Original Article

Biochemistry Section

Analysis of Multiple Myeloma by FTIR Spectroscopy Coupled with Statistical Analysis

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ABSTRACT

Background: Myeloma is a disorder in which malignant plasma cells accumulate in the bone marrow and produce excess immunoglobulin that leads to many complications.

Aim: The aim of the present work was to analyze the difference in the nature of the immunoglobulin protein in healthy human blood plasma and myeloma which was infiltrated by human blood plasma, by employing Fourier Transform Infra Red (FTIR) Spectroscopy which was coupled with statistical analysis.

Materials and Methods: An infrared spectrum was recorded in the mid frequency region, in the 4000 – 450 cm^{-1} range for normal and various types of myeloma which were infiltrated with blood plasma samples, namely IgA and IgG. The proposed method was then quantitatively analyzed by intensity ratio calculation among the spectral absorption peaks and by the Independent t-test.

Results: In Ig A myeloma, the proliferation is more when compared to the normal healthy controls. On contrary, in Ig G myeloma, there observed a reduction in the Ig G immunoglobulins.

Conclusion: From the results, it has been established that IR spectroscopy has the potential to be an efficient and effective diagnostic tool in the analysis of blood.

Key Words: Myeloma, Ig A, Ig G, Blood, FTIR Spectroscopy, Independent "t"-test

INTRODUCTION

Myeloma is an accumulation of malfunctioning or cancerous plasma cells. Most of the plasma cells reside in the bone marrow, and myeloma, accordingly, usually occurs within the marrow which is contained in the large bones of the body. Since they are present throughout the bone marrow, the plasma cells that have undergone malignant transformation do so in clumps and usually at many sites, which explains the terminology, 'myeloma'. The development of myeloma results in an impaired immune system with problems which are associated with antibody overproduction, as well as with those which are associated with any invasive cancer [1, 2]. The normal plasma cells produce antibodies which are also called immunoglobulins (Igs). They are a group of glycoproteins which are secreted by the plasma cells and which function as antibodies in the immune response by binding to specific antigens. There are five classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM. IgD and IgE exist in the monomeric form, while IgA exists as a dimer and IgM exists in the pentameric form [3]. The abnormal plasma cells in myeloma do not produce the normal vast array of different immunoglobulins. Instead, the myeloma cells may produce an abnormal immunoglobulin which is called as the monoclonal protein, or the M protein, where all the proteins which are produced by this cell line have exactly the same identity and the same impaired function, which is essentially a deficiency. The different types of myeloma are classified by the type of immunoglobulins which are produced by the abnormal plasma cells. The immunoglobulin (Ig) is made up of 2 components: a light chain and a heavy chain. If a monoclonal (M) protein is observed in plasma, it means that the immunoglobulin will be comprised of either the heavy chains or the light chains only. The most common monoclonal proteins in myeloma are the IgG, and the IgA types, whereas IgM, IgD, IgE and FLC Lamda are the less commonly prevalent forms of myeloma.

The role of Fourier Transform Infra Red (FTIR) spectroscopy in the clinical analysis has increased tremendously in the recent past, due to the development of sophisticated instruments and efficient data evaluation software. FTIR spectroscopy is a non-invasive, reagent free, diagnostic tool which is used for the analysis of biological fluids. It possesses several advantages over the regular clinical methods, as very small amounts of sample are required, because of its high sensitivity, because it produces instantaneous, accurate and precise results, because of the reliability of its measurement and because of its avoidance of costly disposables and minimum manpower requirement. In the recent past, its diagnostic potential has been tested in the qualitative and quantitative investigation of biological fluids like blood plasma, serum, saliva, urine, etc [4-6]. Several studies have been performed to define the potential of FTIR for accurate quantitative analyses. The multi-component assay of human plasma has been evaluated for the determination of blood substrates [7]. The continuous monitoring of blood samples during chemotherapy in cancer treatment by FTIR spectroscopy was found to be highly informative and useful [8]. FTIR spectroscopy, coupled with statistical calculations, has been employed by researchers for the estimation of plasma proteins [9, 10].

The main aim of this study was to determine the spectral variation of the immunoglobulins, namely IgA and IgG in the myeloma affected blood plasma samples with healthy human blood plasma. The FTIR spectrum of a blood sample exhibits characteristic absorption peaks due to the specific functional groups which are present in the sample. The spectral variations were analyzed quantitatively by the intensity ratio calculation among the absorption peaks. It was observed that the values were significantly different in the normal and the myeloma samples. The results were further validated with statistical analysis by applying the independent 't' test, which indicated that the spectral variations were statistically significant.

Thus, the veracity of FTIR spectroscopy in the clinical analysis of blood could be established.

MATERIALS AND METHODS

Sample Preparation for the FTIR Study

Blood samples were collected from normal healthy subjects who were admitted to a hospital for routine check-up, at a reputed medical centre. The samples were collected from subjects of the age group of 40 - 50 years. Blood samples were also collected from age matched persons with increased immunoglobulin levels, who were admitted to the hospital with severe infection which was diagnosed as myeloma. Due permission was obtained from the institutional ethics committee before the start of the study. Informed consent was obtained from the participants of the study after explaining the objectives of the study to them. In the present work, two different myeloma infiltrated immunoglobulin plasma samples, namely IgA and IgG, were chosen for the analysis. All the sampling procedures were performed between 08.00 to 09.00 am after overnight fasting. The samples were kept as such for two hours. Under each category, 10 plasma samples were used for spectral analysis. The samples were stored in the laboratory in a portable freezer at 4°C until analysis.

Spectral Analysis

The FTIR spectral measurements of all the plasma samples were carried out at the Sophisticated Analytical Instrumentation Facility, Indian Institute of Technology Madras, Chennai-36, by using a Spectrum One Perkin-Elmer FTIR Spectrophotometer. The spectra were recorded in the mid infrared region of 4000 – 400 cm^{-1} in the absorption mode. Fifty microlitres of each sample was spread evenly on the thallium bromide crystals window. The samples were air-dried for water evaporation to eliminate the stray absorption bands which were formed due to the water content. The sampling window was scanned as the background and 32 scans were co-added, with a spectral resolution of 1 cm^{-1}. All the spectra were baseline corrected and normalized to acquire an identical area under the curve.

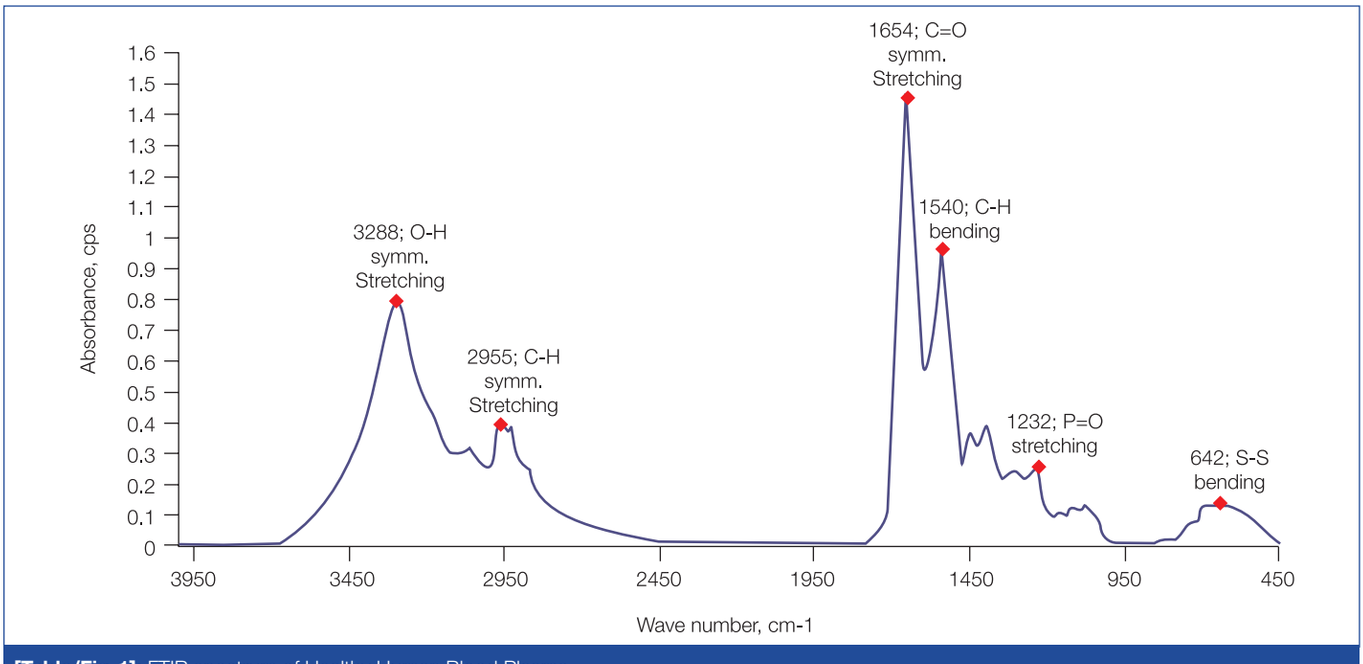
Construction of a Statistical Model

The FTIR spectrum of a sample exhibits characteristic absorption bands, depending on the various functional groups which are present in it. In the present study, the specific vibrational bands that were characteristic of the structure of the immunoglobulin viz. the C=O stretching of the peptide linkage, O-H stretching, which was a component part of the carboxyl group and P=O stretching due to phosphates were noted in the spectrum of each plasma sample. The intensity ratio calculation was carried out for the three functional groups in order to verify the significant level of the FTIR spectral data between the normal and the myeloma infiltrated plasma samples. The independent 't' test was carried out by using SPSS software.

RESULTS AND DISCUSSION

[Table/Fig-1] shows the FTIR spectrum of a normal blood plasma sample. The infrared spectra of the plasma proteins was characterized by the presence of important functional groups viz. the C=O stretching of the peptide linkage at 1654cm^{-1}, the P=O stretching of phosphates at 1232cm^{-1}, and the O-H stretching which was a part of the carboxyl group at 3288cm^{-1} respectively [11],[12]. The corresponding frequency band assignment for the FTIR spectrum is shown in [Table/Fig-2].

[Table/Fig-3] shows the overlaid FTIR spectrum of the healthy blood plasma and the IgA myeloma infiltrated plasma samples. It was observed that though the shape of the FTIR spectra of both looked similar, there was a marked difference in the intensity of absorption between the two spectra, which indicated that a significant change had occurred in the immunoglobulin content in the myeloma infiltrated plasma samples. It was also observed that when the blood plasma was infiltrated with IgA myeloma, there was an abnormal increase in the plasma protein content than that which was found in normal, healthy blood plasma. This was observed by an increase in the intensity of absorption in the characteristic frequency peaks at 1654cm^{-1}, 3288cm^{-1} and 1232cm^{-1} respectively. Thus, the FTIR spectral data qualitatively confirmed the abnormal proliferation of monoclonal IgA plasma proteins throughout the plasma, thereby indicating the presence of myeloma in the blood.



[Table/Fig-1]: FTIR spectrum of Healthy Human Blood Plasma

The statistical method which was used for the validation of the FTIR spectroscopic analysis for the region of 450 cm^{-1} – 4000 cm^{-1} was the Independent Sample 't' test by using the SPSS software package. It is an inferential statistical test that determines whether there is a statistically significant difference between the means in two unrelated variables. The statistical test was carried out for the three characteristic functional groups, namely the peptide group (1654cm^{-1}), the carboxyl group (3288 cm^{-1}) and the phosphate group (1232 cm^{-1}) separately between the normal plasma and two types of myeloma infiltrated plasma samples.

The Independent sample 't' test was first employed for the frequency peak of 3288cm^{-1} between the healthy plasma (variable 1) and IgA myeloma (variable 2) plasma, as shown in [Table/Fig-4].

The result of the test was presented in two sub tables; the Group Statistics Table and the Independent Samples Test Table. The Group Statistics Table provides useful descriptive statistics for the two variables, including the mean and standard deviation.

Vibrational band Assignment	Frequency peak, cm^{-1}
O - H stretching of carboxyl group	3288
C - H stretching of carboxyl group	2955
C = O stretching of peptide linkage	1654
N-H bending and C - H bending, amide II band	1540
Symmetric C - H bending due to methyl group	1395
P = O asymmetric stretching	1232
S – S stretching due to disulphide bond	642

[Table/Fig-2]: Vibrational band assignment of healthy human blood

The Independent Samples Test Table provides the actual results from the independent 't'test and the Levene's Test for the equality of the variances. The Levene's Test for the equality of variances helps in checking whether two variables have similar variances. According to this test, if the variances are equal, then the "Sig." will be greater than 0.05. However, if the "Sig." value is less than 0.05, the variances are unequal. In the present study, the result showed the value of "Sig." to be 0.640, which was greater than 0.05; thus, the variances were found to be similar. The value "Sig" (2-tailed)" gives the level of the statistical significance between the two independent variables. As the value of "Sig" (2) was observed to be 0.001, it meant that the two independent variables were highly significant at a 1% level.

The statistics report was given in the following format;

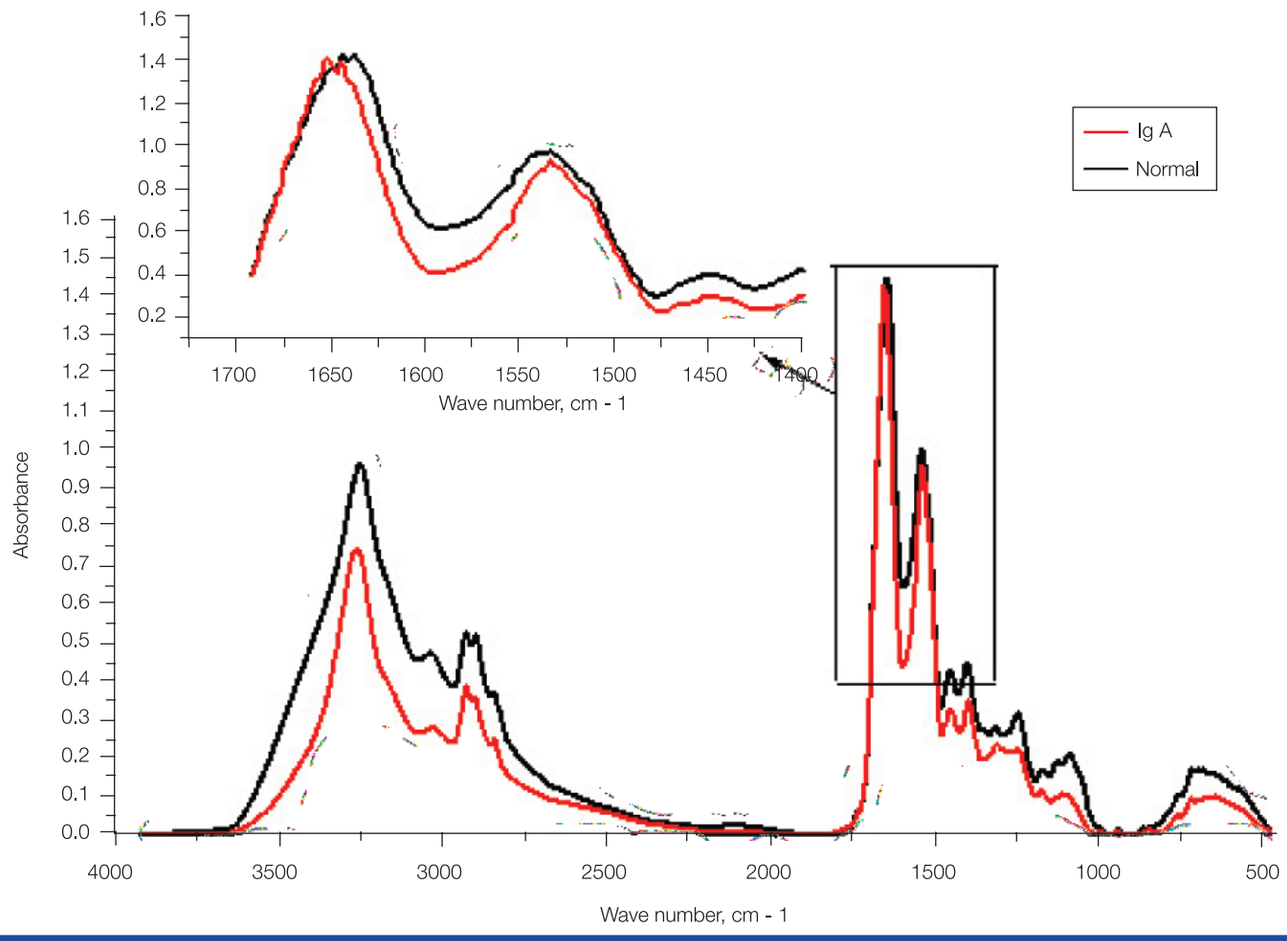
At 3288cm^{-1}, the IgA myeloma infiltrated plasma samples had statistically significant higher levels of carboxyl content (0.5703 ± 0.0245mmol/L) than the normal healthy plasma samples (0.4813 ± 0.0340 mmol/L).

($t(9) = 4.863$, $P = 0.001$).

In a similar manner, the Independent 't' test was calculated for the statistical validation at 1232cm^{-1} and 1654cm^{-1} which corresponded to the phosphate group and the peptide group, as shown in [Table/Fig-5] and [Table/Fig-6] respectively.

From [Table/Fig 5], the statistics report was given in the following format;

At 1232cm^{-1}, the IgA myeloma infiltrated plasma samples had statistically significant higher levels of phosphate content



[Table/Fig-3]: FTIR spectra of healthy and IgA myeloma plasma

Group Statistics

FTIR Spectral Absorabnce	N	Mean	SD	Std. Error Mean
Healthy Plasma f3288	10	0.481	0.034	0.013
Ig A myeloma	10	0.570	0.024	0.011

Independent Sample Test

	Levene's Test for Equality of Variance		t-test for Equality of Means				
	F	Sig.	t	df	Sig (2 tailed)	Mean Difference	Std Error Difference
F3288 Equal variances assumed	0.234	0.640	4.86	9	0.001	0.089	0.0183
F3288 Equal variances not assumed			5.02	8.8	0.001	0.089	0.0177

[Table/Fig 4]: It shows the Independent t test report for healthy and IgA myeloma plasma at 3288cm^{-1}

Group Statistics

FTIR Spectral Absorabnce	N	Mean	SD	Std. Error Mean
Healthy Plasma f1232	10	0.297	0.051	0.020
Ig A myeloma	10	0.417	0.011	0.004

Independent Sample Test

	Levene's Test for Equality of Variance		t-test for Equality of Means				
	F	Sig.	t	df	Sig (2 tailed)	Mean Difference	Std Error Difference
F1232 Equal variances assumed	5.541	0.043	5.12	9	0.001	0.1208	0.0235
F1232 Equal variances not assumed			5.62	8.8	0.002	0.1208	0.0215

[Table/Fig 5]: It shows the Independent t test report for healthy and IgA myeloma plasma at 1232 cm^{-1} Group Statistics

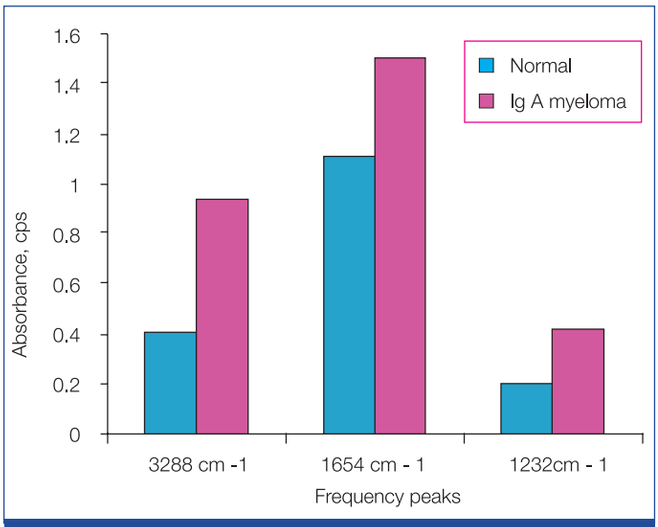
Group Statistics

FTIR Spectral Absorabnce	N	Mean	SD	Std. Error Mean
Healthy Plasma f1654	10	1.436	0.019	0.008
Ig A myeloma	10	0.446	0.018	0.007

Independent Sample Test

	Levene's Test for Equality of Variance		t-test for Equality of Means				
	F	Sig.	t	df	Sig (2 tailed)	Mean Difference	Std Error Difference
F1654 Equal variances assumed	0.169	0.691	0.858	9	0.012	0.0097	0.0113
F1654 Equal variances not assumed			0.854	8.4	0.014	0.0097	0.0101

[Table/Fig 6]: It shows the Independent t test report for healthy and IgA myeloma plasma at 1654cm^{-1} Group Statistics



[Table/Fig-7]: It shows the Bar Graph indicating the absorbance differences between normal and Ig A myeloma plasma

(0.417±0.011mmol/L) than the normal healthy plasma samples (0.297±0.051mmol/L).

($t(9) = 5.12$, $P = 0.001$).

Similarly, from [Table/Fig 6], the statistics report was given in the following format;

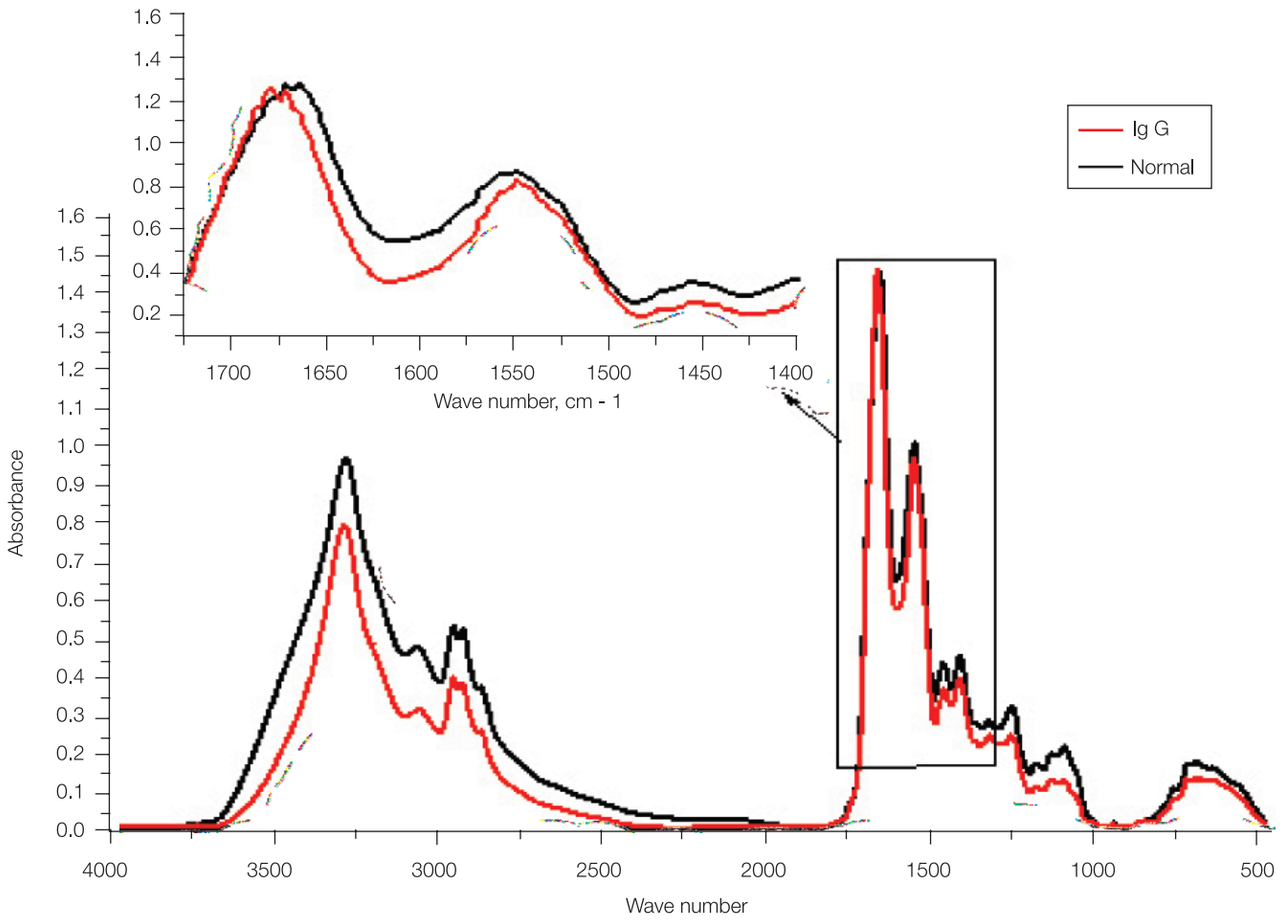
This study found that at 1654cm^{-1}, the IgA myeloma infiltrated plasma samples had statistically significant higher levels of peptide (1.446±0.018mmol/L) than the normal healthy plasma samples (1.436±0.019mmol/L).

($t(9) = 0.858$, $P = 0.012$).

The statistical bar graph is given in [Table/Fig 7].

Thus from the statistical results, it was quantitatively observed that the intensity of the absorbance which was obtained from the FTIR spectrum of the healthy and IgA myeloma plasma samples were significantly different.

[Table/Fig 8] shows the overlaid FTIR spectrum of the healthy blood plasma and the IgG myeloma infiltrated plasma samples.



[Table/Fig-8]: It shows the FTIR spectra of healthy and Ig G myeloma plasma

Group Statistics

FTIR Spectral Absorabnce	N	Mean	SD	Std. Error Mean
Healthy Plasma f3288	10	0.570	0.024	0.011
Ig G myeloma	10	0.406	0.047	0.013

Independent Sample Test

	Levene's Test for Equality of Variance		t-test for Equality of Means				
	F	Sig.	t	df	Sig (2 tailed)	Mean Difference	Std Error Difference
F3288 Equal variances assumed	3.984	0.063	7.26	9	0.001	0.1642	0.0226
F3288 Equal variances not assumed			9.56	8.5	0.002	0.1642	0.0171

[Table/Fig 9]: It shows the Independent t test report for healthy and Ig G myeloma plasma at 3288cm^{-1}

Group Statistics

FTIR Spectral Absorabnce	N	Mean	SD	Std. Error Mean
Healthy Plasma f3288	10	0.417	0.011	0.0049
Ig G myeloma	10	0.266	0.410	0.113

Independent Sample Test

	Levene's Test for Equality of Variance		t-test for Equality of Means				
	F	Sig.	t	df	Sig (2 tailed)	Mean Difference	Std Error Difference
F3288 Equal variances assumed	1.790	0.20	8.03	9	0.004	0.1519	0.0189
F3288 Equal variances not assumed			5.23	8.4	0.006	0.1519	0.0124

[Table/Fig 10]: It shows the Independent t test report for healthy and Ig G myeloma plasma at 1232 cm^{-1}

Group Statistics

FTIR Spectral Absorabnce	N	Mean	SD	Std. Error Mean
Healthy Plasma f3288	10	1.092	0.050	0.013
Ig G myeloma	10	0.978	0.061	0.027

Independent Sample Test

	Levene's Test for Equality of Variance		t-test for Equality of Means				
	F	Sig.	t	Df	Sig (2 tailed)	Mean Difference	Std Error Difference
F3288 Equal variances assumed	0.183	0.674	4.07	9	0.001	0.1139	0.0279
F3288 Equal variances not assumed			3.70	8.20	0.010	0.1139	0.0307

[Table/Fig 11]: It shows the Independent t test report for healthy and Ig G myeloma plasma at 1654cm^{-1} Group Statistics.

It was observed that though the shape of the FTIR spectra of both looked similar, there was a marked difference in the intensity of absorption between the two spectra, which indicated that a significant change had occurred in the immnunoglobulin content in the myeloma infiltrated plasma samples. It was also observed that when the blood plasma was infiltrated with IgG myeloma, there was a reduction in the production of the IgG protein content than that which was found in the normal, healthy blood plasma. This was observed by a decrease in the intensity of the absorption in the characteristic frequency peaks at 1654cm^{-1}, 3288cm^{-1} and 1232cm^{-1} respectively. Thus, the FTIR spectral data qualitatively explained the deterioration in the production of healthy IgG protein and in the formation of monoclonal IgG throughout the plasma, thereby indicating the presence of myeloma in the blood.

The statistical test was carried for the three characteristic functional groups, namely the peptide group (1654cm^{-1}), the carboxyl group (3288 cm^{-1}) and the phosphate group (1232 cm^{-1}) separately between the normal plasma and the two types of myeloma infiltrated plasma samples.

The Independent sample 't' test was first employed for the frequency peak at 3288cm^{-1} between the healthy plasma (variable 1) and the IgG myeloma (variable 2) plasma, as shown in [Table/Fig-9].

From [Table/Fig 9], the statistics report was given in the following format;

At 3288cm^{-1}, the IgG myeloma infiltrated plasma samples had statistically significant lower levels of carboxyl content (0.4061 ± 0.0475mmol/L) than the normal healthy plasma samples (0.5703 ± 0.0245 mmol/L). (t(9) =7.264, P =0.001).

From [Table/Fig 10], the statistics report was given in the following format;

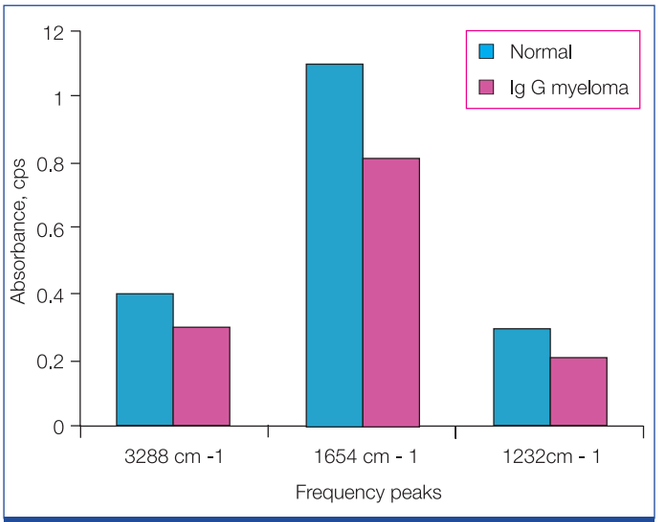
At 1232cm^{-1}, the IgG myeloma infiltrated plasma samples had statistically significant lower levels of phosphate content (0.266 ± 0.410mmol/L) than the normal healthy plasma samples (0.411 ± 0.011mmol/L).

(t(9) = 8.030, P = 0.004).

Similarly from [Table/Fig 11], the statistics report was given in the following format;

At 1654cm^{-1}, the IgG myeloma infiltrated plasma samples had statistically significant lower levels of peptide content (0.978±0.061mmol/L) than the normal healthy plasma samples (1.092±0.050mmol/L).

(t(9) =4.072, P =0.001).



[Table/Fig-12]: It shows the Bar Graph indicating the absorbance differences between normal and Ig G myeloma plasma

The statistical bar graph is given in [Table/Fig-12].

Thus from the statistical results, it was quantitatively observed that the intensity of the absorbance which was obtained from the FTIR spectrum of the healthy and the IgA myeloma plasma samples were significantly different.

DISCUSSION

Several studies had been carried out with respect to the estimation of the plasma protein concentrations, especially the immunoglobulins, IgG and IgA [10], [12], [13]. Petibois et al [10] have estimated them by comparing the FTIR spectrum of the pure compound and by integrating the FTIR spectrum of the plasma FTIR spectrum. The contents of different immunoglobulins in blood have been analyzed both qualitatively and quantitatively by Sankari et al [12] by employing the intensity ratio calculation among some specific absorption peaks in the FTIR spectrum of blood. Lamia Benezzeddine-Boussaidi et al [13] have combined FTIR spectroscopy and Partial Least Square (PLS) analysis for the quantification of the several immunoglobulins in blood plasma. Thus, in the present work, an attempt has been made for the estimation of the two different monoclonal proteins, IgG, and IgA in myeloma affected blood by employing FTIR spectroscopy. The spectral results were validated by applying statistical calculations, namely the independent't' test. Unlike the regular intensity ratio calculation, the independent't' test was able to provide a better significant ratio level for the IgG and IgA myeloma samples for a given spectral peak in the entire frequency region of 4000–400 cm^{-1}.

The results of the statistical bar graphs in [Table/Fig 7] and [Table/Fig 8], clearly indicate that a significant level was obtained at 1% (p=0.001) between IgA myeloma and the normal samples and between IgG myeloma and the healthy serum samples respectively. Thus, in the present work, it has been demonstrated that the independent 't' test can be very well combined with FTIR spectroscopy for the analysis of immunoglobulins in the normal and myeloma affected blood samples.

CONCLUSION

The role of FTIR spectroscopy in the clinical analysis of healthy, IgA and IgG myeloma blood plasma samples has been clearly demonstrated both qualitatively and quantitatively. It has been observed that a proliferation of monoclonal IgA leads to the formation of IgA myeloma and that a reduction in the production of the IgG proteins leads to IgG myeloma in the blood. Thus, this technique may be considered as one of the methods for analyzing the diagnosis of the myeloma activity.

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