CHAPTER 1

Spectroscopy in the analysis of Pharmaceutical Materials and Biomolecules

1.1 Introduction

Spectroscopy is the branch of science dealing with the study of interaction of electromagnetic radiation with matter. The most important consequence of such interaction is that energy is absorbed or emitted by the matter in discrete amounts called quanta. A number of analytical methods, both qualitative and quantitative, involve the interaction of radiant energy with matter. The different ways in which the frequencies of radiation (emitted or absorbed) are measured experimentally and the energy levels deduced from these comprise the practice of spectroscopy. The various branches of spectroscopy generally involve measurement of two important experimental parameters. They are the energy of the radiation absorbed or emitted by the system and the intensity of the spectral lines (1).

Vibrational spectroscopy encompasses the technique of infrared (IR) spectroscopy and Raman spectroscopy. Both are techniques that have been in existence for more than sixty years, but both have shown remarkable growth in the past ten year or so because of advances in instrumentation technology (2). Both IR and Raman produce a spectrum which reflects the vibrational modes of the sample and is therefore characteristic of its molecular structure. The
principle difference between these two types of vibrational spectroscopy is that IR spectroscopy detects vibrations during which the electrical dipole moment changes, while Raman spectroscopy is based on the detection of vibrations during which the electrical polarizability changes.

The IR spectroscopy is one of the most versatile analytical chemical techniques. Since the introduction of Fourier Transform Infrared (FTIR) spectroscopy, this technique is now increasingly used for quantitative and qualitative analysis in many diverse applications. Among these are the analysis of pharmaceutical compounds, biomolecules, biomembranes, biopolymers and microbiological applications. The FTIR spectrometer with its enhanced frequency accuracy, high signal to noise ratio, high data acquisition speed combined with modern computational methods has opened up ways to investigate biochemical systems in more detail (3).

The advent of Fourier Transform Raman (FTRaman) spectrometers has enabled the examination of a wider range of sample types. The recent advances made in the instrumentation of Raman Spectrometers has made it the most sought after instrument for applications as diverse as minerals, polymers, superconductors, forensic analysis and biological samples (4).

Another instrumental method of chemical analysis is UV-Visible spectrophotometry which finds a variety of application. The application of UV-Visible spectra for the identification of degradation and testing the purity in
pharmaceutical and biological research is a popular technique. It also provides a useful source of supporting evidence in the elucidation of structures of organic compounds. Also highly absorbing impurities in non-absorbing media can be detected, which helps in the detection of contaminants (5). A variety of inorganic and organic species absorb in the UV and visible region of the electromagnetic spectrum and thus susceptible to quantitative analysis. Even many non-absorbing species can be analysed after converting them to absorbing species by making use of suitable reagents, thus making this technique gain wide usage.

The applications of spectroscopy are extremely diverse and the techniques are making a contribution to many areas of science. The most interesting areas are in pharmaceutical sciences, mineral sciences and in biomedical research where the early detection of cancer in human tissue is a goal. The present chapter provides a vivid picture about the role of IR, Raman and UV-Visible spectroscopy in the field of pharmaceutical and biological sciences.

1.2 Spectroscopy in the analysis of pharmaceutical materials

Spectroscopy finds a very wide application in pharmacy due to its possibilities for solving many important problems. Fig. 1.1 gives a schematic presentation of the role of vibrational spectroscopy in the field of
pharmaceutical science. IR spectroscopy and IR techniques have well known advantages in comparison with other analytical methods (6, 7).

**Fig. 1.1 Role of vibrational spectroscopy in the analysis of Pharmaceutical materials**

IR and Raman spectroscopies are complementary techniques which together can provide a more complete impression of the information within the sample. Furthermore, these two methods differ such that each is capable of providing information not easily obtainable by the other.

**1.2.1 Verification of drug’s identity**

The need for efficient and sure drug identification methods in pharmacy has led to the choice of IR and Raman Spectroscopy. There are three ways of IR spectral identification of the drugs (6, 7). They are (a) comparison of wave
numbers and relative intensity of the particular absorption bands; (b) comparison of the spectrum with a published reference spectrum; (c) comparison of spectrum of reference standard (obtained in identical conditions of sample preparation and their recording). The third method is the most sure when the above mentioned requirements are taken into consideration as well as the isomorphism of the two drug samples. For example, the last British Pharmacopoeia of 1998 gives the possibility of drug identification in (b) with over 420 published drug reference IR spectra. For the first time IR spectroscopy (8) has been introduced in US Pharmacopoeia (USP XVI) for identification of a great number of drugs since 1960. It was followed by British Pharmacopoeia (BP-1968). Today, IR spectroscopy has an obligatory character in all Pharmacopoeias in the world.

The foregoing facts confirm that spectroscopy in pharmacy is a very useful technique in drug identification based on the up-to-dated principles, procedures, standards and requirements assisting both manufacturers and drug regulators in their control.

1.2.2 Testing purity

IR and Raman spectroscopy secures simultaneously in verification of drug identity and examination of drug test purity. Usually the drug identification precedes the drug test purity. The drugs are the finest products of the chemico-pharmaceutical industry. Nevertheless, they can contain minimal
amounts of pollutants, intermediate and/or disruptive products. Each of these can cause unwanted alteration in drug therapeutic activity (9). Their discovery is an obligation of the spectroscopist-analysts as their removal is a care of the manufacturer. For example, the appearance of the band at 1745cm⁻¹ in IR spectrum of the Chloramphenicol was shown as pollution with dichlor acetic acid. This peak was defined as analytical band of the pollution (6, 7).

Therefore spectroscopy has an important role in examining drug test purity. This, enables the leading pharmaceutical companies to ensure that the drugs responsible for the wanted therapeutic activity are of high quality.

1.2.3 Structural investigation

The deciphering of the structural formula of antibiotic penicillin was made by means of IR spectroscopy (6, 7). Three possible structural formulae have been proposed on the basis of the investigation of large groups of chemists. The study of IR spectrum of the crystal penicillin proved the presence of a strong band at 1780cm⁻¹ related to CO group and the position is corresponding to the structure of β-lactum with condensed cycles. Specially synthesized more simple compounds with analogical structures having an absorption band at the same frequency 1780cm⁻¹, proved the rightness of the choice of the formula. Also the structure of the antibiotic mycomycin and its isomers, were established using their IR spectra. Structural investigations of
synthesized compounds and their derivatives are being carried out in IR laboratories.

The alkaloids with a cyclohexadienone or cyclohexanone ring have been studied by means of IR and UV spectroscopy (10). These compounds are of interest because of their appearance in medicinal plants and are intermediate products in the biosynthesis of aporhine and morphinane alkaloids. UV-Visible spectroscopy can also be used to determine the concentration of a chromophore in a mixture using the Beer Lambert’s law. For this reason this method serves as one of the most useful tool for quantitative analysis.

Numerous papers in literature illustrate spectroscopy’s applications in solving an important pharmaceutical problem – drug crystalline structures (polymorphism/pseudo polymorphism). The unexpected changes of drug crystalline structures can alter their therapeutic activity. For this reason the WHO (section 9) has prescribed in 1973 as a basic research need the study of drug’s polymorphism as an important factor of their activity. It has been reported that 80% of drug substances are polymorphic (11). Drug polymorphism is very important for pharmaceutical applications, especially in pharmaceutical industry. There are a number of techniques used to study polymorphism. Among them IR spectroscopy has been employed in identification of different polymorphs (in solid state) as well as that it is both quantitative and qualitative technique.
IR spectroscopy is a reliable technique for the identification of the antibiotic Cefamandole nafate and its two new crystalline modifications, which is likely to be of importance in an industrial context (12). The range 700-900 cm\(^{-1}\) has been shown as one having the greatest analytical value. IR spectroscopy has been evaluated as a guiding physical method in the studies on the super molecular structures. Thus IR spectroscopy will become a very useful technique in the pharmaceutical industry for technological and analytical control of drugs and its crystalline modifications.

1.2.4 Drug Interaction

Spectroscopy offers possibilities in solving problems in a comparatively new scientific area in pharmacy – to investigate the interactions between active medicaments and excipients. The interactions (mainly complexation, hydrogen bonding) can modify the physicochemical, pharmacological and pharmacokinetic behaviour of active medicaments. Today, pharmaceutical technology and pharmaceutical industry, using this knowledge, have at their disposal a new strategy for development of existing drugs and search of new effective medicaments, aiming at the improvement of the solubility, stability, alteration of therapeutic activity and the decrease of unwanted side effects. The combination of IR spectroscopy and X-ray diffraction patterns offers a successful application in proving the above mentioned interactions. Therefore in this context, the importance of spectroscopy is indisputable. Using Fourier
Transform Infrared Spectroscopy (FTIR) and Attenuated total reflectance method the drug excipients have been characterised (13). The excipients were identified as monosubstances and in mixtures or formulations. The authors evaluated FTIR/ATR as an excellent method for rapid in-process control during manufacturing of drug formulations.

UV-Visible spectroscopy is a well accepted and well documented technique with many pharmaceutical applications. The determination of drug content in pharmaceutical formulations and their interaction with trace elements employing spectrophotometry is an efficient and widespread analytical method (14, 15).

1.3 Spectroscopy in the analysis of biomolecules

Biomedical utilization of the electromagnetic spectrum of light has revolutionized the practice of medicine over the centuries, most recently through the dramatic healthcare advances afforded by the development of magnetic resonance imaging. The last region of the spectrum to be applied to the practice of medicine is the infrared region. The IR spectroscopy has been increasingly utilized in multiple biomedical settings. IR spectroscopy can distinguish differences in the characteristics of diverse molecules by probing chemical bond vibrations and use these molecular and sub-molecular profiles to define and differentiate ‘diseased’ and ‘healthy’ tissues (16). As covalent bonds
vibrate, they absorb energy in the form of infra red light. The wavelength of light that is absorbed depends on the nature of the covalent bond (e.g. C=O, N-H), the type of vibration (bending, stretching etc), and the environment of the bond. The IR spectrum of a biofluid or tissue sample can be regarded as molecular fingerprint of the biofluid or the tissue. If this molecular fingerprint is modified by a disease process, then IR spectroscopy can be used to detect and monitor the disease process. Fig. 1.2 gives a schematic presentation of the role of vibrational spectroscopy in the analysis of biomolecules.

![Diagram of Vibrational Spectroscopy](image)

**Fig. 1.2 Role of vibrational spectroscopy in the analysis of biomolecules**

UV-Visible spectroscopy has been investigated as a novel way to characterize and differentiate the blood types based on spectral differences
which appear throughout portions of both the ultraviolet and visible range. Recently, Gunasekaran and Sankari have employed this method successfully to differentiate diseased and healthy samples (17).

1.3.1 Analysis of structure of biomolecules

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 (18). Since a biomolecule is determined by unique structure, unique FTIR spectrum will be exhibited by the biomolecule, representing its structural fingertip. Furthermore, every biomolecular family present in the sample will exhibit almost similar and overlapping FTIR absorptions. FTIR analytical applications allowed blood contents determination using various materials and sample preparations. Concentrations of glucose (19), total proteins, creatinine, urea, triglycerides and cholesterol (20) in blood, plasma or serum have been determined with clinical accuracy.

Nucleic acids

Although all biomolecules are important, the nucleic acids RNA and DNA are especially important because they carry within their structure the hereditary information that determines the identity and structure of proteins. Each protein, unique in its structure and hence in its function, then participates in the processes that characterize the individuality of the cell. Blout et al.
reported the first IR spectra of nucleic acids (21) and since then IR has been used in applications such as conformational transitions, identification of base composition, effect of base pairing, and DNA-ligand interaction studies. It has also been used in industry for the quality control of products based on DNA, such as fluorescence probes.

**Proteins**

It is a fundamental belief of structural biology that protein function or dysfunction is related to its structure or change in structure. Both IR and Raman provide information on the secondary structure of proteins, ligand interactions and folding (22). In the vibrational spectra, the amide bonds of proteins form so-called chromophores that give rise to nine strong characteristic bands that are named amide A, amide B and amides I- VII. Among these bands, amide I, which is due mostly to the C=O stretching vibration of the peptide backbone, is by far the best characterized. It gives rise to an IR band in the 1600-1700 cm\(^{-1}\) region and has been used the most for structural studies due to its high sensitivity to small changes in molecular geometry and hydrogen bonding of the peptide group. The amide II band, due to its coupling of C-N stretching and in-plane bending of the N-H group, is extremely weak in the Raman Effect. Though it is strong in the IR giving rise to band in the 1480-1575 cm\(^{-1}\) region the amide II band is not often used for structural studies as it is less sensitive and is subject to interference from absorption bands of amino acid side chain
vibrations. The amide III band, arising from coupling of C-N stretching and N-H bending, give rise to bands that are weak in the IR but quite strong in Raman and can also be mixed with vibrations of side chains (23).

**Lipids**

Lipids are critical to all biological media by forming the cell walls that keep biological media organized in their necessary compartments. Lipids of various kinds also help regulate the flow of needed biological molecules from one side of a lipid barrier to the other, often assisted by imbedded protein that form the passages for these molecules called channels. The major absorption bands are at 1738, 1465, 1255, 1168, 1095, 1057 and 968 cm⁻¹ (24). Gunasekaran and Renuga Devi have characterized and compared the blood samples of various disorders of lipid metabolism such as Hyperlipidemia, Diabetes mellitus, Chronic renal failure etc. with the healthy subjects using UV-Visible and FTIR spectroscopic techniques (25). The cardiac risk ratio and atherogenic index values have been calculated with lipid peaks corresponding to total cholesterol, triglyceride and high density lipoprotein (26). The comparison of these values shows that the spectra are not similar when compared down to finer details.

**Carbohydrates**

The most common carbohydrates are sugars, or saccharides. Sugars are present in biological media primarily as hexose sugars, such as glucose, where
they are an immediate energy source. Polysaccharides in the body are found either in a free state or combined with proteins, in a complex known as glycoproteins. The search for a simple and accurate analytical method to determine glucose concentration is a problem of major importance for clinical laboratories. Most physicochemical analytical methods, such as Raman spectroscopy, mass spectrometry and nuclear magnetic resonance spectroscopy have been evaluated as tools to determine glucose concentration in whole blood, plasma or serum (27). With FTIR spectroscopy, the spectrum of any molecule shows its characteristic absorptions. For glucose, the following absorption bands may be found: \( \nu(\text{O-H}) \) between 3570 and 3120 cm\(^{-1}\), \( \nu(=\text{C-H}) \) between 3085 and 3020 cm\(^{-1}\), \( \nu(\text{C-O}) \) between 1230 and 1000 cm\(^{-1}\) and \( \nu(\text{C-O-C}) \) between 1275 and 800 cm\(^{-1}\) (28, 29). When using this technique to determine the glucose concentration, accurate results can be obtained by normalizations or standardizations of the spectra.

**Urea**

Urea and creatinine are the waste products which will turn toxic if not eliminated from the blood by the kidneys periodically. Anthony Shaw *et al.* (30) have done an extensive study on urine thin films. They have predicted the range 1400 -1800 cm\(^{-1}\) for creatinine and 3100 – 3550 cm\(^{-1}\) for urea and the predominant peaks in these regions are employed for the analysis of blood plasma of patients with renal disorder.
1.3.2 Analysis of cells and tissues

The basic unit of life is the cell. The essence of a cell is to grow and divide to produce daughter cells, which are likewise capable of generating new cellular molecules and replicating themselves. Cells are chemically very sophisticated – even the simplest cells contain about 1000 different molecules. These molecules include various sugars, amino acids, fatty acids, proteins and nucleic acids. There are over 200 different types of cells in the human body such as heart cells, muscle cells, liver cells, retina cells, red and white blood cells. These cells are assembled into four types of tissue: epithelial, connective, muscular and nerve.

Studies in the mid-infrared are often done on isolated cells that are dried (31) or on dried whole blood or blood serum samples. Shaw et al. and independently Wener et al. (32) used blood samples for distinguishing healthy versus diseased patients with mid-infrared light. Over the last decade, studies using vibrational spectroscopy have been conducted on samples from a variety of organs, including brain, breast, colon, cervix, endometrium, heart, liver, lung, lymph system, prostate, skin and thyroid. The results of all these studies indicate that normal and malignant tissues can be differentiated on the order of 80-100% accuracy with the use of some statistical analysis. Hence cancer research employing spectroscopic techniques have enormously gained momentum over the past decade.
Analysis of Leukemia cells

Leukemia is a type of cancer that involves the blood-forming lineage of cells. FTIR spectroscopy has been employed to examine the blood plasma of leukemia patients. The results showed that specific spectral peaks were significantly reduced in patients when compared to healthy individuals, indicating that these spectral parameters might serve as biomarkers for monitoring and identification of leukemia patients (33).

Analysis of breast cancer tissues

Breast cancer is the most frequently diagnosed cancer, after cancer among women. In 1991 Alfano et al. (34) recorded the first Raman spectrum of breast tissue, using 1064-nm excitation and a FTRaman spectrometer. They analyzed 14 breast tissues (three normal, four benign and seven malignant) and observed that malignant tissues showed only two bands, at 1651 cm\(^{-1}\) and 1445 cm\(^{-1}\) out of four that were observed for normal tissues. Missing were bands at 1300 cm\(^{-1}\) and at 1078 cm\(^{-1}\). They concluded that the relative intensity of bands at 1651 and 1445 cm\(^{-1}\) could be correlated with disease.

In a later study, Frank and McCreery (35) compared spectra of normal tissue, benign tissue (fibrocystic disease) and malignant tissue (infiltrating ductal carcinoma). In agreement with Alfano studies, using the area ratio of 1654/1439 cm\(^{-1}\) band, they were able to differentiate between malignant and normal tissue. They also noted a shift in frequency from 1439 cm\(^{-1}\) in normal
tissue to 1450 cm\(^{-1}\) in infiltrating ductal carcinoma. However, they were unable to differentiate between carcinoma and fibrocystic disease. The latest studies on breast tissue with Raman come from Feld’s group. Manoharan et al. (36) collected data from 61 specimens taken from 13 patients (15 normal, 15 benign and 31 malignant) using a macroscopic Raman system with an excitation wavelength of 830nm, 80mW laser power and sampling a 1-mm diameter spot size. Consistent with previous studies mentioned above, it was found that normal tissue spectra were dominated by Raman bands of fatty acids (1657, 1444 and 1300 cm\(^{-1}\)), while the Raman spectra of benign and malignant tissue were dominated by protein bands (1667, 1452, 1260, 890 and 820 cm\(^{-1}\)). Using principal component analysis, 14 of 15 normal, 13 of 15 benign and 31 of 31 malignant samples were correctly diagnosed. Gunasekaran et al. have also done an extensive work in the analysis of breast cancer tissue using FTIR spectroscopy (37).

1.3.3 Identification of bacterial isolates

There is a growing concern in the medical field over developing antibiotic resistance. Bacteria can develop resistance to antibiotics when they are exposed to them through several mechanisms. They also have the ability to pass this resistance on to other bacteria that have not been exposed to the antibiotic. Hence, there is a delay in the diagnosis of the specific bacteria and to reduce the pain suffered by the patient, physicians usually prescribe broad
spectrum antibiotics that can cover a wide variety of bacteria until the specific strain of bacteria is identified. After identification, the antibiotics are usually changed to those necessary to treat the infection.

The diagnosis of bacterial organism in a biological fluid can be achieved using spectroscopic techniques. Goodacre et al. (38) disclose the use of pyrolysis mass spectrometry (PyMS), FTIR and dispersive Raman spectroscopy to analyze a group of bacterial isolates associated with urinary tract infections. Ivan et al. have (39) invented a diagnostic method for detecting specific strain of bacteria in biological sample without subjecting the sample to culturing. Preferably FTIR, fluorescence and Raman spectroscopy are used to detect bacteria. More preferably, FTIR is used to diagnose the bacteria. It has been found that by using spectroscopic techniques, a rapid determination of the specific bacterial strains can be made, thus allowing for targeted treatment and avoiding the use of broad spectrum antibiotics. Such targeted treatment eliminates the medical costs associated with prescribing broad spectrum antibiotics and then represcribing antibiotics specific to the infection.

1.3.4 Efficacy studies

Efficacy of a drug is the ability to produce a desired amount of a desired effect. In medical context it indicates the therapeutic effect of a given intervention. Drugs are released into market after an extensive research on its therapeutic effect and other side effects. Recently spectroscopy has been
successfully employed in studying the efficacy of drugs on various diseases. Gunasekaran et al. have done an extensive work using FTIR spectroscopy on the efficacy of Statins and Fibrates on patients with lipid disorders (25). The same group has also worked with the efficacy of vaccines on cattle using IR and UV-Visible spectroscopy (40-42).

The spectroscopic applications that have been discussed are presented as proofs for its importance in pharmaceutical research and pharmaceutical industry involving drug design and manufacturing. The dynamic possibilities of spectroscopy have been well established in certain specific pharmaceutical areas such as drug identity, testing purity and drug interactions. It has been verified that Infrared and Raman spectra provide valuable information on both molecular and sub molecular structure of drugs. The continuous innovations in spectroscopic techniques, the growth of pharmaceutical industry and the care for high quality drugs would determine spectroscopy’s increased role in this area. Similarly in the field of medicine spectroscopy seems to be ideally suited for disease screening procedures. It is inexpensive, can be automated, the measurements are fast and the instruments fairly inexpensive. At the cellular level it is very essential for the spectroscopist to analyse the spectra more carefully as they are full of invaluable information waiting to be interpreted.
References


