FTIR and UV-Visible Spectral Study on Normal and Jaundice Blood Samples

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Passing from normal tissue to pathological tissue, cellular biochemistry changes. From a diagnostic and therapeutic point of view, it is fundamental to study the physical and chemical changes occurring in tissues and cells due to disease. Spectroscopy has received quite a lot of attention not only for understanding the biological nature of the disease, but also for the diagnosis of the disease in recent years. FTIR spectroscopy technique is employed to study the spectral differences in the serum of healthy and jaundice affected subjects. Based on the differences in the spectral signatures, four intensity ratio parameters are used in this study. The set of samples that have been subjected to FTIR study were subjected to UV-visible spectral studies too. The results from the latter one are used to ascertain the former results. Student's t-test has been used to anlayse the set of data obtained and to characterize the sera as healthy and diseased.

Key Words: Blood, Serum, Jaundice, FTIR Spectrum, UV-Visible spectrum.

INTRODUCTION

The human body may be likened to a chemical laboratory in which a wide variety of chemical reactions take place, each one nicely balanced in its relation to the other in a sequence. The difference between health and disease, organically speaking, usually means that disease is the unbalancing of these reactions and this unbalancing can most readily be detected by chemical examinations of the body fluids or excreta[1]. The fast and reliable determination of concentration of blood, plasma or serum constituents is the major requirement of clinical chemistry. The blood sera of jaundice affected people show high level of bilirubin. As blood undergoes physical and biochemical changes due to jaundice, the spectra show variations that can be related to the jaundice condition in the subjects. FTIR spectroscopy

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is able to detect biochemical changes caused by pathologies[2,3] also at a very early stage of the disease, due to the fact that any biochemical change in the tissue must precede any morphological manifestation of the disease itself. The ability to diagnose the early onset of disease, rapidly, non-invasively and unequivocally has multiple benefits. The present work is an attempt to characterize the blood serum as normal or jaundice affected with the aide of FTIR spectral techniques, while the UV-Visible spectra have been used to substantiate the results.

EXPERIMENTAL

2 mL of blood sample was collected by vein puncture from 20 healthy volunteers and 40 jaundice patients. Each blood sample was left to coagulate naturally without adding any anticoagulant agents for about 20 to 30 min. The serum was separated from every sample and centrifuged at a speed of 1200 rpm in REMI electric centrifuge. Each clear serum sample that separates after centrifugation was divided into three parts and transferred into sterile vials.

Clinical method: The bilirubin level in each sample stored in one set of vials was determined with the Bilirubin kit from Beacon diagnostics Pvt. Ltd. at Balaji Clinical Laboratory, Chennai. The bilirubin level was determined by adding the reagents with the serum in prescribed quantities and incubated in Biochem thermostat at 35 ºC. By using Photochem colorimeter, the absorbance of the serum reagent mixtures at 540 nm was used to quantify the total bilirubin level in both healthy and jaundice samples.

FTIR: For the FTIR spectral studies, to remove the water content, the second set of serum was lyophilized using freeze drier at Centralized Instrumentation Laboratory, Madras Veterinary College, Chennai. FTIR spectra of the lyophilized samples were recorded by KBr pellet method in the region 4000-400 cm^{-1} using FTIR ABB Bomem MB series at Dr. CEEAL Analytical lab, Chennai. Each measurement was repeated to ensure the reproducibility of the spectra. All the infrared spectra were baseline corrected and normalized. To account for the hygroscopic character of KBr substrate, absorbance scan on the KBr substrate, before and immediately after the measurements on the samples was performed. In this way, the absence of any unwanted wet trace that eventually could affect the analyzed spectra was confirmed.

UV-Visible: For UV-Visible spectroscopic measurements, the third set of each serum sample was diluted with normal saline to a concentration of 2 μL/mL and the spectra were recorded in the region 200-400 nm using UV-1601 Shimadzu UV-Visible spectrophotometer at Dr. CEEAL Analytical lab, Chennai.

RESULTS AND DISCUSSION

Many researchers have been working on the spectra of human blood and blood serum in order to characterize the blood[4-6] and attempted to understand the pathophysiological conditions of the body. Based on these studies, various important groups of vibrations were identified in the FTIR spectrum of blood sera (Fig. 1), interpreted and presented in Table-1.
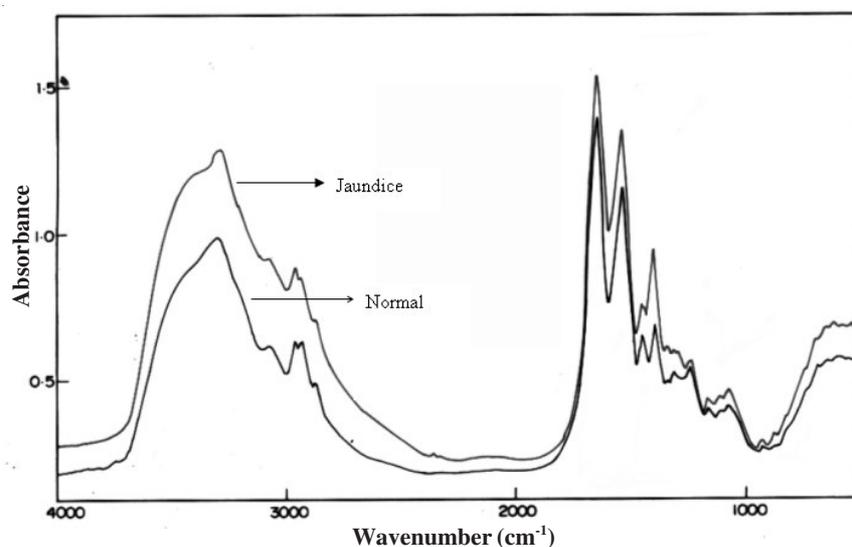

Fig. 1. Overlay of FTIR spectrum of healthy and jaundice blood serum samples

A N-H stretching is a localized mode, which usually appears as a doublet called as amide-A and amide-A′ or sometimes amide-B, respectively in the spectra of sera samples. The amide-A band is caused by the N-H stretching vibration and the amide-B is the first overtone of the amide II vibration. A strong band that is observed at about 3300 cm^{-1} and weaker one in the 3068 cm^{-1} region are designated as amide-A and amide-B bands, respectively. In hydrated state and/or in water solution the N-H stretching is partially screened with O-H stretching of H_2O molecules. A N-H stretching region around 3200 cm^{-1} in the infrared spectrum is usually strongly overlapped with the O-H stretching band of hydrating H_2O molecules. The serum samples having been subjected to lyophilization in this study, do not have any water component in them and hence the bands in this region could be attributed to amide vibrations themselves. The absorption of double bonds in acyl chains gives rise to a small band at 3010 cm^{-1}. In biological tissues, this absorption can be observed due to the presence of fatty acids, ceramides, triglycerides or phospholipids[7].

TABLE -1
INFRARED BAND ASSIGNMENT OF HUMAN SERUM

Absorption band (cm^{-1})	Assignment
3303	Amide-A band due to N-H stretching vibration
3068	Amide- B band due to overtone of Amide I band
2960	Asymmetric stretching vibrations of CH_3 of proteins and lipids
2873	Symmetric stretching vibrations of methyl group of proteins and lipids
2927	Asymmetric stretching vibrations of methylene group of proteins and lipids
2852	Symmetric stretching vibrations of methylene group of proteins and lipids
1652	Amide-I band mainly due to C=O stretching vibration amide groups
1543	Amide-II band due to the N-H bending vibration strongly coupled to the C-N stretching vibration of protein.
1455	Deformation vibrational modes of methyl groups
1403	Deformational vibrational modes methyl groups
1319	Deformational vibrational modes methyl groups
1240	Asymmetric PO_2 stretching vibration of phospolipids
1170	Ring vibrational mode of C-O-H and C-O-C bonds
1080	C-O stretch of glucose region
1035	C-O stretch of glucose region, a major glucose band
970	P-O symmetric stretching mode due to phospolipids
726	N-H out of plane bending with the contribution of C-N torsional vibration

In the spectral region between 3000 and 2800 cm^{-1} absorptions are detected arising from the presence of hydrocarbon chains-the asymmetric and symmetric stretching vibration of methyl group is located at 2960 and 2873 cm^{-1}, respectively, whereas the corresponding methylene stretching vibrations are observed at 2927 and 2852 cm^{-1} as weak intensity bands. The positions of the symmetric and asymmetric methylene stretch are diagnostic bands for the assessment of the phase state of the corresponding lipids in the tissue (discrimination between the gel and liquid crystalline phase) and fluidity of the hydrocarbon chains[8]. Manimegalai[9] has chosen the ratio between the absorbance of the two methyl bands as a criterion to separate diabetic from normal samples. There is a marked difference as reported by Gunasekaran and Sankari[10] in the intensity ratio between these methyl bands that has been used as a discriminating factor between the healthy subjects and those affected with some diseases like diabetes, cholesterol, thyroid and urea. The spectral region between 2700 and 1800 cm^{-1} is devoid of vibrations. Depending on whether the infrared spectral reording is performed under ambient air conditions or the infrared chamber

is flushed with dry nitrogen, more or less intense bands are detected between 2400 and 2300 cm^{-1}. These bands arise from the absorptions of carbondioxide[11].

Two intense absorptions arising from the amide group of proteins are detected between 1700 and 1480 cm^{-1}. The amide I mode is primarily a C=O stretching band. The amide II mode is an out-of-phase combination of largely NH in plane bending and C-N stretching and smaller contributions from C=O in-plane bending and N-C stretching. The intensity of these two bands varies considerably indicating the protein profile of the subjects. For this reason, Menikh *et al.*[12] have studied the bands in this region to understand the orientation of a peptide in a protein called Shiga toxin by employing polarized ATR-FTIR technique. The amide III mode is the in-phase combination of N-H in-plane bending and C-N stretching. Less intensive is contribution from C-C stretching and C-O bending.

In current study, the lower wavenumber region starts with a very intensive amide I band centered at 1652 cm^{-1}. The deformation motion of the NH group has dominant contribution in next band near 1543 cm^{-1} assigned as amide II band. Further, bands at 1455, 1403 and 1314 cm^{-1} in infrared spectrum are less intensive and belong to additional vibrations of amino groups and alkyl side chains present in proteins. For secondary structure determination, amide III band in the region near 1300 cm^{-1} is very important. The amide I and amide II bands of amino acids and hence that of proteins traced at 1656 and 1545 cm^{-1} and the bands at 1403 cm^{-1} and 1315 cm^{-1} which are due to the deformation vibrations of methyl groups of protein and lipids have been chosen by Gunasekaran and Sankari[10] to differentiate the healthy sera from the diseased ones.

Zeller *et al.*[13], Kajiwara *et al.*[14] and Back and Polavarapu[15] have identified several characteristic frequencies in the MIR glucose absorption spectra. Specific frequencies identified in blood by Zeller and co-workers[13] are 1365, 1152, 1109, 1080 and 1035 cm^{-1} for the study of glucose component. The 1365 cm^{-1} peak has been identified due to albumin, hemoglobin and other constituents, while 1080 cm^{-1} peak is identified in albumin and hemoglobin and the 1109 cm^{-1} peak is identified in hemoglobin. A band at 1035 cm^{-1} is mainly due to the glucose concentration in the blood analyzed. Also, in a quantitative relationship between glucose concentrations and corresponding absorption spectra changes within normal physiological ranges demon-strated by Blake *et al.*[16], 1035 cm^{-1} has been declared as the best predictor of glucose.

Below 1280 cm^{-1}, bands arising from the asymmetric and symmetric stretching of phosphate have been detected. The infrared band characteristic of phospholipids originates from the vibrations of phosphate moiety is centered at 1240 cm^{-1} and is due to the asymmetric stretching of P-O bond.

This has been used as an evidence to confirm the alteration caused by CCl_4 that originates the formation of fat in the liver[17]. A weak absorption near 960 cm^{-1} is considered to be due to P-O symmetric stretching of phospholipids.

UV-Visible: The UV-Visible spectroscopic study of blood in healthy and diseased has already been reported[10,18,19]. Various plasma and cellular constituents reflect physiological and pathological changes that take place in the tissues. This variation has been explored for understanding the spectral UV-Visible spectral differences between normal healthy sera and that affected with certain diseases. Proteins in the sera absorb strongly at about 280 nm due to the amino acids like tyrosine and tryptophan. The amide backbone of the proteins present in the blood absorbs[10] at 210 nm. The spectra of the sera from healthy and jaundice subjects showed peaks at these two positions, but with varying intensity.

Characterization of healthy and jaundice blood sera: The characteristic spectral features and patterns have been identified as the basis to distinguish spectra corresponding to healthy subjects from those corresponding to diseased patients. Hence, the infrared bands are used to derive qualitative information about the serum samples of healthy and various diseases. Moreover, there is a considerable difference in the absorbance levels of these bands between the normal and diseased sera, which aid quantitative treatment of these bands that can be readily noted from the overlay of the two FTIR spectra.

Researchers have tapped these differences in order to quantify several metabolites in blood and other biological tissues. In the current work, the complete spectral region was divided into three, which corresponds to the glucose region (925-1300 cm^{-1}), protein region (1500-1700 cm^{-1}) and lipids or fat region (2800-3300 cm^{-1}). In order to quantify the spectral differences in these regions, four intensity ratio parameters that show significant difference in Student's t-test have been chosen. As a support to the FTIR analysis, UV-Visible spectroscopic data too have been subjected to t-test. The internal standard calculations of the healthy blood serum samples and diseased blood serum samples treatment, along with the t-test results are presented in Table-2.

Data analysis: The intensity of amide A and amide B bands, which are due to the protein component of blood show considerable change in their intensity, but not in their respective positions in the healthy and jaundice sera samples. These bands can be located in the FTIR spectrum of pure bilirubin sample also. The intensity ratio parameter $R_1 = I_{3303}/I_{3068}$ is introduced to understand this change. It can be seen that the average value of ratio is 1.7130 with a standard deviation of ± 0.0297 for the healthy subjects. A conspicuous variation can be seen for the jaundice patients

TABLE-2
A COMPARISON OF INTENSITY RATIO PARAMETERS OF SERA
FROM HEALTHY SUBJECTS AND JAUNDICE PATIENTS AND
t-TEST RESULTS

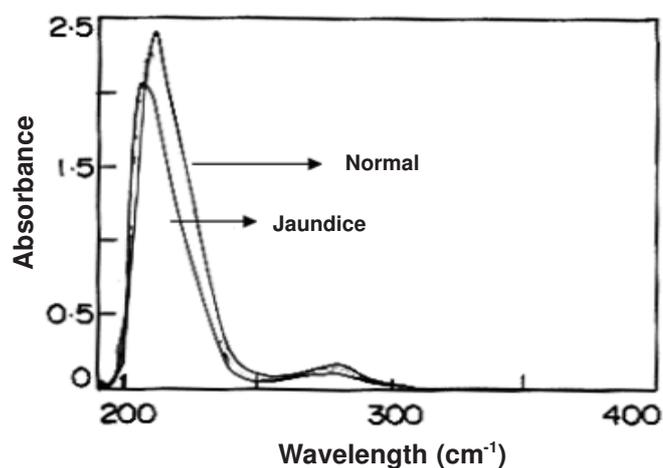| Intensity ratio parameters	Healthy samples		Jaundice samples		p-Value
	Mean	SD	Mean	SD	
$R_1 = I_{3303}/I_{3068}$	1.7130	0.0297	1.4613	0.1053	0.0003
$R_2 = I_{2963}/I_{2873}$	1.3806	0.0279	1.2157	0.0604	0.0002
$R_3 = I_{1652}/I_{1543}$	1.1565	0.0290	1.3193	0.0704	0.0001
$R_4 = I_{1170}/I_{1080}$	0.9296	0.0176	1.0072	0.0322	0.0004
$R = I_{280}/I_{210}$	0.0639	0.0026	0.0466	0.0040	0.0001

Fig. 2. Overlay of UV-Visible spectra of healthy and jaundice blood serum samples

with an average of 1.4613 ± 0.1053. This large difference can be traced to their very high bilirubin content. The mean value between the two sets is 1.5872 that can serve as a criterion to differentiate these two groups. The second intensity ratio parameter $R_2 = I_{2963}/I_{2873}$ is the ratio of the asymmetric and symmetric stretching of methyl groups can be seen in the spectrum of bilirubin too, as the latter contains four methyl groups attached to its four pyrrole rings. The amide I and amide II bands are the two most intense bands in the spectra of sera samples. With the onset of jaundice, liver loses its capacity to convert bilirubin into readily excretable derivatives. This increases the bilirubin level in the blood, which serves as a primary indicator of jaundice condition. This may be the reason for an increase in the absorbance

values of these two amide bands. A change in the internal ratio parameter $R_3 = I_{1652}/I_{1543}$ is hence may be perceived as an indicator of the said condition. Zeller and co-workers[13] have identified 1080 cm^{-1} peak in blood characteristic of albumin and hemoglobin. The 1170 cm^{-1} peak is mainly due to urea content in blood[11]. Though these bands are not very strong bands, they show a remarkable change in the spectra of jaundice sera compared to normal ones. The change in the value of the ratio $R_4 = I_{1170}/I_{1080}$, when subjected to Student's t-test, is highly significant for the classification of sera as healthy and jaundice inflicted. The UV-Visible absorbance values of the blood sera at 210 and 280 nm vary between healthy and jaundice sera samples. To study this variation, intensity ratio parameter $R = I_{280}/I_{210}$ was introduced. The average value of every intensity ratio parameter for healthy subjects varies from that of jaundice patients. p-Value from Student's t-test indicates that there is a significant difference between the means of the intensity ratio parameter calculated for healthy and diseased blood serum and hence stands as an evidence for the method adopted.

Conclusion

In this study, it has been demonstrated that the study of FTIR spectra of lyophilized sera may be used to differentiate between the healthy and jaundice patients. Some clear differences are observed in terms of spectral profiles, absorption bands, wave numbers and the intensity ratio parameters and satisfactory analysis has been made. The results from the UV-Visible spectral study substantiate the results obtained from FTIR spectral study for the characterization of sera as healthy and jaundice affected.

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ACKNOWLEDGEMENTS

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