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Serum based analysis of Asthma and Study of Efficacy of Drug Therapy using FTIR-ATR technique

P. Sasi Rekha[1], S.Gunasekaran[2]

Full Time Research Scholar, St.Peter's University, Avadi, Chennai, Tamil Nadu, India[1].

Dean, Research and Development, St.Peter's University, Avadi, Chennai, Tamil Nadu, India[2].

ABSTRACT: This study attempts to evaluate the spectral differences between healthy and asthmatic disease blood sera and to find the efficacy of the drug in asthmatic patients using FTIR-ATR spectroscopic technique with a minimally invasive sample. Ten asthmatic patients of two different grades with five mild, five severe cases and with ten references were enrolled in the study. FTIR-ATR spectra were recorded in the mid-infrared region of 4000cm^{-1} - 450cm^{-1}. In order to find the efficacy of the drug, the quantity of bio-molecules such as protein, DNA, HDL, amide I, amide II, LDL components present in blood sera of pre and post treatments in terms of absorption values of specific modes of vibration in FTIR spectra were significantly different and the post treatment values were very close to the healthy blood samples. Based on the differences in the spectral signatures, six intensity ratio parameters among the absorption peaks were computed and it was found to be an indicator to differentiate the diseased subjects from pre and post drug therapy and also with the healthy one. The overall interpretation of the spectra appears to provide a direct link to the assessment of the state of the efficacy of the asthmatic drug. The results were further validated with statistical analysis by applying the paired t-test which indicated that the spectral variations were statistically significant.

KEYWORDS: **BLOOD SERA, ASTHMATIC DISEASE, BIO-MOLECULES, INTERNAL RATIO PARAMETER, FTIR-ATR SPECTROSCOPY.**

I. INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways characterized by the airway hyper-responsiveness (AHR) and reversible air flow obstruction that fluctuates overtime (1). Airway obstruction and allergic inflammation during the disease occur due to release of Immunoglobulin E (IgE) and pro-inflammatory cytokines such as interleukin (IL)-4 ,(IL)-5,(IL)-9 and (IL)-13 by T- helper cell type2 (Th2) and other immune effector cells producing toxic inflammatory molecules that ultimately elicit obstruction(2,3). Based on clinical parameters, patients are assigned to distinct categories as mild, moderate, severe and very severe. Current diagnosis of asthma is based on the history of wheeze, shortness of breath and cough, which are variable in severity and overtime (1,4).

II. MATERIALS AND METHODS

Healthy blood samples were collected from ten healthy volunteers of age group between 18-25 years. Asthmatic blood samples were collected from ten asthmatic volunteers (age group between 40-50 years), after their written consent. A quantity of 1ml blood was collected from each subject in a centrifuge tube before and after drug therapy. After centrifugation of blood in a refrigerated centrifuge, the serum samples were transported to the laboratory in portable freezer spectra and kept at 0^0c until analyzed, and for few hours samples were allowed to thaw passively, following which FTIR spectra were acquired.

The measurement of serum samples with FTIR-ATR spectroscopy were carried out at Saif -SPU, Chennai-54, using Perkin-Elmer Spectrum two FTIR/ATR spectrophotometer. Each spectrum was measured in 4000cm^{-1}- 450cm^{-1} range with a 4cm^{-1} resolution and with 16 scans. For each sample, 30µL serum was dropped onto ATR diamond crystal in the apparatus and evenly spread out. The samples were air dried up with an air dryer for water evaporation. The dried samples were immediately measured and the spectra were constructed using the software 'Spectrum', provided with

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FTIR Spectrum Two Spectrometer. The measurement of serum samples with FTIR-ATR spectroscopy were carried out at Saif -SPU, Chennai-54, using Perkin-Elmer Spectrum two FTIR/ATR spectrophotometer.

III. STATISTICAL ANALYSIS

Analysis of variance was implemented to identify the spectral variations that were statistically significant. The t-test is one of the most rapid procedures for classification of biological data (10). In current study, the t-test was used to differentiate certain regions of the FTIR spectra of the examined healthy sera and diseased sera samples of the Asthmatic patients. For entire spectrum from 4000 to 450 cm-^{1}, statistical analysis was performed by t-test and it shows the full successful classification to distinguish pre-treated from post-treated blood sera samples. In t-test analysis, considering the mean difference variance of the analysis, the t-test was carried out. The absorbance values observed gives a macroscopic value difference as compared to the minute variance observed in the serum analysis using FTIR spectroscopic technique.

The following are the intensity ratio parameters of pre and post-treated serum samples R_1, R_2, R_3, R_4, R_5, and R_6. Using FTIR, serum samples were analyzed and the results were compared and statistically analyzed using the t-test. The mean variance and standard deviation was observed and showed that there is a significant difference between pre and post serum samples values. This test is used for correlating the means of two samples, even if they have unequal numbers of replicates.

In simple terms, comparing the actual difference between two means in relation to the variation in the data expressed as the standard deviation of the difference between the means using the t-test. Statistical tests allow for making statements with a higher degree of precision, but cannot actually proving or disproving anything. Significant results at the 95% probability level make perfect data, which is good enough to support a conclusion with 95% confidence (but there is a 1 in 20 chance of being wrong). In biological work, to maintain and accepts this level of significance as being reasonable (10).

IV. RESULTS AND DISCUSSIONS

The spectral analysis undertaken in this work primarily focuses upon qualitative and quantitative study of efficacy of asthmatic drug. The FTIR spectrum exhibits vibrational band characteristics of the various group frequencies, of the spectrum of a healthy serum sample and that of an asthmatic serum sample, which are the same with respect to the positions of the peaks but different in of the absorption level of the peaks(5). It is clear that, the absorption peaks of proteins, lipids, glucose, phospholipids and etc, for asthmatic patient is more than that of the healthy individuals as because several substances like histamines, leukotrienes, prostaglandins, eosinophils and cytokines are being released by mast cells during an asthma attack(1). FTIR absorption spectrum of normal blood sample is shown in Figure1. Figures2 &3 represents the overlaid FTIR spectra of normal with pre and post drug therapy asthmatic diseased samples for severe and mild grades respectively.

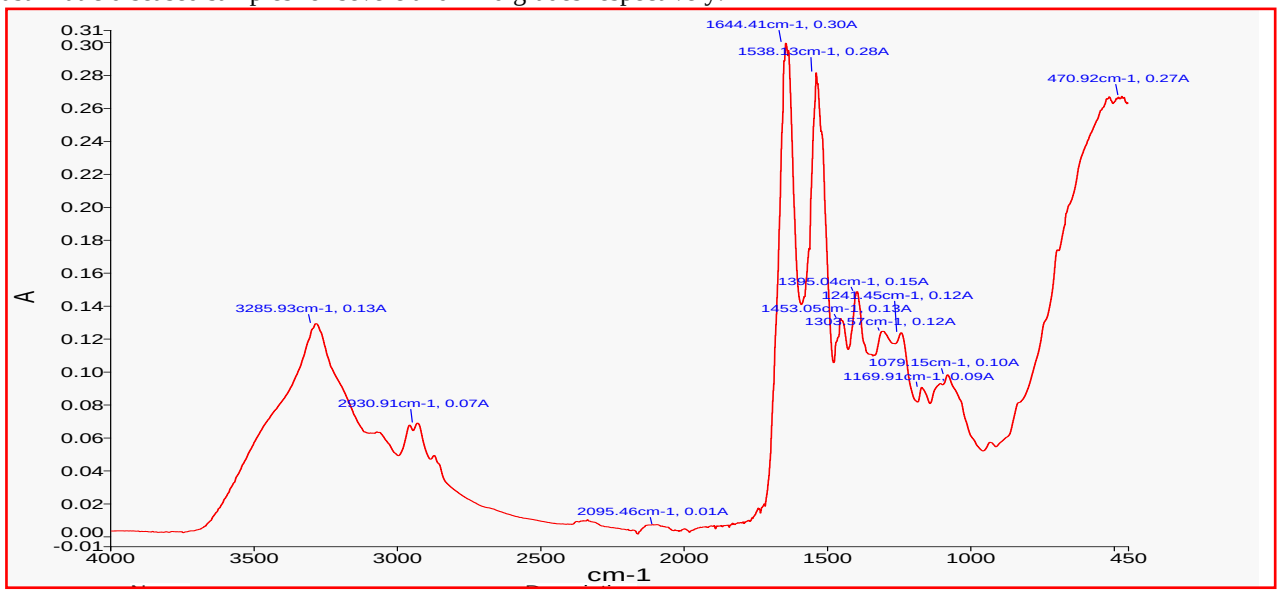

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FIG 1. FTIR SPECTRA OF HEALTHY BLOOD SERA

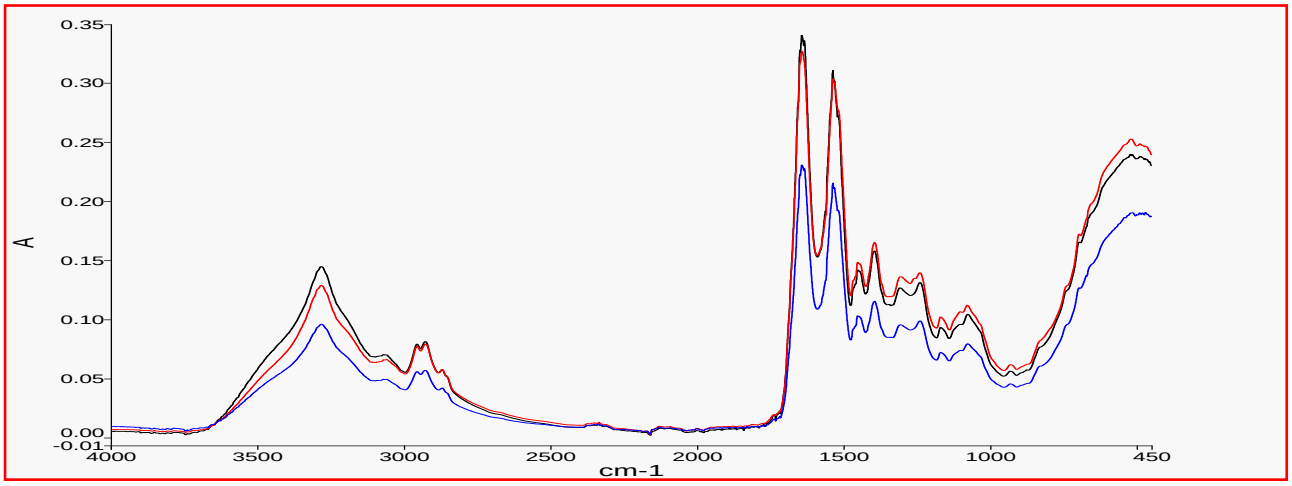

FIG 2. OVERLAID SPECTRA OF HEALTHY, PRE AND POST DRUG THERAPY SERA SAMPLES OF SEVERE ASTHMA

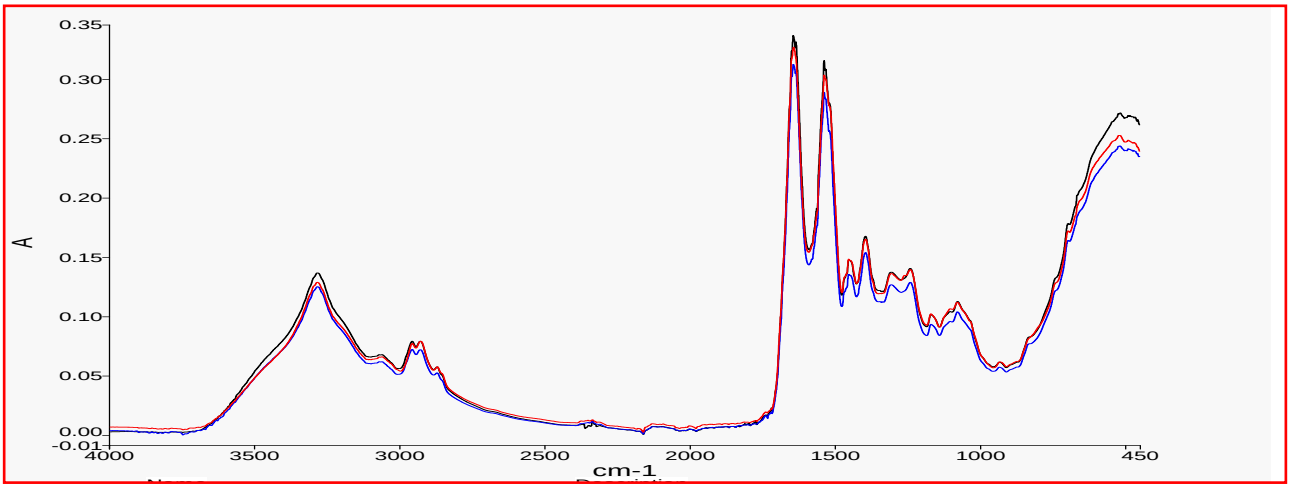

FIG 3.OVERLAID SPECTRA OF HEALTHY, PRE AND POST DRUG THERAPY SERA SAMPLES OF MILD ASTHMA

Table 1: Intensity ratio parameters for Healthy individual Blood Sera

Sample	R_1	R_2	R_3	R_4	R_5	R_6
1	3.047	2.864	1.335	1.513	1.268	1.259
2	2.727	2.545	1.272	1.363	1.181	1.180
3	2.925	2.652	1.460	1.528	1.245	1.223
4	3.120	2.946	1.394	1.650	1.392	1.389
5	3.333	3.311	1.444	1.666	1.333	1.333
6	3.000	2.666	1.166	1.540	1.000	1.000
7	3.233	2.919	1.293	1.598	1.178	1.162
Mean	**3.055**	**2.843**	**1.338**	**1.551**	**1.228**	**1.221**
Standard Deviation	**0.200**	**0.256**	**0.104**	**0.102**	**0.127**	**0.127**

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Table 2: Intensity ratio parameters for Asthmatic individual Blood Sera

Sample	R_1	R_2	R_3	R_4	R_5	R_6
1	3.33	3.08	1.41	1.583	1.25	1.33
2	3.875	3.125	1.375	1.5	1.25	1.25
3	3.284	3.027	1.354	1.55	1.257	1.268
4	3.612	3.329	1.489	1.705	1.382	1.39
5	3.647	3.362	1.504	1.722	1.396	1.404
6	3.644	3.359	1.503	1.721	1.395	1.403
7	3.812	3.16	1.325	1.45	1.21	1.26
8	3.14	2.981	1.325	1.574	1.256	1.259
9	3.627	3.334	1.467	1.624	1.357	1.385
10	3.52	3.064	1.285	1.689	1.216	1.414
Mean	**3.549**	**3.182**	**1.404**	**1.612**	**1.297**	**1.336**
Standard Deviation	**0.233**	**0.149**	**0.082**	**0.096**	**0.076**	**0.070**

Table 3: Intensity ratio parameters of Pre and Post drug therapy for Asthmatic Blood Sera

Sample	R_1		R_2		R_3		R_4		R_5		R_6	
	Amide I(1644cm^{-1})/ Glucose(1080cm^{-1})		Amide II (1538cm^{-1})/ Glucose(1080cm^{-1})		LDL(1450cm^{-1})/ Glucose (1080cm^{-1})		Methyl (1395cm^{-1})/ Glucose (1080cm^{-1})		Methyl (1303cm^{-1}) / Glucose(1080cm^{-1})		Phospholipid (1240cm^{-1})/ Glucose (1080cm^{-1})	
	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post
1	3.330	3.214	3.080	2.928	1.410	1.357	1.583	1.500	1.250	1.214	1.330	1.200
2	3.875	3.214	3.125	2.928	1.375	1.350	1.500	1.440	1.250	1.200	1.250	1.210
3	3.284	3.150	3.027	2.951	1.354	1.340	1.550	1.540	1.257	1.240	1.268	1.264
4	3.612	3.481	3.329	3.324	1.489	1.468	1.705	1.687	1.382	1.357	1.390	1.389
5	3.647	3.515	3.362	3.266	1.504	1.483	1.722	1.704	1.396	1.373	1.404	1.403
6	3.644	3.484	3.359	3.237	1.503	1.470	1.721	1.689	1.395	1.360	1.403	1.390
7	3.812	3.420	3.160	3.125	1.325	1.302	1.450	1.392	1.210	1.192	1.260	1.201
8	3.140	3.091	2.981	2.640	1.325	1.297	1.574	1.553	1.256	1.121	1.259	1.242
9	3.627	3.541	3.334	3.232	1.467	1.450	1.624	1.525	1.357	1.294	1.385	1.306
10	3.520	3.489	3.064	2.982	1.285	1.269	1.689	1.551	1.216	1.193	1.414	1.390

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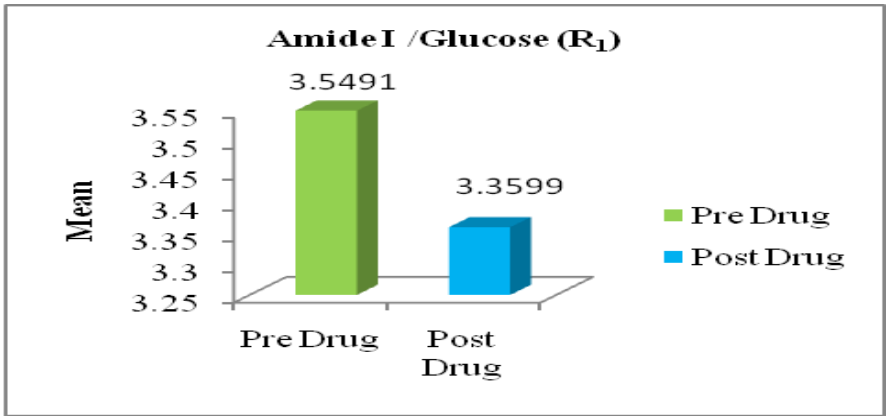

FIGURE 4: MEAN INTENSITY RATIO OF AMIDE I (1644 CM^{-1})/GLUCOSE (1080 CM^{-1}) FOR PRE & POST ASTHMATIC INDIVIDUAL

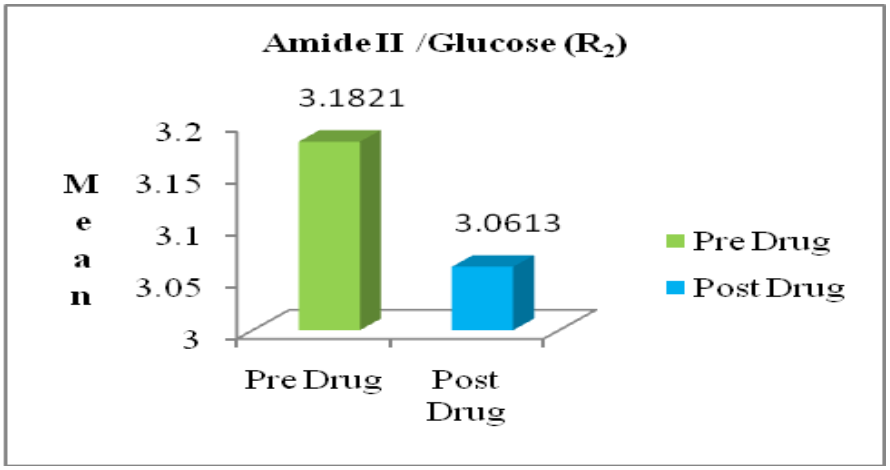

FIGURE 5: MEAN INTENSITY RATIO OF AMIDE II (1538 CM^{-1})/GLUCOSE (1080 CM^{-1}) FOR PRE & POST ASTHMATIC INDIVIDUAL

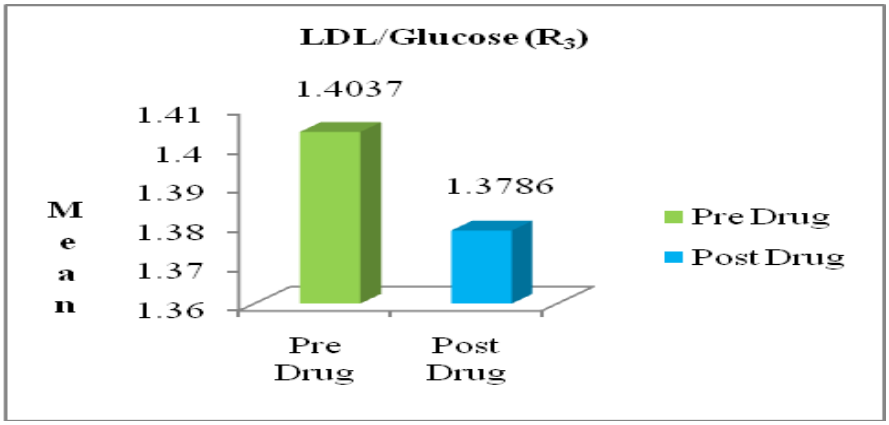

FIGURE 6: MEAN INTENSITY RATIO OF LDL (1450 CM^{-1})/GLUCOSE (1080 CM^{-1}) FOR PRE & POST ASTHMATIC INDIVIDUAL

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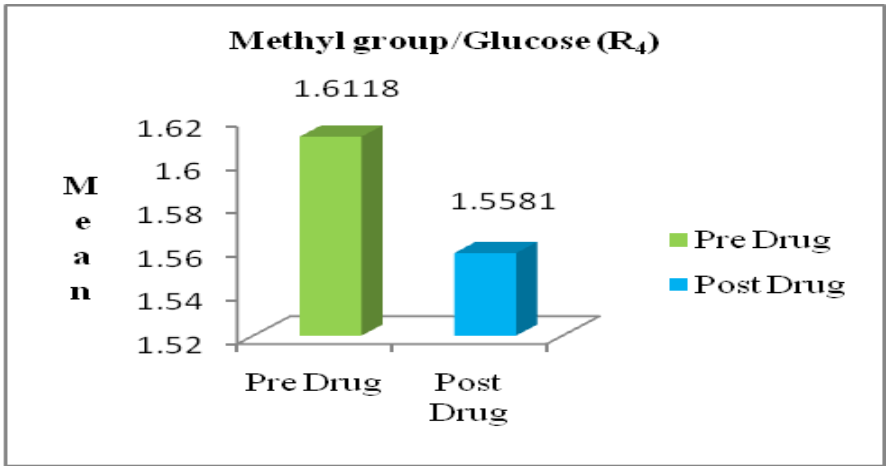

FIGURE 7: MEAN INTENSITY RATIO OF METHYL (1395 CM^{-1})/GLUCOSE (1080 CM^{-1}) FOR PRE & POST ASTHMATIC INDIVIDUAL

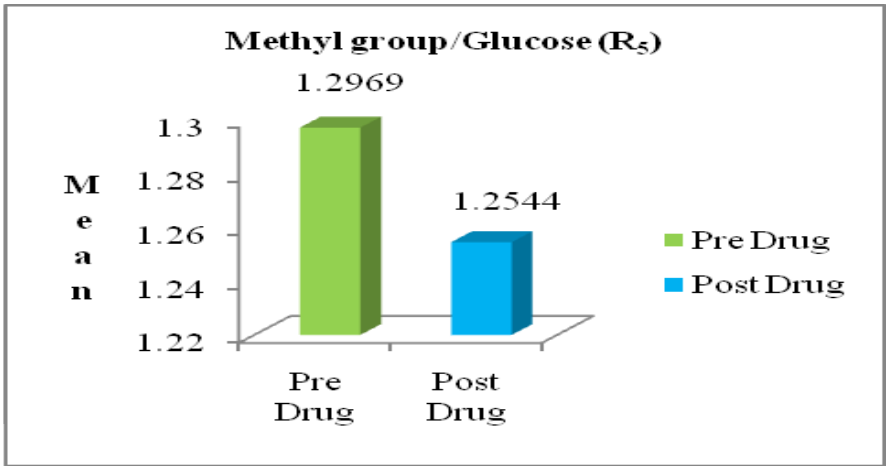

FIGURE 8: MEAN INTENSITY RATIO OF METHYL (1303 CM^{-1})/GLUCOSE (1080 CM^{-1}) FOR PRE & POST ASTHMATIC INDIVIDUAL

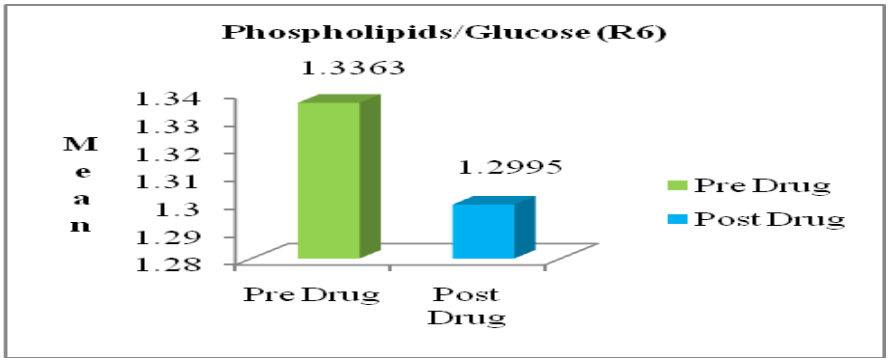

FIGURE 9: MEAN INTENSITY RATIO OF PHOSPHOLIPIDS (1240 CM^{-1})/GLUCOSE (1080 CM^{-1}) FOR PRE & POST ASTHMATIC INDIVIDUAL

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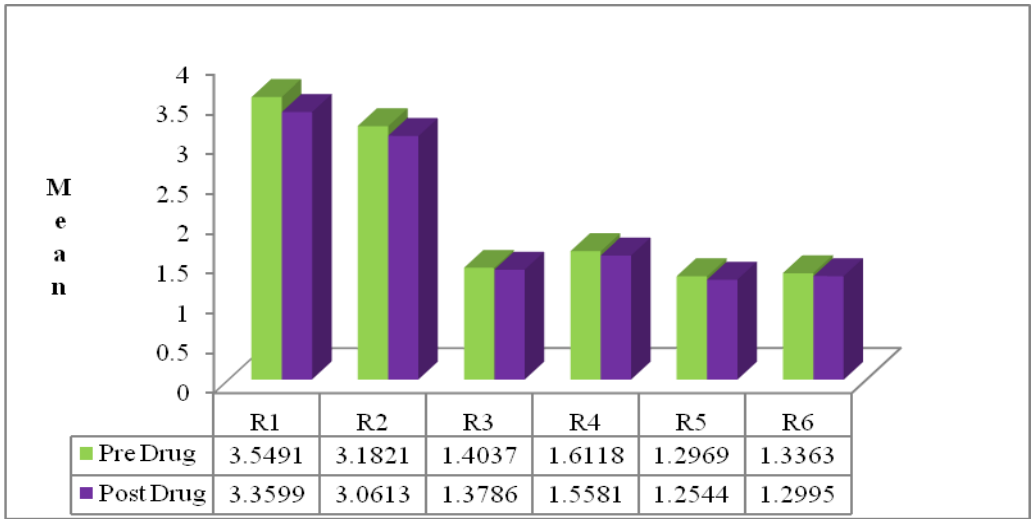

FIGURE 10: COMPARISON OF MEAN INTENSITY RATIO FOR PRE DRUG AND POST DRUG THERAPY FOR ASTHMATIC INDIVIDUAL

In order to quantify the spectral differences, six intensity ratio parameters R_1 Amide I (1644cm^{-1}) /Glucose (1080cm^{-1}), R_2 Amide II (1538cm^{-1}) /Glucose (1080cm^{-1}), R_3 LDL (1450cm^{-1})/ Glucose(1080cm^{-1}), R_4 Methyl (1395cm^{-1})/ Glucose (1080cm^{-1}), R_5 Methyl (1303cm^{-1})/Glucose (1080cm^{-1}), and R_6 Phospholipid (1240cm^{-1})/Glucose (1080cm^{-1}) have been introduced and calculated for healthy and pre, post asthmatic drug samples which are tabulated in Tables 1, 2 & 3 (6,7). Figures 4, 5, 6, 7, 8 & 9 shows the histogram of Mean intensity ratios of pre and post drug therapy of Asthmatic individuals. Figure 10 is a histogram which shows the comparison of mean intensity ratio for pre and post drug therapy asthmatic individuals.

For entire spectrum from 4000 to 450 cm^{-1}, statistical analysis was performed by t-test, considering the mean difference variance of the analysis and it shows the full successful classification to distinguish Pre and Post drug therapy treatment of asthmatic individuals as shown in Tables 4 & 5. The low p-value (< 0.05) indicates that there is a significant difference between the means of the intensity ratio parameter calculated for pre and post drug blood serum and hence stands as an evidence for the method adopted (7,8).

Table 4:Mean Intensity ratio parameters of Pre and post drug therapy for Asthmatic individuals using t-test

	Pre drug therapy				Post drug therapy			
	N	Mean IRP	Standard Deviation	Standard Error Mean	N	Mean IRP	Standard Deviation	Standard Error Mean
Amide I (1644cm^{-1}) / Glucose(1080cm^{-1})	10	3.549	0.233437	0.073819	10	3.3599	0.171962	0.054379
Amide II(1538cm^{-1})/ Glucose (1080cm^{-1})	10	3.182	0.149417	0.04725	10	3.0613	0.212516	0.067203
LDL (1450cm^{-1})/ Glucose (1080cm^{-1})	10	1.404	0.082337	0.026037	10	1.3786	0.081416	0.025746
Methyl (1395cm^{-1})/ Glucose (1080cm^{-1})	10	1.612	0.09639	0.030481	10	1.5581	0.10617	0.033574
Methyl(1303cm^{-1})/ Glucose (1080cm^{-1})	10	1.297	0.076055	0.024051	10	1.2544	0.086648	0.0274
Phospholipid (1240cm^{-1}) / Glucose (1080cm^{-1})	10	1.336	0.070138	0.02218	10	1.2995	0.08649	0.027351

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Table 5 : t Test RESULTS OF INTENSITY RATIO PARAMETERS OF SERA FROM PRE AND POST DRUG THERAPY FOR ASTHMATIC INDIVIDUALS

	Degrees of Freedom	t Stat	P(T<=t) one-tail	Pearson Correlation
Amide I (1644cm^{-1}) / Glucose(1080cm^{-1})	9	3.10125	0.00635	0.58347939
Amide II(1538cm^{-1})/ Glucose (1080cm^{-1})	9	4.03861	0.00147	0.92181317
LDL (1450cm^{-1})/ Glucose (1080cm^{-1})	9	6.99475	0.000032	0.99045886
Methyl (1395cm^{-1})/ Glucose (1080cm^{-1})	9	4.01821	0.00151	0.9174124
Methyl(1303cm^{-1})/ Glucose (1080cm^{-1})	9	3.76822	0.00221	0.91199919
Phospholipid (1240cm^{-1}) / Glucose (1080cm^{-1})	9	2.78344	0.01064	0.87796601

V. CONCLUSION

Biological fluids are being widely investigated with the use of molecular spectroscopy for clinical diagnosis of various diseases. The method newly introduced in the last decades for such investigations is the FTIR-ATR spectroscopic technique. FTIR spectroscopy of body fluids has shown potential in a multitude of biomedical applications, including pharmacological and forensic applications (9). Thus the FTIR spectra of body fluids could be used to monitor metabolic changes occurring with the disease. In light, of need for better diagnostic tools which attributes to rapidity, objectivity and the use of minimally invasive samples, FTIR spectroscopy serves usefully for the detection of asthma. Thus, the study was carried out to explore the potential applicability of FTIR spectroscopy in detecting disease related perturbations in sera of mild and severe asthma as well as healthy subjects. The internal standard method is adopted in characterizing the samples quantitatively.

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