CHAPTER 8

Summary and Conclusion

The combination of powerful new computational tools and rapid growth of instrumental facilities are leading to a new era in vibrational spectroscopy. Spectroscopy is extensively used as a powerful tool in medicine, pollution control, pharmacy along with physical and chemical sciences. The successive improvements and innovations made in measurement technique and data evaluation has enabled infrared spectroscopy to be employed for analysis of pharmaceutical compounds, assay of biomolecules and determining the safety and efficacy of medicines. FTIR spectrometry has proved to be a global, sensitive and highly reproducible physico-chemical analytical technique with which structural biomolecular moieties are characterized by their infrared absorption. The recent advances made in the instrumentation of Raman Spectrometers has made it possible to detect small structural changes that has been useful to discriminate between healthy and unhealthy tissues, or to determine the degree of progress of certain diseases. The application of UV-Visible spectra for the identification of degradation and testing the purity in pharmaceutical and biological research is a popular technique.

Liquid chromatography is used extensively in research, developmental and manufacturing sectors of the pharmaceutical industry. Chromatographic
techniques play a vital role in measuring the levels of active drugs, synthetic byproducts, or degradation products in pharmaceutical dosage forms. It is now the premiere method for the separation of closely related chemical species. In addition, it is used for qualitative identification and quantitative determination of separated species.

The present thesis is a research work done on the application of infrared, Raman, UV-Visible spectroscopic methods and high performance liquid chromatography technique towards analyzing materials of pharmaceutical and biological interest.

Chapter one gives an overall survey of the role of spectroscopy in pharmaceutical and biological sciences. It discusses about the various areas in which spectroscopy is widely used as an investigation tool. It gives a brief idea about the scope of spectroscopy in industry and medicine owing to the advancements made in the instrumentation and measurement techniques.

Chapter two outlines the theory and instrumentation of FTIR, FTRaman, UV-Visible spectroscopy and HPLC techniques, which have been applied in the current work. A brief note on the various sampling techniques adopted for the above mentioned instruments are also presented.

The innovations made in spectroscopic techniques have enabled us to exploit it as an excellent quality control tool in pharmaceutical laboratories. In chapter three, qualitative and quantitative analysis of a few antianginal and
anti-psychotic drugs has been carried out by employing FTIR, FTRaman and UV-Visible spectroscopic techniques. The antianginal drugs chosen for the study are Metoprolol Tartrate and Trimetazidine hydrochloride and the anti-psychotic drugs are Sertraline hydrochloride and Olanzapine. The vibrational band assignment of the drugs has been made based on the position, shape and relative intensity of the recorded spectra and in correlation with the vibrational bands of structurally related molecules. A satisfactory vibrational assignment by both FTIR and FTRaman spectra confirms the presence of various functional groups in the compounds ascertaining its purity factor. The drugs were stored in conditions other than the labeled condition. The internal standard ratio calculated among the various absorption bands for these drugs clearly states the change in the quality of drugs due to the alteration in the storage condition. UV-Visible spectroscopy also serves as an excellent quality control tool in routine pharmaceutical analysis. The absorbance values for Metoprolol Tartrate has been noted as 274nm and 222nm, while for Trimetazidine the maximum absorption is found to occur at 362nm and 270nm. In case of Sertraline, \( \lambda_{\text{max}} \) have been identified at 281nm and 273 nm, whereas olanzapine has its \( \lambda_{\text{max}} \) at 259nm. Calibration curve of all the four drugs have been obtained by linear regressions to figure out the linearity range in which the drugs obey Beer-Lambert’s law. In order to support the qualitative analysis done by the FTIR method, UV-Visible spectroscopic approach has been
adopted to study the variation in the light absorption properties of the drugs. A glance at the absorbance values again emphasizes the fact that change in storage condition leads to a change in the behaviour of drugs. This change has been represented in the numerical form as Q-factor or the intensity ratio among the absorption peaks for all the drugs. Thus these two spectroscopic methods have been employed in identifying the quality change that occurs due to improper and careless storing of drugs, consequently making them ineffective in treating patients.

The measure of the biological activity of a drug is called its potency. Assay is the estimation of the potency of the active principle in the unit quantity of medicinal preparation. In the present work medicines of metoprolol, trimetazidine, sertraline and olanzapine in the form of tablets were subjected to quantitative estimation of the drug substance in the tablet using UV-Visible spectroscopic technique. The tablets Meto-Er 50mg, Taz 20mg, Serta 25mg and Oleanz10mg containing the above mentioned drugs as the active ingredient were used for the analysis. The UV-Visible spectroscopic method was used to find the amount of drug present in tablet formulations. The quantity of active substance, metoprolol and trimetazidine present in tablets Meto-Er 50mg and Taz 20mg were found to be 50.05 mg and 20.07 mg respectively. Tablets Serta 25mg and Oleanz 10mg were found to contain 24.77mg of sertraline and 10.08mg of olanzapine as the active substance.
Shelf life is the time a product is expected to remain stable when stored under reasonable conditions and generally retains more than 90% of potency. All medications tend to lose their potency over time and hence bear an expiration date. Assays proved an exact result which allows an accurate statement on the content or potency of the analyte in a sample. Chapter four deals with the estimation of Atenolol, Verapamil and Losartan in pharmaceutical formulations, before and 10-12 months after expiry period by reversed phase HPLC technique. The above mentioned drugs are used to treat cardiovascular disorders. The regression of each of the drug concentration over the mean peak area obtained by HPLC method has been used to quantify the amount of drug present in the tablet forms. The HPLC results indicate that Atenolol contains 99.75% of drug in the pharmaceutical dosage form, whereas the drug present is only 89.45% of the labeled amount, twelve months after expiry period. In the case of Verapamil, the drug content reduces to 83.52% after the expiry period from the actual drug content of 99.50%, while in Losartan, the amount of drug present is only 86.44% of the labeled value. It can be seen that the drug content reduces after the expiry period from the measured actual value, indicating the deterioration in the quality of the drug after the stipulated shelf life period. Recovery studies have been carried out to ensure the accuracy of the procedure adopted in each case. The high recovery percentage highlights the accuracy of the method followed. The low percentage
of relative standard deviation value indicates the suitability of this method for routine analysis of the drugs in pharmaceutical dosage form. UV-Visible spectroscopic analysis of the same samples confirms the results obtained by the former method. Recovery studies to ensure the accuracy of the UV-Visible spectroscopic method has also been carried out. From the current work it is very clear that the drugs tend to deteriorate and hence they may lose their efficacy after the stipulated period of shelf life. Hence, it is recommended that the expired drugs have to be removed from the consumption circuit as they lose their potency over the period of time. This study concludes that HPLC and UV-Visible spectroscopic methods are simple, cost-effective, accurate, precise and less time consuming and can be successfully employed for the estimation of drugs in pharmaceutical dosage form. It is highly suitable for routine drug analysis so as to effectively monitor and control the quality of the drug products.

Chapter five presents the semi-empirical and density functional computations of the vibrational spectra of Methionine, Homocysteine and Cysteine. Methionine, a sulphur containing essential amino acid is used for protein synthesis and belongs to the group of compounds called lipotropics. Homocysteine is an intermediate non-essential amino acid produced during the metabolism of methionine to cysteine. Cysteine is a non essential amino acid biosynthesized by the body. The FTIR and FTRaman spectra of these
molecules were recorded in the regions 4000-400cm\(^{-1}\) and 4000-100cm\(^{-1}\) respectively. The Modified Neglect of Differential Overlap (MNDO) and Austin Model 1 (AM1) were adopted in semi-empirical analysis. The ab initio and density functional study based on Hartree-Fock and Becke3-Lee-Yang-Parr(B3LYP) level using 6-31G(d,p) basis set was also performed. The entire calculations performed at MNDO, AM1, HF and B3LYP/6-31G(d,p) levels were on an AMD 4000+/3.2 GHz personal computer using Gaussian03 program package. The geometrical parameters such as the bond lengths and the bond angles of the molecules have been studied at semi-empirical and DFT levels and a comparison have been made with the experimental values. Statistical treatment of these data reveal that for the bond lengths B3LYP/6-31G(d,p) is much better than the MNDO and AM1 geometry. The correlation coefficient for bond length in the case of methionine was 0.9985 using the B3LYP/6-31G(d,p) method. For homocysteine and cysteine molecules, again the B3LYP/6-31G(d,p) method leads to geometry parameters which are close to experimental data and the correlation coefficient for the bond length were 0.9985 and 0.9988 respectively. The optimized structural parameters were used in the vibrational wave number calculations at the MNDO, AM1, HF and B3LYP/6-31G(d,p) levels to characterize all stationary points as minima. Then, vibrationally averaged nuclear positions of methionine, homocysteine and cysteine were used for harmonic vibrational wave number calculations.
resulting in IR and Raman wave numbers together with intensities. Vibrational wave numbers computed by DFT methods have been adjudicated to be more reliable than those obtained by the MNDO and AM1 semi-empirical methods. The assignment of the calculated wave numbers was supported by the animation option of chemcraft, a graphical interface for Gaussian programs, which gives a visual presentation of the shape of the vibrational modes. Mulliken charges of methionine, homocysteine and cysteine molecules at different levels were calculated. The thermodynamic parameters of these molecules such as thermal energy, zero point energy and entropy were calculated. Scale factors were recommended for an accurate prediction in determining the zero-point vibrational energies and the entropy. The variation in the zero point vibrational energies seems to be insignificant. The changes in the total entropy of methionine, homocysteine and cysteine at room temperature found using different semi-empirical and density functional methods are only marginal.

Chapter six is an attempt to use FTIR spectroscopy for the analysis of blood plasma to detect elevated levels of homocysteine. Hyperhomocysteinemia is an independent risk factor for atherosclerosis. Those with cardiovascular disease, renal failure, smokers and alcoholics are prone to have elevated levels of plasma homocysteine. Homocysteine is an unstable amino acid, which undergoes auto oxidation to produce oxygen free radicals.
Hyperhomocysteinemia thus causes increased production of free oxygen radicals and an oxidative stress. This contributes to atherosclerosis in two ways. The free oxygen radicals convert the Low Density Lipoprotein (LDLc) deposited in the sub-endothelial tissue to oxidized LDLc (oxLDLc). The oxLDLc then acts as the key mediator of the inflammatory process in atherosclerosis.

Plasma homocysteine levels are elevated in renal failure patients. Kidney eliminates about 70% of the daily homocysteine burden and this indicates why homocysteinemia levels are high in predialysis as well as renal transplant patients. Also among dialysis patients the homocysteine levels are high because the water soluble vitamins like folic acid, vitamins B6, vitamin B12 are all removed with dialysis. These vitamins act as the cofactors and substrates in the metabolism of methionine and homocysteine.

Smoking is associated with an increase in plasma homocysteine levels. Smoking destroys the B complex vitamins that are the cofactors for homocysteine metabolism. Smokers generally have lower levels of folic acid and vitamin B12. Thus cigarette smoking which is one of the leading and avoidable causes of death and morbidity has been associated with increased homocysteine levels.

Chronic alcohol intake among alcohol dependent patients redounds to markedly elevated homocysteine plasma concentrations. The data indicates that
actively drinking alcoholics had twice the level of homocysteine in their plasma than did the healthy controls. It has been proposed that ethanol-induced hyperhomocysteinemia may be a significant factor in the increased incidence of coronary artery disease and stroke related to high alcohol consumption.

For the present study ten patients from each of the above mentioned categories were chosen. 2 ml of blood of each individual were collected in EDTA vacutainers. The blood was centrifuged immediately and the plasma was separated. One part of the blood plasma was subjected to conventional clinical diagnosis (Immunoassay-chemiluminescence) to estimate the plasma homocysteine level, while another part was used for FTIR spectral analysis. A volume of 1ml of serum was spread evenly over the surface of a thallium chromide pellet and air dried for thirty minutes prior to measuring the spectra in the range 400 – 4000 cm⁻¹. The analysis led to the identification of specific modes of vibration pertaining to homocysteine in blood plasma. The absorbance values at these specific modes of vibration were significantly increased for those having elevated homocysteine levels when compared to healthy individuals with optimal levels of homocysteine. The internal ratio parameter was calculated using the absorbance values of the wavelength corresponding to specific modes of vibration. It provided an excellent classification between healthy individuals and those with elevated homocysteine levels, which correlated completely with the clinical data. This
indicates that spectroscopic methods can be successfully applied for studying pathological changes in blood and characterization of blood as healthy and diseased.

Efficacy is the ability of the drugs to produce a desired amount of the desired effect. In medical context it indicates the therapeutic effect of the chosen drug administered during a given interval. Chapter seven is entirely devoted to study the efficacy of multivitamins on patients with elevated homocysteine levels. Homocysteine is metabolized via remethylation, resulting in the formation of methionine, or via transulfuration, resulting in the formation of cysteine and finally taurine. The remethylation pathway of homocysteine is strongly dependent on the availability of folic acid in the active form of 5-methyltetrahydorfolate and vitamin B12. The latter is essential for optimal activity of the enzyme methionine synthase, which is responsible for methylation of homocysteine to methionine. In the transulfuration pathway an essential cofactor is the active form of vitamin B6, pyridoxal -5’-phosphate P5P. Deficiency of any of these vitamins is associated with hyperhomocysteinemia. Many drugs increase the level of homocysteine either by interfering with the metabolism of folate or vitamin B6 or vitamin B12. Certain lipid lowering drugs administered in patients with high levels of plasmatic lipids and anti-hypertensive drugs belonging to the family of thiazides can increase homocysteine levels. Elevated levels of plasma
homocysteine have been documented in epileptic patients after chronic treatment with anti epileptic drugs.

For the present study five patients from each of the following four categories were chosen: (i) atherosclerosis (ii) hypertension (iii) hyperlipidemia and (iv) epilepsy. Before the initiation of vitamin supplements along with their regular medication the FTIR spectra of the blood plasma was recorded and their homocysteine levels were clinically tested. They were orally administered a daily dosage of folic acid(5 mg), vitamin B12(250mcg) and vitamin B6(25mg) supplements for a period of two months. Efficacy of these vitamin supplements were analyzed both clinically and spectroscopically. The FTIR spectra were recorded at the end of the first and the second month and also the homocysteine levels were clinically tested. The absorption values of the specific modes of vibration pertaining to homocysteine of both pre and post-treatment spectra were noted and the percentage of efficacy of the multivitamins was calculated. The plasma homocysteine levels had decreased with the progress of the treatment. The spectroscopical outcome was substantiated with the clinical results. This study forms a promising basis for employing spectroscopy in the follow-up of patients undergoing treatment for various ailments. It is much cost effective when compared to clinical tests. It is therefore worthwhile to continue developing spectroscopy as an effective and reliable tool for the diagnosis and follow-up of disease pattern.
chapter eight is the final chapter that summarizes the results and conclusions arrived at, on the spectroscopic investigation carried out on a few samples of pharmaceutical and biological significance.

Thus, the thesis presents a systematic approach that is followed in investigating some pharmaceutical samples and biomolecules. The above discussed spectroscopical applications have been presented as proofs for its importance in pharmaceutical sciences and pharmaceutical industry. The great possibilities of spectroscopy have been established in specific pharmaceutical areas such as drug identity, purity, and quantitative analysis. Similarly in the field of biology, especially medicine, spectroscopy seems to be ideally suited for disease screening procedures. Thus a systematic work was carried out in understanding the role of spectroscopy in the study of pharmaceutical compounds and biomolecules. The computations, findings and their inferences were presented and discussed in detail.