5447	0.0194	0.0043	15.89	8.564 x 10 ⁻⁹
Triglycerides/ Glucose I2845/1080	50	0.9267	0.0459	0.0103	50	0.4612	0.0369	0.0082	20.39	7.945 x 10 ⁻⁹
Total cholesterol/ Glucose I1640/1080	20	5.371	0.1855	0.0415	20	3.186	0.1901	0.0425	22.24	7.546 x 10 ⁻⁹

Table 3 Mean, standard deviation, t value and p value of intensity ratio parameters of hyperthyroid blood samples

IRP Ratios	Pre treatment				Post-treatment				t-value	p-value
	N	Mean IRP	Standard Deviation	Standard Error	N	Mean IRP	Standard Deviation	Standard Error		
LDL/ Glucose I2900/1 080	20	0.8286	0.0978	0.0219	20	1.3625	0.1539	0.0344	17.86	7.65703×10^{-9}
Triglycerides/ Glucose I2845/1 080	20	1.5045	0.2340	0.0523	20	2.3397	0.4445	0.0994	20.12	6.83148×10^{-9}
Total cholesterol/ Glucose I1640/1 080	20	2.2972	0.3006	0.0672	20	3.5545	0.5021	0.1123	24.44	3.55317×10^{-9}

Conclusion

The role of FTIR-ATR spectroscopic technique in the analysis of human blood samples in the treatment of thyroid disorders before and after treatment has been evidently demonstrated by both qualitative and quantitative methods. Blood from patients with hypothyroid disorder clearly shows an increase in the peak height ratios of LDL/Glucose [$R_1(I_{1460}/I_{1080})$], Triglycerides/Glucose [$R_2(I_{3060}/I_{1080})$] and Total cholesterol/Glucose [$R_3(I_{2933}/I_{1080})$] and with hyperthyroid disorder clearly shows a decrease in the peak height ratios of viz. LDL/Glucose [$R_1(I_{1460}/I_{1080})$], Triglycerides/Glucose [$R_2(I_{3060}/I_{1080})$] and Total cholesterol/Glucose [$R_3(I_{2933}/I_{1080})$] of LDL, triglycerides and total cholesterol bands and efficacy of Ayurvedic drug, thyronil in the thyroid disorder subjects indicates the progress towards the healthy subjects. From the results obtained from the spectroscopic technique, it can be observed that the ayurvedic drug thyronil yielded satisfactory results in the treatment of thyroid disorder.

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