ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: https://ijrps.com

FTIR- ATR, biochemical and histopathological studies on phenylhydrazine-induced hyperbilirubinemia in rats: A comparative study of diagnostic approaches

Saira Khatheeja*[1], Safiullah A[2], Chintaginjala Venkateswara Rao[3], Murad Alsawalha[4], Vishnu Priya V[5], Prabhakaran AR[6], Surapaneni Krishna Mohan[7]

[1]Department of Physics, JBAS College for Women, Chennai 600 018, Tamil Nadu, India
[2]S.S. Diagnostic Centre, Royapettah, Chennai-600 014, Tamil Nadu, India
[3]Department of General Studies, [4]Department of Chemical and Process Engineering Technology, Jubail Industrial College (JIC), P.O.Box 10099, Jubail Industrial City 31961, Kingdom of Saudi Arabia
[5]Department of Biochemistry, Saveetha Dental College & Hospitals, Saveetha Institute of Medical & Technical Sciences (SIMATS), Saveetha University, Chennai – 600 077, Tamil Nadu, India
[6]Department of Physics, Pachaiyappa's College, Chennai-600 030, Tamil Nadu, India
[7]Department of Biochemistry, Chettinad Hospital & Research Institute, Chettinad Academy of Research & Education (CARE), Rajiv Gandhi Salai (OMR), Kelambakkam, Chennai - 603 103, Tamil Nadu, India

Article History:

Received on: 09.06.2018
Revised on: 22.09.2018
Accepted on: 25.09.2018

Keywords:

FTIR-ATR,
Spectroscopic Studies,
Hyperbilirubinemia,
Wistar Rats,
Phenyl Hydrazine,
Induction

ABSTRACT

FTIR spectroscopy might be the right method to be focused as a diagnostic tool in detecting various diseases / clinical conditions by analyzing various functional groups concern with different components. The chemical bonding stretches reveal biochemical composition in blood/serum, different tissue homogenate including liver, kidney, heart, skeletal muscle, etc., helps in determining the detection and severity of various disease conditions. FTIR- ATR spectroscopic techniques were employed to evaluate biomarkers by studying the variations on biomolecule composition in blood/serum and organs of control and experimental animals. The absorbance peak ratio for serum of Wistar rats induced with hyperbilirubinemia was studied and I_{1453}/I_{1076} considered as a biomarker to evaluate hyperbilirubinemia in male Wistar rats induced with phenylhydrazine. Similarly, the peak ratio absorption with FTIR-ATR spectral studies for I_{2931}/I_{1453}, I_{2879}/I_{1240}, I_{1634}/I_{1538} and I_{1395}/I_{1040} shows that the organs like liver and kidney were highly significant in evaluating hyperbilirubinemia in male Wistar rats induced with phenylhydrazine. FTIR-ATR can be an alternate as well as an additional tool in clinical diagnostics compared to other techniques for understanding the biological nature of the disease condition for prognosis and management.

* Corresponding Author

Name: Mrs. Saira Khatheeja
Phone: +91-9962203047
Email: sairajbas@gmail.com

ISSN: 0975-7538
DOI: https://doi.org/10.26452/ijrps.v9i4.1725

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INTRODUCTION

Bilirubin is the degradation product of heme, which is derived from the haemoglobin of senescent erythrocytes and hepatic hemoproteins. Bilirubin is potentially toxic but is normally rendered harmless by binding to plasma albumin and efficient hepatic clearance. Excessive accumulation of bilirubin, as a result of enhanced production or impaired elimination, results in yellow discolouration of the skin, sclera, and mucous membranes, which is termed jaundice / icterus (Weizheng W *et al.,* 2005). The yellowish pigmentation of the skin,

the conjunctival membranes over the sclerae (whites of the eyes) and other mucous membranes are caused by hyperbilirubinemia (increased levels of bilirubin in the blood) (Weizheng W *et al.,* 2005).

Diagnostic tests are often used to evaluate the patient's pathological conditions and even to guide the selection of further tests and treatments. Over the years technological advances and automation have made tests easier and led to very accurate, more precise and timely results via. ELISA (Enzyme Linked Immuno Sorbent Assay), RIA (Radio Immuno Assay), IF (Immuno Fluorescence Assay), CLIA (Chemical Luminance Immuno Assay) and Spectrophotometer for clinical correlation (Dehghani F *et al.,* 2011; Rajasekaran *et al.,* 2013; Roos A *et al.,* 2005; Saud Alarifi *et al.,* 2012; Singer EA *et al.,* 1995; Swati R *et al.,* 2014). But these existing methods involve specific reagents and equipment and the costs of which have increased tremendously over the years. Further to perform tests, skilled experts are required to run the calibration and quality control. Also, these sophisticated equipment need special care and proper maintenance. Moreover, the multiple parameter analysis in research might require different analytical techniques with different methodologies. Hence researchers seek alternative methods to analyze those samples timely and economically with accuracy, precision and user-friendly.

Spectroscopic techniques can serve as right methods to focus as a diagnostic tool in detecting diseases via analyzing various functional groups concern with different components. Body fluids/tissues are considered as an attractive source for clinical markers. Since the constitution of body fluids have highly specific functional groups and because of their molecular structure analysis of these body fluids/tissues aids in diagnosing the mechanism diseases Spectroscopic techniques can be effectively employed as a diagnostic tool in clinical chemistry and it can be an alternate as well as an additional tool in clinical analysis. The study on biochemical composition evaluation by spectroscopic techniques can be used not only for understanding the biological nature of the disease, but also for the diagnosis of the disease. Spectroscopic studies on both blood parameters and organs in various disease conditions are very much limited. In recent years the analytical tools which has shown more consideration in biological sciences include Immunosensors (Peter *et al.,* 2001), Ultra Violet (UV)-Visible (Gunasekaran S., 2003; Gunasekaran S *et al.,* 2008), Raman (Hanlon EB *et al.,* 2000; Kees Maquelin *et al.,* 2000), NMR (Bales *et al.,* 1984; Bruce L., 1991), Vibrational spectroscopy (Catherine Kendall *et al.,* 2009), IR (Gamze Hosafc *et al.,* 2007; Heise HM *et al.,* 2000; Herbert Michael Heise A and Bittner Ralf Marbacj., 1998); Fourier transform infrared spectroscopy (Zanyar Movasaghi *et al.,* 2008) etc., FTIR-ATR spectroscopic imaging has significant advantages composed to many other imaging methods for the characterization of biomedical molecules because it relies on the characteristic absorbance of corresponding molecular vibration in the sample functional group of chemical compounds such as carbohydrates, cholesterol, triglycerides, albumin, proteins, as well as inter atom chemical bonds. The great advantage of FTIR spectroscopy is a high sensibility that permits the determination of many components even in a very small amount (Cyril P *et al.,* 2001; Frank CMD., 1993). The spectral based analysis is that it can be rapidly and simultaneously qualify several components without any specific reagents (Deleris G and Petibois C., 2003; Heise HM *et al.,* 1998; Shaw RA *et al.,* 1998; Yan-Ping Zhou *et al.,* 2007). FTIR has been used as a research tool to analyze the chemical components of gallstone (Kleiner J *et al.,* 2002), diagnosis of diabetes (Saira Khatheeja *et al.,* 2016), analysis of serum immunoglobulins (G Sankari *et al.,* 2010), blood samples of renal failure patients (Renugadevi *et al.,* 2009; Saira Khatheeja *et al.,* 2016), as diagnostic tool in hypothyroidism (Prabhakaran AR *et al.,* 2016), analysis of multiple myeloma (Sankari G *et al.,* 2011), hypercholesterolemia (Saira Khatheeja *et al.,* 2018), protein plasma in blood (Kleiner J *et al.,* 2002), breast cancer diagnosis (Dimitrova M *et al.,* 2009) and other diseases (Dimitrova m *ET AL.,* 2009; G Sankari *et al.,* 2010; Gunasekaran S *et al.,* 2008; Gunasekaran S *et al.,* 2010; Haas SL *et al.,* 2010; Kmatchi S *et al.,* 2016; Mackanos MA and Contag CH., 2009; Renuga Devi TSR *et al.,* 2009). Based on certain drawbacks and advantages FTIR-ATR spectroscopic method is chosen among the other spectroscopic methods in diseases diagnosis. This study objective was to determine morpho-functional alterations in blood serum and different organs (liver, kidney, lung, heart and muscle) using FTIR-ATR spectral analysis of animals with the hypothyroid condition in addition to biochemical, histopathological studies.

MATERIALS AND METHODS

Procurement of Animals

For the experimental purpose male Wistar rats weighing about 100-150 gm were purchased from Sapthagiri Livestock and Organic Research Farm, Tamilnadu, India, were housed and maintained in Animal House of the Saveetha Medical College, Saveetha University, Thandalam Chennai, India according to the guidelines for care and use of experimental animals and approved by Committee for the Purpose of Control and Supervision of

Experiments on Animals (CPCSEA). Study proposal was approved by the Institutional Animal Ethical Committee. The animal house was maintained at an average temperature (24.0°C±2°C) and 30-70 % RH, with 12hr. light-dark cycle (lights on from 8.00 a.m. to 8.00 p.m.). The experimental rats received human care and were fed with commercial pellet diet and the animals were acclimatized for one week before the start of the experiment.

Experimental Design of Induction Study

Six rats per cage were housed in polypropylene cages (32.5×21×14) cm lined with raw husk which was renewed every 48 h. In the current study, experimental animals were divided into two groups. The group 1 is the healthy control rats were provided with normal saline and with fed with normal diet and maintained parallelly till the completion the study period and group 2 Wistar rat to induce hyperbilirubinemia and was achieved by subcutaneous injection of Phenylhydrazine (75mgs/kg body weight) daily for 14 days as per the standardized procedure of Lang Zhang, 2015. The phenylhydrazine used is 97% pure (100gm) obtained from Sigma Aldrich, Mumbai to conduct induction study.

Collection and Processing of Blood Samples

At the end of each experiment, the Wistar rats have fasted overnight. Blood samples of the Wistar rats were withdrawn on from the heart under mild anaesthesia before killing and collected in plain tubes. Blood serum was separated by centrifugation at 3000rpmfor15 min preserved for further biochemical analysis. For FTIR-ATR spectral analysis, the serum samples were properly preserved in ice bags and immediately transported to the wet lab for spectral studies.

Collection and Processing of Tissue Samples

The fresh homogenization leads to loss of cell structure A high-quality preparation of a tissue homogenate represents an optimum compromise in diagnosis Instead of homogenization of whole tissue, lyophilization was one to analyse the spectral variations at molecules level and so it is necessary properly handle excision of the organ. At postmortem, heart, liver, Lungs, kidney Muscle, etc., were immediately excised from adult rats and washed with saline and refrigerated (-20 ^{o} C). Lyophilization of organs tissue was done by Scanvac cool, safe, 55-9 Denmark vacuum concentrator at Central Institute of Brackishwater Aquaculture, Indian Council of Agricultural Research, Govt. of India, Chennai. Further, the frozen, dried samples grounded to a powder using mortar and pestle preserved in desiccators containing silica gel till FTIR-ATR spectral analysis. For histological studies, excised organs after saline wash were fixed in 10 % buffered neutral formalin for further processing and analysis

Assay of Blood serum and Organs of Hyperbilirubinemia experimental Wistar

Biochemical analyses are useful in disease diagnosis studies because the animal cells/tissues are made up of basic components like protein, lipid, carbohydrates, vitamins, minerals, water etc. When certain types of cells are damaged, they may leak enzymes into the blood, where they can be measured as indicators of cell damage including the heart, muscle, kidney, liver, and lung etc. The amount of biomolecules in the blood is directly related to the extent of tissue damage**.**

Quantification of Biomolecules by Routine Methods

The quantitative analysis of blood components, as well as tissues components of different organs, is a major field in clinical chemistry. The composition/components are the preferred indicators with respect to the pathophysiological condition of the system. The blood serum was analyzed in a reputed clinical laboratory in Chennai. Quantitative analysis of biomolecules carried out include glucose, urea, creatinine, calcium, phosphorus, uric acid, total bilirubin, SGOT, SGPT, total protein, albumin, cholesterol, triglyceride, HDL (High-Density Lipoprotein) etc., by enzymatic assay method using respective commercial diagnostic kits [35-46]]The serum total T4, T3 and TSH concentrations were determined by ELISA method (detection kits provided by Transasia, Zemun, SCG) (Berger AJ *et al.,* 1997; Voller A *et al.,* 1978).

FTIR – ATR Analysis of blood serum and organs

FTIR-ATR spectral measurements of serum samples and lyophilized tissues of different organs of experimental male Wistar rats were carried out at Sophisticated Analytical Instrumentation Facility (SAIF-SPU), St. Peter's University, Avadi, and Chennai-600 054, using Perkin Elmer Spectrum-Two FTIR Spectrophotometer with Attenuated Total Reflectance accessory having highly reliable and single bounce diamond as its Internal Reflectance Element (IRE).

50 ul Serum sample was placed on the IRE crystal and the water content on the serum samples was air dried for water evaporation to eliminate the stray absorption bands due to water and holder is mounted in sample window of the spectrophotometer. The sampling window is scanned as the background and 32 scans are co-added with a spectral resolution of 1cm^{-1}. As the sample absorbs IR radiation at certain frequencies, the resultant reflected radiation(or) evanescent wave will be attenuated (altered) in regions of the infrared spectrum where

Table 1: Changes in biochemical composition levels in Blood serum of control and rat induced with Phenyl Hydrazine

Biochemical Parameters	Control	Phenyl Hydrazine Injected
Total Bilirubin (mg/dl)	0.67 ±0.39	3.91±.81***
Direct Bilirubin (mg/dl)	0.23 ± 0.11	1.91. ±11***
SGOT (IU/L)	19.65 ±8.7	51.7 ±12.81**
SGPT (IU/L]	23.33±9.1	70.5 ±14.1**
ALK.P	76.7±10.8	159.8± 15.8**
Total Protein (gm/dl)	7.16±2.89	7.31±1.81*
Albumin(gm/dl)	4.35±1.11	4.5±.96†
Globulin (gm/dl)	2.86±0.77	3.06±0.50†
Plasma Glucose mg /dl	112.7±5.42	129±2.10†
Uric acid (mg /dl)	4.8±1.11	4.85± 0.3†
Calcium(mg /dl)	7.9±2.43	8.19±1.72†
Total Cholesterol (mg /dl)	167±31.10	188±5.72†
Triglyceride(mg /dl)	120±30.89	144±3.11†
HDL Cholesterol(mg /dl)	47±9.80	39±3.09†
T3(ng/dl)	161±15.87	178+7.32†
T4(µ/dl)	5.9±1.20	5.99+.90†
TSH (mIU/dl)	4.8±1.33	4.97± 4.05†
Urea(mg/dl)	40±7.10	41±6.40†
Creatinine (mg/dl)	0.88±0.4	0.91±0.5†

Table 2: FTIR – ATR Vibration Band assignment of blood serum of control and phenylhydrazine-induced male Wistar rat blood serum

Wave Number (cm^{-1})	Vibration Band assignment
3283	N-H stretch due to protein and Urea
3071	Amide B band due to an overtone of Amide I band and olefinic group C-H stretch Lipids of Unsaturated fatty acid
2961	C-O-C Asymmetric / Symmetric stretch vibrations of Methyl group of Protein and C-H Lipids (Fatty acids and TGL)
2931	Asymmetric stretching vibrations of Methylene group of protein and lipids
2879	Symmetric stretching vibrations of Methylene group of protein and lipids
1742	C=O group of cholesterol ester (HDL)
1634	Aryl substituted C=C Amide I band mainly due to C=O, C=N and N-H stretching
1538	Amide II band due to NH vibrations stretching coupled with C-N stretching vibrations in protein.
1453	Asymmetric bending vibrations of lipids, proteins of CH3 groups.
1395	Free Amino Acid and Fatty Acids;
1313	Amide III erythrocyte
1240	Amide III and Asymmetric PO2 stretching vibration mode of Nucleic acid
1165	Ring vibration mode of C-O-H and C-O-C bonds (CO-O-C) asymmetric Cholesterol ester, Phosphoric acid
1115	Stretching vibration of glycogen
1076	C-O characterization stretching of glucose
1040	Primary alcohol C-O stretch glucoseMuco Polysaccharide
934	Ribose, Phospholipids
532	Poly sulfidic S-S stretch in cystic acid

the sample absorbs energy (Baulsir CF and Simler RJ., 1996; Katon JE., 1996).

Histopathological Study

The 10 % formalin fixed different tissue samples of organs including Lung, Heart, Liver, Kidney Muscle, etc., were dehydrated in ascending grades of ethyl alcohol, cleared in xylene (Palmero S *et al.,* 1989; Krasilnikova OA *et al.,* 2002) and embedded in paraffin wax. Sections of 5µm thickness were cut by rotator microtome.

At least 25 tissue sections for each organ were assessed. Tissue Sections of 5µm thickness (Palmero S *et al.,* 1989) were cut by rotator microtome. The sections were processed and passed through graded alcohol series, stained (Bancroft JD and Stevens A., 1999 Luna LG.,1968). The selections were

processed and passed through graded alcohol series stained with hematoxylin in and eosin, cleaned in xylene and coverslipped in DPX. Histological examination was done under 10X magnification using Trinocular Research Zeiss Microscope (Gottingen, Germany) and further obtained from 10 random microscopic fields per animal at X45 and X100objective.

Statistical Analysis

All statistical analysis was performed using 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). 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. The obtained data sets were statistically evaluated and focused on the spectral ranges that correspond to the structure and conformation of proteins and other biomolecules.

RESULTS AND DISCUSSION

Biochemical Evaluation

Hemolysis induced by phenylhydrazine has been used to evaluate the increased bilirubin production. Exposure to phenylhydrazine cause damage to red blood cells, potentially resulting in anaemia and consequentially hyperbilirubinemia (Chakrabarti S *et al.,* 1990; Itano HA *et al.,* 1976; Stern A., 1989). The induction study results implicated oxidation products of phenylhydrazine and not proteolytic degradation as major contributors to phenylhydrazine-induced protein damage in red blood cells ghosts. Phenylhydrazine directly oxidizes haemoglobins and has a property to exacerbate G6PD deficiency and the precipitation of unstable haemoglobins. Treatment of rats with phenylhydrazine 5mg / kg body weight for two weeks resulted in the development of hyperbilirubinemia as a result the total bilirubin and direct bilirubin level were found to be higher (3.9 1±0.81 mg/dl and 1.91± 0.65) and statistically highly significant ($p<0.001$) among control and hyperbilirubinemia experimental male Wistar rat. This study also reports that phenyl hydrazine induces hepatic damage resulted moderately raised serum AST, ALT and ALP (51.7±12.81, 70.5±14.1 and 159.8±15.8) respectively and the values obtained are statistically significant ($p<0.01$). The increased serum ALT, AST and ALP indicating chemical induced hepatocellular toxicity is significant and showed as a specific marker of liver injury due to toxic drugs, alcohol and virus (Sherlock S and Dolley J., 2002), paracetamol (Sastry AVS *et al.,* 2011), acetaminophen (Suresh Kumar S V *ET AL.,* 2006). In contrast, another literature documented that phenyl hydrazine did not increase AST and ALT levels in serum of rats (Lan Zhang *et al.,* 2015).

Further treated with phenylhydrazine developed significant hepatic damage and oxidative stress which was observed from a substantial increase in serum cholesterol and triglycerides level also significantly elevated. This is indicative cellular leakage and loss of functions integrity of the cell membrane in the liver (Raju S *et al.,* 2011; Sapakal V *et al.,* 2008). Moreover, phenylhydrazine administration in rats significantly increased the level ($p<0.05$) of serum creatinine (1.6±0.5) and urea (58±6.4) as compared to control rat (serum creatinine0.88± 0.5 and urea - 40±7.10) which support the earlier studies (Ahlfors CE and Shapiro SM., 2001; Brodersen R., 1980). Of the other parameters like total protein, albumin, globulin, glucose, uric acid, calcium, HDL Cholesterol, T3, T4, TSH, etc., studied were do not show significant difference among control and phenylhydrazine injected rats (Table 1).

FTIR-ATR Spectral studies

FTIR-ATR spectral analysis carried out to identify the various analytes in the experimental samples of blood serum for phenylhydrazine-induced hyperbilirubinemia in male Wistar rat. To evaluate the clinical status of the hyperbilirubinemia, overlaid spectral pattern of blood serum of control and induced hyperbilirubinemia in male Wistar are represented in Figure 1.

FTIR Vibration Band assignment of blood serum of control and phenylhydrazine-induced male Wistar rat blood serum and tissues given in Table 2. The study shows that the amide I band is caused by N-H stretching vibrations and amide II band is caused by N-H stretching coupled with C-N stretching vibrations of protein are recorded at 1634 cm^{-1} and 1538 cm^{-1} respectively.

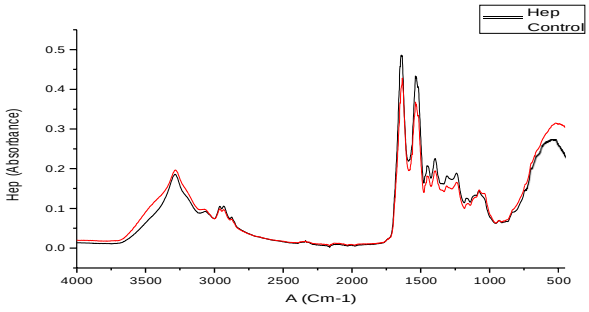

Figure 1: FTIR-ATR spectral overlaid pattern of blood serum of healthy control and phenylhydrazine-induced hyperbilirubinemia in experimental male Wistar rat

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

Peak ratio	Wave Number (cm $^{-1}$)	Absorbance		P Value
		Control	HBI	
(Protein) $_{sym\&asym}$vib-(Lipids –FA-TGL) /HDL cholesterol ester	$I_{2961}/_{1742}$	4.2393±0.177	5.3080±0.179	0.0001
Amide I / Ribose –phospholipid	$I_{1634}/_{934}$	6.1594±0.174	6.9971±0.179	0.001
Amide II/ Mucopoly-Glu-str.	$I_{1538}/_{1040}$	2.6661±0.177	3.4345±0.175	0.0003
$(Lipoprotein)_{asym}$.vib./Glucose-str	$I_{1453}/_{1076}$	1.2394±0.176	1.4406±0.176	0.0005

The p values calculated are 0.0001, 0.001, 0.0003 and 0.0005. These findings were almost similar to that of earlier studies [11, 31] Based on this (Protein) $_{sym\&asym}$ vib- (Lipids –FA-TGL) / HDL cholesterol ester, amide II / Mucopoly-Glu-str. and $(Lipoprotein)_{asym}$.vib./ Glucose-str is considered as a biomarker to evaluate hyperbilirubinemia in male Wistar rat.

Table 4a: The changes in the FTIR-ATR band internal peak ratio calculation for various molecules in the lyophilized organs of control and induced HB experimental rats

Peaks ratio	KIDNEY Absorbances		P value	LIVER Absorbances		p-value	LUNG Absorbances		p-value
	Cont.	HBI		Cont.	HBI		Cont.	HBI	
I_{2931} $(Lipoprotein)_{sym}$ I_{1453} $(Lipoprotein)_{asym}$.vib.	0.8694 + 0.008	1.3694 ± 0.011	0.0001	0.7192 ± 0.008	0.8720 ± 0.0098	0.0005	0.7799 ± 0.048	0.7143 ± 0.07	0.017
I_{2879} $(Lioprotein)_{sym.}$vib I_{1240} - (Nucleoprotein-Amide III)$_{asym.}PO_4$	0.6635 ± 0.010	1.0475 ± 0.024	0.0002	0.8703 ± 0.013	0.9640 ± 0.013	0.0059	0.5237 ± 0.075	0.5008 ± 0.040	0.3911
I_{1634} Amide I I_{1538} Amide II	0.6513 ± 0.008	0.9510 ± 0.008	0.0001	0.6047 ± 0.010	0.7941 ± 0.005	0.0008	0.6221 ± 0.061	0.6644 ± 0.060	0.1259
I_{1395} FAA-FFA I_{1040} (Cystic acid)s-s –str.	0.8508 ± 0.008	0.9403 ± 0.007	0.0008	0.8307 ± 0.036	1.0045 ± 0.190	0.0079	0.7123 ± 0.031	0.6843 ± 0.054	0.0255

Table 5b: The changes in the FTIR-ATR band internal peak ratio calculation for various molecules in the lyophilized organs of control and induced HB experimental rats

Peaks ratio	HEART Absorbances		P value	MUSCLE Absorbances		P value
	Cont.	HBI		Cont.	HBI	
I_{2931} $(Lipoprotein)_{sym}$ I_{1453} $(Lipoprotein)_{asym}$.vib.	0.8476± 0.019	0.8214± 0.020	0.0026	0.8715± 0.014	0.8546± 0.013	0.0005
I_{2879} $(Lioprotein)_{sym.}$vib I_{1240} - (Nucleoprotein-Amide III)$_{asym.}PO_4$	0.5582± 0.014	0.5168± 0.020	0.00012	0.5930± 0.015	0.5732± 0.016	0.0021
I_{1634} Amide I I_{1538} Amide II	0.5910± 0.440	0.5732± 0.010	0.3363	0.6831± 0.014	0.6536± 0.016	0.0686
I_{1395} FAA-FFA I_{1040} (Cystic acid)s-s –str.	0.7424± 0.019	0.7264± 0.020	0.0082	1.1748± 0.41	1.1286± 0.015	0.2432

These observations were a contrast to the study of earlier literature (Gunasekaran S *et al.,* 2008) documented that the amide –A band is caused by the N-H stretching vibration and the amide-B was the first overtone of the amide II vibration. Specific frequencies identified in the blood (Zeller H *et al.,* 1989) 1365, 1152, 1109, 1080 and 1035cm^{-1} for the study of glucose component and this study shows specific frequencies identified from 1076 - 1040 cm^{-1}. Also, in a quantitative relationship between glucose concentrations and corresponding absorption spectra changes within normal physiological ranges demonstrated below 1240 cm^{-1}. A weak absorption near 934cm^{-1} is considered due to P-O symmetric stretching of phospholipids where the authors observed that the absorption due to the presence of fatty acids, ceramides and ribose and phospholipids (Vishnu Priya V *et al.,* 2010).

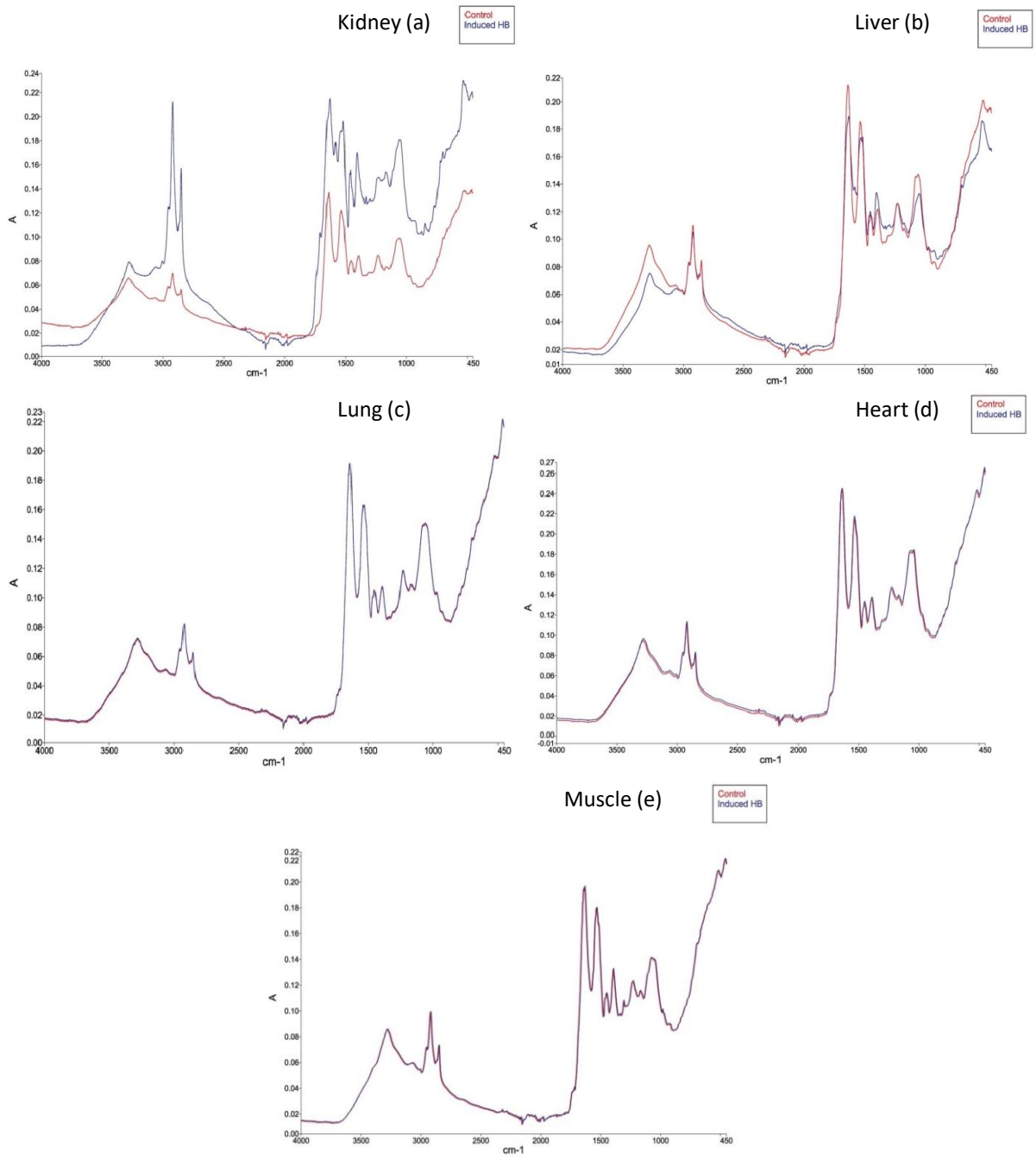

Figure 2(a-e): Histological studies on phenylhydrazine-induced Hyperbilirubinemia in male Wistar rat

The study also documented that elevated biomolecules like bilirubin, AST, ALT, ALP, cholesterol and triglycerides which are needed ostensibly for the repair of damaged cell organelles and tissue regeneration

Internal peak ratio calculation for serum

The ratio of internal parameter calculation for absorbance is predominantly higher in phenylhydrazine-induced hyperbilirubinemia than the control indicates the changes in biochemical variations after induction with phenylhydrazine injection (Table 3). In order to quantify spectral differences in these regions, four intensity ratio parameters show highly significant (P<0.001). The absorbance obtained in hyperbilirubinemia rat are significantly high for (Protein) $_{sym\ \&\ asym}$ vib-(Lipids –FA-TGL) / HDL cholesterol ester (I_{2961}/I_{1742}), amide I / Ribose –phospholipid (I_{1634}/I_{934}), amide II / Mucopoly-Glu-str. (I_{1538}/I_{1040}) as well as $(Lipoprotein)_{asym}$vib. and Glucose-str. (I_{1453}/I_{1076}) compared with absorbance in control healthy rats.

Internal peak ratio calculation for organs

The overlaid FTIR-ATR spectral bands in different organs of Wistar rat control and induced hyperbilirubinemia disease are represented in Figure 2 (a-e). The internal ratio calculated for the lyophilized organs are $(lipoprotein)_{asym}$ str./ $(lipoprotein)_{asym}$.vib. (I_{2931}/I_{1453}), $(lipoprotein)_{sym.}$vib/

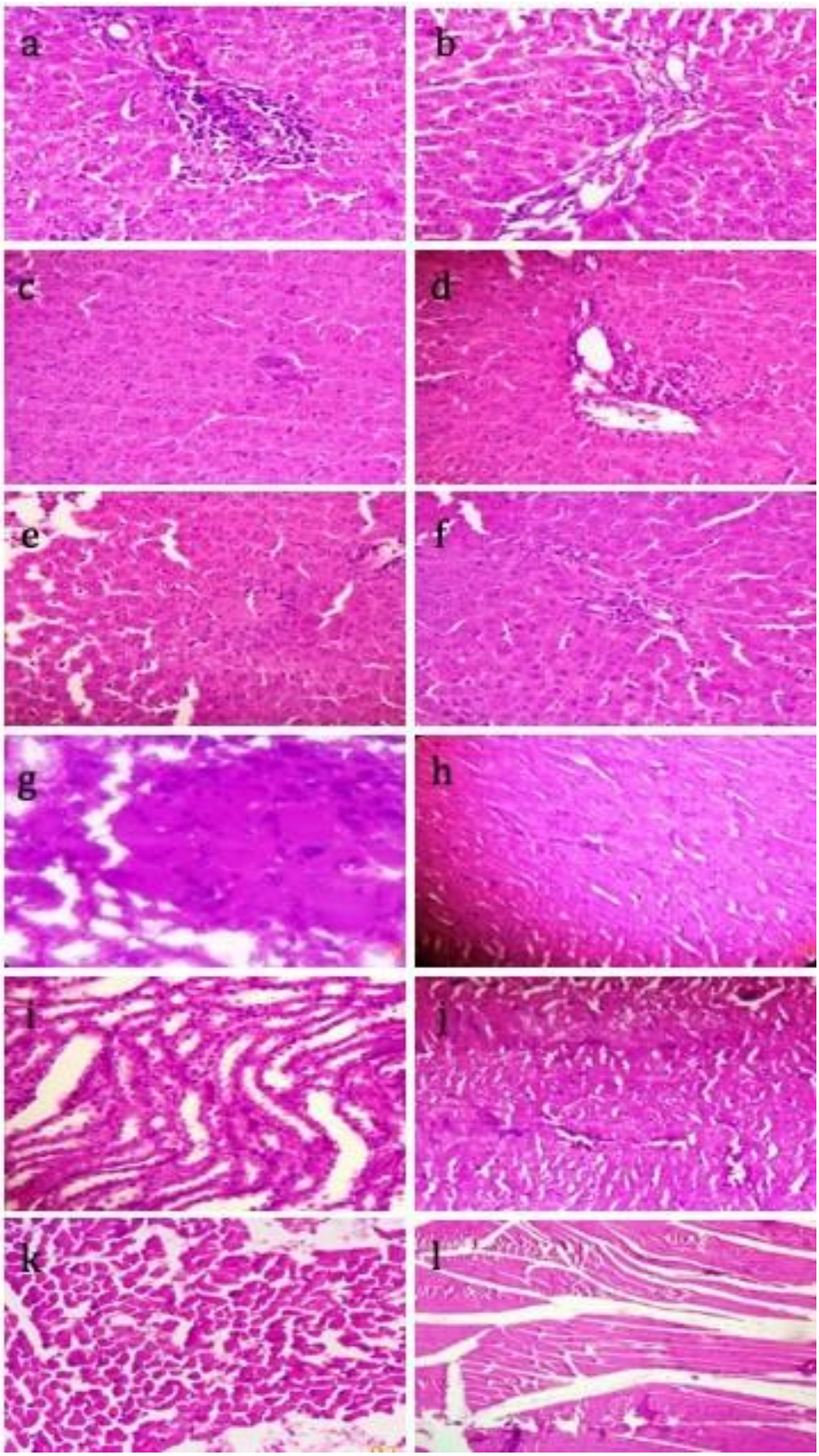

Figure 3(a-i): Histological studies on phenylhydrazine-induced Hyperbilirubinemia in male Wistar rat

(amide III)$_{asym}PO_4$of NA, ($I_{2879}/_{1240}$), amide I /amide II(I_{1654}/I_{1538}) and FAA-FFA / mucopoly glu-str. (I_{1395}/I_{1040}) shows that the liver and kidney were affected by cellular damages and the same was agree with histopathological studies (Table 4a&b). The statistical analysis shows that the p values obtained were <0.001 for all the intensity ratio parameter or liver and kidney. The organs like lung,

heart and muscle do not show major cellular changes, but minor changes at cellular level might be due to exposure of phenylhydrazine during the experimental period. Though the lung, heart and muscle were not affected much on exposure with phenylhydrazine, statistically the values obtained for most of the intensities absorbances peak ratio were significant for heart and muscle not significant for lung.

Histological studies on phenylhydrazine-induced HB in male Wistar rat

The chemical composition of the normal tissue entirely different from pathological tissue due to cellular biochemistry changes on different conditions. Phenylhydrazine induced jaundice in Wistar rat liver showing moderate to severe periportal inflammation and delicate Periportal fibrosis. Besides liver cell necrosis with inflammation and bridging necrosis extending between central to the portal area also noticed where they observed that paracetamol (acetaminophen) induced hepatotoxicity. The liver sections of phenylhydrazine treated rats showed centrilobular necrosis (Figure 2 a-e).

Further hepatotoxic effect of phenylhydrazine is due to binding to the macromolecule and induces peroxidative degradation of the membrane lipids of the endoplasmic reticulum that rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxidase, which in turn produces toxic aldehyde that causes liver damage (Sapakal V *et al.,* 2008). This was evident by an increase lipid peroxidation in phenylhydrazine induction. The lung shows aggregate induction with foamy histiocytes (Figure 3 j) The other organs like kidney, heart and muscle Figures 3 i, k&l) does not show any significant changes with phenylhydrazine treatment.

CONCLUSION

Induction of rats with phenylhydrazine showed the development of hyperbilirubinemia with significantly increased total bilirubin and direct bilirubin level ($p<0.001$), AST, ALT and ALP ($p<0.01$) and urea and creatinine ($p<0.05$) compared with control male Wistar rat concludes that the phenylhydrazine induces significant hepatic cellular toxicity. The absorbance peak ratio for hyperbilirubinemia studied were (protein) $_{sym\ \&\ asym}$ vib-(lipids –FA-TGL) / HDL cholesterol ester (I_{2961}/I_{1742}), amide I /ribose–phospholipid (I_{1634}/I_{934}), amide II / mucopoly-glu-str. ($I_{1538}/_{1040}$) as well as $(lipoprotein)_{asym}$.vib. and glucose-str. (I_{1435}/I_{1076}) compared with absorbance in control healthy rats. Based on these I_{1453}/I_{1076} considered as a biomarker to evaluate hyperbilirubinemia in male Wistar rat induced with phenylhydrazine. The internal peak ratio calculated for $(Lipoprotein)_{asym}$.str./ $(lipoprotein)_{asym}$.vib. (I_{2931}/I_{1453}), $(lipoprotein)_{sym}$.vib/ $(amideIII)_{asym}.PO_4$ of NA, ($I_{2879}/_{1240}$), amide I /amide II(I_{1654}/I_{1538}) and FAA-FFA /mucopoly glu-str. (I_{1395}/I_{1040}) concluded that the marked improvement in the chemical composition changes in the liver and kidney as a result of treatment with phenylhydrazine. The liver sections of phenylhydrazine showed centrilobular necrosis hepatotoxic effect. The peak ratio absorption with FTIR-ATR spectral studies for I_{2931}/I_{1453}, I_{2879}/I_{1240}, I_{1634}/I_{1538} and I_{1395}/I_{1040} shows that the organs like liver and kidney were highly significant in evaluating hyperbilirubinemia in male Wistar rat induced with phenylhydrazine.

Acknowledgement

The Authors would like to thank the Saveetha University for providing Animal House facility to conduct experiments and the Dean, Sophisticated Analytical Instrumentation Facility (SAIF- SPU), St. Peter's University, Avadi and Chennai-600054, using PerkinElmer Spectrum-Two FTIR Spectrophotometer with Attenuated Total Reflectance accessory.

SUMMARY AND CONCLUSION

In this study, the experimental animals were induced with phenylhydrazine agents to induce hyperbilirubinemia. 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. The absorbance peak ratio for serum of hyperbilirubinemia Wistar rats studied and I_{1453}/I_{1076} considered as a biomarker to evaluate hyperbilirubinemia in male Wistar rat induced with phenylhydrazine. Similarly, The peak ratio absorption with FTIR-ATR spectral studies for I_{2931}/I_{1453}, I_{2879}/I_{1240}, I_{1634}/I_{1538} and I_{1395}/I_{1040} shows that the organs like liver and kidney were highly significant in evaluating hyperbilirubinemia in male Wistar rat induced with phenylhydrazine. By virtue of this research study, it has been evident that the FTIR-ATR can be an alternate as well as an additional tool in clinical analysis compared with other techniques for understanding the biological nature of the disease for prognosis and management.

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