Original Article

# Evaluation of metformin hydrochloride in Wistar rats by FTIR-ATR spectroscopy: A convenient tool in the clinical study of diabetes

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## Abstract

**Introduction:** The IR absorption patterns (in $cm^{-1}$) provide the basis to distinguish among the constituents and to separately quantify as well as qualify them and they possess many advantages such as very small sample volume requirement, good precision over entire physiological range, avoid of costly disposables, wealth of information from a single spectral measurement. The efficacy of anti-diabetic drug metformin hydrochloride as used to treat diabetic-induced Wistar rats and their sera were analyzed by FT-IR (ATR) in absorption mode. **Materials and Methods:** The present work was attempted in the study of normal and antidiabetic regimen-treated rat blood samples using FTIR spectroscopy by the attenuated total refl ectance (ATR) sampling technique. The biomolecule characteristics were measured as intensity ratio parameter (IRP) values and interpreted. **Results:** To quantify the results three IRPs such as R1, R2 and R3 were calculated, respectively, for lipid, protein, and glucose. The glucose IRP value R3 showed, 0.3802, 0.3304, and 0.2847, respectively, for diseased, metformin-treated, and normal rats. **Conclusion:** The IRP values for glucose are compared to the glucose level obtained by using a glucometer. This study can be conveniently used in diagnostic procedures, patient compliance assessment, and efficacy evaluation of metformin hydrochlorides.

**Key words:** Fourier transform, Infrared spectroscopy, antidiabetic, metformin, blood glucose

## INTRODUCTION

Infrared (IR) spectroscopy has proved to be a powerful tool for studying biological molecules, and its applications to biological problems are continually expanding. One of the great advantages of IR spectroscopy is that virtually any sample, at any state, can be studied. Biomolecules such as proteins, carbohydrates, peptides, lipids, biomembranes, pharmaceuticals, foods, and both plant and animal tissues have all been successfully characterized by using IR spectroscopy without any reagents. The obtained results can be used in the qualitative and quantitative investigation of blood, serum, saliva, urine, etc.[1-5]

The quantitative analysis of blood is a major field in the clinical chemistry, and its composition is the preferred indicator with respect to the pathophysiological condition of the system. IR analysis of biomolecules is based on the rich IR absorption patterns that characterize the analytes themselves. These absorption patterns (in $cm^{-1}$) provide the basis to distinguish among the constituents and to separately quantify as well as qualify them. They possess many advantages such as very small sample volume requirement, good precision over entire physiological range, avoid of costly disposables, wealth of information from a single spectral measurement.[6-12]

The literature revealed that the multicomponent assay of human plasma has been evaluated for the determination of blood substrates.[13] Shaw *et al.* has used IR analysis for the

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quantification of urea, creatinine, and total protein from the dried blood sample.[14] Petibois *et al.* determined glucose in the serum samples.[15] Gunasekaran *et al.* studied lipid disorder in women's blood samples and renal failure blood samples.[16] Moreover, continuous monitoring of blood samples during chemotherapy in cancer treatment by FTIR spectroscopy is found to be highly informative and useful.[17] FTIR spectroscopy coupled with statistical calculation has been employed by the researchers in the estimation of plasma proteins. Clinically, immunofixation electrophoresis is carried out for the estimation of immunoglobulin levels in blood, which involves a lot of analytical reagents, costly disposables, and manpower.[18,19] The present work was attempted in the study of normal and antidiabetic regimen-treated rat blood samples using FTIR spectroscopy by the attenuated total reflectance (ATR) sampling technique.

ATR is a technique for obtaining IR spectra of samples that are difficult to deal with, such as solids of limited solubility, films, threads, pastes, adhesives, and powders. In this process, a beam of radiation entering a crystal will undergo total internal reflection when the angle of incidence at the interface between the sample and the crystal is greater than the critical angle. The critical angle is a function of the refractive indices of the two surfaces. The beam penetrates a fraction of a wavelength beyond the reflecting surface and when a material that selectively absorbs radiation is in close contact with the reflecting surface, the beam loses energy at the wavelength where the material absorbs. The resultant attenuated radiation is measured and plotted as a function of the wavelength by the spectrometer and gives rise to the absorption spectral characteristics of the sample.

Metformin hydrochloride [Figure 1] improves hyperglycemia primarily by suppressing glucose production by the liver (hepatic gluconeogenesis). The "average" person with type 2 diabetes has three times the normal rate of gluconeogenesis; metformin treatment reduces this by over one third. Metformin activates AMP-activated protein kinase (AMPK), an enzyme that plays an important role in insulin signaling, whole body energy balance, and the metabolism of glucose and fats; activation of AMPK is required for metformin's inhibitory effect on the production of glucose by liver cells.

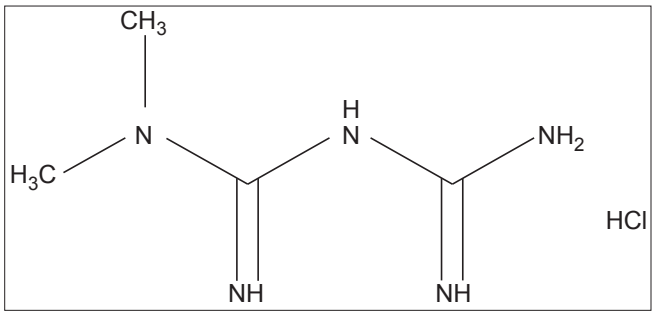


**Figure 1:** Structure of metformin hydrochloride

## MATERIALS AND METHODS

A FT-IR spectrophotometer BRUKER-Alpha E was used. The standard metformin was obtained from Hetero Drugs Pvt. Ltd with certificate of analysis with 99.94%. An Accu-Chek Active glucometer was used to detect the glucose level. All experiments and protocols described in this study were approved by the Institutional Animal Ethical Committee (IAEC) (Protocol No: 878/ac/05/CPCSEA/008/2013, Govt. of India**)**.

### Animals

Wistar albino rats (male) were housed in groups of three (I, II, and III) and maintained under standard conditions (27 ± 2°C, relative humidity 44-56%, and light and dark cycles of 10 and 14 h, respectively) and fed with standard rat diet and purified drinking water *ad libitum* for 1 week before and during the experiments. In this study, diabetes was induced in group I and II, by using subcutaneous injection of alloxan (100 mg/kg) as per the procedure reported by Katsumata *et al.* The animals were allowed to drink glucose solution overnight to overcome the drug-induced hypoglycaemia. The equivalent dose of metformin was given in saline to group II, and remaining group I was kept as disease control. The glucose levels for all the groups were estimated by the glucometer for every 30 min over the period for 2 h and periodically blood samples were withdrawn.

### Sample collection

The blood sample was collected from group I, II, and III at 30 min, 1 h, 2 h intervals. The blood samples were centrifuged immediately to get serum. The serum was separated and stored at − 10°C prior to analysis.

### Spectral measurement for serum by FTIR spectroscopy and ATR technique

First, the instrument was calibrated and cleaned properly and the background correction was done for air and carbon dioxide. The IR spectrum was recorded for various serum samples obtained at different intervals, and all the spectra were recorded in absorption mode and overlapped for interpretation. The region and absorption band for glucose, protein, and lipid were identified based on Tables 2 and 3.

### Statistics

Data were statistically converted to mean ± SD and the IRP for the normal, disease, and treated groups was calculated. There were three parameters calculated namely R1, R2, and R3, respectively, for the lipid region (2961), protein region (1637), and glucose region (1109): R1 = I (2961)/I (2846)–IRP for lipid; R2 = I (1645)/I (1551))–IRP for protein;

**Table 1: Comparative IR band assignment of Rat Serum and Human Serum**

| Rat serum | | Human serum | |
|---|---|---|---|
| Frequency ($cm^{-1}$) | Assignments | Frequency ($cm^{-1}$) | Assignments |
| 702 | N–H out of plane deformation of protein | 702 | N–H out of plane deformation of protein |
| 1018 | C–O stretch of β-anomer | 1035 | C–O stretch of β-anomer |
| 1079 | C–O stretch of α-anomer | 1079 | C–O stretch of α-anomer |
| 1109 | Endocyclic C–O–C vibration | 1107 | Endocyclic C–O–C vibration |
| 1153 | Ring vibration modes of C–O–C and C–O–H bonds | 1153 | Ring vibration modes of C–O–C and C–O–H bonds |
| 1169 | C–O stretch of COH tyrosine protein | 1169 | C–O stretch of COH tyrosine protein |
| 1315 | $CH_2$ vibrations of α- anomer | 1315 | $CH_2$ vibrations of α- anomer |
| 1365 | $CH_2$ vibrations of β -anomer | 1365 | $CH_2$ vibrations of β-anomer |
| 1400 | $CH_3$ symmetric bending vibration of protein | 1400 | $CH_3$ symmetric bending vibration of protein |
| 1435 | C–H bending | 1435 | C–H bending |
| 1456 | $CH_3$ asymmetric bending vibration of protein | 1456 | $CH_3$ asymmetric bending vibration of protein |
| 1645 | C=O stretching/C–N stretching/N–H bending of proteins (amide I band) | 1655 | C=O stretching/C–N stretching/N–H bending of proteins (amide I band) |
| 1551 | N–H bending strongly coupled with C–N stretching (amide II band) | 1548 | N–H bending strongly coupled with C–N stretching (amide II band) |
| 2846 | $CH_2$ symmetric stretching | 2851 | $CH_2$ symmetric stretching |
| 2871 | $CH_2$ asymmetric stretching | 2871 | $CH_2$ asymmetric stretching |
| 2922 | $CH_3$ symmetric stretching of proteins and lipids | 2922 | $CH_3$ symmetric stretching of proteins and lipids |
| 2961 | $CH_3$ asymmetric stretching of proteins and lipids | 2956 | $CH_3$ asymmetric stretching of proteins and lipids |
| 3366 | N–H asymmetric stretching of secondary amides of proteins | 3400 | N–H asymmetric stretching of secondary amides of proteins |

**Table 2: Absorbance value for different absorption region at 1 hr after metformin treatment**

| Animals ($n$ = 5) | R1 (Lipid) | | R2 (Protein) | | R3 (Glucose) | |
|---|---|---|---|---|---|---|
| | 2961 $cm^{-1}$ | 2846 $cm^{-1}$ | 1645 $cm^{-1}$ | 1551 $cm^{-1}$ | 1109 $cm^{-1}$ | 1018 $cm^{-1}$ |
| I | 0.071 ± 0.002 | 0.064 ± 0.001 | 0.289 ± 0.003 | 0.153 ± 0.001 | 0.081 ± 0.001 | 0.213 ± 0.001 |
| II | 0.055 ± 0.001 | 0.054 ± 0.001 | 0.215 ± 0.002 | 0.101 ± 0.002 | 0.062 ± 0.001 | 0.182 ± 0.002 |
| III | 0.051 ± 0.002 | 0.053 ± 0.002 | 0.114 ± 0.001 | 0.054 ± 0.001 | 0.043 ± 0.001 | 0.151 ± 0.001 |

**Table 3: Intensity ratio parameters (IRP value)**

| Animals ($n$ = 5) | R1 (Lipid) | R2 (Protein) | R3 (Glucose) |
|---|---|---|---|
| I (Disease: A) | 1.1093 | 1.8888 | 0.3802 |
| II (Metformin: B) | 1.0183 | 2.0287 | 0.3304 |
| III (Normal: C) | 0.9444 | 2.1111 | 0.2847 |

R3 = I (1109)/I (1018)–IRP for glucose. The results are shown in Tables 2 and 3.

## RESULTS AND DISCUSSION

The mid-IR spectral region (4000 to 400 $cm^{-1}$) was used largely for both qualitative and quantitative analysis of lipids, proteins, and glucose. IR spectra for rat serum were analyzed in absorption mode. Assessment of rat serum for respective R1, R2, and R3 absorption region for lipid, protein, and glucose was carried out based on human serum. Assessment of IR region for human and rat serum is shown in Table 1. The IR spectrum is the essence of reflection of the IR color pattern characteristics of the sample. The basis of quantification is that each constituent contributes a unique absorption pattern to the overall spectrum governed by the unique set of molecular vibration characteristics of each distinct molecular specimen. The quantitative measurement is carried out by the relative intensities of the various contributing spectra to the unique absorption profile. A representative FTIR absorption spectrum of the rat serum sample is shown in Figure 2. The vibrational peaks are mainly dominated by the protein constituents of the sample rather than glucose and lipids.

### IR spectral analysis of healthy sera

The vibrational band at 3400 $cm^{-1}$ is due to N–H stretching vibration of the secondary amides of protein. The asymmetric and symmetric stretching vibrations of the methyl group of the proteins and lipids are present in the region 2800-3050 $cm^{-1}$. It emerges from C–H stretching vibrations of fatty acyl chains of all cellular lipids. The other two vibrational bands in the C–H stretching region are found to be present near 2922 and 2851 $cm^{-1}$, which are due to the asymmetric and symmetric stretching vibrations of the methylene group. The essential amide bands dominate in the region 1500-1700 $cm^{-1}$. The strong absorption band at 1655 $cm^{-1}$ is assigned to the C = O stretching of amide I of the proteins. The presence of a band at 1548 $cm^{-1}$ is due

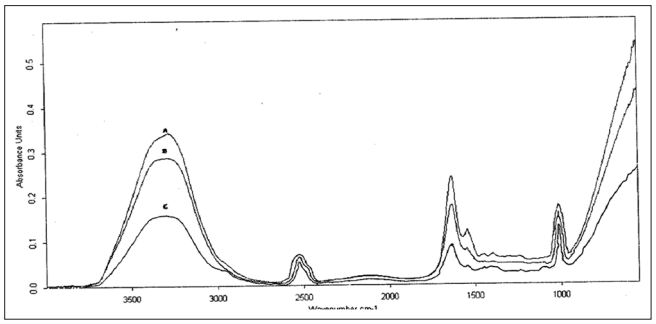


**Figure 2:** Overline IR-ATR spectra. (a) Disease control; (b) metformin treated (1 h); and (c) normal control

to the N–H bending vibrations of amide that are strongly coupled to the C–N stretching vibrations of the protein amide group. The peaks at 1456, 1400, and 1315 $cm^{-1}$ arise mainly from the asymmetry and symmetry deformations of methyl groups of proteins. The peak at 1400 $cm^{-1}$ may also be due to the COO- stretching of ionized amino acid chains, suggesting an increased contribution from carboxylate. The lipid phosphate band due to the asymmetric P–O stretching of $PO_2$ occurs at 1240 $cm^{-1}$. The absorption bands at 1325, 1365, and 1435 $cm^{-1}$ arise due to the C–H bending of $CH_2$ groups in $\alpha$ and $\beta$ anomers. For glucose, the optional frequency range of 925-1250 $cm^{-1}$ is used, since the mid-IR spectrum of glucose includes several strong absorption bands in this region. The absorption peaks present at 1169, 1153, 1107, 1079, and 1035 $cm^{-1}$ are due to the different C–O stretching vibrations of C–O–H and C–O–C bonds. The medium strength vibrational band present at 702 $cm^{-1}$ is assigned to N–H out of plane bending with the contribution of C–N torsional vibrations. Comparative characteristic absorption value in $cm^{-1}$ for human and rat serum was shown in Table 1.

As the IR spectrum exhibits vibration band characteristics of the various group frequencies, the spectrum of a normal Wistar rat serum, metformin-treated rat serum, and diseased rat serum were recorded and their over line spectra are shown in Figure 2. To quantify the results three IRPs such as R1, R2 and R3 were calculated, respectively, for lipid, protein, and glucose. The intensity ratio was calculated with respect to wave number based on the absorbance using the following formula:

- R1 = I (2961)/I (2846) – IRP for lipid
- R2 = I (1645)/I (1551) – IRP for protein
- R3 = I (1109)/I (1018) – IRP for glucose.

The results of the intensity ratio are shown in Tables 2 and 3 as well as in Figure 3. R1 and R2, respectively, for lipid and protein were 1.109 and 1.888 for diabetic induced rats and 0.9944 and 2.111 for normal rats. In the case of metformin-treated rats, the IR*P* value for R1 is very nearer to disease-induced rats and indicated the ineffectiveness

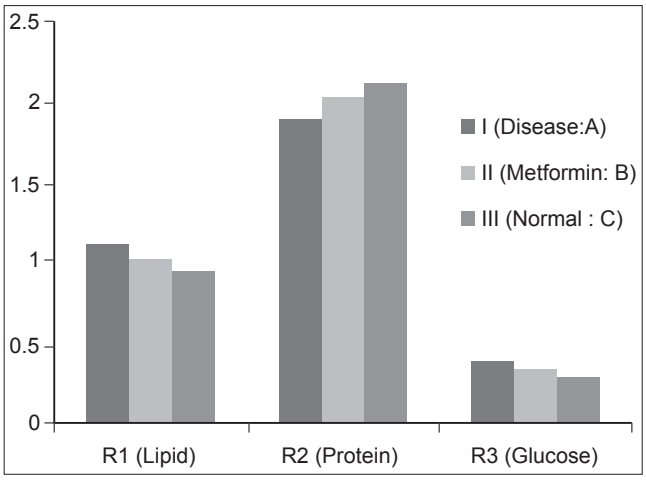


**Figure 3:** IRP values (R1(lipid), R2 R3) for the treatment schedule

of metformin on the lipid level in serum. However, the R2 value was nearer to the normal rats. The glucose IR*P* value R3 showed, 0.3802, 0.3304, and 0.2847, respectively, for diseased, metformin-treated, and normal rats. In comparison with normal rats, it indicated the elevated blood sugar level in the diabetic condition and efficacy of metformin by reduction in the blood glucose level of diabetic-induced rats. The results of IR*P* values were compared with the results obtained by using the glucometer.

## CONCLUSION

The role of FTIR spectroscopy in the clinical analysis of normal and diabetic blood samples is clearly demonstrated. The use of the ATR sampling technique provides us the FTIR tool as the most convenient diagnostic tool as well as evaluating in diabetes. Compared to IR*P* values among rats, it is clearly indicated the elevated blood sugar level in the diabetic condition and efficacy of metformin in treatment of diabetic-induced rats. The IR*P* values were compared with the glucose level obtained using the glucometer. This can be more conveniently employed in diagnostic procedures, patient compliance assessment, and efficacy evaluation of the antidiabetic drug in diabetes.

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