Deanship of Graduate Studies
Al-Quds University

Study the interaction of Human Serum Albumin with Aspirin Using Spectroscopic Techniques

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M.Sc. Thesis

Jerusalem – Palestine

2018 /1440

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A thesis Submitted in Partial fulfillment of requirement for the degree of Master of Science from the Department of physics, Faculty of Science and Technology, Al-Quds University.

2018 /1440

Al-Quds University
Deanship of Graduate Studies
Physics Department

Thesis Approval

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Master thesis submitted and accepted, Date: 12 /12 / 2018

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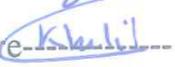
Jerusalem-Palestine

2018/1440

Dedication

To the of my husband, father, mother, brothers and sisters, my family
with love and respect.

Declaration:

I Certify that this thesis submitted for the degree of Master, is the result of my own research, except where otherwise acknowledged, and that This study (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed:

Kholoud Ali Awad Khalipha

Date: 12 /12   / 2018

Acknowledgements

At first and last my great commendation and thanks to Allah, who gave me the ability to accomplish this work.

I am very pleased to express my deep gratitude to my supervisor Dr. Husain Alsamamra for their supervision, guidance and insightful suggestions.

My sincere thanks go to my dear husband, lovely family and my friends for their unlimited support.

Kholoud Ali Awad Khalipha

Abstract

Aspirin is an important for reduce the risk of heart attack and stroke. Human serum albumin is the major soluble protein constituent of the circulatory system and has many physiological functions including transport of a variety of compounds. In this work, interaction between Aspirin with human serum albumin was investigated by using fluorescence spectroscopy and UV absorption spectrum. From spectral analysis, Aspirin showed a strong ability to quench the intrinsic fluorescence of human serum albumin through a static quenching procedure. The binding constant (k) is estimated as k=2.02×10^4 M^{-1} for HSA-Aspirin. In addition The Stern-Volmer constant is calculated at room temperature for Aspirin.FT-IR spectroscopy was used to determine the protein secondary structure. The observed spectral changes indicates an increase of intensity for HSA-Aspirin interaction.. This variation of intensity is related indirectly to the formation of H-bonding in the complex molecules.

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List of Symbols

symbol	Description
N	Degree of freedom
R	the different part of 20 amino acids
c	speed of light
v	Frequency
λ	Wavelength
E	Energy
h	Planck's constant
E_{total}	total energy
E_{ele}	energy of the molecule's electrons
E_{vib}	vibrational energy
E_{rot}	rotational energy
I	the intensity of light transmitted
I_0	the intensity of light incident
ε	the molar absorption coefficient
C	concentration of absorbing molecule in the sample
l	length of the light path
A	Absorbance
F_x	restoring force
f	the spring or force constant
Δx	isplacement of the spheres along the x-axis from equilibrium position
m$_A$	Mass of atom A
m$_B$	Mass of atom B
V	potential energy
T	kinetic energy
μ	reduced mass
ω	circular frequency
E_v	The potential energy for diatomic molecule for harmonic oscillator

D_{eq}	dissociation energy
r_{eq}	Equilibrium position
r	Position
δ	constant for a particular molecule
E_n	allowed vibration energy levels
n	Interger
W_e	oscillating frequency
$\dot{W}_e$	oscillation frequency in wave number
X_e	an-harmonicity constant
a	absorptivity of the molecule
b	distance that the light travels through the sample
T	Transmittance
mM	Mile molar
n	nonbonding occupied molecular orbital
π	Pi bonding occupied molecular orbital
σ	Sigma bonding occupied molecular orbital
π^*	Pi anti-bonding unoccupied molecular orbital
σ^*	Sigma anti-bonding unoccupied molecular orbital
A	recorded absorption at different concentrations
A_∞	the final absorption of the ligated protein
A_0	the initial absorption of protein at 280 nm in the absence of ligand
L	Concentration of ligand
k_q	biomolecular quenching constant
$[l]$	the quencher concentration
k_{sv}	Stern-Volmer quenching constant
τ_0	unquenched lifetime
F_0	the HSA fluorescence intensities in the absence of quencher
F	the HSA fluorescence intensities in the presence of quencher

List of Abreviations

abbreviation	Representation
G	Gram
IR	Infra-Red
VIS-UV	Visible Ultra Violet
ALB gene	Albumin gene
α-helix	alpha helix
β-pleated sheet	Beta pleated sheets
N-terminus	Nitrogen terminus
C-terminus	Carbon terminus
DNA	Deoxyribonucleic acid
FTIR	Fourier Transform Infrared
HSA	Human Serum Albumin
pH	potential of hydrogen
GLO	gulonolactone oxidase

PA	pernicious anemia
BSA	bovine serum albumin
DPPH	α, α-diphenyl-β-picrylhydrazyl
ITC	isothermal titration calorimetry
EMW	Electromagnetic waves
Far-IR	Far Infra-Red
Mid-IR	Middle Infra-Red
EM	Electromagnetic
D_2O	Deuterium oxide
H_2O	Water
HOMO	Highest Occupied Molecular Orbital
LOMO	Lowest Unoccupied Molecular Orbital
LED	light emitting diodes
CCD	charge-coupled device
PC	personal computer
OPUS	Optical User Software
Eq	Equation

Chapter One

Introduction

1.1 Introduction

A technique used for the vibration of the atoms of a molecule is called Infrared (IR) spectroscopy. To obtain an infrared spectrum, determine what fraction of the incident radiation is absorbed at a particular energy when infrared radiation is passed through a sample. The appearance of any energy peak in an absorption spectrum corresponds to the frequency of a part of a sample molecule **[Banwell, 1972]**.

Infrared Spectroscopy is an absorption method pertaining to wavelengths in the region of 1 to 100 μm, extending the region of visible light to longer wavelengths and shorter frequencies (or energies). The IR light does not have sufficient energy to induce transitions of valence electrons, but can excite vibrational and rotational motions in molecules. Noted that the principle of IR spectroscopy are similar to ultra violet (VIS-UV) spectroscopy or other spectroscopic techniques except the differences in energy transfer from radiation to the molecules**[Wilson et al., 1955]**.

The length of a bond will vary in length when atoms move relatively to each other causing the bonds to stretch, or bend when atoms move out of plane relatively to one another. Reported linear frequencies to have a resonance frequency of 3N-5 and non-linear molecules have a frequency of 3N-6, where N is the degree of freedom, and some of these will interact with incident infrared radiation **[Hollowood and Miramontos, 2011]**.

Serum albumin, also known as blood albumin, is a type of globular protein found in vertebrate's blood. The albumin gene (ALB gene) is used to encode Human serum albumin and is similar to other mammalian forms such as bovine serum. They are all chemically similar **[Hawkins, 1982]**.

1.2 Proteins

Proteins are complex macromolecules. They are made up of successive amino acids, are covalently bonded together in a head-to-tail arrangement with substituted amide linkages called peptide bonds. The building blocks of proteins include 20 amino acids, which differ in the structure of their R-groups, and may be hydrophilic or hydrophobic, acidic, basic, or neutral. Proteins have the same basic structure, which is an amine group (NH_2), central carbon atom (alpha-carbon) and a carboxyl group (COOH), with the only difference on the side chain labeled R in the figure 1.1. **[Rosenberg, 2005].**
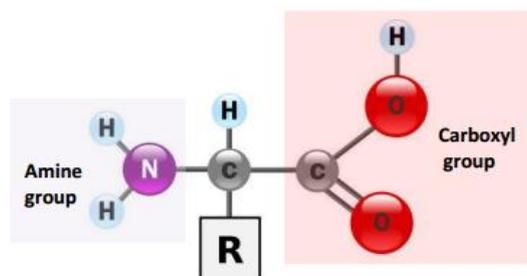
Figure 1.1:   general structure of all amino acids **[Nelson, 2005]**.

Proteins are known as polypeptides because each protein molecule is made up of a long chain of amino acids, and each molecule is attached to it neighboring molecule through a covalent peptide bond. A large number of different proteins are known, with each type of protein showing a unique sequence of amino acids (see figure 1.2). This is exactly the same from one molecule to the next, with each with its own particular amino acid sequence **[Alberts et al., 2002]**.
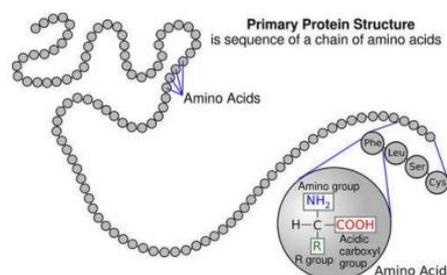
Figure 1.2: polypeptide (a chain made up of many linked amino acids).

1.2.1 Protein Structure

Noted that protein structure has four levels that determine protein function. These structures are primary, secondary, tertiary, and quaternary **[Stabler, 2013]**.

1.2.2 Primary Structure

A primary structure is the simplest level of protein structure. The sequence of amino acids is in a polypeptide chain and it's unique for each protein **[Richardson, 1981]**. See Figure 1.3.
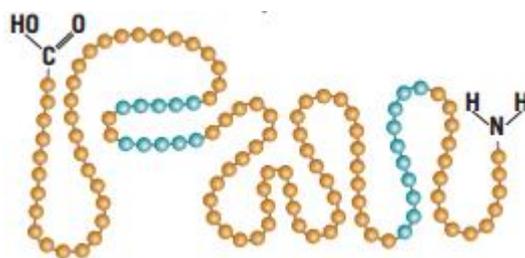
Figure 1.3:   Primary Structure of protein **[Smith et al., 2005].**

1.2.3 Secondary Structure

 A secondary structure of protein is formed when local folded structures form within a polypeptide as a result of interactions between the carbonyl of one amino acid, and the amino hydrogen of another. Alpha helix (α-helix) and the beta pleated sheet (β-pleated sheet) are the most common types of secondary structures as depicted in figures 1.4 and 1.5 respectively. Both of these structures are held in shape by hydrogen bonds **[Smith et al., 2005]**.

The structure of a α-helix involves the carbonyl (C=O) of one amino acid to be hydrogen bonded to the amino H (N-H) of an amino acid, that is four down the chain. The R groups of the amino acids stick outward from the α-helix, **[Richardson, 1981].**
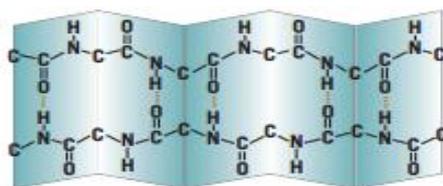
Figure 1.4: Beta Sheet **[Smith, 2005]**.

In a β-pleated sheet, two or more segments of a polypeptide chain line up next to each other, forming a sheet-like structure held together by hydrogen bonds and the R groups extends above and below the plane of the sheet. The strands of a β-pleated sheet may be parallel, pointing in the same direction (meaning that their N- and C-termini match up).In antiparallel sheets, the pointing is in the opposite directions (meaning that the N-terminus of one strand is positioned next to the C-terminus of the other **[Nelson, 2005]**.
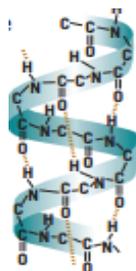
Figure 1.5: Alpha helix **[Smith, 2005]**.

1.2.4 Tertiary Structure

Tertiary structures involve a three dimensional space. Interactions between different secondary structures elements and R groups of the amino acid, which make up the protein, result in tertiary structures. The interactions can include hydrogen bonding, ionic bonding, dipole-dipole interactions, and London dispersion forces, producing a linear or spherical structure as in figure 1.6 **[Beatty et al., 2016]**.
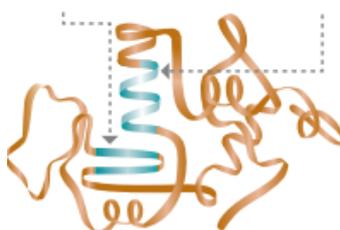
Figure 1.6: Tertiary Structure **[Smith et al., 2005]**.

1.2.5 Quaternary Structure

Many proteins are made up of a single polypeptide chain and have only three levels of structure (as outlined above). However, some proteins are made up of multiple

polypeptide chains, also known as subunits. When these subunits come together, they give the protein its quaternary structure as in figure 1.7. For example, hemoglobin and Deoxyribonucleic acid (DNA) polymerase **[Gregory and Dagmar, 2004]**. Figure 1.8 shows the Fourier transformation infrared (FTIR) spectrum for a protein and the bands.

Figure 1.7: Quaternary Structure **[Smith et al., 2005]**.
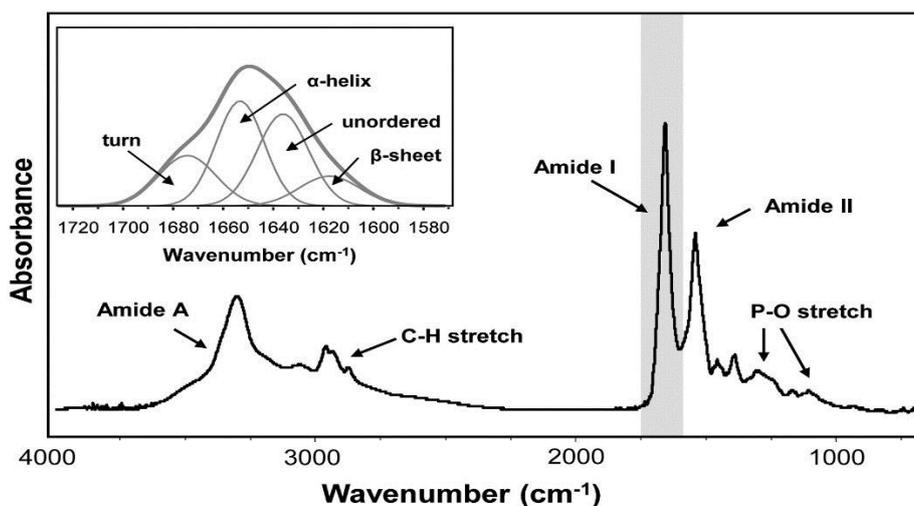
Figure 1.8:   FTIR spectrum of a typical protein illustrating the Amide I and Amide II bands **[yang et al., 2012]**.

1.3 Human Serum Albumin

The most abundant protein in blood plasma is Human Serum Albumin (HSA). HSA is able to bind and thereby transport various compounds such as fatty acids, hormones, bilirubin, tryptophan, steroids, metal ions, therapeutic agents and a large number of drugs. It serves as the major soluble protein constituent of the circulatory system.

HSA contributes to colloid osmotic blood pressure, and it can bind and carry drugs which are poorly soluble in water [**Abu Teir et al., 2011; Alsamamra et al., 2017**]. HSA binds a wide variety of hydrophobic ligands including fatty acids, bilirubin, thyroxine and hemin and also drugs [**Carter et al., 1989**].

Abu Teir stated that a protein plays a significant role in bringing solutes into blood stream and delivering them to the target organs, as well as to maintain the potential of hydrogen (pH) and osmotic pressure of plasma. HSA concentration in blood plasma is 40 mg/ml. To determine the three dimensional structure of HAS, an x-ray crystallographic measurements is applied [**Carter et al., 1989**].

Human Serum Albumin is composed of a single polypeptide chain of 585 amino acids as shown in figure 1.9. It contains 35cysteines, forming 17 disulfide bridges. The Thiols group of the unpaired cysteine at the amino acid position 34 plays an important roles in transporting and serving as reservoir for nitric oxide and binds to many small metal ions such as (Cu^{++}, Cd^{++}, Hg^{++}, Ag^{+} and Au^{+}). Moreover, a single tryptophan at amino acid position 214 is important as a research tool for protein chemists and biochemists because of the single tryptophan residue. For this reason, many spectroscopic and fluorescence studies, including molar concentration determination, fluorescence quenching studies, fluorescence lifetime studies also use it. HSA also contains many cysteine residues and charged amino acids, and it has small numbers of glycine, methionine [**XM and Carter, 1992**].

```
DAHKSE VAHRFKDLGE ENFKALVLIA FAQYLQQCPF
EDHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT
VATLRETYGE MADCCAKQEP ERNECFLQHK DDNPNLPRLV
RPEVDVMCTA FHDNEETFLK KYLYEIARRH PYFYAPELLF
FAKRYKAAFT ECCQAADKAA CLLPKLDELR DEGKASSAKQ
RLKCASLQKF GERAFKAWAV ARLSQRFPKA EFAEVSKLVT
DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK
ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVESKDVC
KNYAEAKDVF LGMFLYEYAR RHPDYSVVLL LRLAKTYETT
LEKCCAAADP HECYAKVFDE FKPLVEEPQN LIKQNCELFE
QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV SRNLGKVGSK
CCKHPEAKRM PCAEDYLSVV LNQLCVLHEK TPVSDRVTKC
CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL
SEKERQIKKQ TALVELVKHK PKATKEQLKA VMDDFAAFVE
KCCKADDKET CFAEEGKKLV AASQAALGL
```

Figure 1.9:   Amino acid sequence of the protein HSA [**Manuel and Bhusan, 2012**].

HSA contains three homologous domains in a heart-shaped conformation, named as domain I, domain II, and domain III, (see figure 1.10). Each domain has two separate helical subdomains (named A and B), connected by random coil. These subdomains A and B consist of 10 alpha helixes: alpha helix (1 - 6) forms subdomain A, and helixes (7 - 10) forms subdomain B **[Peters, 1998]**.
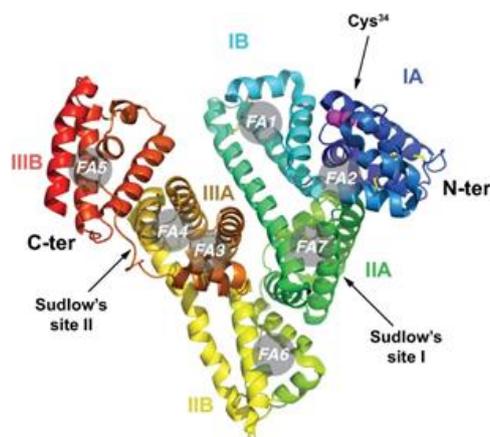
Figure 1.10: Molecular structure of HSA. Crystal structure of HSA with an indication of its subdomains (IA, IB, IIA, IIB, IIIA and IIIB), of the N and C termini **[Bhattacharya et al., 2000]**.

The N terminal end attaches the Cu^{+2} and Ni^{+2} ions. Domain III is the site of the two high affinity fatty acid attachments. Other fatty acid sites are located in the center of Domains I and II. Fatty acid significantly increases conformational stability. The overall denaturation temperature is increased from about Co_{60} (defatted) to Co_{80} (fatted) at pH 7.0, which must reflect an increase in stability in Domain III. Domain I has the highest net charge (-9) **[Fujiwara, 2006]**.

Aromatic and heterocyclic ligands bind within two hydrophobic pockets in subdomains IIA and IIIA, which are site I and site II. Site I is dominated by strong hydrophobic interaction with most neutral, bulky, heterocyclic compounds, while site II mainly dipole-dipole, van de Waals, and/or hydrogen-bonding interactions with many aromatic carboxylic acids **[Ouameur et al., 2004]**.

HSA contained a single tryptophan residue (Trp 214) in domain IIA and its intrinsic fluorescence is sensitive to the ligands bounded nearby **[Il'ichev et al., 2002]**. For this reason, it is often used as a probe to investigate the binding properties of drugs with HSA. Kang and colleagues have shown that the distribution free concentration and the metabolism of various drugs can be significantly altered as a result of their binding to HSA **[Kang et al., 2004]**. Research literature confirms multiple drug binding sites for HSA **[Bhattacharya et al., 2000]**.The binding properties of HAS depend on the three dimensional structure of the binding sites, which are distributed over the molecule. Strong binding can decrease the concentration of free drugs in plasma, whereas weak binding results in a short lifetime or poor distribution. HAS's remarkable capacity to bind a variety of drugs has led to its prevailing role in drug pharmacokinetics and pharmaco dynamics **[Kandagal et al., 2007]**.

1.4 Aspirin

Aspirin known as Acetylsalicylic Acid is the prototypical analgesic used in the treatment of mild to moderate pain. The molecular formula for Aspirin is C9H8O4 (see figure 1.11 for the chemical structure) and has anti-inflammatory and antipyretic properties. It acts as an inhibitor of cyclooxygenase, which results in the inhibition of the biosynthesis of prostaglandins **[Carswell et al., 1975]**.
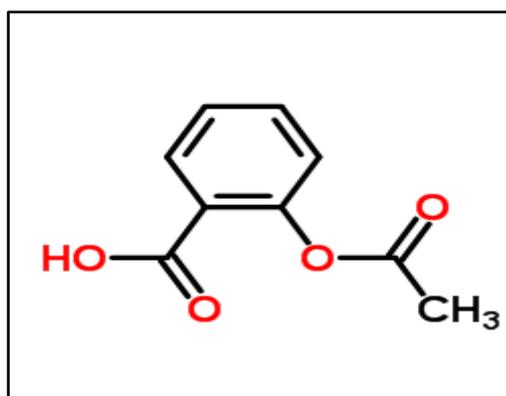
Figure 1.11: chemical structure of Aspirin

Acetylsalicylic acid is a weak acid (pK $_a$ = 3.5) and for this reason it can be absorbed across the mucosal lining of the stomach. However, most of the drug is absorbed from the upper regions of the small intestine. Upon entering the bloodstream Aspirin is hydrolyzed to acetic acid and salicylic acid [**Carswell et al., 1975**].

The most common use of Aspirin is to treat mild to moderate pain or to reduce fever. Aspirin is also prescribed to individuals suffering from arthritis and osteoarthritis because of its anti-inflammatory action. In addition to its anti-inflammatory, antipyretic, and analgesic properties, aspirin is also prescribed to patients at high risk for heart attack [**Patrignani, 2016**].

Aspirin in high doses use prescribed in the treatment and management of rheumatic fever, rheumatic arthritis, other inflammatory joint conditions and pericarditis. In low doses, it is useful in preventing blood clots from forming, reducing the risk of a transient ischemic attack (TIA) and unstable angina. It is useful in preventing myocardial infarction in patients with cardiovascular disease, by preventing clot formation. It is helpful as a preventative medicine for stroke and colorectal cancer, but not to treat a stroke [**Avorn, 1983**].

1.5 Recent Studies

Studied the competitive binding of vitamin C and aspirin to bovine serum albumin, using constant protein concentration and various drug concentrations at pH 7.2. FTIR and UV−Vis spectroscopic methods were used to analyze vitamin C and aspirin binding modes, the binding constants and the effects of drug complication on BSA stability and conformation. These authors reported that at low drugs concentrations, no major protein conformational changes occurred, whereas at high drugs contents, significant decreases of protein a-helix and b-sheet structures were observed. This is indicative of a partial destabilization of protein secondary structure at high drug concentrations. This study is interesting in the sense that BSA can be considered as a good carrier for transportation of vitamin C and aspirin in vitro [**Nafisi and Sadeghi, 2011**].

Research into the reaction mechanism between aspirin and bovine serum albumin (BSA) at different temperatures (293 K, 303 K, 310 K) and two different methods: method 1, the fluorescence spectroscopy focusing on the fluorescence change of protein; and method 2, the fluorescence spectroscopy, focusing on the fluorescence change of the drug, under simulated physiological conditions (pH = 7.4) revealed that the electrostatic forces played an important role on the conjugation reaction between aspirin and BSA **[Li et al., 2014]**.

Investigation on the interaction between aspirin and HSA by fluorescence spectroscopy, and from the interaction, constants K_D of human serum albumin and aspirin were determined at different temperatures according to double reciprocal Line weaver-Burk plot. Wang and colleagues discuss the main binding force through thermodynamic equations. Where constants K_D at 37°C 25°C was 1.44×10^{-3} and 1.96×10^{-3} mol.L^{-1} respectively **[yang et al., 2008]**.

Carried out research on the concurrent binding behavior of indomethacin to HSA under the effect of aspirin-mediated protein acetylation. They also explored the esterase-like catalytic property of the modified proteins, as binary or ternary systems, by using various spectroscopic and molecular docking techniques **[Esmaeili, 2017]**.

1.6 Research Statement

Infrared spectroscopy provides measurements of molecular vibrations through specific absorption of infrared radiation by chemical bonds. The form and frequency of the Amide I band, which is assigned to the C=O stretching vibration within the peptide bonds, is very characteristic for the structure of the studied protein. From the band secondary structure, components peaks (α-helix, β-strand) can be derived and analysis of this single band allows clarification of conformational changes with high sensitivity **[Darwish et al., 2010; Alsamamra et al., 2017]**.

This study is limited to the mid-range infrared, covering the frequency range from 4000 to 400 cm^{-1}. This wavelength region includes bands that arise from three conformational sensitive vibrations within the peptide backbone (Amides I, II and III) of these vibrations. Amide I is the most widely used and can provide information on secondary structure composition and structural stability **[Cui et al., 2008; Kang et al., 2004; Rondeau et al., 2007; Abu Teir et al., 2014; Alsamamra et al., 2017]**.

Other spectroscopic techniques used for studying the interaction between drugs and proteins include fluorescence and UV-VIS spectroscopy. These have the advantage of offering high sensitivity, rapidity and ease of implementation. **[Wybranowski et al., 2008; Li et al., 2008; Li et al., 2006].**

All the above-mentioned techniques will be used to study the interaction between HSA and Aspirin.

Finally, this thesis is comprised of five chapters: chapter two will discuss the theoretical aspects to guide the reader to the important ideas of this study. Chapter three includes details of the experimental, procedures, and instruments used. In chapter four the results are presented and discussed. The final chapter contains conclusions and future work.

Chapter Two

Theoretical Background

2.1 Introduction

In this chapter, I will outline spectroscopic techniques used in this thesis, along with the physical principle for each technique. In particular, Fourier transformation infrared spectroscopy and ultra-violet spectroscopy and florescence spectroscopy will be outlined.

2.2 Electromagnetic Waves

Electromagnetic waves (EMW) are transverse oscillating waves composed of electric and magnetic fields perpendicular to each other and perpendicular to the direction of propagation (see figure 2.1). EMW propagate by way of a sine or cosine waves at the speed of light in vacuum **[Stuart, 2004]**. Included in the electromagnetic spectrum are: radio waves, microwaves, infrared radiation, visible light, ultraviolet radiation, X-rays and gamma rays (see figure 2.2).
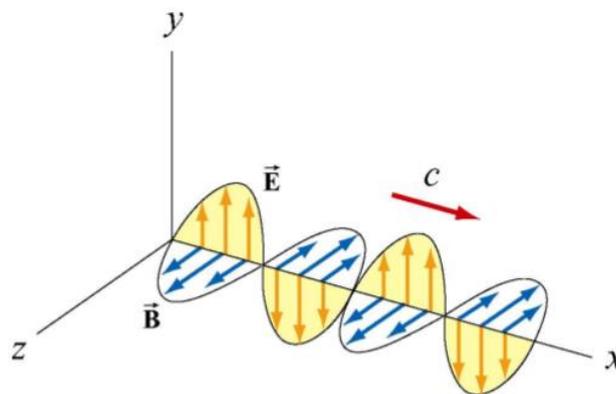
Figure 2.1: Plane electromagnetic wave propagating **[Sharma, 2007]**.
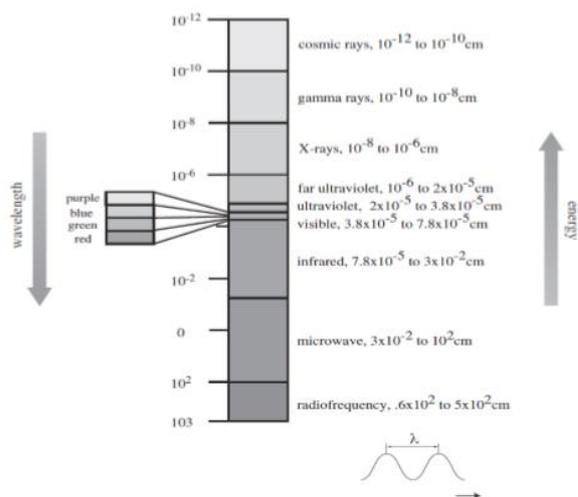
Figure 2.2: Electromagnetic spectrum **[Shernan, 2014]**.

In vacuum, IR radiation has a wavelength between ≈ (780 nm - 1 mm). It spans three orders of the Mid-IR region from 4000 to 400 cm^{-1}, where vibrational and rotational bands are observed, and the UV-VIS region is from 10-800 nm **[Hollas, 2004].**
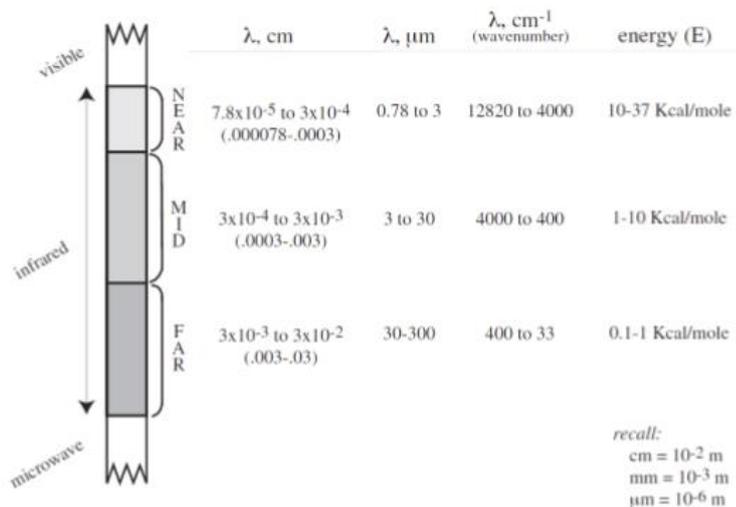
Figure 2.3: The IR region of electromagnetic spectrum **[Shernan, 2014]**.

The wavelength λ is inversely proportional to frequency v and is governed by the relation:

$$\lambda = \frac{c}{v} \qquad (2.1)$$

15

Where c is speed of light and ν is the frequency.

Electromagnetic radiation comprises multiple electromagnetic waves or photons, which carry energy, momentum and angular momentum. The energy of each photon is given by Planck–Einstein equation:

$$E = h\nu = h\frac{c}{\lambda} \tag{2.2}$$

Where E is the energy and h is Planck's constant and ν is frequencyand λ is wavelength **[Yadav, 2005; Williams, 1976; Ball, 2001].**

outlined that atomic spectra arise from the transition of electron between atomic energy levels, while molecular spectra arise from three types of energy transitions due to molecular rotation, molecular vibrational, and electronic transition **[Williams, 1976]**.

According to Born Oppenheimer approximation, the total energy of the molecule is given by:

$$E_{total} = E_{ele} + E_{vib} + E_{rot} + \cdots \tag{2.3}$$

Where:

E_{rot} : is rotational energy due to the molecule rotation about the axis passing the center of gravity for the molecule, E_{vib}: is vibrational energy due to the periodic displacement of atoms around their equilibrium positions , E_{ele}: is related to the energy of the molecule's electrons **[Sharma, 2007].**

When radiation falls on a sample it may be absorbed and occurs when the energy of radiation matches the difference in energy levels of the sample, otherwise it may be either transmitted or scattered by the sample.

A simplified representation of the quantized electronic and vibrational states is represented in figure 2.4. Research findings have confirmed that transitions between electronic energy states require more energy than transitions between vibrational energy states **[Hollas, 2004; Turro, 1991; Ball, 2001].**

IR radiation does not have enough energy to induce electronic transitions as seen with UV-VIS. Absorption of IR is appropriate to excite vibrational and rotational states of a molecule as shown in figure 2.4.
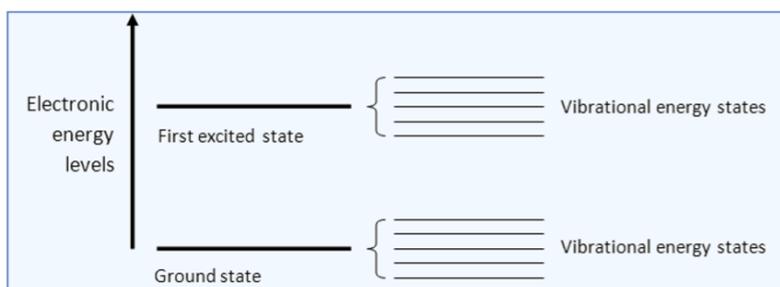
Figure 2.4: A schematic representation of the quantized electronic and vibrational energy levels of a molecule.

Intensity of the light absorbed to produce a given transition is given by Beer-Lambert law:

$$\frac{I}{I_0} = 10^{-\varepsilon Cl} \tag{2.4}$$

In which I and I_0 are the intensity of light transmitted through the absorber and incident upon it respectively.

ε : is the molar absorption coefficient or (molar extinction coefficient).

C: is the concentration of absorbing molecule in the sample.

l: is the length of the light path in the sample.

Equation 2.4 can be represented in logarithmic form:

$$A = \log_{10} \frac{I}{I_0} = \varepsilon(v)Cl \tag{2.5}$$

Where A is called Absorbance **[Hollas, 2004; Schulman, 1977].**

2.2.1 Infrared (IR) Spectroscopy

To obtain an infrared spectrum, infrared radiation is passed through a sample and determining the fraction of the incident radiation absorption at a particular energy. The energy at which any peak in an absorption spectrum appears corresponds to the frequency of a vibration of a part of a sample molecule **[Stuart, 2004].**

Infrared spectroscopy ($\bar{\upsilon}$= 400 to 4000 cm^{-1}) measures the changes in the vibrational and rotation movements of molecules. It is commonly used to show the presence or absence of functional groups, which have specific vibration frequencies for example, C=O, NH$_2$, OH, CH, C-O etc., **[Subodh, 2006].**

In order to for a molecule to absorb radiation, incoming infrared radiation has to be of the same frequency as one of the fundamental modes of vibration of the molecule. Resulting in the increase of the vibrational motion of a small part of the molecule whilst the rest of the molecule is unchanged. Vibrations can lead to either a change in bond length (stretching) or bond angle (bending), and some bonds can stretch in-phase (symmetrical stretching) or out-of-phase (asymmetric stretching) **[Stuart, 2004].**

Infrared radiation covers wavenumbers approximately from 13,000 to 10 cm^{-1} of the electromagnetic spectrum, or wavelengths from 0.78 to about 1000 μm. At high frequencies, it is bound in the red end of the visible region and at low frequency it is bound in the microwave region. The IR region is further separated into three smaller areas: Near IR, Mid IR and Far- IR. Mid-IR, between 4000 to 400 cm^{-1}, is the most frequently used region **[Shernan, 2014]**. Therefore, the Mid-IR range is selected for this study. As an experimental and analytical technique, IR spectroscopy is one of the oldest procedures available to contemporary researchers **[Settle, 1997].**

The key purpose of IR spectroscopic analysis is to determine the chemical functional groups in a sample. It works by positioning a sample in the path of an IR beam and absorption measurement of different IR frequencies is taken. Different functional groups absorb characteristic frequencies of IR radiation. Using various sampling accessories, IR spectrometers works on a range of sample types such as gases, liquids and solids, and makes the ideal tool for structural elucidation and compound identification **[Shernan, 2014].** IR spectroscopy provides measurements of molecular vibrations due to the specific absorption of infrared radiation by chemical bonds **[Narhi, 2013]**. The energy at which peak in absorption spectrum appears ,it tallies with the frequency of vibration of a part of a sample molecule. Molecules in an array of point masses that are connected with each other by massless springs representing the intra-molecular interactions between the atoms are known as Harmonic vibrations **[Wilson et al., 1955].**

The simplest case is given by two masses, m_A and m_B, corresponding to diatomic molecule A-B upon displacement of the spheres along the x-axis from equilibrium position by Δx, a restoring force F_x acts on the spheres, which according to Hooke's law, is given by :

$$F_x = -f \Delta x \tag{2.6}$$

Here f is the spring or force constant, which is a measure of the rigidity of the spring, that is, the strength of the bond [**Hildebrandt and Siebert, 2008**].

The potential energy V depends on the square of the displacement from the equilibrium position,

$$V = \frac{1}{2} f \Delta x^2 \tag{2.7}$$

As when spring (bond) is stretching it will affect the equilibrium distance (bond length) so potential energy will be affected.

The kinetic energy T of the oscillating motion is:

$$T = \frac{1}{2} \mu (\acute{X})^2 \tag{2.8}$$

Where μ is the reduced mass defined by

$$\mu = \frac{(M_A \cdot M_B)}{(M_A + M_B)} \tag{2.9}$$

Because of the conservation of energy, the sum of V and T must be constant such that the sum of the first derivatives of V and T is equal to zero. This leads eventually to the Newton equation of motion:

$$\frac{d^2 \Delta x}{dt^2} + \frac{f}{\mu} \Delta x = 0 \tag{2.10}$$

This equation represents the differential equation for a harmonic motion. Solving this equation leads to:

$$\omega = \sqrt{\frac{f}{\mu}} \tag{2.11}$$

The above equation describes what one intuitively expects when the circular frequency of the harmonic vibration increases, the rigidity of the spring (or the strength the bond) increases but decreases with increasing masses of the atoms (spheres).

2.2.2 Normal Modes of Vibration

Outlines that the normal modes of a system are the vibrational motions of the system, such that each coordinate of the system oscillates under simple harmonic motion with the same frequency **[Rosman, 2008].**

In Cartesian coordinate system, each atom can be displaced in the x, y and z directions, corresponding to three degrees of freedom. Thus, a molecule of N atoms has in total 3N degrees of freedom as shown in table 2.1, but not all of them correspond to vibrational degrees of freedom **[Settle, 1997].**

A nonlinear molecule (where the atoms are not located in straight line) has three rotational degrees of freedom, whereas a linear molecule has only two. The remaining 3N-6 and 3N-5 degrees of freedom correspond to the vibrations of a nonlinear and a linear molecule as shown in table 2.1 , respectively **[Hildebrandt and Siebert, 2008].**

Table 2.1: Degrees of freedom for polyatomic molecules **[Stuart, 1997]**.

Type of degree of freedom	Linear	Non-linear
Translational	3	3
Rotational	2	3
Vibrational	3N-5	3N-6
Total	3N	3N

Stretching and bending are two types of molecular vibrations corresponding to the normal mode of molecule. Stretching is rhythmical movement along the bond axis and can be symmetric or anti-symmetric **[Settle, 1997].** Bending vibrations arise when there is a change in bond angle between two atoms or when a group of atoms move, relative to the reminder of the molecule **[Mirabeela, 1998].**

In order to determine which functional groups are present or absent, use the frequency of normal modes, which is a characteristic of the presence of certain functional group. By examination of this frequency one can determine which functional groups are present or absent **[Shernan, 2014].**

There is a wide range of variation in many of the group frequencies. This is because the bands arise from complex interacting vibrations within the molecule. Absorption bands may, however, represent predominantly a single vibrational mode. Certain absorption bands, for example, those arising from C-H, O-H, and C=O stretching modes, remain within fairly narrow regions of the spectrum **[Settle, 1997].**

2.2.3 Quantum Mechanical Treatment of Vibration

The harmonic oscillator approximation treats a diatomic as if the nuclei were held together by a spring. The potential energy of classical harmonic oscillator depends upon the square of the displacement from equilibrium and the strength of the spring. All values of energy are allowed classically. The quantum mechanical solution to the harmonic oscillator equation of motion predicts that only certain energies are allowed

$$E_v = \frac{h}{2\pi}\sqrt{\frac{f}{\mu}}\left(v + \frac{1}{2}\right) \tag{2.12}$$

The potential energy for diatomic molecule for harmonic oscillator approximation is shown below in figure 2.5.

2.2.4 The An-Harmonic Oscillator

Real molecules follow less precisely the simple harmonic motion and do not adhere to Hooks law because they are not so elastic. For example, if a bond stretches 60% of its real length then a molecular complicated situation should be assumed **[Banwell, 1972].**

The Morse curve, see (fig. 2.5) for a molecule undergoing harmonic extensional compression a purely empirical expression, which fits this curve to good approximation was derived by Morse and is called the Morse function **[Settle, 1997].**

$$E = D_{eq}\big[1 - \exp(\delta(r_{eq} - r))\big]^2 \tag{2.13}$$

Where :

δ: constant for a particular molecule, D_{eq}:the dissociation energy, D_{eq}:the dissociation energy.

When it is treated using Schrodinger equation and using $E = \frac{1}{2}f\left(r - r_{eq}\right)^2$ then the pattern of the allowed vibration energy levels are found to be :

$$E_n = \left(n + \frac{1}{2}\right)\grave{W}_e - \left(n + \frac{1}{2}\right)^2 W_e X_e \tag{2.14}$$

Where:

$$n = 0,1,2,\dots$$

W_e: is an oscillating frequency, $\grave{W}_e$: is the oscillation frequency in wave number, X_e: is the corresponding an-harmonic it y constant which is positive and small for bond stretching ($\approx$+0.01) .this means that the vibration levels crowded more closely with increasing n **[Banwell, 1972].**
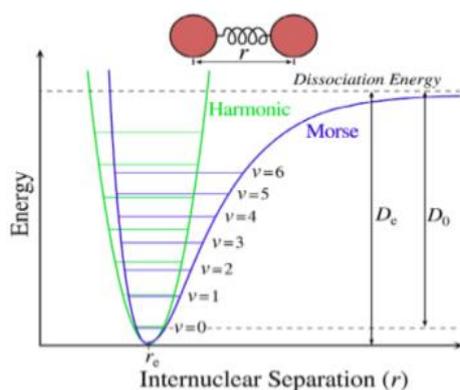
Figure 2.5: potential energy of a diatomic molecule as a function of atomic displacement (inter-nuclear separation) during vibration. The Morse potential (blue) and harmonic oscillator potential (green) **[Settle, 1997]**.

2.3 Fourier Transform Infrared (FT-IR) Spectroscopy

According to Settle (1977), FT-IR spectroscopy is a measurement of wavelength and intensity of the absorption of IR radiation by a sample. Historically, spectroscopy was heavily dependent on dispersion elements such as prisms or gratings .Hence different components of light (λ, ν) were allowed into the sample separately, while FT-IR

allows simultaneous measurements at all frequencies and can be applied to both emission or absorption **[Banwell, 1972].**

Infrared spectrometers capabilities have been significantly improved by the introduction of Fourier Transform spectrometers and have been applied to many areas that are very difficult or nearly impossible to analyze by dispersive instruments **[Shernan, 2014].** The most important advantage of FT-IR spectroscopy for biological studies is that spectra of almost any biological system can be obtained in a wide variety of environments **[Li et al., 2007].**

2.3.1 Principle of IR Absorption

At the very core of IR spectroscopy is the Principle of IR absorption by molecules. Absorption is the process by which the energy of a photon is taken up by the matter. Several types of physical processes are involved in absorption, depending on the quantum energy of the particular frequency of electromagnetic (EM) radiation. For example, electronic transitions and ionization **[Settle, 1997].** During energy absorption, molecules are excited to a higher energy states including IR absorption. IR radiation lacks enough energy to induce electronic transitions compared to UV-VIS. It corresponds to energy changes on the order of 8 to 40 KJ/mole **[Shernan, 2014].**

According to Settle (1997), the absorption of IR is restricted to excite vibrational and rotational states of a molecule. IR's energy range corresponds to the range encompassing the stretching and bending vibrational frequencies of the bonds in the most covalent molecules. A change in the amplitude of molecular vibration occurs when the frequency of the radiation matches the vibrational frequency of the molecule and radiation is absorbed. However not all bonds in a molecule are capable of infrared energy absorption .Only when the vibrations or rotations within a molecule cause a net change in the dipole moment of the molecule, then the bond is capable to absorb IR **[Pavia et al., 2009].**

IR spectrum represents a thumbprint of a sample with absorption peaks, corresponding to the frequencies of vibrations between bonds of the atoms making up the material. Since every material has its unique combination of atoms, no two compounds generate the exact same infrared spectrum **[Settle, 1997].** This is why IR spectroscopy is so useful for obtaining positive identification (qualitative analysis) of

different kind of material. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present (quantitative analysis) **[Thermo Nicolet, 2001].**

The height of the peaks is defined by the Beer-Lambert relationship. It states that the concentration C is directly proportional to the absorbance A.

That is:

$$A = abC \tag{2.15}$$

Where

a: is the absorptivity of the molecule.

b: is the path length or distance that the light travels through the sample **[Workman, 1998].**

2.3.2 IR Spectrum Presentation

Presentation of IR absorption information is generally takes form of a spectrum with wavelength or wave-number as the x-axis, and absorption intensity or percent transmittance as the y-axis. Transmittance, T, is the ratio of radiant power transmitted by the sample I to the radiant power incident on the sample I_0. Absorbance A is the logarithm to the base 10 of the reciprocal of the transmittance T .

$$A = \log_{10} \left(\frac{1}{T} \right) = -\log_{10} T = -\log_{10} \left(\frac{I}{I_0} \right) \tag{2.16}$$

The transmittance spectra provide better contrast between intensities of strong and weak bands because transmittance ranges from 0 to 100% T, on the other hand, absorbance range is from infinity to zero **[Shernan, 2014].**

2.3.3 Theory of FT-IR Spectroscopy

The most preferred method for IR spectroscopy is FT-IR. After IR is passes through a sample, the resulting spectrum represents the molecular absorption and transmission creating a molecular fingerprint of the sample **[Thermo Nicolet, 2001].**

A simple optical device called an interferometer is involved in the mechanism of a FT-IR spectrometer. As shown in the figure 2.6 below, the interferometer requires two mirrors, an infrared light source, an infrared detector and a beam splitter.
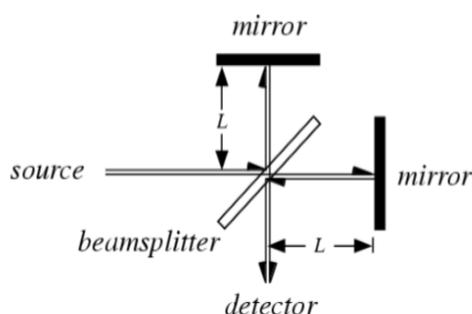
Figure 2.6: The Michelson interferometer **[Viji, 2006]**.

At the heart of an interferometer is a beam-splitter. This is a half-silvered mirror, the beam-splitter reflects about half of an incident light beam while simultaneously transmitting the remaining half. One half of this split light beam travels to the interferometer's moving mirror while the other half travels to the interferometer's stationary mirror **[Hsieh, 2008].** Both beams are reflected back by the two mirrors towards the beam-splitter, upon where each of the two beams is again half reflected and half transmitted. This results in two output beams. One travelling to the detector and the other one travels to the source **[Viji, 2006].**

When the returning two beams reach the beam-splitter, an interference pattern, or interferogram, is generated. This interference pattern varies with displacement of the moving mirror, with the difference in path length in the two arms of the interferometer. The interference pattern, detected by the infrared detector as variations in the infrared energy level, is what ultimately yields, spectral information **[Hsieh, 2008].** The interferogram is Fourier transformed with the help of a computer to convert the space domain into wave number domain **[Viji, 2006].**

A very simple optical system operates in the FTIR spectrometer (Fig. 2.7). The interferometer requires two mirrors, an infrared light source, an infrared detector, and a beam splitter.
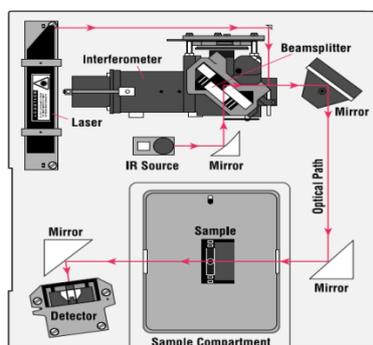
Figure 2.7: A Simple Spectrometer Layout **[Thermo Nicolet, 2001].**

An infrared source is polychromatic, and the light emitted is over a broad range of frequencies. Each frequency yields a unique cosine signal and the resulting interferogram represents the sum of all cosine waves generated by each individual infrared frequency. Importantly, however, only at the point where both mirrors are at an equal distances from the beam splitter does complete constructive interference occur, with simultaneous constructive interference of all wavelengths. At all other points, only some wavelengths interfere constructively, whilst others do not. The Fourier transform resolves the frequency and intensity of each cosine wave in the interferogram. That is, the algorithm converts the measured intensity versus mirror-displacement signal (the interferogram) into a plot of intensity versus frequency (a spectrum) **[Hsieh, 2008].**
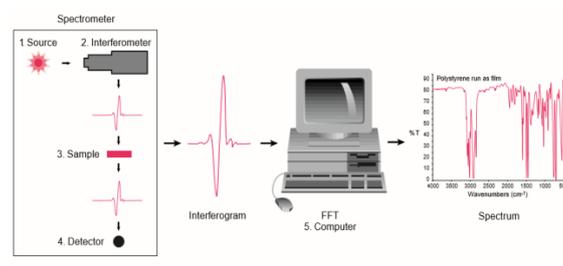
Figure 2.8: FT-IR spectrometer layout and basic components **[chemwiki]**.

2.3.4 Amide Bands

A polypeptide, or a protein for that matter, has nine infrared absorption bands. These include amide A, B, and I-VII. The amide I and II bands are the two most prominent vibrational bands of the protein backbone. The most sensitive spectral region for the protein secondary structure is the amide I band (1600 to 1700 cm^{-1}), which is due almost entirely to the C=O stretching vibrations. The amide II is mainly from the in-plane NH bending vibration and from CN stretching vibrations **[Smith, 2011].**

Amide vibrational bands in other categories are very complex depending on the details of the force field, the nature of side chains, and hydrogen bonding. So they are rarely used in the studying of protein structure. All characteristic amide bands are shown in (Table 2.2) **[Kong and Yu, 2007;Raaman, 2006].**

Table 2.2: Characteristic amide bands of peptide linkage **[Kong and Yu, 2007; Smith 2011].**

Designation	Approximate frequency	Description
Amide A	3300	NH stretching
Amide B	3100	NH stretching
Amide I	1600-1690	C=O stretching
Amide II	1480-1575	CN stretching, NH bending
Amide III	1229-1301	CN stretching, NHbending
Amide IV	625-767	OCN bending
Amide V	640-800	Out-of-plane NH bending
Amide VI	537-606	Out-of-plane C=O bending
Amide VII	200	Skeletal torsion

In amide I region (1600 to 1700 cm^{-1}), molecular geometry and hydrogen bonding pattern will give rise to different C=O stretching vibration. The amide I band contour consists of overlapping component bands (α-helix, parallel β-pleated sheet, anti-parallel β-pleated sheet, random coils, and β-turns).characteristic software is used to assign each component band **[Kong and Yu, 2007].**

Deuterium oxide (D_2O) is employed in infrared studies. Water (H_2O) is less frequently used because water absorbs strongly in the spectral region that overlap with amide I band and therefore it can affect the spectra. Conversely, D_2O has relatively low absorbance in the region between 1400 to1800 cm^{-1} **[Bai and Nussinov, 2007].**

Some proteins frequencies and the assigned secondary structural element in amide I band when employing H_2O or D_2O are shown in (Table 2.3)

Table 2.3: DE convoluted amide I band frequencies and assignments to secondary structure for protein in D_2O and H_2O media **[Kong and Yu, 2007]**.

H2O		D2O	
Mean frequencies	Assignment	Mean frequencies	Assignment
1624±1.0	β-sheet	1624±4.0	β-sheet
1627±2.0	β-sheet		
1633±2.0	β-sheet	1631±3.0	β-sheet
1638±2.0	β-sheet	1637±3.0	β-sheet
1642±1.0	β-sheet	1641±2.0	3-helix
1648±2.0	random	1645±4.0	Random
1656±2.0	α-helix	1653±4.0	α-helix
1663±3.0	3-helix	1663±4.0	β-turn
1667±1.0	β-turn	1671±3.0	β-turn
1675±1.0	β-turn	1675±5.0	β-sheet
1680±2.0	β-turn	1683±2.0	β-turn
1685±2.0	β-turn	1689±2.0	β-turn
1691±2.0	β-sheet	1694±2.0	β-turn
1696±2.0	β-sheet		

2.4 Ultraviolet

UV-VIS spectroscopy (λ = 200 - 800 nm) measures the changes in electronic energy levels within the molecule arising as a result of transfer of electrons from π- or non-bonding orbitals. It provides the knowledge about π-electron systems, conjugated unsaturation, aromatic compounds and conjugated non-bonding electron systems etc**., [Subodh, 2006].**

This absorption spectroscopy uses electromagnetic radiations between 190nm to 800 nm. It is divided into the ultraviolet (UV 190-400 nm) and visible (VIS 400-800 nm) regions and the absorption of ultraviolet or visible radiation by often referred as electronic spectroscopy.

Energy absorbed in the UV regions causes changes in the electronic energy of a molecule. As a molecule absorbs energy, an electron is promoted from an occupied molecular orbital (usually a non-bonding or bonding π orbital) to an unoccupied molecular orbital (an anti-bonding π^* or σ^* orbital) of greater potential energy, as in figure 2.9 **[Smith, 2011].**
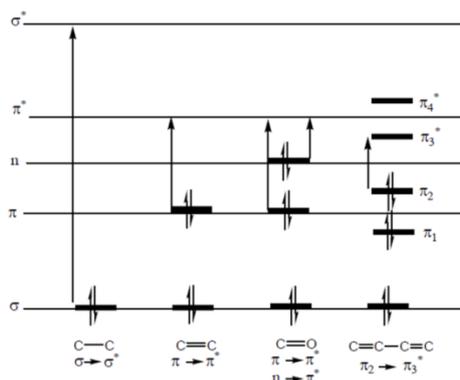
Figure 2.9: Relative energies of orbitals most commonly involved in electronic spectroscopy of organic molecules **[Subodh, 2006]**

For the vast majority of molecules, the lowest energy occupied molecular orbitals are σ orbitals, which correspond to σ bonds. Thus likely electronic transitions are:

$$n \to \pi^* < n \to \sigma^* < \pi \to \pi^* < \sigma \to \pi^* < \sigma \to \sigma^* \qquad (2.24)$$

When radiation energy is absorbed during excitation of electrons from ground state to excited state, this process principally depends on the nuclei that hold the electrons together in a bond. The group of atoms containing electrons responsible for the absorption is called Chromophore **[Hildebrandt and Siebert, 2008].**
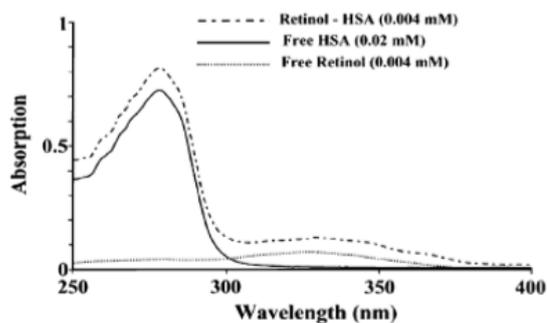
Figure 2.10: UV-absorption spectra of free HSA (0.02 mM), free retinol (0.004 mM) and their protein complexes **[Subodh, 2006]**.

The wavelength of the radiation that will be absorbed by organic molecule is contingent upon the difference in energy between ground state and the various excited electronic states of the molecule [Smith, 2011].

Atoms in organic molecules are bonded through σ and π bonds and the possible transitions between them are shown in (Fig. 2.11). Principally, the transition occurs from Highest Occupied Molecular Orbital (HOMO) to Lowest Unoccupied Molecular Orbital (LUMO). Of all the six transitions shown in (Fig 2.11), only the two of lowest energy ($n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$) can be achieved with radiation available in the range 200-650 nm which corresponds to UV-Vis region [Yadav, 2005 ; Kalsi, 2004; Raaman, 2006] .
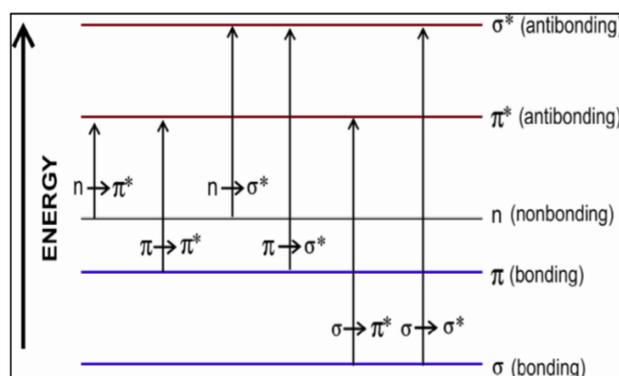
Figure 2.11: Generalized molecular orbital energy level diagram and possible transitions for organic compounds [Yadav, 2005; Raaman, 2006].

The $\pi \rightarrow \pi^*$ transitions are generally intense while $n \rightarrow \pi^*$ transitions are weak. Only molecules that have π bonds and atoms with nonbonding electrons absorb light in the range 200-700nm and it is called chromophores. Examples of chromopheric bonds and their absorption characteristics are given in (Table 2.4), and absorption ranges for various electronic transitions are shown in (Fig 2.12) [Kalsi, 2004].
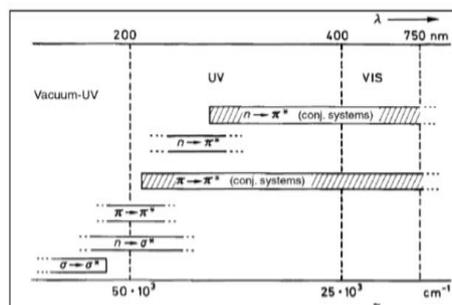
Figure 2.12: Absorption ranges for various electronic transitions [Yadav, 2005].

Table 2.4: Absorption characteristics of some common chromophoric groups [Kalsi, 2004].

chromophore	λ_{max}	Type of transition
>C=C<	177	$\pi \to \pi^*$
-C=C-	178	$\pi \to \pi^*$
>C=O	186	$n \to \sigma^*$
	280	$n \to \pi^*$
	180	$n \to \sigma^*$
	293	$n \to \pi^*$
-COOH	204	$n \to \pi^*$
-CONH$_2$	214	$n \to \pi^*$
-N=N-	339	$n \to \pi^*$
-NO$_2$	280	$n \to \pi^*$
-N=O	300	$n \to \pi^*$
	665	$n \to \pi^*$

The absorption data were treated using linear double reciprocal plots based on the following equation [Rosman, 2008]:

$$\frac{1}{A - A_0} = \frac{1}{A_\infty - A_0} + \frac{1}{K[A_\infty - A_0]} \cdot \frac{1}{L} \tag{2.25}$$

for ligand, A_∞ is the final absorption of the ligated protein, and A is the recorded absorption at different concentrations (L).

The double reciprocal plot of $\frac{1}{A-A_0}$ vs $\frac{1}{L}$ is linear and the binding constant K can be estimated from the ratio of the intercept to the slope. In order to determine whether the bidding between HAS and Aspirin is weak or strong, compare the value obtained

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with the complex constants for strongly bound ligand-protein complexes vary within the range 10^6 to 10^8 M^{-1} **[Hornaback, 2006].**

To measure the intensity of a light beam as a function of wavelength, a spectrometer is used. Spectrophotometers, for the measurement of absorbance in the UV-VIS range, come in a variety of configurations. The most common routine laboratory instruments are single or double beam devices made up of a light source, monochromatic, sample compartment, detector, data processor and display **[Cooper, 2004].**

Spectrophotometers components include a light source (UV and VIS), monochromator (wavelength selector), sample holder, a detector, signal processor and readout. The radiation source used is often a tungsten filament, a deuterium arc lamp, which is continuous over the ultraviolet region, and more recently light emitting diodes (LED) and xenon arc lamps for the visible wavelengths. The detector is commonly a photodiode or a charge-coupled device (CCD). Photodiodes are used with mono chromators, which filter the light resulting in a single wavelength reaching the detector. When measuring absorbance at the UV spectrum, the other lamp has to be turned off. The same goes when measuring visible light absorbance **[Nakanishi et al., 1998].**

The absorption of UV light by proteins has been research thoroughly and proposed as a structural probe from the start of molecular biology. The absorption of proteins in the UV arises mainly from electronic bands in aromatic amino acid side chains (tryptophan, tyrosine, phenylalanine) and ,to a lesser extent, cysteine residues, close to 280 n **[Serduyk et al., 2007].**
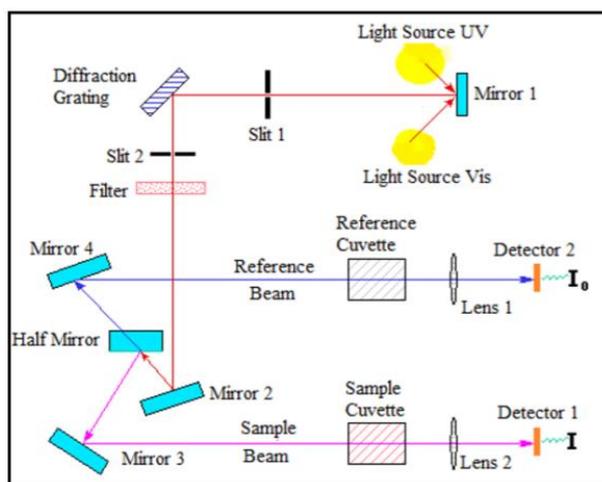
Figure 2.13: Schematic diagram of UV-VIS−NIR Spectrophotometer [**Nakanishi et al., 1998**].

The light source is a mono chromate splits the light into two equal intensity beams by a half mirrored device before it reaches the sample. Beam one is label led sample beam and beam two is label led reference. The sample beam passes through a small transparent container (cuvette) containing a solution compound being studies in a transparent solvent. The reference beam passes through an identical cuvette containing only the solvent. The containers for the sample and reference solution must be transparent to the radiation which will pass through them. Spectroscopy in the UV-Vis−NIR region requires quartz or fused silica cuvettes. The light sensitive detector follows the sample chamber and measures the intensity of light transmitted from the cuvettes and passes the information to a meter that records and displays the value to the operator on a screen. Electronic detectors are used to measure the intensities of these light beams and compared [**Nakanishi et al., 1998**].

2.5 Fluorescence

Luminescence is emission of light by a substance not resulting from heat. It is therefore essentially a form of cold body radiation. It can be divided into categories: fluorescence and phosphorescence, depending on the nature of the excited states **[Melhuish et al., 1981].**

Fluorescence and phosphorescence are photon emission processes. They occur during molecular relaxation from electronic excited states. The emission of light by a substance that has absorbed light or other electromagnetic radiation is a process called Fluorescence **[Johnson and Spence, 2010].** An example of a molecule that is capable

of fluorescing is Fluorophore, which in its ground state has relatively low energy, stable configuration and it does not fluoresce. However, at high energy configurations, fluorophore is unstable and will eventually adopt the lowest-energy excited state, which is semi-stable **[Melhuish et al., 1981].**

The mechanisms by which fluorescence of fluorescent probes and other fluorophores takes place is depicted by the simple electronic-state diagram (Jablonski diagram) shown in Figure (2.14) below
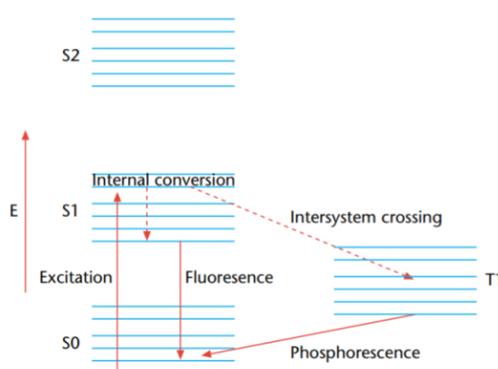
 Figure 2.14:   The Jablonski diagram of fluorophore excitation **[Dong and So, 2002]**.

In the above figure E denotes the energy scale; S_0 is the ground singlet electronic state, S_1 and S_2 are the successively higher energy excited singlet electronic states. T_1 is the lowest energy triplet state **[Johnson and Spence, 2010].**

Spectrochemical method of analysis or Fluorescence occurs when the molecules of the analyze are excited by irradiation at a certain wavelength and emit radiation of a different wavelength. Both qualitative and quantitative information is derived from the emission spectrum **[Melhuish et al., 1981].**

When a molecule absorbs light at a particular wavelength, the electronic state of the molecule changes from ground state to one of many vibrational levels in one of the excited electronic states. This is usually in the first excited singlet state.

When the molecule is in an excited state, relaxation can occur via several processes. For example, the process of Fluorescence can occur resulting in light emission **[Johnson and Spence, 2010]**. And this relates with the relaxation of the molecule from the singlet excited state to the singlet ground state with emission of light. Since fluorescence has short lifetime ($\sim 10^{-8}$ sec), in many molecules it can compete

favorably with collisional deactivation, intersystem crossing and phosphorescence. The wavelength (and thus the energy) of the light emitted is dependent on the energy gap between the ground state and the singlet excited state **[O'Reilly et al., 1975].**

The most important characteristics of a fluorophore are fluorescence lifetime and quantum yield. In order to work out the Quantum yield, take away the number of emitted photons relative to the number of absorbed photons. The lifetime is determined by the time available for the fluorophore to interact with or diffuse in its environment, and hence the information available from its emission **[Lakowicz, 2006].**

After the excitation occurs in a molecule, the excited state exists for a finite time (typically 1^{-10} nanoseconds). The fluorophore undergoes conformational changes during this time and is also subject to a multitude of possible interactions with its molecular environment. There are two important consequences of these processes :firstly, the energy of S_2 is partially dissipated, yielding a relaxed singlet excited state S_1 from which fluorescence emission originates; and secondly, not all the molecules initially excited by absorption return to the ground state S_0 by fluorescence emission **[O'Reilly et al., 1975].**

At Fluorescence Emission stage a photon of energy $h\upsilon_{EM}$ is emitted, returning the fluorophore to its ground state S_0. When energy is depleted during the excited-state lifetime, the energy of this photon is lower, and therefore of a longer wavelength compared to the excitation photon $h\upsilon_{EX}$. The difference in energy or wavelength represented by $(h\upsilon_{EX} - h\upsilon_{EM})$ is called the Stokes shift **[Johnson and Spence, 2010].**

There are two main categories for Fluorophores. Intrinsic fluorophores occur naturally, for example, aromatic amino acids. Eextrinsic fluorophore are added to the sample to provide fluorescence when none exists, or to change the spectral properties of the sample **[Lakowicz, 2006].**

2.5.1 Quenching

Fluorescence quenching is a bimolecular process, which reduces the fluorescence intensity without changing the fluorescence emission spectrum. It is a consequence of transient excited-state interactions (collisional quenching) or from formation of non-fluorescent ground-state species. Although, this rarely occurs, a decrease of

fluorescence intensity by interaction of the excited state of the fluorophores with its surroundings is known as quenching. Several mechanisms may involve quenching, for instance, collisional quenching occurs when exited state fluorophores are deactivated upon contact with some other molecule in solution, which is called a quencher **[Sheehan, 2009].**

For collisional quenching the decrease in intensity is given by Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + k_{sv}[L] = 1 + k_q \tau_0 [L] \qquad (2.26)$$

Where k_{sv} is Stern-Volmer quenching constant, k_q is bimolecular quenching constant, τ_0 is the unquenched lifetime, and $[l]$ is the quencher concentration. The quenching constant indicates the sensitivity of the fluorophores to the quencher. A wide variety of molecules can act as a collisional quencher. Examples include oxygen, halogen, amines, and electron deficient molecules like acryl amide **[Turro, 1991].**

Different mechanisms for quenching may be involved depending on the fluorophores quencher pair. For instance, quenching of in dole by acryl amide place as a consequence of electron transfer from in dole to acryl amide, whilst quenching by halogens and heavy atoms occurs as a result of spin orbit coupling and intersystem crossing to the triplet state **[Lakowicz, 2006; Sheehan, 2009].**

Static quenching is another type of quenching. It occurs in the ground state and does not rely on diffusion or molecular collisions. It occurs as a result of complex formation in the ground state between fluorophores and quencher **[Turro, 1991].**

2.5.2 Phosphorescence

When an electron is promoted into a higher orbital because of production of excited states, the direction of spin of the electrons is preserved. This happened because most of the molecules have an even number of electrons and they will be arranged in pairs. It is also possible for the spin of the promoted electron to be reversed, so pairing does not occur and leaving the molecule with two independent electrons of the spin in different orbitals **[Lakowicz, 2006].** Quantum theory underscores that such a molecule can exist and found mainly in three forms that differ slightly. However,

these are normally indistinguishable in their energy, and the molecule is described to exist in a triplet state. Intersystem crossing is said to occur when the indirect process of conversion takes place from the excited state, which is the produced by absorption energy from singlet state, to a triplet state (see Fig. 2.13).This can take place in various substances and occurs when the lowest vibrational level of the excited singlet state, S1, has the same energy level as an upper vibrational level of the triplet state [Sheehan, 2009].

It is theoretically forbidden for the direct transition from the ground state, usually singlet state, for a molecule with an even number of electrons, to an excited triplet state . This implies that w the reverse transition from triplet to ground state will be difficult. Moreover, while the transition from an excited singlet state, for example, S_1, to the ground state with the emission of fluorescence can take place easily and within 10^{-9}- 10^{-6} seconds, the transition from an excited triplet state to the ground state with the emission of phosphorescence requires at least 10^{-4} seconds and may take as long as 10^2 seconds. Therefore, describing phosphorescence as the transitions from the triplet state to the ground state [Sharma, 2007; Lakowicz, 2006].

In absorption spectroscopy, the fundamental process is the absorption of a discrete amount of energy. However, the mechanism of absorption of energy is different in the ultraviolet, infrared and nuclear magnetic resonance regions. The energy required for the transition from a state of lower energy (E_1) to state of higher energy (E_2) is exactly equivalent to the energy of electromagnetic radiation that causes transition [Sheehan, 2009].

A molecule can only absorb a particular frequency provided the molecule contains an energy transition of magnitude $E = hv$. In order to understand matter; virtually all parts of the electromagnetic spectrum are used. However, in organic chemistry we are mainly concerned with energy absorption from only the ultraviolet and visible, infrared, microwave and radiofrequency regions [Subodh, 2006].

Chapter Three

Experimental Part

3.1 Introduction

Section two is about dealing with samples, and film preparation. Section three describes the spectrometers used in this work, which are UV-VIS spectrophotometer (Nano Drop ND-1000), and Bruker IFS 66/S FT-IR. Section four presents the experimental procedure in details.

3.2 Samples and Materials

Human Serum Albumin, Aspirin were purchased from Sigma Aldrich chemical company and used without further purifications. The data were collected using samples in the form of thin films for FT-IR measurements and liquid form for UV-VIS. Preparations of the thin film samples required three stock solutions as described below:

3.2.1 Preparation of HSA Stock Solution

HSA was dissolved in phosphate buffer Saline and at physiological (pH 7.4), to a concentration of (40 mg/ml), and used at final concentration of (40 mg/ml) in the final Aspirin - HSA solution.

3.2.2 Preparation of Aspirin Stock Solution

Aspirin with molecular weight of (180.157 g.mol^{-1}), was dissolved in phosphate buffer Saline and, then the solution was placed in ultrasonic water path (SIBATA AU-3T) for one hour to ensure that all the amount of Aspirin was completely dissolved.

3.2.3 HSA-Aspirin Samples

The final concentrations of HSA-Aspirin solutions were prepared by mixing equal volume from HSA to equal volume from different concentration of Aspirin. HSA concentration in all samples kept at 40 mg.ml^{-1}. However, the final concentrations of aspirin in mM are (0.5, 0.7, 0.9, 1.2 and 1.3).

3.2.4 Thin Film Preparations

Silicon windows (NICODOM Ltd) were used as spectroscopic cell windows. The optical transmission is high with little or no distortion of the transmitted signal. The 100% line of a NICODOM silicon window shows that the silicon bands in the mid-IR region do not exhibit total absorption and can be easily subtracted. 40 μl of each sample of HSA-Aspirin was spread on a silicon widow and an incubator was used to evaporate the solvent, to obtain a transparent thin film on the silicon window. All solutions were prepared at the same time for one run at room temperature 25°C.

3.3 Instruments

Many instruments can be used in studying the interaction of HSA with drugs. In this work the following instruments have been used in taking the measurements.

3.3.1 FT-IR Spectrometer

The FT-IR measurements were obtained on a Bruker IFS 66/S spectrophotometer equipped with a liquid nitrogen-cooled MCT detector and a KBrbeam splitter. The spectrometer was continuously purged with dry air during the measurements.

3.3.2 UV-VIS Spectrophotometer

The absorption spectra were obtained by the use of a NanoDrop ND-1000 spectrophotometer. It is used to measure the absorption spectrum of the samples in the range between 220-750 nm, with high accuracy and reproducibility.

3.3.3 Fluorospectrometer

The fluorescence measurements were performed by a NanoDrop ND-3300 Fluorospectrophotometer at 25°C. The excitation source comes from one of three solid-state LED's. The excitation source options include: UV LED with maximum excitation 365 nm, Blue LED with excitation 470 nm, and white LED from 500 to 650nm excitation. A 2048-element CCD array detector covering 400-750 nm is connected by an optical fiber to the optical measurement surface. The excitation is done at the wavelength of 360 nm and the maximum emission wavelength is at 439 nm. Other equipment such as Digital balance, pH meter, Vortex, Plate stirand Micropipettes were used **[NanoDrop 3300 Fluorospectrometer V2.7 user's Manual 2008]**.

3.4 Experimental Procedure

3.4.1 UV-VIS Spectrophotometer Experimental Procedures

Procedure of UV-VIS spectrophotometer was followed as described in NanoDrop1000 Spectrophotometer V3.7, 2008, User's Manual **[NanoDrop 1000 Spectrophotometer V3.7, User's Manual, 2008]**, which is as follows:

A (10) μl sample of Aspirin is pipetted into the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap is controlled to both 1mm and 0.2 mm paths. A pulsed xenon flash lamp provides the light source and a spectrophotometer utilizing a linear CCD array is used to analyze the light after passing through the sample. The instrument is controlled by personal computer (PC) based software, and the data is logged in an active file on the PC.

Before taking the samples absorbance on the NanoDrop 1000 Spectrophotometer "blanked". When the NanoDrop 1000 Spectrophotometer is "blanked", spectrum is taken of a reference material (blank) and stored in memory of the instrument as an array of light intensities by wavelength. When a measurement of a sample is taken, the intensity of light that has transmitted through the sample is recorded.

The sample intensities along with the blank intensities are used to calculate the sample absorbance according to the following equation:

$$\text{Absorbance} = -\log(\text{Intensity sample}/\text{Intensity blank}) \tag{3.1}$$

Thus, the measured light intensity of both the sample and of the blank are required calculating the absorbance at a given wavelength, and Beer-Lambert equation is used to correlate the calculated absorbance with concentration.

Basic Use: the main steps for using the sample retention system are listed below:

1- With the sampling arm open, pipette the sample onto the lower measurement pedestal see photo no.1 of figure3.1.

2- Close the sampling arm and initiate a spectral measurement using the operating software on the PC. The sample column is automatically drawn

between the upper and lower measurement pedestals and the spectral measurement made see photo no.2 of figure 3.1.

3- When the measurement is complete, open the sampling arm and wipe the sample from both the upper and lower pedestals using a soft laboratory wipe. Simple wiping prevents sample carryover in successive measurements for samples varying by more than 1000 fold in concentration see photo no.3 of figure 3.1.
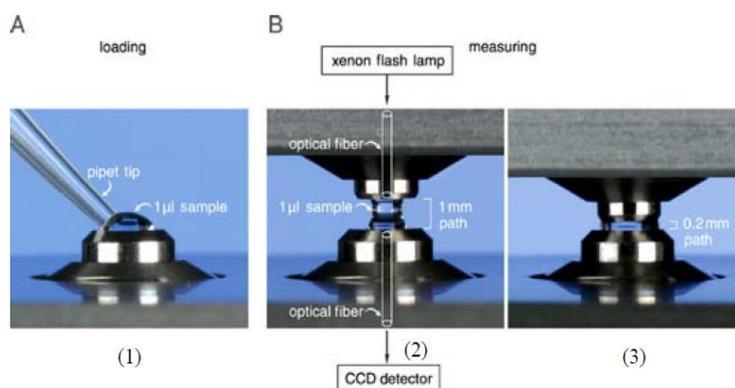
Figure3.1: Main steps for using the sample UV-VIS spectrometer **[NanoDrop 1000 Spectrophotometer V3.7, User's Manual, 2008].**

3.4.2 Fluorospectrophotometer Experimental Procedures

Procedure of Fluorospectrophotometer was followed as described in NanoDrop 3300 Fluorospectrometer V2.7,2008 User's Manual,**[NanoDrop 3300 Fluorospectrometer V2.7 User's Manual, 2008]**, which is as follows:

Before taking the measurements of samples the NanoDrop 3300 Fluorospectrometer was "balnked".

A (10) µl sample of Aspirin is pipetted onto the end of the lower measurement pedestal (the receiving fiber). A non-reflective "bushing" attached to the arm is then brought into contact with the liquid sample causing the liquid to bridge the gap between it and the receiving fiber. The gap, or path-length, is controlled to 1mm. following excitation with one of the three LEDs; emitted light from the sample passing through the receiving fiber is captured by the spectrophotometer. The

NanoDrop 3300 is controlled by software run from a PC. All data is logged and archived in a folder at a user defined location.

Basic Use: The main steps for making a measurement are listed below:

1. With the sampling arm open, pipette the sample into the lower measurement pedestal see photo no. 1 of figure 3.2.

2. Close the sampling arm and initiate a measurement using the operating software on the PC. The sample column is automatically drawn between the upper bushing and the lower measurement pedestal and the measurement is made see photo no. 2 of figure 3.2.

3. When the measurement is complete, open the sampling arm and wipe the sample from both the upper bushing and the lower pedestal using low lint laboratory wipe see photo no. 3 of figure 3.2.
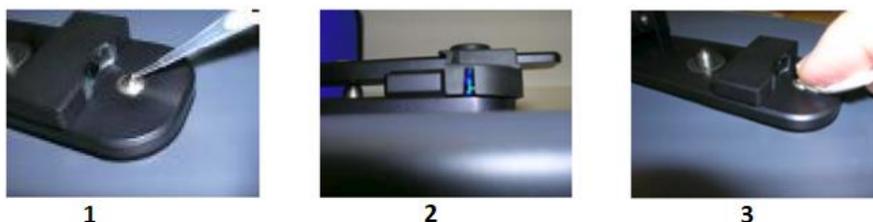
Figure 3.2: Main steps for using the sample fluorescence spectrometer **[NanoDrop 3300 Fluorospectrometer]**.

3.4.3 FT-IR Spectrometer experimental procedures

The absorption spectra were obtained in the wave number range of 400-4000 cm^{-1}. A spectrum was taken as an average of 60 scans to increase the signal to noise ratio, and the spectral resolution was at 4 cm^{-1}. The aperture used in this study was 8 mm, since we found that this aperture gives best signal to noise ratio. Baseline correction, normalization and peak areas calculations were performed for all the spectra by Optic User (OPUS) software. The peak positions were determined using the second derivative of the spectra.

The infrared spectra of HSA, Aspirin-HSA complexes were obtained in the region of 1000-1800 cm^{-1}. The FT-IR spectrum of free HSA was acquired by subtracting the absorption spectrum of the buffer solution from the spectrum of the protein solution. For the net interaction effect, the difference spectra {(protein and Aspirin solution)-

(protein solution)} were generated using the featureless region of the protein solution 1800-2200 cm^{-1} as an internal standard **[Surewicz et al. 1993].**

3.4.4 FT-IR Data Processing

The analysis of IR spectra in terms of protein structure is not straightforward and presents serious conceptual and practical problems, despite the well-recognized conformational sensitivity of the IR-active bonds. Bands in amide I, amide II and amide III regions are broad, not resolved into individual components corresponding to different secondary structure elements.

Resolution enhancement or band-narrowing methods are applied to resolve broad overlapped bands into individual bands. FT-IR spectroscopy presents several advantages over conventional dispersive techniques for this type of analysis through the application of second derivative, peak picking, spectral subtraction, baseline correction, smoothing, integration, curve fitting and Fourier self-deconvolution. In the present study several data processing tasks were used, such as:

3.4.4.1 Baseline Correction

The baseline correction method applied here includes two steps.

The first step is to recognize the baseline; this is done by selecting a point from spectral points on the spectrum. Then adding or subtracting intensity value from the point or points to correct the baseline offset. Baseline correction task is used to bring the minimum point to zero. This is done automatically using Optic User Software (OPUS) and successfully removes most baseline offsets **[Griffiths et al., 2007; OPUS Bruker manual, 2004].**

3.4.4.2 Peak Picking

Automated peak picking involves two steps: (1) the recognition of peaks, and (2) the determination of the wave-number values of maximum or minimum absorbance. A threshold absorbance value is usually set so that weak bands are not measured **[Griffiths et al., 2007].**

3.4.4.3 Second Derivative

Increased separation of the overlapping bands can be achieved by calculating the second derivative rate of change of slope of the absorption spectrum,

Second derivative procedure has been successfully applied in the qualitative study of a large number of proteins **[Haris et al., 1999].**

3.4.4.4 Fourier Self-Deconvolution

The Fourier deconvolution procedure, sometimes referred to as 'resolution enhancement' is the most widely used bands narrowing technique in infrared spectroscopy of biological materials. Both second derivative and deconvolution procedures have been successfully applied in the qualitative study of a large number of proteins **[Workman, 1998; Kauppinnen et al., 1981].** In addition to providing valuable information about their secondary structure, the method has been shown to be useful for detecting conformational changes arising as a result of a ligand binding, pH, temperature, organic solvents, detergents,.etc. In many cases results obtained using this approach has been later supported by studies using other techniques such as X-ray diffraction and Nuclear Magnetic Resonance (NMR). However, both derivative and deconvolution techniques should be applied with care since they amplify the noise significantly **[Haris et al., 1999].**

3.4.4.5 Spectral Subtraction

Difference spectroscopy is another approach that is very useful for investigating subtle difference in protein structure. The principle of difference spectroscopy involves the subtraction of a protein absorbance spectrum in state A from that of the protein in state B. The resultant difference spectrum only shows peaks that are associated with those groups involved in the conformational change **[Goormaghtigh et al., 2006 ; Haris et al., 1999].** The accuracy of this subtraction method is tested using several control samples with the same protein or drug concentrations, which resulted into a flat base line formation.

3.4.4.6 Curve Fitting

The Curve Fit command allows calculating single components in a system of overlapping bands. A model consisting of an estimated number of bands and a baseline should be generated before the fitting calculation is started. The model can be set up interactively on the display and is optimized during the calculation **[OPUS Bruker manual, 2004].**

Chapter Four

Result and Discussion

In the first section, UV-VIS spectrophotometer results are discussed and analyzed. The next section deals with fluorescence spectrophotometer results. In the final section, FT-IR graphs and data analysis are given.

4.1 UV-VIS

UV-absorption spectroscopy was used to determine the binding constants between HSA and a drug (Aspirin). The strength of interaction between HSA and drugs is dependent on the binding constant which can be calculated using graphical analysis of the absorbance spectrum.

The excitation has been done on 210 nm and the absorption is recorded at 235 nm for Aspirin. The absorption spectra of different concentrations of Aspirin (Fig.4.1) with HSA showed an increase of the intensity as the Aspirin concentration increases; this is due to major ligand protein interaction at protein surface which does not limit the mobility of ligand around HSA molecule.
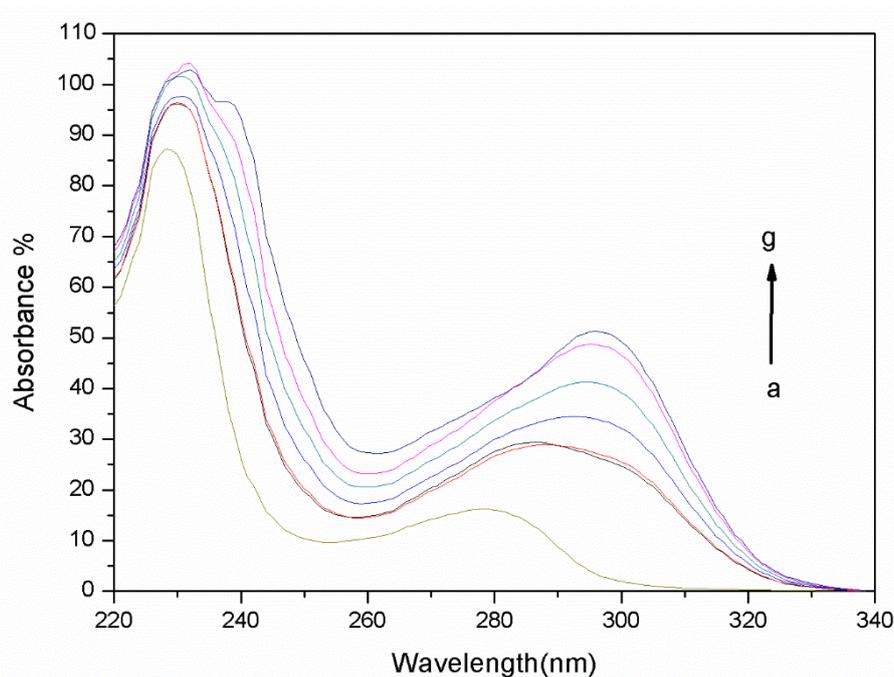

Figure 4.1: UV-absorbance spectra of HSA with different concentrations of Aspirin (a=freeAspirin,b=freeHSA,c=0.5mM,d=0.7mM,e=0.9mM,f=1.2mM,g=1.3mM)

This result support that the peak shifts between free HSA solution and Aspirin-HSA complexes are due to the interaction between Aspirin and HSA.

4.1.1 Binding Constant

Aspirin-HSA complexes binding constant were determined using UV-VIS spectrophotometer results according to published method **[Stephanos, 1996; Koltz et al., 1971; Ouameur et al., 2004]**, by assuming that there is only one type of interaction between Aspirin and HSA in aqueous solution, which leads to establish equation as follows:

$$K = [\text{ Aspirin:HSA }] / [\text{ Aspirin }][\text{ HSA }] \qquad (4.1)$$

The absorption data were treated using linear reciprocal plots based on the following equation **[Lakowicz, 2006].**

$$\frac{1}{A - A_0} = \frac{1}{A_\infty - A_0} + \frac{1}{K[A_\infty - A_0]} \cdot \frac{1}{L}$$

Where A_0 corresponds to the initial absorption of protein at 280 nm in the absence of ligand, A_∞ is the final absorption of the ligated protein, and A is the recorded absorption at different concentrations (L).
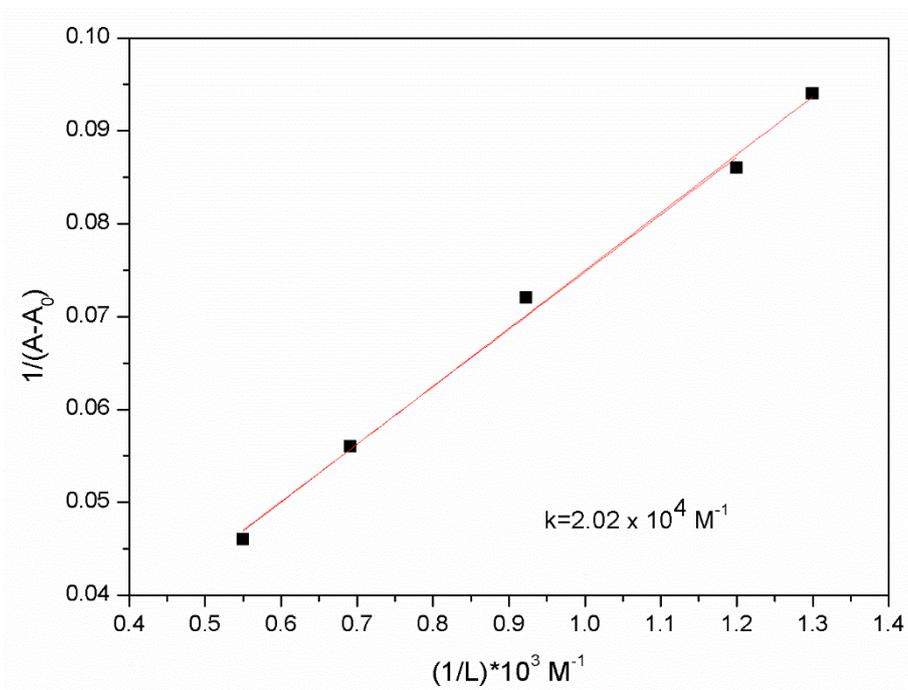

Figure 4.2: The plot of $1/(A-A_0)$ vs. $1/L$ for HSA with different concentrations of aspirin.

Figure 4.2 represents the double reciprocal plot of $1/(A - A_0)$ vs. $1/L$ for HSA-Aspirin complexes respectively. The binding constant (K) can be estimated from the ratio of the intercept to the slope. The obtained values of the binding constants indicates a relatively weak interaction of Aspirin (K= 2.02×10^4 M^{-1}) when compared to other drug-HSA complexes with binding constants in the range of 10^5 and 10^6 M^{-1} **[Pourgonabadi et al., 2011].** The reason for the low stability can be attributed to the presence of mainly hydrogen-bonding interaction **[Boulkanz et al., 1995].**

4.2 Fluorescence

Fluorescence spectroscopy can be applied to a wide range of problems in the chemical and biological sciences. The measurements can provide information on a wide range of molecular processes, including the interactions of solvent molecules with fluorophores, conformational changes, and binding interactions **[Lakowicz, 2006].**

Various molecular interactions can decrease the fluorescence intensity of a compound such as molecular rearrangements, exited state reactions, energy transfer, ground state complex formation, and collisional quenching **[Turro, 1991; Sheehan, 2009].**

The fluorescence of HSA results from the tryptophan, tyrosine, and phenylalanine residues. The intrinsic fluorescence of many proteins is mainly contributed by tryptophan alone, because phenylalanine has very low quantum yield and the fluorescence of tyrosine is almost totally quenched if it is ionized or near an amino group, a carboxyl group, or a tryptophan residue **[Darwish et al., 2010].**

As was observed, the HSA fluorescence spectrum exhibiting the peak maximum at 448 nm (Fig.4.3) and the intensity increases as the aspirin concentration decreased.
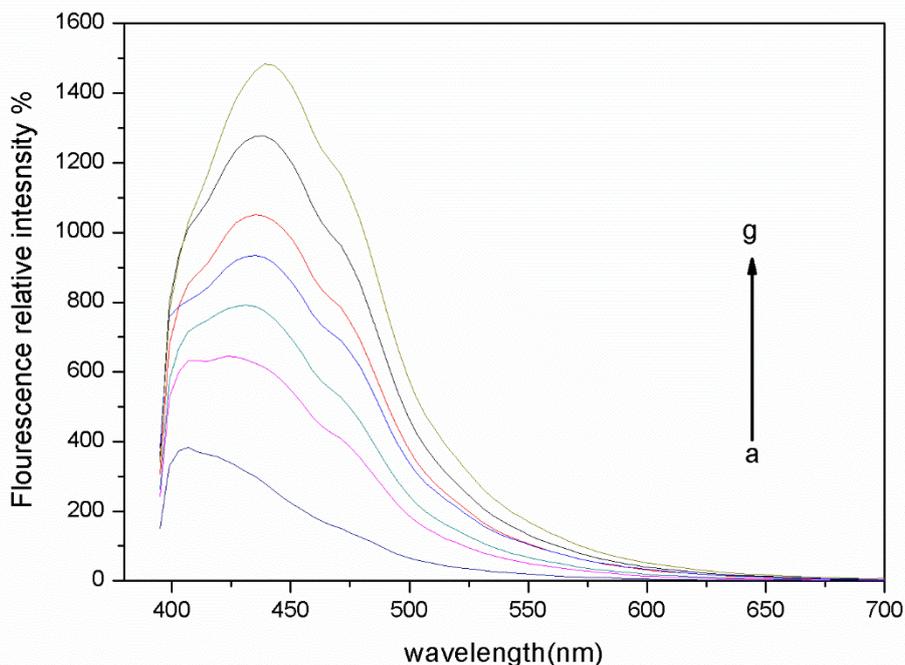
Figure 4.3: Fluorescence emission spectra of HSA in the absence and presence of Aspirin in these concentrations (a=free aspirin, b=1.3mM, c=1.2mM, d=0.9mM, e=0.7mM,f=0.5mM,g=free HSA).

4.2.1 Stern-Volmer quenching constants (k_{sv}) and the quenching rate constant of the biomolecule (k_q)

Fluorescence quenching can be defined as a bimolecular process that reduces the fluorescence intensity without changing the fluorescence emission spectrum; it can result from transient excited-state interactions (collisional quenching) or from formation of non-fluorescent ground-state species.

To elucidate the mechanism of fluorescence quenching, the steady state fluorescence quenching data were examined based on the classic Stern-Volmer equation **[Krimm and Bandekar, 1986]:**

$$\frac{F_0}{F} = 1 + k_{sv}[L] = 1 + k_q \tau_0 [L] \qquad (4.2)$$

where F_0 and F are the HSA fluorescence intensity in the absence and presence of quencher (Aspirin); k_q is the HSA bimolecular quenching rate constant; τ_0 is the average fluorescence lifetime of the HSA molecule without quencher (of the order of

10^{-8}s) **[Byler and Susi, 1986]**; $[L]$ is the concentration of quencher, k_{sv} is Stern-Volmer fluorescence quenching constant.

Linear curve was plotted according to the Stern-Volmer equation as shown in figure 4.4.
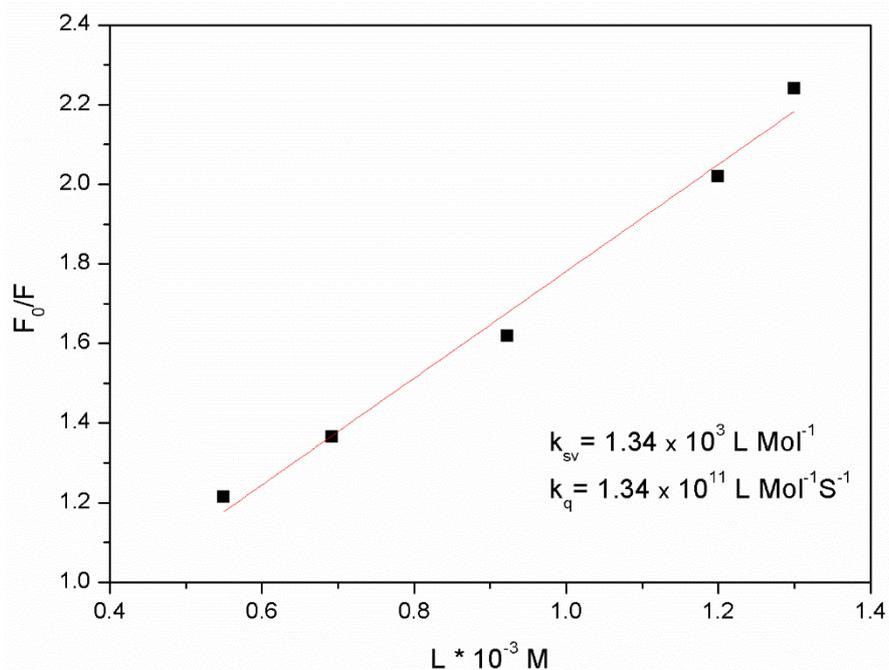
Figure 4.4: The Stern-Volmer plot for Aspirin-HSA complex.

Fig.4.4 represents the Stern-Volmer plot of HSA fluorescence intensity of Aspirin respectively. The curve is linear, suggesting the existence of a single type of quenching (dynamic or static) and/or a single binding site for Aspirin in the HSA neighborhood , The Stern-Volmer fluorescence quenching constant determined applying Eq. (4.2) is equal to (1.34×10^3) L mol^{-1} for Aspirin respectively , The Stern – Volmer quenching constant k_{sv} was obtained by the slope of the curveobtained in figure 4.4, The value is much lower than other k_{sv} values for the similar systems signalized earlier in literature **[Bhattacharya et al., 2000 ; Bai et al., 2008].** The value of k_q, which is equal to (1.34×10^{11}) L mol^{-1} s^{-1} for Aspirin From equation (4.2) the value of $k_{sv} = k_q\tau_0$ from which we can calculate the value of k_q using the fluorescence life time of 10^{-8} s for HSA. This value confirms clearly the existence of

static (diffusion- independent) mechanism of fluorescence quenching [**Zsila et al., 2003**].

4.2.2 Determination of the Binding Constant Using Fluorescence Spectrophotometer

When static quenching is dominant the modified Stern-Volmer equation could be used **[Yang, 1994].**

$$\frac{1}{F_0 - F} = \frac{1}{F_0 KL} + \frac{1}{F_0}$$

Where K is the binding constant of Aspirin with HSA, and can be calculated by plotting $\frac{1}{F_0 - F}$ vs $\frac{1}{L}$, see figure 4.5 .
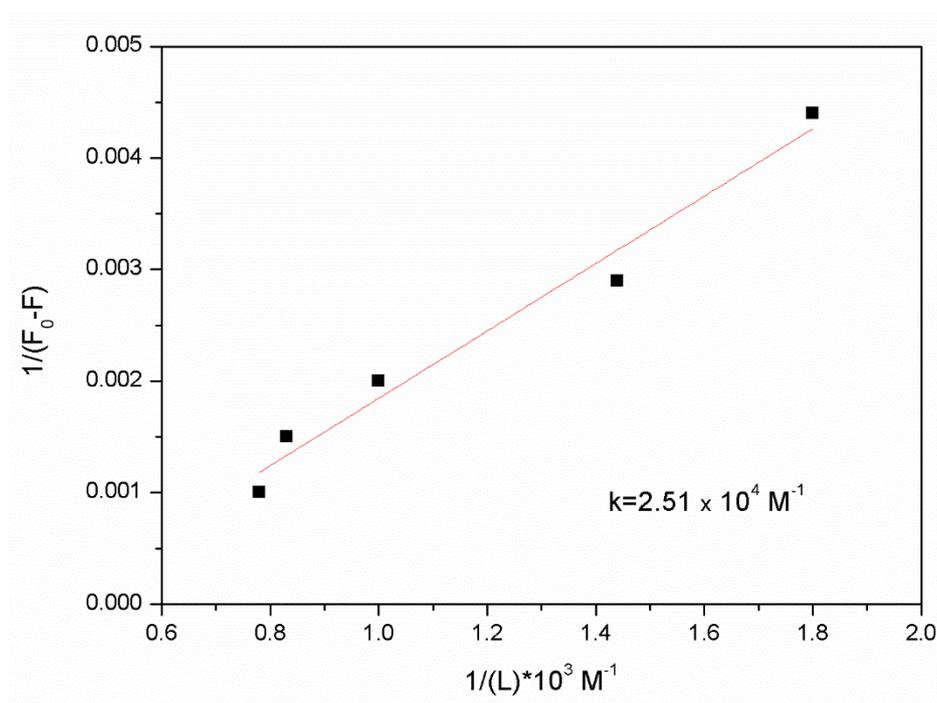
Figure 4.5: The plot of $1/(F_0-F)$ vs $1/[L*10^3]$ for Aspirin-HSA complex.

To determine the binding constant of HSA- Aspirin system, a plot of $\frac{1}{F_0-F}$ vs $\frac{1}{L}$ for different Aspirin concentrations are made and shown in Fig .4.5 for Aspirin respectively. The plot is linear and has a slope of $\frac{1}{F_0 K}$ and intercept $\frac{1}{F_0}$ according to eq. (4.3). The value of K which equal the ratio of the intercept to the slope was found to

be $(2.51 \times 10^4 \text{ M}^{-1})$, which agree well with the value obtained earlier by UV spectroscopy and support the effective role of static quenching.

4.3 FT-IR Spectroscopy

FT-IR spectroscopy is a powerful technique for the study of hydrogen bonding **[Li et al., 2006],** and has been identified as one of the few techniques that is established in the determination of protein secondary structure at different physiological systems **[Arrondo et al., 1993].** The information on the secondary structure of proteins could be deduced from the infrared spectra. Proteins exhibit a number of amide bands, which represent different vibrations of the peptide moiety. The amide group of proteins and polypeptides presents characteristic vibrational modes (amide modes) that are sensitive to the protein conformation and largely been constrained to group frequency interpretations **[Lakowicz, 2006].**

The modes most widely used in protein structural studies are amide I, amide II and amide III. Amide I band ranging from 1600 to 1700 cm^{-1} and arises principally from the C=O stretching **[Vanden bussche G et al., 1992],** has been widely accepted to be used **[Workman, 1998]**. The amide II band is primarily N-H bending with a contribution from C-N stretching vibrations; amide II ranging from 1480 to 1600 cm^{-1} while amide III band ranging from 1220 to 1330 cm^{-1} which is due to the C-N stretching mode coupled to the in-plane N-H bending mode **[Arrondo et al., 1993].**

The second derivative of free HSA is shown in Fig.4.6, where the spectra is dominated by absorbance bands of amide I and amide II at peak positions 1660 and 1540 cm^{-1}. Figure (4.7) respectively show the spectrum of HSA-Aspirin complexes with different concentrations.
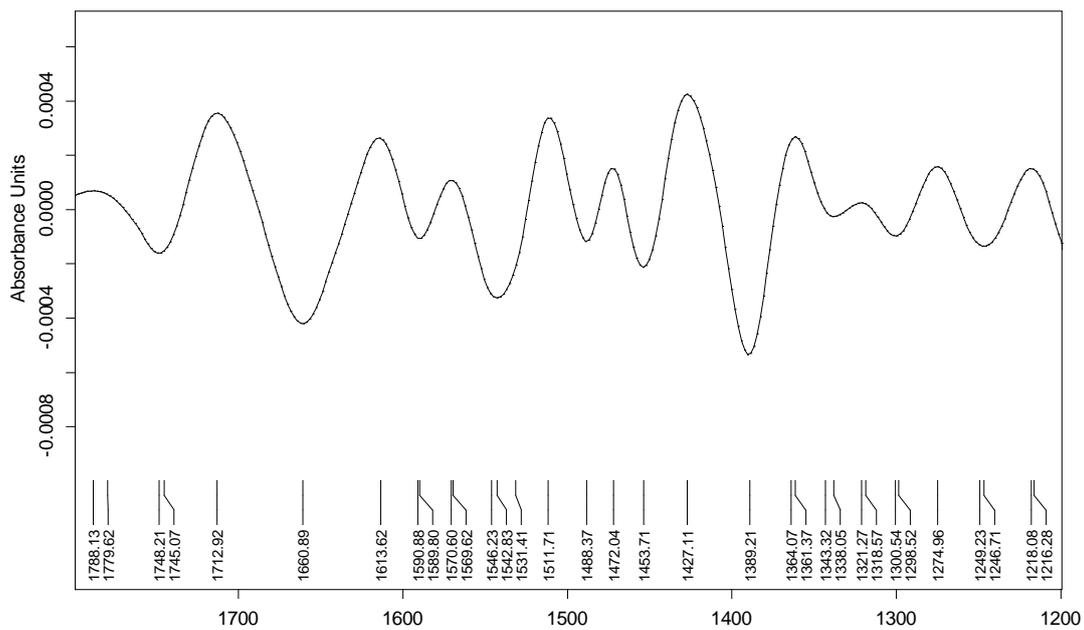
Figure 4.6: Second derivative of free HSA
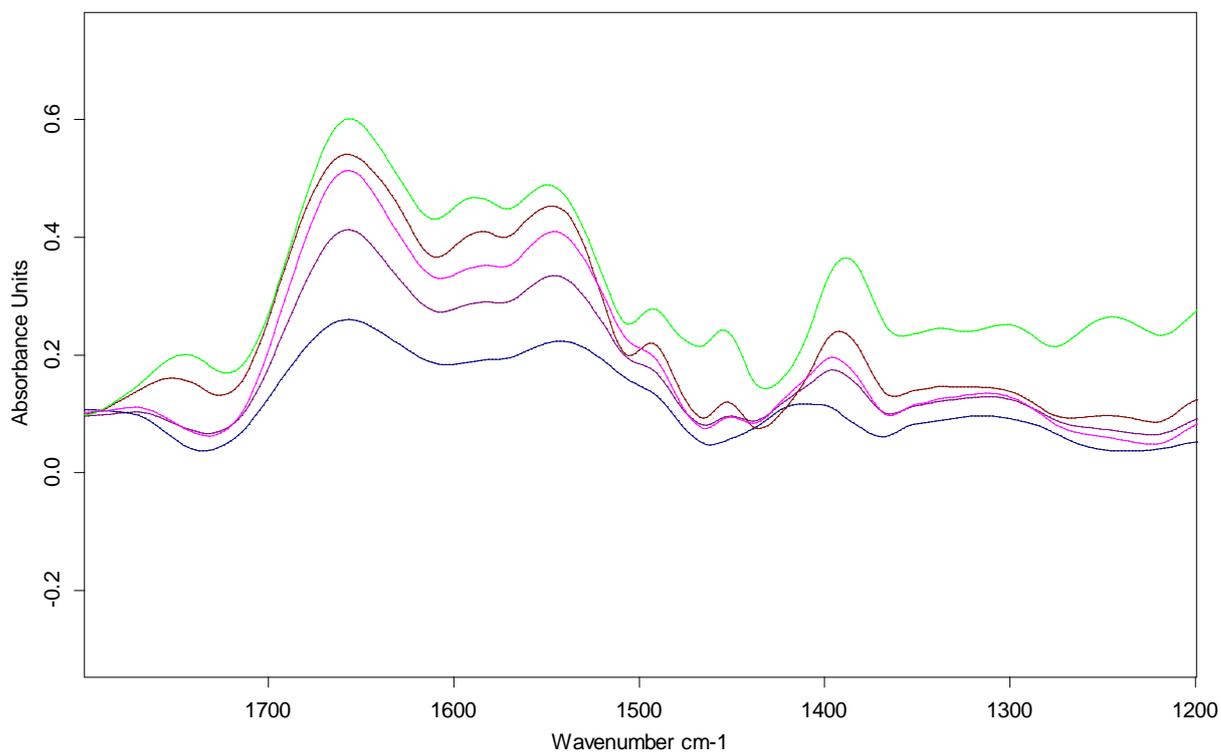
Figure 4.7: Different spectra of HSA and its complexes with different Aspirin concentrations in the region 1800-1200 cm^{-1}.

The peak position of HSA with different concentrations of Aspirin is listed in table 4.1 respectively. It is clearly that the amide bands of HSA infrared spectrum are shifted in two different manners.

Table 4.1: Band assignment in the absorbance spectra of HSA with different Aspirin concentrations for Amid I-III regions

Band regions (cm^{-1})	Free HSA	0.5 mM	0.7 mM	0.9 mM	1.2 mM	1.3 mM
Amid I (1600-1700)	1661	1660	1658	1657	1655	1653
	1614	1610	1608	1607	1605	1604
Amid II (1480-1600)	1571	1574	1578	1581	1582	1584
	1543	1540	1538	1534	1534	1533
	1512	1514	1517	1517	1518	1519
	1472	1471	1471	1469	1468	1468
Amid III (1220-1330)	1343	1345	1347	1348	1350	1354
	1299	1293	1291	1290	1289	1288
	1247	1246	1245	1244	1242	1240

An intensity increase in the difference spectra of the Aspirin-HSA for amide I , amide II and amid III bands , this happen due to drug binding to protein C=O,C-N and N-H groups.

From table 4.1, noticed that the change in the peak position indicates that the second structure of HSA change after interacted with Aspirin drug.

In summary, the binding of Aspirin to HSA has been investigated by UV-absorption spectroscopy, fluorescence spectroscopy and by FT-IR spectroscopy. The binding constant value indicates a relatively weak binding of Aspirin with HSA and the quenching constant indicate that the intrinsic fluorescence of HSA was quenched by Aspirin through static quenching mechanism. Analysis of FT-IR spectra reveals that HSA-Aspirin interaction induces intensity reduction.

Chapter Five

Conclusion

In my work the interaction of aspirin with HSA was investigated by means of UV-VIS spectrophotometer, and FT-IR spectroscopy. I have determined the binding parameter for binding of Aspirin with HSA: For aspirin-HSA the binding constant by using UV-absorption and fluorescence spectroscopy are estimated to be K= $(2.02\times10^4$ $M^{-1})$respectively$(2.45\times10^4$), The experimental result The binding constant value indicate a relatively weak binding of aspirin with HSA and the quenching constant indicate that the intrinsic fluorescence of HSA was quenched by Aspirin through static quenching mechanism.

Analysis of FT-IR spectrum indicated that HSA-Aspirin interaction induces intensity reduction and a change of the secondary structure of HSA in different bands was happened due its interaction of aspirin drug.

This research need further studies to be a useful guide for synthesis of efficient drugs that interact with HSA such as the determinations of binding sites, binding location, and thermodynamic parameters (enthalpy, free energy, entropy) at different temperatures to deduce the type of the acting force for the binding reaction between drugs and HSA.

Furthermore, it is needed to investigate the effect of ions on the binding constants, because of the existence of metal ions that can directly influence the binding force of drug with protein. Thus, affecting the storage time of the drug in blood plasma and enhancing the maximum effectiveness of the drug.

For future work we investigate the interaction using other spectroscopic techniques such as circular dichroism spectroscopy under simulative physiological conditions ,isothermal titration calorimetry (ITC), molecular docking and molecular dynamics simulation(MDS), we can also study the alteration of HSA secondary structure after the interaction with Aspirin, and also make thermodynamic investigations and electrochemical investigation for the interaction of Aspirin with HSA, and study the interaction with more protein transporters.

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دراسة التفاعل بين الأسبرين مع مصل البيومين البشري باستخدام التقنيات المطيافية

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ملخص:

الأسبرين مهم في تقليل خطر الضربة القلبية والسكتة الدماغية ,البيومين المصل البشري هو البروتين الذائب الرئيسي وعنصر أساسي في الجهاز الدوراني وله الكثير من الوظائف الفسيولوجية تشمل نقل الكثير من المركبات الكيميائية. في هذا البحث تم دراسة التفاعل بين الأسبرين مع البيومين المصل البشري وذلك باستخدام تقنيات التحليل الطيفي الفلوري وايضا التحليل الطيفي لأشعة الفوق البنفسجية، ومن خلال التحليل الطيفي أظهرالأسبرين قدرة عالية على اخماد الطيف الفلوري لالبيومين المصل البشري من خلال اجراء الاخماد الاستاتيكي, تم حساب ثابت الربط للأسبرين والبيومين المصل البشري حيث كان (2.02×10^4 M^{-1}),وقد تم حساب ثابت شتيرن فولمر عند درجة حرارة الغرفة للأسبرين، أما التحليل الطيفي باستخدام تقنية تحويل فورييه للاشعة تحت الحمراء فتم استعمالها في تحديد بنية البروتين الثانوية، و ملاحظة التغيرات في الطيف وأشارت لزيادة التفاعل بين الأسبرين والبيومين المصل البشري، وهذا الاختلاف في الشدة يتعلق بطريقة غيرمباشرة لتكون روابط هيدروجينية في الجزيء المعقد.