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COMPARISON OF THERAPEUTIC EFFICACY OF METFORMIN AND GLIBENCLAMIDE AS A SINGLE AGENT OR IN COMBINATION BY FOURIER TRANSFORM INFRARED SPECTROMETRY

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ABSTRACT

FTIR spectroscopy has emerged in recent years as the analytical method of choice in understanding the biological nature of the disease and as a biodiagnostics tool. Since blood serves as the primary metabolic transport system in the human body, its composition is the preferred indicator with respect to the pathophysiological conditions of the patient. The biochemical changes in diabetic blood have been determined using FTIR spectroscopy. This article explains the IR spectroscopy based analytical method to compare the efficacy of monotherapy and combination therapy of type-2 diabetes drugs, namely metformin and glibenclamide.The technique uses the IR spectra of air dried fasting and post prandial sera treated with metformin, glibenclmide and their combinations, respectively. The characteristic spectral features and patterns have been identified as the basis to distinguish spectra corresponding to healthy subjects from those corresponding to diabetic patients. The therapeutic efficacy of the two drugs is analyzed by comparing the absorption level of the diabetic sera treated with either metformin or glibenclamide or the combination. To quantify the spectral differences between healthy, fasting and post prandial sera, three intensity ratio parameters are introduced. Also, to substantiate the findings, one way ANOVA analysis has been carried out. The combination therapy was found to be better than monotherapy. Results matched with those obtained by clinical methods. FTIR spectroscopy is a sensitive analytical tool to compare the therapeutic efficacy of drugs by quantitative analysis of the spectra of diseased sera, treated with the respective drugs.

Key words: FT-IR, Metformim, Glibenclamide, Diabetic.

INTRODUCTION

Fourier transform infrared (FTIR) spectrometry is a global, sensitive and highly reproducible physicochemical analytical technique that identifies structural moieties of

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biomolecules on the basis of their IR absorption.[1,2] Because biomolecules are determined by their unique structure, each biomolecule will exhibit a unique FTIR spectrum, representing the vibrations of its structural bonds.[3] Blood being the primary metabolic transport system in the body, its composition indicates the metabolic condition of the patient. These biochemical changes of blood are especially significant in the case of diabetes mellitus. FTIR analytical applications have allowed determination of blood contents using various materials and sample preparations. Concentrations of glucose,[1, 2, 4, 5] total proteins, creatinine, urea, triglycerides[1] and cholesterol[2] in blood, plasma or serum have been determined with clinical accuracy. Elimination of water by drying the fluids makes it feasible to employ FTIR spectroscopy for analysis, without using expensive cuvettes or attenuated total reflection accessories.

Type 2 diabetes is a multifactorial metabolic disease characterized by abnormalities at multiple organ sites. These defects include insulin resistance and insulin deficiency[6,7]. When lifestyle changes fail to reduce glucose levels to the desirable range, pharmacological therapy is often necessary to achieve optimal glycemic control in the management of type 2 diabetes. Inzucchi[8] has made a systematic review of oral antihyperglycemic agents for type 2 diabetes assessing the efficacy of monotherapy and combination therapy of several drugs using evidence based treatment strategies.[8] Also therapeutic comparison studies of metformin with sulfonylurea was done by Hermann et al.[9] This article focuses on the use of FTIR technique to compare the efficacy of monotherapy and combination therapy of metformin and glibenclamide, the most commonly used antihyperglycemic agents.. This analytical method is based on the comparative study of the intensity ratio parameters (IRP) introduced in the FTIR spectra of healthy, fasting and post prandial air dried sera treated with metformin, glibenclamide or combination of both respectively.

EXPERIMENTAL

Sample collection

Serum samples were collected from seven healthy adult volunteers and twenty one type-2 diabetes patients in Swami Vivekananda Diagnostic Laboratory, Chennai, India. The mean age of the patients was 51.7619 ± 5.8813. (range 40-65 years). Fasting samples were collected between 0730 and 0830 hours, after an overnight fast of 10 hours. Post prandial sampling was done from the same set of patients on the same day exactly after 2 hours of their breakfast. Since better results are obtained from the sera, the blood samples were centrifuged immediately for 10 minutes to get sera. A set of seven diabetic patients

were undergoing treatment with metformin 500 mg twice daily. Another set were consuming glibenclamide 5 mg twice daily. The dosage level for combination therapy was 500 mg metformin and 2.5 mg glibenclamide twice daily.

Sample preparation

A volume of 1 mL of serum was diluted with an equal volume of 4 mg/L aqueous potassium thiocyanate (KSCN) solution. 20 µL of each diluted sample was spread evenly over the surface of a circular KBr window (9 mm diameter and 2 mm thickness). All the specimens were air dried for 30 minutes prior to recording IR spectra. The serum samples are air dried to form a thin uniform film on KBr pellets, in order to avoid the strong broad absorption bands of water over wide portion of the mid IR region.[1]

Spectral measurements and analysis

The FTIR spectra have been recorded in the range 4000 - 400 cm^{-1} using BRUCKER IFS 66 V FTIR spectrophotometer for air dried sera at Sophisticated Analytical Instrumentation Facility, IIT, Chennai, India. The FTIR module has globar and mercury vapor lamp as the sources and the interferometer chamber has KBr and Mylar beam splitters. The sampling technique used was KBr pellet method. The FTIR spectrometer had a resolution of 1 to 0.1cm^{-1} with the facilities of signal averaging, signal enhancement; baseline correction and other spectral manipulations are also possible with multitasking OPUS software on the dedicated PC/INTEL-4.

The spectra of the sera were baseline corrected and they were normalized to acquire identical area under the curves. The maximum values of the corresponding characteristic bands were noted.

Statistics

Data are expressed as mean ± SD. Generally spectral investigations have been made using different univariate methods involving ratio of peak intensities, [10] shift in band position,[11] and area under the curve.[12] According to Beer-Lambert's law, the absorbance of a constituent is directly proportional to its concentration. Assuming that the law is obeyed here, the present work involves one way ANOVA of IRP and the statistical significant difference among the drugs were obtained using Tukey HSD test having $p < 0.05$.

RESULTS AND DISCUSSION

Clinical data in the study of efficacy

The combination of an insulin sensitizer, e.g. metformin, and an insulin secretagogue, e.g. glibenclamide, represents a rational choice of agents for inclusion within a combination tablet, as they address the underlying endocrine disturbances present in patients with type 2 diabetes. In addition, the pharmacokinetics of metformin and glibenclamide both support twice-daily dosing. Furthermore, the co-administration of metformin and glibenclamide is proven to effectively control glycaemia in patients with type 2 diabetes.[13,14] Indeed, metformin and glibenclamide probably represent the most widely used free combination of oral antidiabetic agents worldwide.

Clinical studies have evaluated metformin-glibenclamide combination tablets in patients with type 2 diabetes with uncontrolled glycaemia despite previous treatment with metformin,[15] sulphonylureas,[16]or diet and exercise[17]. The combination tablet controlled HbA_{1C} and plasma glucose significantly more effectively than monotherapy in each of these populations.[15,17, 18] All of the studies involved titration of study treatments to achieve an optimal glycaemic response. In each case, the greater efficacy of the metformin-glibenclamide combination tablets was achieved with lower mean final doses of metformin and glibenclamide, compared with metformin and glibenclamide monotherapy. This suggests that synergy between metformin and glibenclamide within the combination tablets led to greater blood glucose-lowering efficacy, compared with monotherapy, despite a lower exposure to oral antidiabetic drugs. Moreover, the combination tablets controlled post prandial plasma glucose more effectively compared with each component monotherapy[16,18] consistent with the timing of delivery of glibenclamide to the blood stream.

FTIR spectral acquisition

For the set of 28 air dried diabetic and 10 healthy sera FTIR spectra, the mean signal to noise ratio in the 2100-2200 cm^{-1} region was 680 ± 20. The baselines between repeated samples were also homogenous, varying by 0.011 ± 0.006 at 4000 cm^{-1}. Results were comparable for the set of FTIR spectra of the air dried sera samples from 21 patients.

FTIR spectral analysis of healthy sera

The representatives normalized FTIR absorption overlay spectrum of normal, fasting and post prandial diabetic serum samples treated with metformin, glibenclamide and their combination in the frequency region 4000 - 400 cm^{-1} are presented in Fig. 1-3,

respectively.

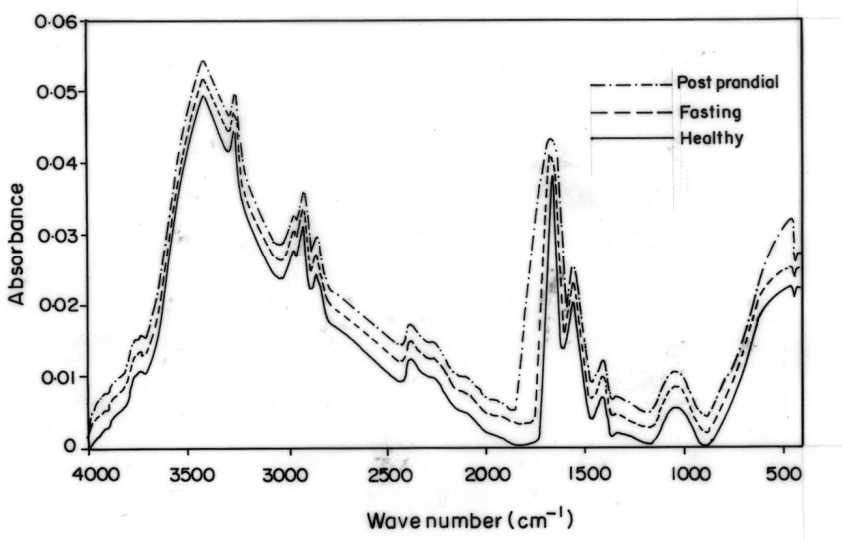

Fig. 1: FTIR overlay spectrum of blood sera treated with metformin

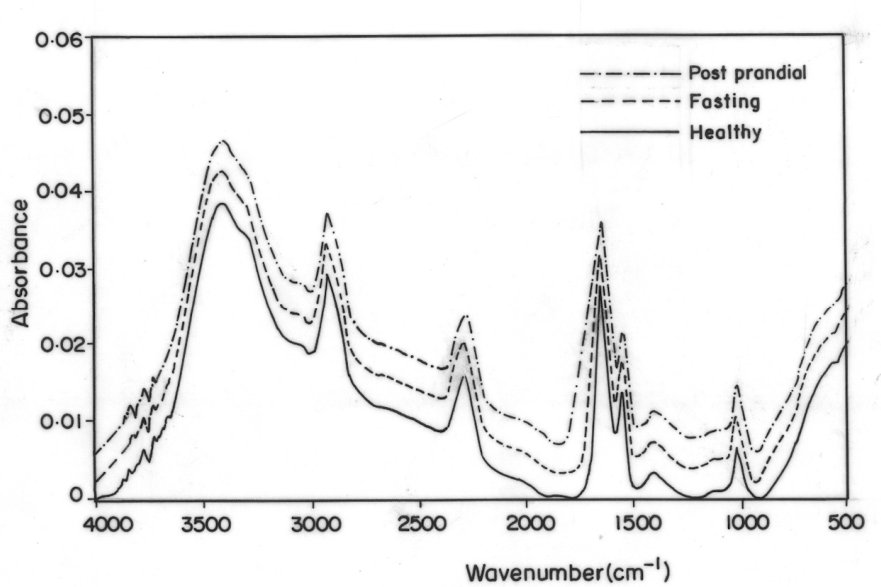

Fig. 2: FTIR overlay spectrum of blood sera treated with glibenclamide

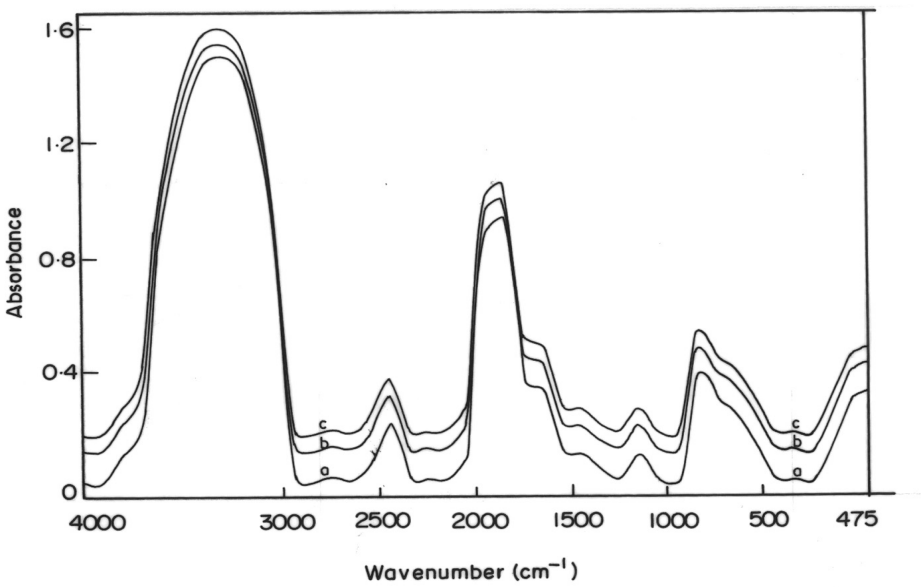

Fig. 3: FTIR overlay spectrum of blood sera treated with metformin and glibenclamide in combination

The infrared spectrum of serum provides useful information of biomolecules like structure, functional groups, types of bonds and its interactions. A satisfactory vibrational band assignment of absorption bands of the spectra is done with the idea of the group frequency of the various constituents of the serum samples.[19, 20, 21] Table 1 presents the vibrational band assignment of human serum.

Table 1. Infrared band assignment of human serum

Frequency (cm^{-1})	Assignments
702	N-H out of plane deformation of protein
1035	C-O stretch of β-anomer, (major glucose band)
1079	C-O stretch of α- anomer
1107	Endocyclic C-O-C vibration
1153	Ring vibrational modes of C-O-C and C-O-H bonds
1169	C-O stretch of COH tyrosine protein

Cont…

Frequency (cm^{-1})	Assignments
1315	CH_2 vibrations of α- anomer
1365	CH_2 vibrations of β-anomer
1400	CH_3 symmetric bending vibration of protein
1435	C-H bending
1456	CH_3 asymmetric bending vibration of protein
1655	C=O stretching/ C-N stretching/ N-H bending of proteins (Amide I band)
1548	N-H bending strongly coupled with C-N stretching (Amide II band)
2851	CH_2 symmetric stretching
2871	CH_2 asymmetric stretching
2922	CH_3 symmetric stretching of proteins and lipids
2956	CH_3 asymmetric stretching of proteins and lipids
3400	N-H asymmetric stretching of secondary amides of proteins

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 CH stretching vibrations of fatty acyl chains of all cellular lipids.[21] 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.[22] The essential amide bands dominate in the region 1500 - 1700 cm^{-1}. The strong absorption band at 1655 cm^{-1} is assigned to C=O stretching of amide I of the proteins.[23] The presence of band at 1548 cm^{-1} is due to the N-H bending vibrations of amide II that are strongly coupled to the C-N stretching vibrations of the protein amide group. The peaks at 1456 cm^{-1}, 1400 and 1315 cm^{-1} arise mainly from the asymmetric and symmetric deformations of methyl groups of proteins.[24] The peak at 1400 cm^{-1} may also be considered due to COO^{-} stretch of ionized amino acid chains, suggesting an increased contribution from carboxylate.[25] 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 CH bending of CH_2 groups in α-and β-anomers.[26] For glucose the optimal 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 considered to be 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.

FTIR spectral analysis of diabetic sera

Insulin, being an anabolic hormone, has profound effects on the metabolism of carbohydrates, fats and proteins, significant changes in serum composition with regard to the latter classes of substances are expected. The IR spectroscopy can provide qualitative and quantitative information on such biomolecules. In this study, the complete mid IR spectral region 4000-400 cm^{-1} was divided into three, namely glucose region (925-1259 cm^{-1}), protein region (1500-1700 cm^{-1}) and lipid region (2800-3400 cm^{-1}).

Table 2. Results of one way ANOVA for the sera treated with metformin

Region	Serum			f Value	p Value
	Healthy	Fasting	Post prandial		
Lipid	1.7170 ± 0.1341^{b}	1.3460 ± 0.0742^{a}	1.3916 ± 0.0752^{a}	29.503	0.000^{**}
Amide	1.9292 ± 0.1933^{b}	1.7719 ± 0.1337^{b}	1.5512 ± 0.1341^{a}	13.406	0.003^{**}
Glucose	0.7187 ± 0.1036^{a}	0.8170 ± 0.1069^{ab}	0.8595 ± 0.0653^{b}	4.149	0.033^{*}

** denotes p value ≤ 0.01 significance @ 1% level

* denotes 0.011≤ p value ≤ 0.05 significance @ 5% level

Different alphabets denote significance @ 5% level

Considerable spectral differences have been observed between healthy, fasting and post prandial sera. Based on these differences in spectral signatures, three intensity ratio parameters (IRP) were introduced. They were $R_1 = I_{2926}/I_{2856}$ in lipid region, $R_2 = I_{1654}/I_{1547}$ in protein region and $R_3 = I_{1135}/I_{1020}$ in glucose region, respectively. For the discrimination of healthy, fasting and post prandial sera, univariate statistical analysis of IRP was used. Tables 2-4 present the results of one way ANOVA calculations with f and p values of

statistical significance determined by Tukey HSD test.

Table 3. Results of one way ANOVA for the sera treated with glibenclamide

Region	Serum			f Value	p Value
	Healthy	Fasting	Post prandial		
Lipid	1.7170 ± 0.1341^{a}	1.4074 ± 0.3717^{a}	1.4953 ± 0.0894^{a}	3.257	0.062
Amide	1.9292 ± 0.1933^{b}	1.7786 ± 0.056437^{b}	1.5209 ± 0.1656^{a}	13.176	0.0003^{**}
Glucose	0.7187 ± 0.1036^{a}	0.78460 ± 0.0697^{ab}	0.8394 ± 0.0723^{b}	3.686	0.0455^{*}

** denotes p value ≤ 0.01 significance @ 1% level

* denotes $0.011 \leq$ p value ≤ 0.05 significance @ 5% level

Different alphabets denote significance @ 5% level

Table 4. Results of one way ANOVA for the sera treated with combination of metformin and glibenclamide

Region	Serum			f Value	p Value
	Healthy	Fasting	Post prandial		
Lipid	1.7170 ± 0.1341^{c}	0.7801 ± 0.0759^{a}	1.3987 ± 0.2541^{b}	52.564	0.000^{**}
Amide	1.9292 ± 0.1933^{a}	2.0206 ± 0.2650^{a}	3.1425 ± 0.1985^{b}	65.202	0.000^{**}
Glucose	0.7187 ± 0.1036^{a}	0.7476 ± 0.1078^{a}	0.905 ± 0.0776^{b}	7.442	0.004^{**}

** denotes p value ≤ 0.01 significance @ 1% level

* denotes $0.011 \leq$ p value ≤ 0.05 significance @ 5% level

Different alphabets denote significance @ 5% level

It is known that non – enzymatic glycation is responsible for the secondary

complications present in diabetic pathology and aging related problems.[27] Earlier, spectral investigations have been carried out to confirm that the higher intensity at 3400 cm^{-1} is due to glycated proteins, where Otero de Joshi et al.[28] have demonstrated that the increase in absorption around 3400 cm^{-1} is the increase in O-H stretching modes attributable to the presence of reducing sugars like glucose.[28] IRP $R_1 = I_{2926}/I_{2856}$ is introduced in this sector, indicating the change in conformation structure of methylene chains of triglycerides of diabetic fasting and post prandial sera. From Table 4, it is clearly seen that the combination of the two hyperglycemic agents have produced pronounced difference among the healthy, fasting and post prandial sera ($p = 0.000$) in the lipid region of the FTIR spectra.

The intensity of protein bands amide I around 1653 cm^{-1} and amide II around 1548 cm^{-1} vary considerably indicating that the protein profiles get altered in diabetic serum compared to healthy serum, The IRP $R_2 = I_{1654}/I_{1547}$ is introduced in this spectral window.. The change in absorption ratio for the fasting and post prandial sera in this amide spectral window confirms that the secondary structure of proteins gets altered.[23] Similar alphabets in the superscripts of the healthy and fasting sera IRP data in Tables 2-4 in the amide region indicate that fasting blood glucose is controlled well by the two drugs.

Petibois et al.[29, 30] validated the fact that the spectral band sited at 1033 cm^{-1} is the major IR glucose absorbance band in the C-O region and the one at 1127 cm^{-1} is the most specific for the lactate.[29,30] Wolfgang Petrich et al.[31] could establish the disease specific deviations between the spectra of healthy subjects and diabetes by selecting the spectral region 940-1060 and 1140-1180 cm^{-1} [31]. In this study, significant spectral differences were obtained in the glucose sector of FTIR spectra. To confirm this report, the IRP $R_3 = I_{1135}/I_{1020}$ was introduced in this spectral zone. Comparing the results of the univariate statistical analysis of the sera treated with metformin, glibenclamide and their combination, it was seen that in the glucose region, the combination therapy had better results in fasting blood glucose reduction. The different alphabets in the superscript indicate statistically significant difference at 5% level. The Table 4 clearly shows that there was no statistical significant difference between healthy and fasting sera. This indicates that the combination drug has reduced fasting glucose level in blood. Moreover, the statistical significant results in the three regions of the mid IR spectra, among the sera treated with the drugs as single agent and in combination, were obtained for the combination therapy.

In conclusion, the present study has demonstrated that FTIR spectrometry is a tool for comparing the therapeutic efficacies of drugs, by analyzing and quantifying the sera of the diseased blood.

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