Deanship of Graduate Studies
Al-Quds University

Study the interaction of Bovine Serum Albumin with Vitamin C and Vitamin B_{12} Using Spectroscopic Techniques

Isra' Ibrahim Mahmoud AbuSrour

M.Sc. Thesis

Jerusalem – Palestine

1439/2018

Study the interaction of Bovine Serum Albumin with Vitamin C and Vitamin B_{12} Using Spectroscopic Techniques

Prepared By:

Isra' Ibrahim Mahmoud AbuSrour

B.Sc.: Physics Science, Al-Quds University, Palestine.

Supervisor: Dr. Husain Alsamamra

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

1439/2018

Al-Quds University
Deanship of Graduate Studies
Physics Department

Thesis Approval

Study the interaction of Bovine Serum Albumin with Vitamin C and Vitamin B_{12} Using Spectroscopic Techniques

Prepared by: Isra' Ibrahim Abu Srour
Registration No: 21510646

Supervisor: Dr. Husain Alsamamra

Master thesis submitted and accepted, Date: 7/7/2018

The names and signatures of the examining committee members:

1. Head of committee: Dr. Husain Alsamamra signature:.....
2. Internal Examiner: Dr. Elias Elias signature:.....
3. External Examiner: Dr. Mustafa Abu Safa signature:.....

Jerusalem-Palestine

1439/2018

Dedication

For myself who is still alive in this world and that is still dreaming despite the constant underestimating by others.

For the piano piece that I kept listening to while writing this.

And for the lasting question of my mother which is (did you pray?).

Declaration:

I Certify that this thesis is 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 of any other university or institution.

Singed:

Isra' Ibrahim Mahmoud Abu Srour

Date: 7/7/2018

Acknowledgments

At the beginning I want to thank Allahfor giving me the strength and patience to complete this work. Of course, this work would not have been possible without the help of some people so it's a great pleasure for me to take this opportunity to thank them for standing with me to finish my thesis.

First, I would like to thank my advisor Dr. Husain Alsamamra for his guidance during my university studies,hegiving me this opportunity to fulfill this thesis, he gave me moral support and guided in different matters regarding to my project, he had been patient on me in the time that I should finishing write my thesis, thanks him for his overall support.

Thanks to Ibrahim Hawaren for his help in preparing the samples, and analyze the data,Asmaa AbuHammad, Dua' Salama, Mohammad Abualaanfor their help and support; and gratitudeto Miss Naila Qissi for her kindness and beautiful smile that comes in her face when she meets us.

Finally, I want to thank my family: My Father,for earning an honest living for us and for supporting and encouraging me to complete my education and for being the number one for me, my Mother, a strong and gentle soul who taught me to trust in myself and to achieving my dreams, to my brothers whom I have always been able to depend on whenever I needed; toall my sisters and to my youngest aunt Marwa forher incrediblelove and support at difficult times. I am thankful to my friends especially Nour, Haneen and Maram for being so close despite of concerns of life,to be part of my life and memories and to bear all my complaints and for solving my problems. Ultimately, I'm indebted to all my teachers and to all who stood by me in any way.

Isra' AbuSrour

Abstract

Vitamin C (ascorbic acid) and Vitamin B_{12} (Cobalamin) are belong to a hydrophilic vitamins which carried by blood proteins. Bovine serum albumin (BSA) is the major soluble protein constituent of the circulatory system and has many physiological function including transport of a variety of a compound.

Binding of vitamin C and vitamin B_{12} to bovine serum albumin (BSA) has been studied using Fluorescence spectroscopy, Ultraviolet-Visible (UV-Vis) absorption spectroscopy, and Fourier transform infrared (FT-IR) spectroscopy using constant protein concentration and various vitamins concentrations at pH 7.4.

UV-Vis spectroscopy was used to analyze vitamin C and vitamin B_{12} binding modes, the binding constant and the effect of the drug complexation on BSA stability and conformation, from UV-Vis spectral determined that the binding constant of vitamin C and B_{12} with interaction of BSA (1.39×10^4 M^{-1} and 1.61×10^4 M^{-1}, respectively)

Fluorescence spectroscopy was used to analyze the emission spectra of BSA in the absence and presence of vitamin C and vitamin B_{12}, and to determine the Stern-Volmer quenching constant $\boldsymbol{k_{sv}}$and the bimolecular quenching constant $\boldsymbol{k_q}$is (4.78×10^3 $L.mol^{-1}$ and 4.78×10^{11} $L.mol^{-1}.s^{-1}$, respectively) for vitamin C and (2.42×10^4 $L.mol^{-1}$ and 2.42×10^{12} $L.mlo^{-1}.s^{-1}$, respectively) for vitamin B_{12}. In addition, determined the values of binding constant from the Lehrer equation for binding of vitamin C and vitamin B_{12} with BSA complex which are equals ($1.6\times10^4 M^{-1}$ and 1.44×10^4 M^{-1}, respectively).

Where FT-IR was used to determine the protein secondary structure, which show a change in the secondary structure of BSA after interacting with vitamin C and vitamin B_{12}.

Table of Contents:

List of Tables

List of Figures

List of Symbols

symbol	Description
N	Degree of freedom
$\boldsymbol{c}$	speed of light
$\boldsymbol{v}$	Frequency
$\boldsymbol{\lambda}$	Wavelength
$\boldsymbol{E}$	Energy
$\boldsymbol{h}$	Planck's constant
$\boldsymbol{E_{total}}$	total energy
$\boldsymbol{E_{ele}}$	energy of the molecule's electrons
$\boldsymbol{E_{vib}}$	vibrational energy
$\boldsymbol{E_{rot}}$	rotational energy
P	the intensity of light transmitted
$\boldsymbol{P_0}$	the intensity of light incident
ε	the molar absorption coefficient
$\boldsymbol{C}$	concentration of absorbing molecule in the sample
$\boldsymbol{b}$	length of the light path
$\boldsymbol{A}$	Absorbance
$\boldsymbol{T}$	Transmittance
$\boldsymbol{n}$	nonbonding occupied molecular orbital
$\boldsymbol{\pi}$	Pi bonding occupied molecular orbital
$\boldsymbol{\sigma}$	Sigma bonding occupied molecular orbital
$\boldsymbol{\pi^*}$	Pi anti-bonding unoccupied molecular orbital
$\boldsymbol{\sigma^*}$	Sigma anti-bonding unoccupied molecular orbital
$\boldsymbol{A}$	recorded absorption at different concentrations
$\boldsymbol{A_\infty}$	the final absorption of the ligated protein
$\boldsymbol{A_0}$	the initial absorption of protein at 280 nm in the absence of ligand
$\boldsymbol{L}$	Concentration of ligand
$\boldsymbol{k_q}$	bimolecular quenching constant
$[\boldsymbol{l}]$	the quencher concentration
$\boldsymbol{k_{sv}}$	Stern-Volmer quenching constant
$\boldsymbol{\tau_0}$	unquenched lifetime
$\boldsymbol{F_0}$	the BSA fluorescence intensities in the absence of quencher
$\boldsymbol{F}$	the BSA fluorescence intensities in the presence of quencher

List of Abbreviations

abbreviation	Representation
g	gram
IR	Infrared
UV-Vis	Ultraviolet Visible
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
FT-IR	Fourier Transform Infrared
BSA	Bovine Serum Albumin
pH	potential of hydrogen
BSA	bovine serum albumin
DPPH	α, α-diphenyl-β-picrylhydrazyl
ITC	isothermal titration calorimetry
EM	Electromagnetic waves
Far-IR	Far Infrared
Mid-IR	Middle Infrared
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
3D	3 dimensional

Chapter one

Introduction

1.1 Introduction

In recent years, studies on the mechanisms of interactions between drugs and proteins have been performed with techniques such as spectroscopy, chromatography, and electrochemical and atomic force microscopy. Several factors can affect these mechanisms, such as concentration, temperature, and pH of solution [Guo et al.,2009].

Serum albumin is the most abundant protein in blood plasma (60%), it serve as depot proteins and transport proteins for a variety of compounds, like fatty acids, amino acids, bile salts, metals, hormones, drugs and pharmaceuticals, which has many physiological functions, such as maintaining the osmotic pressure and pH of blood and scavenging free radicals as an antioxidant. It is an attractive macromolecular carrier, the lack of toxicity and immunogenicity make it an ideal candidate for drug delivery. In addition, serum albumin is the most multifunctional transport protein and plays an important role in the transport and deposition of a variety of endogenous and exogenous substances in blood.

Bovine serum albumin (BSA) has been one of the most extensively studied of this group of proteins, not only because of its medical importance, abundance, low cost, ease of purification, ready availability, unusual ligand-binding properties and it is widely accepted

in the pharmaceutical industry, but also because of its structural homology with human serum albumin (HSA) [Li et al.,2014] .

Vitamins are vital as they play integral roles in hundreds of life-sustaining biochemical reactions and are also catalysts for all reactions using proteins, fats and carbohydrates for energy, growth and cell maintenance.

Vitamin (B_{12}) is needed in many body processes; in the manufacture and the maintenance of red blood cells, the synthesis of DNA, the stimulate of nerve cells, the growth promotion and energy releases and the proper functioning of folic acid. Characteristic signs of vitamin (B_{12}) deficiency include fatigue, weakness, nausea, constipation, flatulence (gas), loss of appetite and weight loss. it is the only vitamin to contain metal ion(cobalt (III)) [Ling,2017] .

Ascorbic acid (vitamin C) represents the major water-soluble antioxidant in plasma and acts as a primary defense in the blood against free radical attack. It has a strong quenching ability for reactive oxygen species such as singlet oxygen and the superoxide anion radical by converting their hydro peroxides into stable products [journal of Photoscience, 2004].

1.2 Proteins

Proteins are biological polymers composed of amino acids. There are 20 different types of amino acids that can be together to produce the protein, these amino acids are connecting together by the peptide bonds to form the polypeptide chain [Zhang et al., 2012].

1.2.1 Protein structure

The structure of protein sets the foundation for its interaction with other molecules in the body and, therefore, determines its function. Protein has four levels of structure which are the primary, secondary, tertiary, quaternary protein structure (figure1.1).

1. Primary protein structure

The primary structure of a protein is a linear specific sequence of amino acid which are bonding together by the covalent bonds, and it’s differ from one protein to another (figure1.1).

2. Secondary protein structure

The secondary structure of a protein refers to the folding or coiling of a polypeptide chain that gives the protein its three dimensional shape. There are two types of secondary structure that found in proteins (figure1.1).

- α-helix: one segment of a polypeptide chain that form a helical structure, these type of structure are bonding together by a hydrogen bonding.

- β- pleated sheet: a polypeptide chain that are folding and pleating over itself , which held together by the hydrogen bonding.

3. Tertiary protein structure

The tertiary structure of protein refers to the three dimensional shape of the entire polypeptide chain, and how all the different secondary structure of proteins are folding in themselves to become a globular 3D- shape of proteins (figure1.1).

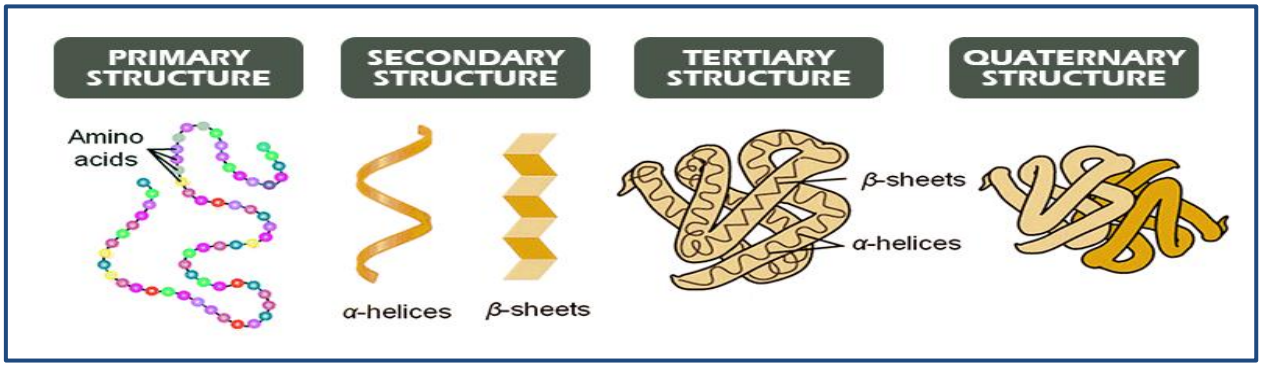

Figure 1.1: The four levels of protein structure.

4. Quaternary protein structure

The quaternary structure of protein represent when having a multiple polypeptide chains to make a particular protein. The quaternary structure doesn't exist in all proteins, only in the proteins that have more than one polypeptide chain (figure1.1)[young et al.,2012]..

1.2.2 Serum albumin

Serum albumin is the most abundant plasma protein in mammals. Serum albumin is a multifunctional protein with extraordinary ligand binding capacity, making it a transporter molecule for a diverse range of metabolites, drugs, nutrients, metals and other molecules. Due to its ligand binding properties, albumins have wide clinical, pharmaceutical, and biochemical applications.

Albumin is protein that essential for maintaining the oncotic pressure needed for proper distribution of body fluidsbetween blood vessels and body tissues. If the albumin doesn't exist, the high pressure in the blood vessels will force more fluid out the body tissues. A proper balance of albumin keep fluid from lacking out of blood vessels, the albumin gives the body the protein that needs to keep growing and preparing tissues[Peters, 1998].

The most important two types of albumin are human serum albumin and bovine serum albumin. In this research I used the bovine serum albumin.

1.2.2.1 Bovine serum albumin (BSA)

The bovine serum albumin is a type of serum albumin which derived from bovine (cows), it is often used in laboratory experiments and science related activities.It is used to diagnose illnesses such as AIDS, malaria or tuberculosis, to study how cancerous tumors behave and grow, and also used in cell culture mediums as a supplement to feed cells and support cell growth[Morrisett et al., 1974].

A structure of BSA can be obtained from its amino acid sequence and crystal structure of related homologous protein. The BSA molecule is made up of three homologous domains (I, II, III) that are divided into nine loops (L1–L9) by 17 disulfide bonds. The loops in each domain are made up of a sequence of large–small–large loops forming a triplet. Each domain in turn is the product of two subdomains (IA, IB, etc.) .BSA has two tryptophan (Trp) residues that possess intrinsic fluorescence. Trp-212 locates within a hydrophobic binding pocket in subdomain IIA, and Trp-134 on

the surface of the albumin molecule in domain I. The principal ligand binding sites of BSA are located in the subdomain IIA and IIIA, which are termed binding, sites I and II (figure1.2) [kajiyama et al.,1998].

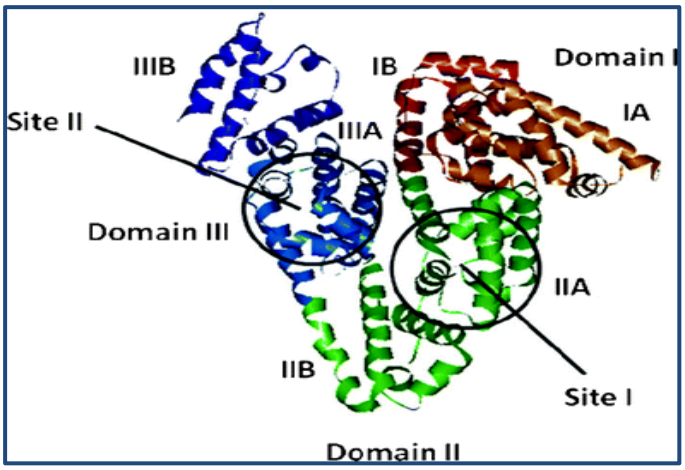

Figure 1.2: Structure of bovine serum albumin (BSA).

1.3 Vitamin

Vitamins are organic compounds that our body needed to inject by a small amount to keep functioning. The human body can't produce vitamins by him, so we have to get it from other sources, we must get them through food or in some cases supplements.

Vitamins are considered as the body builders and maintenance workers that helping to build muscles and bones, making use as nutrients capture to produce energy and heal wounds [Carpenter et al.,1986].

In this research, I used two types of vitamins, vitamin C and vitamin B_{12}.

1.3.1 Vitamin C

Vitamin C (ascorbic acid) is a water-soluble vitamin, The empirical formula of it is $C_6H_8O_6$, it's chemical structure is represented in figure(1.3) and the molecular mass is 176.13 $g.mol^{-1}$ [Wikipedia], vitamin C is necessary in the body to form collagen in bones, cartilage, muscle, and blood vessels and aids in the absorption of iron, prevent scurvy , decrease the bad cholesterol(LDL) in the body, Vitamin C can't be made by the human

body , so it is taken by the food and it can be found in green leafy, vegetables and different citrus fruits. Vitamin C can't be stored in the body, when the human body absorbed the dose that needs it from vitamin C; the body gets rid of it.

The deficiency in vitamin C in the body can lead to resistance in healing cuts, anemia, scurvy and weakness in bones, cartilages and connective tissue [Martirosyan,2015].

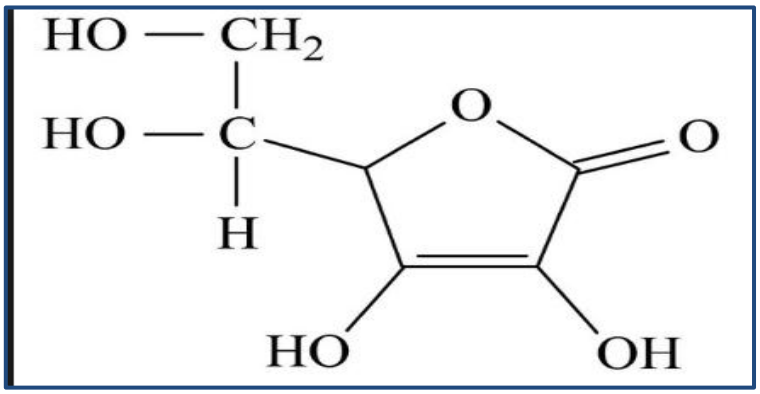

Figure 1.3: The chemical structure for vitamin C.

1.3.2 Vitamin B_{12}

Vitamin B_{12} (cobalamin) is a water-soluble vitamin, the empirical formula of it is $C_{63}H_{88}CoN_{14}O_{14}P$, it's chemical structure represented in figure (1.4) and the molecular mass is 1355.37g.mol^{-1}[Wikipedia], it plays essential roles in red blood cell formation, cell metabolism, nerve function and the production of DNA, it plays a part in the synthesis of fatty acids and energy production. Vitamin B_{12} enables the release of energy by helping the human body absorb folic acid[smith,2008].

Vitamin B_{12} is one of 8 B vitamins. All B vitamins help the body convert food (carbohydrates) into fuel (glucose), which is used to produce energy. These B vitamins often referred to as B complex vitamins, also help the body use fats and protein. B complex vitamins are needed for healthy skin, hair, eyes, and liver. They also help the nervous system function properly [Beatty et al., 2016].

The human body doesn't produce $B_{12,}$ so we get it from the animal based food like eggs, fish, meat, and poultry, or from supplements. When the body absorb the dose that needed from B_{12} , the remaining quantity of B_{12} will be store in the liver until needed. The deficiency

in B_{12} leads to megaloblastic anemia, demyelinating neurologic disease, and damage to brain and nervous system [Stabler,2013].

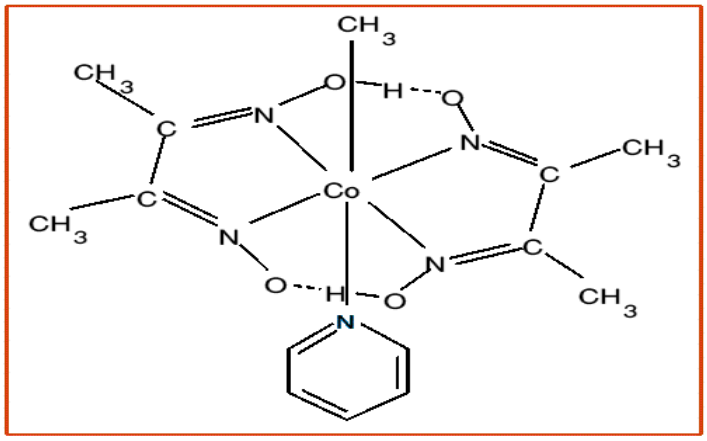

Figure 1.4: The chemical structure for vitamin B_{12}.

1.4 Recent studies

Kamat and Seetharamappa, (2004), investigated the mechanism of interaction of Cyanocobalamin (B_{12}) with bovine serum albumin by specrtofluorescence and circular dichroism methods, Association constant for the CB-BSA system showed that the interaction is non-covalent in nature, Thermodynamic parameters obtained from data at different temperatures showed that the binding of CB to BSA involves hydrophobic bonds predominantly. Significant increase in concentration of free drug was observed for CB in presence of paracetamol.

Xu and Leu, (2009), investigated the mechanism of binding of vitamin C and Bovine serum albumin by using spectroscopic methods under simulated physiological conditions, the binding constants *K*A, and the number of binding sites *n*, and corresponding thermodynamic parameters ΔG^{θ}, $\Delta H^{\theta}$and ΔS^{θ}between VC and BSA were calculated at different temperatures. The primary binding pattern between VC and BSA was interpreted as being a hydrophobic interaction.

Nafisi and Sadeghi,(2011), studied the competitive binding of vitamin C and aspirin to bovine serum albumin using constant protein concentration and various drug concentration at pH 7.2, FT-IR 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, this study show 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.

Li, Wang et al., (2014), studied the binding of ascorbic acid (water-soluble antioxidant) and α-tocopherol (lipid-soluble antioxidant) to bovine serum albumin using isothermal calorimetry (ITC) in combination with fluorescence spectroscopy, UV-Vis spectroscopy and Fourier transform infrared (FT-IR) spectroscopy, Thermodynamic investigations reveal that ascorbic acid/a-tocopherol binding to BSA is driven by favorable enthalpy and unfavorable entropy, and the major driving forces are hydrogen bonding and van der Waals forces. In addition, shown by the UV-Vis absorption, synchronous fluorescence spectroscopy, and FT-IR, ascorbic acid and a-tocopherol may induce conformational and microenvironmental changes of BSA.

Makarska, (2017), investigated the binding affinity between vitamin (B_{12}) and bovine serum albumin in aqueous solution at pH=7.4, by employing UV-Vis absorption and steady state, synchronous and three-dimensional fluorescence spectra technique. Representative effects noted for BSA intrinsic fluorescence resulting from the interactions with vitamin B_{12} confirm the formation of π-π stacked non-covalent and non-fluorescent complexes in the system vitamin (B_{12})-BSA. The interaction with vitamin B_{12} induces folding of the polypeptide chains around Tryptophan residues of BSA, resulting in a more hydrophobic surrounding.

1.5 Motivations

In this thesis, I studied the binding interaction between bovine serum albumin (BSA) with vitamin C and vitamin B_{12}. In my thesis, I chose to study the interaction by using vitamin C and vitamin B_{12}, because the two vitamins are hydrophilic vitamins which are water soluble that found in water part of foods and move into bloodstream on absorption. In addition, our body can't produce and create them.

The mechanism of interaction of vitamin C and vitamin B_{12} with bovine serum albumin (BSA) has been investigated using constant protein concentration (40mg/ml) and various drug concentrations (1mg/ml to 40mg/ml) at pH of 7.4 by FT-IR spectrometer,UV-Vis spectrophotometer and Fluorospectrometer.

In this thesis, I will find the absorbance spectrum, the quenching value and analyze the binding mechanism between the BSA with vitamin C and vitamin $B_{12.}$

Chapter Two:

Theory:

This chapter discusses a brief review of using physical techniques such as FT-IR, UV-Vis and Fluorescence spectroscopy to elucidate the structural conformations of BSA protein interactions with the vitamins especially vitamin C and vitamin B_{12}. This chapter will start with brief information about electromagnetic radiation. Theory of infrared and absorption spectroscopy will be discussed in section two and will give information about the principles of FT-IR spectroscopy. Finally in section three we will discuss the theory of UV- visible spectroscopy followed by section four that will explain the principle of fluorescence spectroscopy.

2.1 Electromagnetic Radiations

Electromagnetic radiation figure (2.1) isthe flow of energy through free space or through a material medium in the form of the electric and magnetic fields that make up electromagnetic waves such as radio waves, visible light, and gamma rays.Electromagnetic radiation consists of oscillating electric and magnetic fields that propagate through space along a linear path and with a constant velocity[Jaffé et al.,1964], The oscillations in the electric and magnetic fields are perpendicular to each other, and to the direction of the wave's propagation.

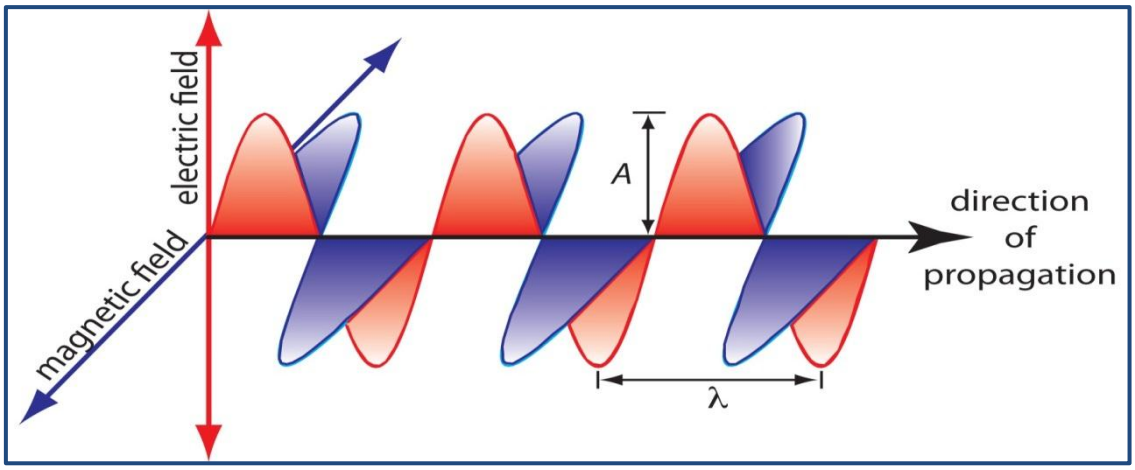

Figure 2.1: Plane-polarized electromagnetic radiation showing the oscillating electric field in red and the oscillating magnetic field in blue.

In a vacuum electromagnetic radiation travels at the speed of light, c, which is 2.99792 $\times 10^8$ m/s. When electromagnetic radiation moves through a medium other than a vacuum its velocity v, isless than the speed of light in a vacuum [Parikh et al.,1974].

When a matter absorbs the electromagnetic radiation will produce a change in energy according to the interaction between the electromagnetic radiation and the matter, the change in energy knows as photons that occurs as a beam of energy, the energy of photon is given by the Planck–Einstein equation:

$$\boldsymbol{E} = \hbar \nu = \hbar \frac{c}{\lambda} \quad (1)$$

Where E is photon energy, $\hbar$ is the plank's constant, which has a value of 6.626×10^{-34} J .s, c is speed of light, which is 2.99792 $\times 10^8$ m/s , λ is wavelength and ν is the frequency[Ball,1994].

The electromagnetic spectrum[figure (2.2)] is a continuous range of wavelengths. The types of radiation that occur in different parts of the spectrum have different uses and dangers, depending on their wavelength and frequency [Stern et al.,1971].

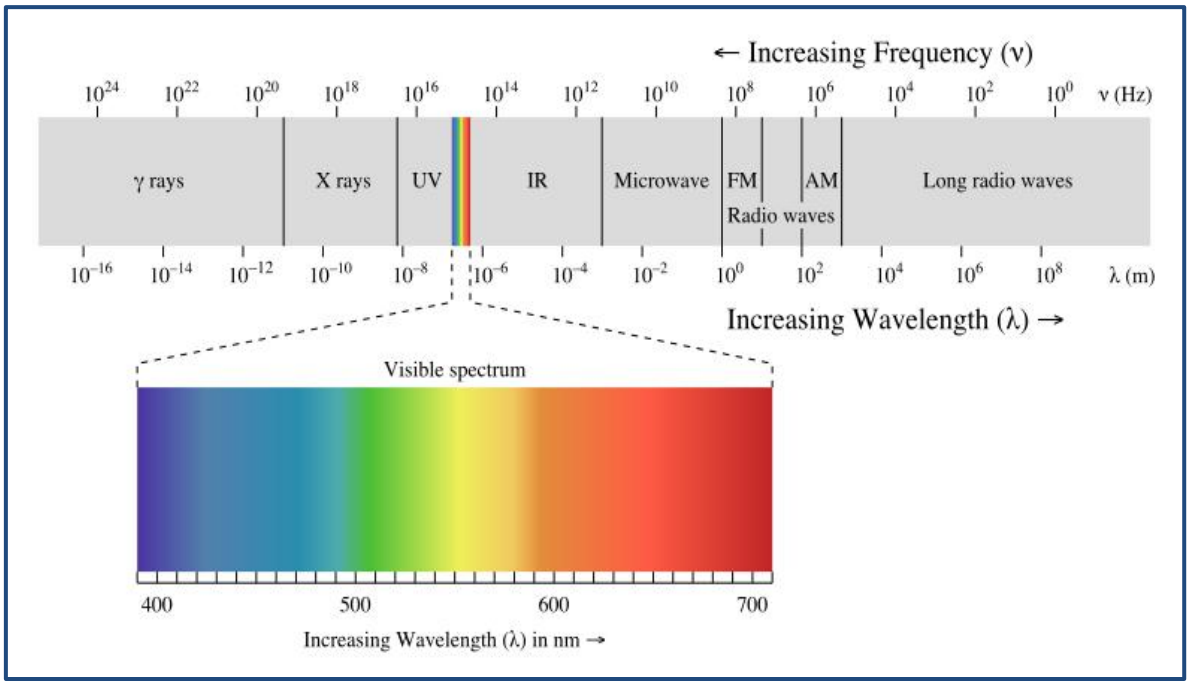

Figure 2.2: The electromagnetic spectrum showing the boundaries between different regions and the type of atomic or molecular transition responsible for the change in energy.

The spectroscopyhas two techniques, the absorption and the emission spectroscopy.

In absorption spectroscopy, a photon is absorbed by an atom or molecule, which undergoes a transition from a lower-energy state to a higher energy, or excited state figure (2.3).

In emission spectroscopy, an atom or molecule in an excited state returns to a lower energy state, the excess energy often is released as a photon(figure (2.3)). There are several ways in which an atom or molecule may end up in an excited state, including thermal energy, absorption of a photon, or by a chemical reaction[Silverstein et al.,2005].

When a photon of energy hν strikes the atom or molecule, absorption may occur if the difference in energy, ΔE, between the ground state E_0 and the excited state E_1 is equal to the photon's energy [Scott,1971]**.**

$$\Delta E_{transsion}=E_1-E_0= h\nu \qquad (2)$$

The ratio of the source radiation's power exiting the sample P_T, to that incident on the sample $P_0$is defined as the Transmittance T:

$$T=P_T/P_0 \qquad (3)$$

While the Absorbance (*A*) is the measurement of the decrease in the number of photons that passes through the sample when an atom or molecules absorbs electromagnetic radiation [Friedel et al.,1951].

$$A = - \log T = - \log (P_T/P_0) \quad (4)$$

Beer's law (beer's-lambert law)is establishing the linear relationship between absorbance and concentration [Graselli et al., 1975].

$$A = \varepsilon bC \quad (5)$$

Where ε is the molar absorptivity (L mol^{-1} cm^{-1}), b is the path length of the cuvette containing the sample (cm), C is the concentration of the sample(mol L^{-1}).

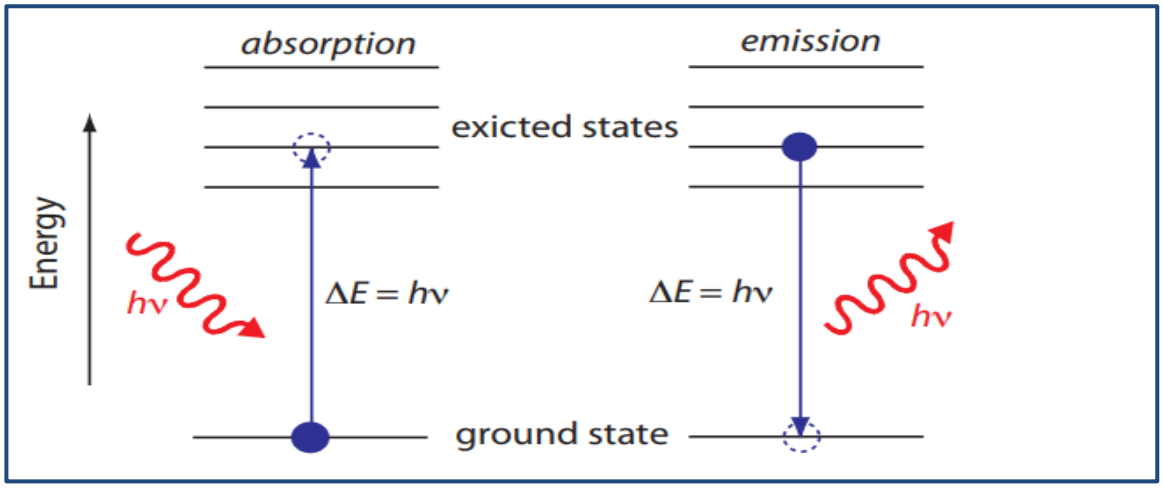

Figure 2.3: Simplified energy diagram showing the absorption and emission of a photon by an atomor a molecule.

2.2 Infrared Spectroscopy (IR)

Spectroscopy deals with measuring and interpreting spectra that arise from the interaction of electromagnetic radiation with matter. It is concerned with the absorption, emission, or scattering of electromagnetic radiation by atoms or molecules [Szymanski,1980].

Infrared spectroscopy deals with the infrared region of the electromagnetic spectrum that is light with a longer wavelength and lower frequency than visible light. IR spectroscopy is an analysis of infrared light interacting with a molecule.

The IR spectroscopy can be analyzed in three ways: by measuring absorption, emission, and reflection. The major use of this technique is in organic and inorganic chemistry to determine functional groups of molecules [Colthrup et al., 1990].

The energy needed to excite the bonds in a molecule to make them vibrate with greater amplitude occurs in the IR region. A bond will only interact with the electromagnetic infrared radiation, however, if it is polar. The presence of separate areas of partial positive and negative charge in a molecule allows the electric field component of the electromagnetic wave to excite the vibrational energy of the molecule. The change in the vibrational energy produces a corresponding change in the dipole moment of the molecule. The intensity of the absorption depends on the polarity of the bond. Symmetrical non-polar bonds in N≡N and O=O don't absorb radiation, as they cannot interact with an electric field [Nakanishi et al.,1998].

Most of the bands that indicate what functional group is present are found in the region, from 4000 cm^{-1} to 1300 cm^{-1}. Their bands can be identified and used to determine the functional group of an unknown compound.Bands that are unique to each molecule, like a fingerprint, and are found in the fingerprint region, from 1300 cm^{-1} to 400 cm^{-1}. These bands are only used to compare the spectra of one compound to another [Macomber et al.,1998].

2.2.1 Molecular vibration

When the frequencies of infrared light and the frequency of vibration coincide, the infrared light is absorbed by a molecular vibration. The Vibration frequency and absorption probability depend on the strength and polarity of the vibrating bounds, which are also influenced by intra- and intermolecular effects[Nelson et al.,2003].

Infrared spectroscopy provides measurements of molecular vibrations [figure (2.4)] due to the specific absorption of infrared radiation by chemical bonds [Pretsch et al., 2000].Mid-IR radiation in most covalent bonds will cause vibrations, these vibrations can involve either a change in bond length (stretching) or bond angle (bending).Some bonds can stretch in-phase (symmetrical stretching) or out-of-phase(asymmetric stretching). Bending vibrations (also called deformations) are divided into two types, in plane bending (scissoring, rocking), and out plane bending (twisting, wagging).

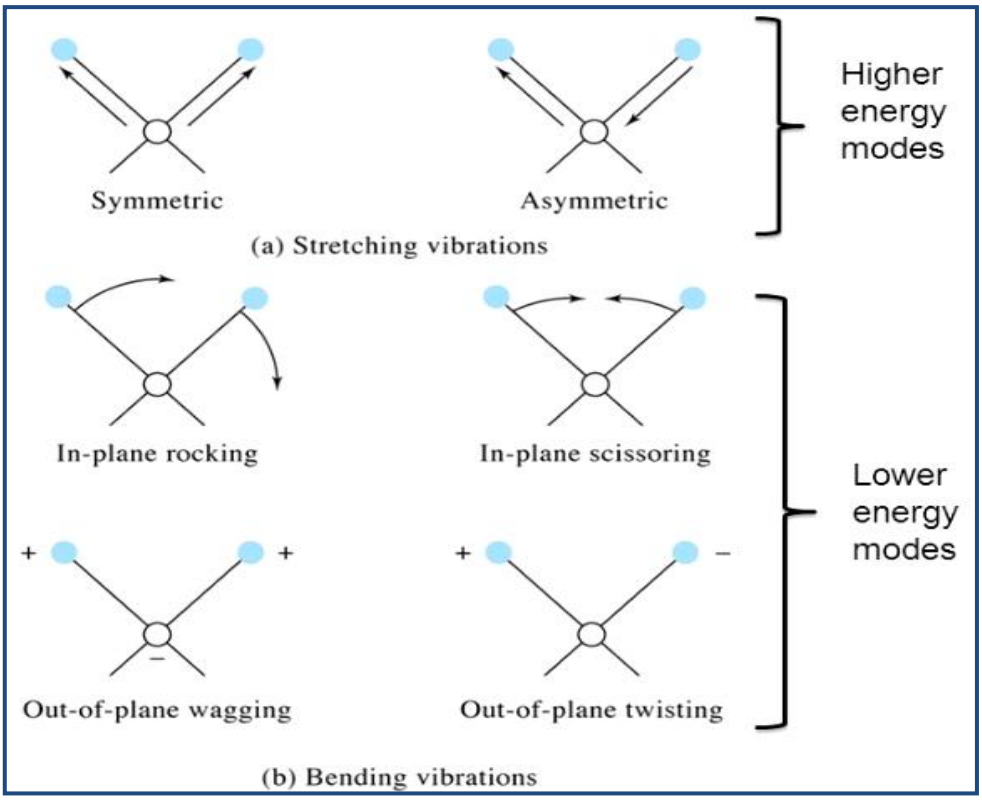

Figure 2.4: Common types of vibration (normal modes).

2.2.2 Number of vibrational modes

Atoms within a molecule are constrained by molecular bonds to move together in a certain specified ways, called degrees of freedom that can be: electronic, translational, rotational and vibrational. In electronic motion, the electrons change energy levels or directions of spins. The translational motion is characterized by a shift of an entire molecule to a new position. The rotational motion is described as a rotation of the molecule around its center of mass. When the individual atoms within a molecule change their relative position then we say that the molecule vibrates [Pople et al.,1959].

If we have a nonlinear molecule consisting of N atoms, we need to specify 3N degrees of freedom that correspond to their locations and orientations as shown in table(2.1). Three of those can be used to specify the centre of mass of the molecule, leaving 3N-3 degrees of freedom for the location of the atoms relative to the centre of mass. For determining the orientation of the molecule we need to specify three angles (if the molecule is linear, only two angles are sufficient) [Roberts, 1959], so leaving 3N-6 degrees of freedom that, when varied, do not change the location of the centre of the mass nor the orientation of the

molecule. These 3N-6 coordinates correspond to different vibrational degrees of freedom of the molecule that can range from the simple coupled motion of the two atoms of a diatomic molecule to the much more complex motion of each atom in a large polyfunctional molecule.

Table 2.1: Degrees of freedom for polyatomic molecules

Type of degrees of freedom	Linear	Nonlinear
Translational	3	3
Rotational	2	3
Vibrational	3N – 5	3N – 6
Total	3N	3N

When a molecule is exposed to wide-spectrum radiation, some distinct parts of it are absorbed by the molecule. The absorbed wavelengths are the ones that match the transitions between the different energy levels of the corresponding degrees of freedom of that molecule. The vibrational transitions are the most important transitions for IR spectroscopy because IR radiation is too low to affect the electrons within the individual atoms and too powerful for rotational and translational transitions[Chatwal,1979].

2.2.3 Fourier Transforms Infrared spectroscopy (FT-IR)

Fourier Transform Infrared Spectroscopy (FT-IR) is one of the techniques that are used today for measuring the intensity of infrared radiation as a function of frequency or wavelength.This technique also used to obtain the information about the chemical structure of a variety of substances by use of infrared electromagnetic radiation[Roberts et al., 2000].

Infrared radiation is invisible electromagnetic radiation just below the red color of the visible electromagnetic spectrum, with wavelength range from 700 nm to 1 mm [kalsi, **1996**].

2.2.3.1 Principle of FT-IR spectroscopy

An FT-IR is based on The Michelson Interferometer, which is shown in figure (2.5).It consists of two mutually perpendicular plane mirrors, one of which can move along the axis that is perpendicular to its plane. In the middle between the fixed and the movable mirror is a beam splitter, a device that ideally, allows 50% of light to pass through to the movable mirror while reflecting the other 50% to the fixed mirror. The beam that travels to the fixed mirror is reflected there and returns to the beam splitter again, after a total optical path length of 2L. The same happens to the beam that is transmitted by the beam splitter in the direction of the movable mirror. That mirror is moving very precisely back and forth by a distance x as it is shown in Figure 1, so the total optical path length of the beam reflected from it is 2(L + x)[Stuart et al.,1997]. When the two beams return to the beam splitter they exhibit an optical retardation of 2x. Because of their spatial coherence they interfere. The result from the interference of the partial waves depends on their optical path difference. At the splitter, both beams are split again, causing the part of radiation reflected from the fixed and moving mirrors to recombine, and go through the sample into the detector, the other part of radiation that was reflected by the mirrors goes back to the radiation source [Chatwal,1979].

Because the two mirrors are setting at equal distance from the splitter, the two beams reach the detector to be in the same phase, and that's which is called a constructive interference. At this point, the radiation that reaching the detector has the highest intensity[Gremlich et al., 2000]. However, if one of the mirrors is moved by a short distance, the intensity of the radiation that reading by the detector, suddenly drops, this is related to the two recombined beams is no longer in the same phase and canceled each other (destructive interference).

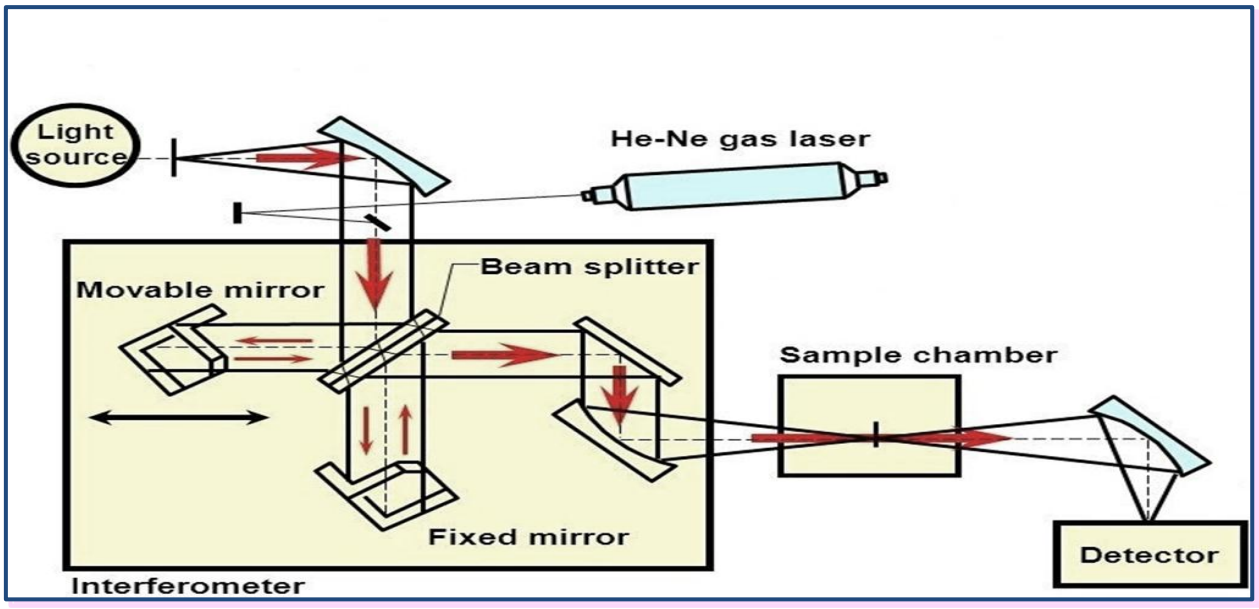

Figure 2.5: Schematic diagram of Fourier Transform Infrared Spectroscopy.

In order to carry out the measurement the movable mirror will move, and according to this action, the interferogram will result, by measuring the intensity of the radiation that reaches the detector. The interferogram are converted into spectrum [figure (2.6)]and combined in order to see what wavelength radiation was absorbed by the substance [Chatwal,1979].

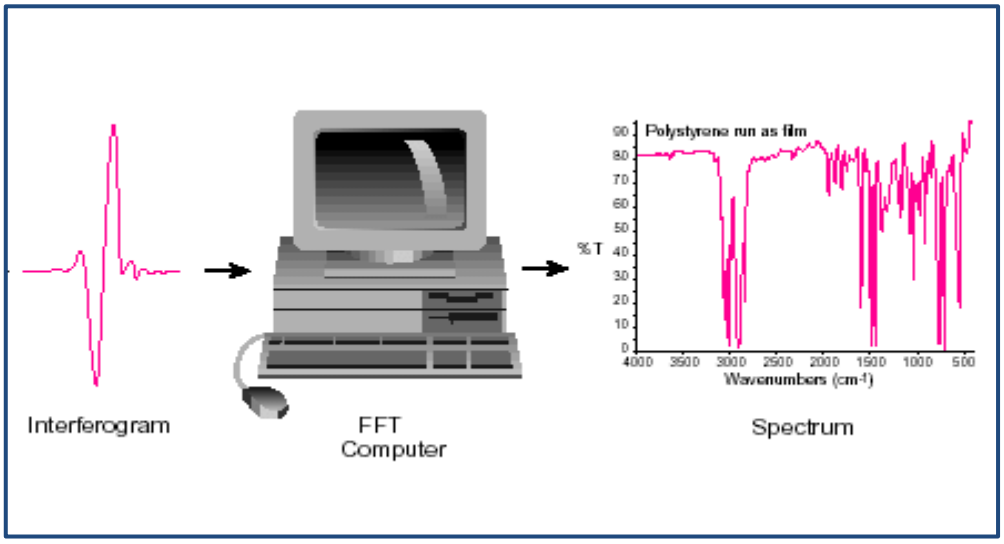

Figure 2.6: Schematic diagram shows the conversion of the interferogram into spectrum.

2.3 Ultraviolet visible (UV-Vis) Spectroscopy

Ultraviolet-Visible spectroscopy is type of absorption spectroscopy and is the measurement of the attenuation of a beam of light after it passes through a sample or after reflection from a sample surface. Absorption measurements can be at a single wavelength or over an extended spectral range [Harris et al., 1989].

The UV region extends from 10 nm to 400 nm and the visible radiation region extends from 400 nm to 800 nm.

2.3.1 Principle of Ultraviolet Visible Spectroscopy

The system consist of two light sources as shown in figure (2.7), one giving out visible light which is usually a tungsten lamp and the other for ultraviolet light which is deuterium lamp. The source lamp is creating a variety of wavelength of different light [Weckhuysen,2000]. This light passes through the first slit of the monochromator, ensuring that all of light photons are travelling along parallel pathways, when they strike the prism, they are refracted into the colors of rainbow, so each wavelength of light is moving to a different place in space, so only one wavelength of light is going to pass through the second slit in monochromator and striking the beam splitter, according to strike, the splitter will produce two beams of equal intensity, one beam passes through the sample cell, while the other passes through the reference cell. Then both sample and reference beams are directed by mirrors onto a detector, which compares their intensities and a signal proportional to the ratio of their intensities to the computer that controls the instrument.

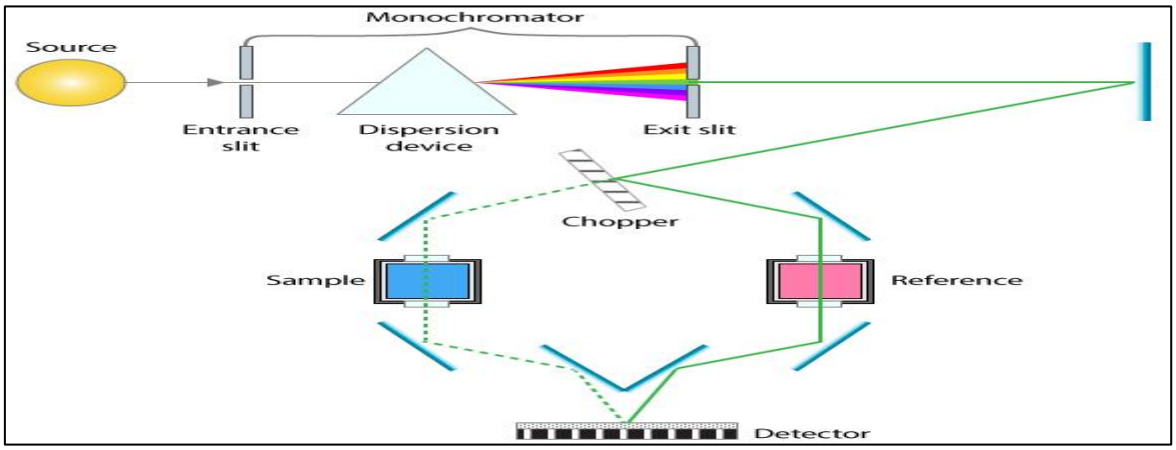

Figure 2.7: The schematic diagram of principle of ultraviolet-visible spectroscopy.

The logarithm of the ratio between the intensities of two beams gives the absorbance, which is measure of how much light is being absorbed by the sample at that particular wavelength [Jaffé et al.,1964].

2.4 Fluorescence Spectroscopy

In a UV-Visible absorption experiment, the samples continue absorbing light. This means that the higher energy molecular orbitals never get saturated. This further implies that after excitation, the molecules somehow get rid of the excess energy and return back to the ground state [Koney,1969]. The electrons can return back to the ground state in different ways such as releasing the excess energy through collisions or through emitting a photon. In fluorescence, the molecules return back to the ground state by emitting a photon. The molecules that show fluorescence are usually referred to as the fluorophores [Wehry,1975]. Various electronic and molecular processes that occur following excitation are usually represented on a Jablonski diagram as shown in (Figure 2.8).

Fluorescence spectroscopy is used to obtain excitation and emission spectrum and to measure the fluoresce intensity. In the fluorescence spectroscopy, the light source produces light photons over a broad energy spectrum, typically ranging from 200 to 900 nm [Miller, 1981].

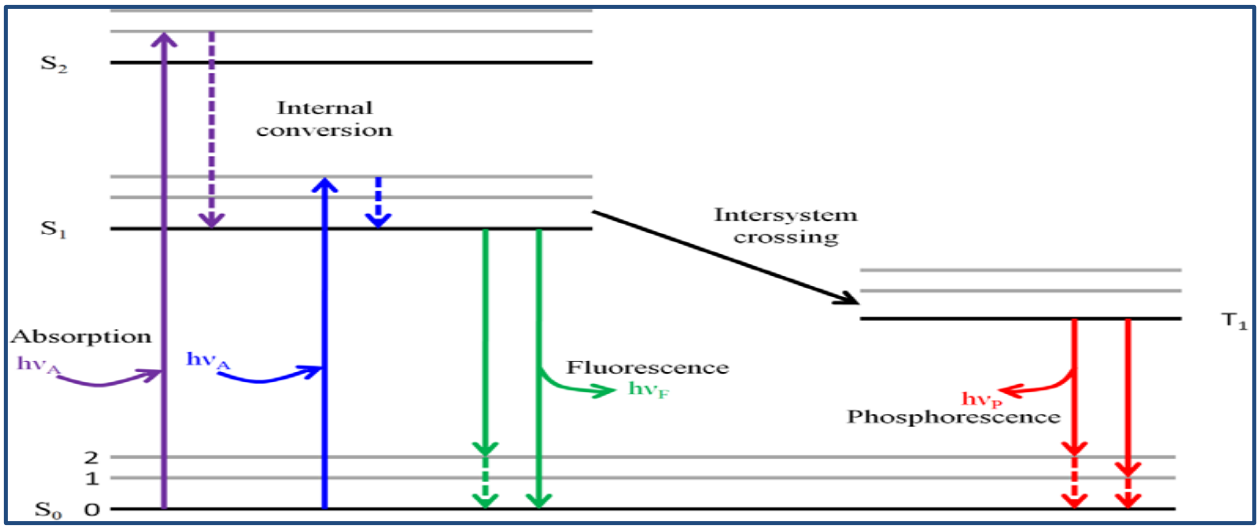

Figure 2.8: Jablonski diagram showing various processes following absorption of light by the fluorophore.

2.4.1 Principle of Fluorescence Spectroscopy

The light source(xenon lamp) emitted light that passes through the exciter monochromator as shown in (figure 2.9), only the amount of radiation that needed will passes through the exit slit. An amount of the incident light is absorbed by the sample, while the remaining light gets transmitted and falls in the transmission detector. The sample will be excited by the incident radiation, and the fluorescence will happens which will having a specific wavelength and will be emitted by the sample, the emitted radiation will pass through analyser monochromator, but the emission filter prevent any stay light to enter next to the fluorescence radiation. When the fluorescence light exist the analyser monochromator, its intensity is measured by the fluorescence detector [Melhuish et al., 1981].

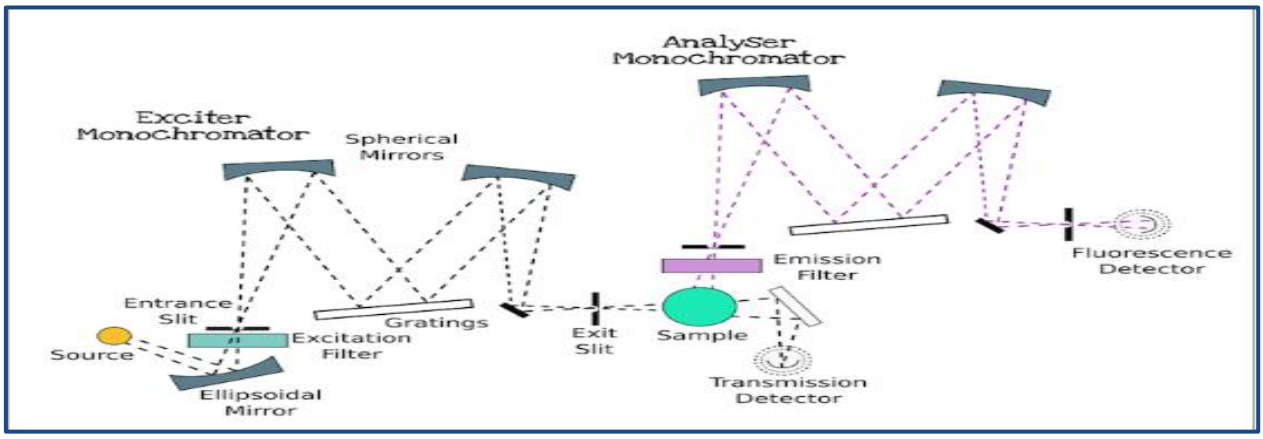

Figure 2.9: Schematic diagram of Fluorescence Spectroscopy.

Chapter Three:

Experimental Setup and Techniques

3.1 Samples preparation

BSA, vitamin C, and vitamin B_{12} 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:

3.1.1 Preparation of BSA stock solution

BSA was dissolved in 25% ethanol in phosphate buffer Saline and at physiological (pH 7.4), to a concentration of (80mg/ml), and used at final concentration of (40 mg/ml) in the final vitamin- BSA solution.

3.1.2 Preparation of vitamin C stock solution

Vitamin C with molecular mass of ($176.13 g.mol^{-1}$), was dissolved in 25% ethanol 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 vitamin C was completely dissolved.

3.1.3 Preparation of vitamin B_{12} stock solution

Vitamin B_{12} with molecular mass (1355.37g.mol^{-1}) was dissolved in 25% ethanol 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 vitamin B_{12} was completely dissolved.

3.1.4 BSA-Vitamin C samples

The final concentrations of BSA-Vitamin C solutions were prepared by mixing equal volume from BSA to equal volume from different concentration of vitamin C. BSA concentration in all samples kept at 40 mg.ml^{-1}. However, the final concentrations of vitamin C in solutions are
(40 mg.ml^{-1} , 20 mg.ml^{-1} , 10 mg.ml^{-1} , 5 mg.ml^{-1} , 2 mg.ml^{-1} and 1 mg.ml^{-1}).

3.1.5 BSA-Vitamin B12samples

The final concentrations of BSA-Vitamin B_{12} solutions were prepared by mixing equal volume from BSA to equal volume from different concentration of vitamin B_{12}. BSA concentration in all samples kept at 40 mg.ml^{-1}. However, the final concentrations of vitamin B_{12} in solutions are (40 mg.ml^{-1} , 20 mg.ml^{-1} , 10 mg.ml^{-1} , 5 mg.ml^{-1} , 2 mg.ml^{-1} and 1 mg.ml^{-1}).

3.1.6 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 BSA - Vitamin C 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.The same procedure was followed for BSA- Vitamin B_{12} films preparation.

3.2 Instruments

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

3.2.1 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 KBr beam splitter. The spectrometer was continuously purged with dry air during the measurements.

3.2.2 UV-Vis Spectrophotometer

The absorption spectra were obtained by the use of a Nano Drop 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.

3.2.3 Fluorospectrometer

The fluorescence measurements were performed by a Nano Drop ND-3300 Fluorospectrophotometer at 25°C. The excitation source comes from one of three solid-state light emitting diodes (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 strand Micropipettes were used [Nano Drop 3300 Fluorospectrometer V2.7 user's Manual, 2008].

3.3 Experimental procedure

3.3.1 UV-Vis spectrophotometer experimental procedures

Procedure of UV-Vis spectrophotometer was followed as described in NanoDrop ND-1000 Spectrophotometer V3.7, 2008, User's Manual [Nano Drop 1000 Spectrophotometer V3.7, User's Manual, 2008].

Which is as follows:

A (10) µl sample of vitamin 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 charge-coupled device array is used to

analyze the light after passing through the sample. The instrument is controlled by personal computer based software, and the data is logged in an active file on the personal computer.

Before taking the samples absorbance on the Nano Drop ND-1000 Spectrophotometer "blanked". When the Nano Drop ND-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:

Absorbance = - log (Intensity sample/Intensity blank) (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.

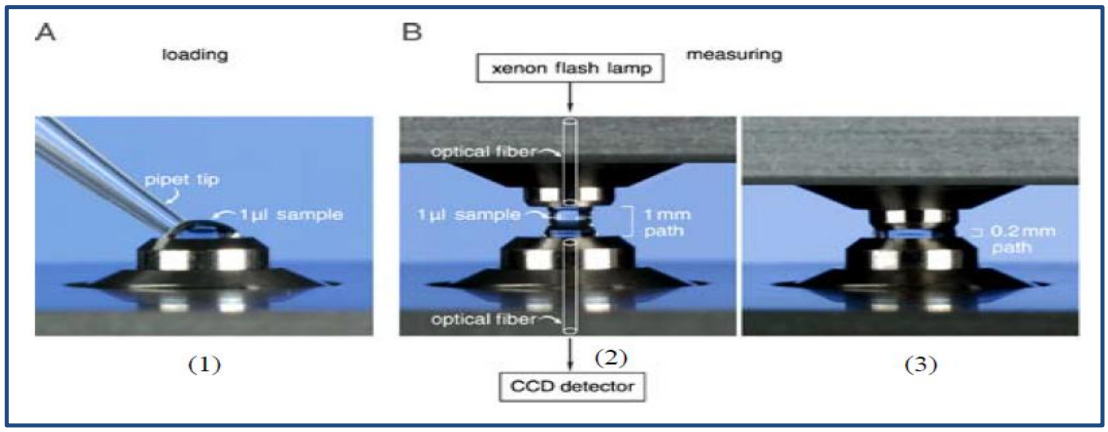

Figure 3.1: Themain steps for using the sample UV-Vis.

3.3.2 Fluorospectrophotometer experimental procedures

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

Before taking the measurements of samples the Nano Drop ND-3300 Fluorospectrometer was "blanked".

A (10) µl sample of vitamin 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 Nano Drop ND-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.

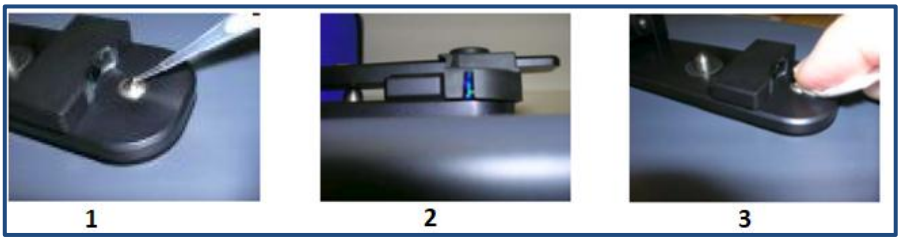

Figure 3.2: Main steps for using the sample fluorescence spectrometer (Nano Drop 3300 Fluorospectrometer).

3.3.3 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 OPUS software. The peak positions were determined using the second derivative of the spectra.

The infrared spectra of BSA, vitamin-BSA complexes were obtained in the region of 1000-1800 cm^{-1}. The FT-IR spectrum of free BSA 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 vitamin 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].

3.3.4 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:

3.3.4.1 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].

3.3.4.2 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].

3.3.4.3 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,Secondderivative procedures have been successfully applied in the qualitative study of a large number of proteins [Haris et al., 1999].

3.3.4.4 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 (Jackson et al. 1981). 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].

3.3.4.5 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.

3.3.4.6 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:

Data Analysis and Discussion

4.1 Absorption spectroscopic studies

The UV-Vis absorption technique can be used to explore the structural changes of proteins and to investigate protein-ligand complex formation [chiand liu, 2011]. UV-Vis absorption spectroscopy used to determine the binding constant between BSA with drugs (vitamin C and vitamin B_{12}) complex. The interaction dependent on the binding constant which can be calculated by using graphical analysis of the absorbance spectrum.

The UV-Vis absorption spectra of BSA in the absence and the presence of ascorbic acid(vitamin C)/cobalamin (vitamin B_{12}) obtained by utilizing a mixture of ascorbic acid/ cobalamin and phosphate buffer at the same concentration as the reference solution are shown in figure 4.1A and figure 4.1B.

In the BSA-Cobalamin complex, BSA has one absorption peak at about 280nm reflect the framework conformation of the protein. With gradual addition of different concentration of cobalamin (vitamin B_{12}) to the BSA solution, the intensity of the peak at 280 nm decreases with no obvious shift[figure 4.1A],due to the existence of aromatic amino acids which being the components of a protein molecule as well as its complicated molecular structure.

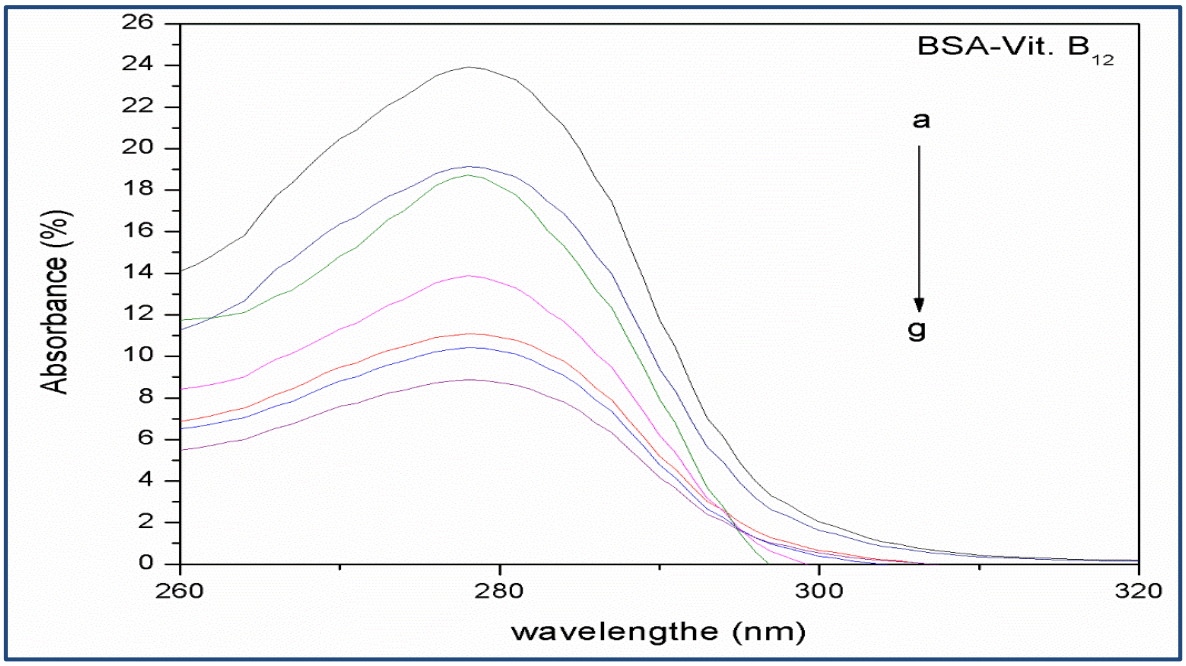

Figure 4.1A: UV-Vis absorbance spectra of BSA with different concentrations of Vitamin B_{12}

(a= Free BSA, b=1mg/ml,c=2mg/ml, d=5mg/ml, e=10mg/ml, f=20mg/ml, g=40mg/ml).

In the BSA-ascorbic acid complex, BSA has one absorption peak at about 268nm reflect the framework conformation of the protein, with gradual addition of different concentration of ascorbic acid (vitamin C) to the BSA solution, the intensity of the peak at 268 nm increase with no obvious shift [figure 4.1B],due to major ligand protein interaction at protein surface which does not limit the mobility of ligand around BSA molecule.

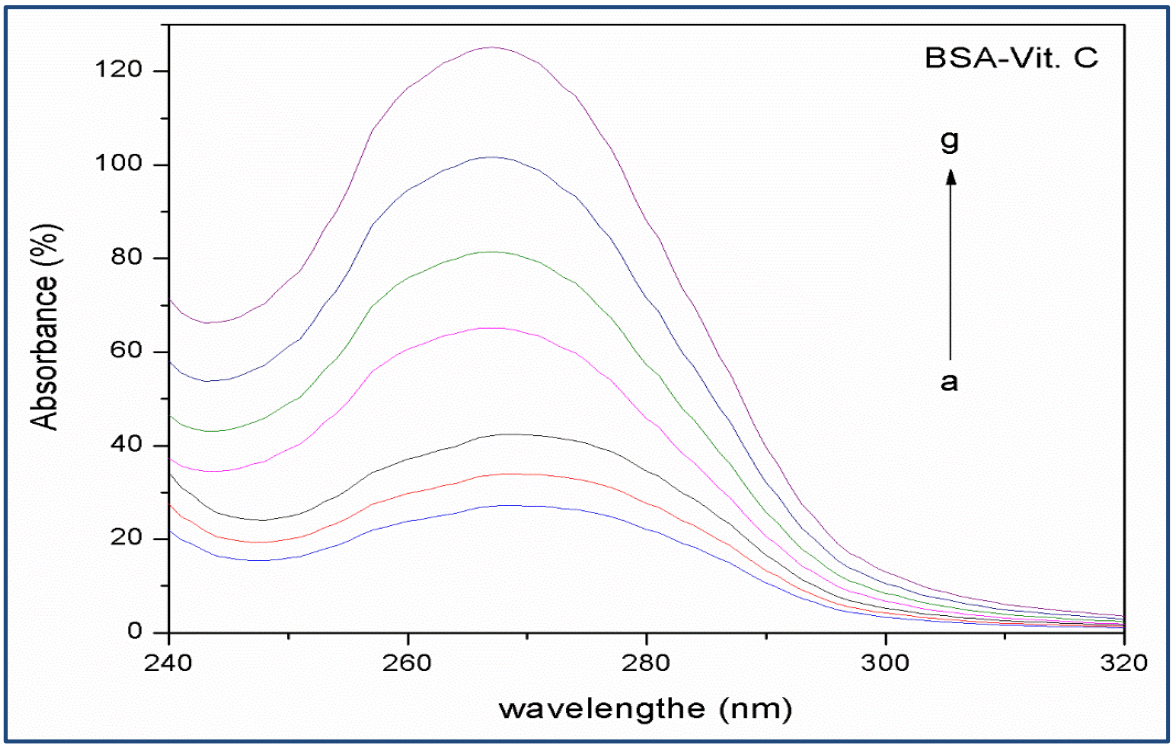

Figure 4.1B: UV-Vis absorbance spectra of BSA with different concentrations of Vitamin C

(a= Free BSA, b=1mg/ml ,c=2mg/ml, d=5mg/ml, e=10mg/ml, f=20mg/ml, g=40mg/ml).

4.1.1 The binding constant

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

k = [vitamin:BSA] /[vitamin][BSA] (4.1)

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

$$\frac{\mathbf{1}}{\boldsymbol{A - A_0}} = \frac{\mathbf{1}}{\boldsymbol{A_\infty - A_0}} + \frac{\mathbf{1}}{\boldsymbol{K[A_\infty - A_0]}} \cdot \frac{\mathbf{1}}{\boldsymbol{L}} \qquad (\mathbf{4.2})$$

where $\boldsymbol{A_0}$is the initial absorption band of free BSA at 280 nm in the absence of ligand , $\boldsymbol{A_\infty}$is the final absorption of BSA, and $\boldsymbol{A}$ is the recorded absorption at different drug(vitamin C/vitamin B_{12}) concentrations (1-40mg/ml) and final BSA concentration at 40mg/ml.

Figure 4.2A and figure 4.2B shows the plot of 1/[drug complex concentration] as a function of 1/[free drug concentration].

In figure 4.2A shows the plot of 1/ ($A-A_0$) vs. 1/ vitamin B_{12} concentration for BSA and its vitamin B_{12} adducts, the figure shows a proportional decrease of 1/[drug complex concentration] as a function of 1/[free drug concentration]. The binding constant from the interaction between BSA-B_{12} complex is estimated to be $k_{\text{vitamin B12-BSA}}=1.61\times10^4\ M^{-1}$.

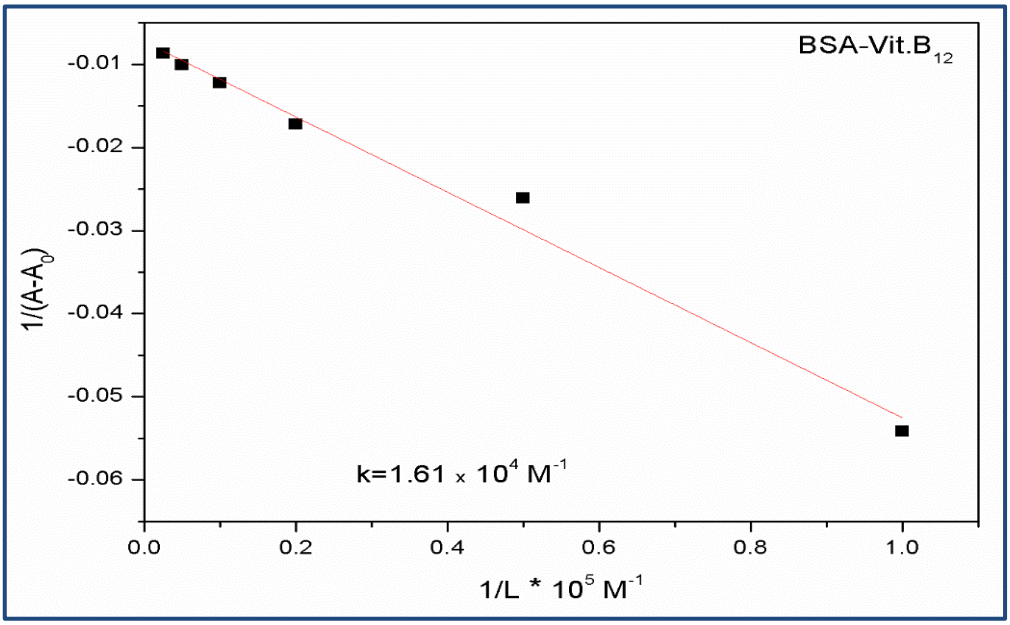

Figure 4.2A: The plot of 1/ ($A-A_0$) vs. 1/L for BSA with different concentrations of vitamin B_{12}.

In figure 4.2B shows the plot of 1/ ($A-A_0$) vs. 1/ vitamin C concentration for BSA and its vitamin C adducts, the figure shows a proportional increase of 1/[drug complex concentration] as a function of 1/[free drug concentration]. The binding constant from the interaction between BSA-C complex is estimated to be $k_{\text{vitamin C-BSA}}=1.39\times10^4\ M^{-1}$.

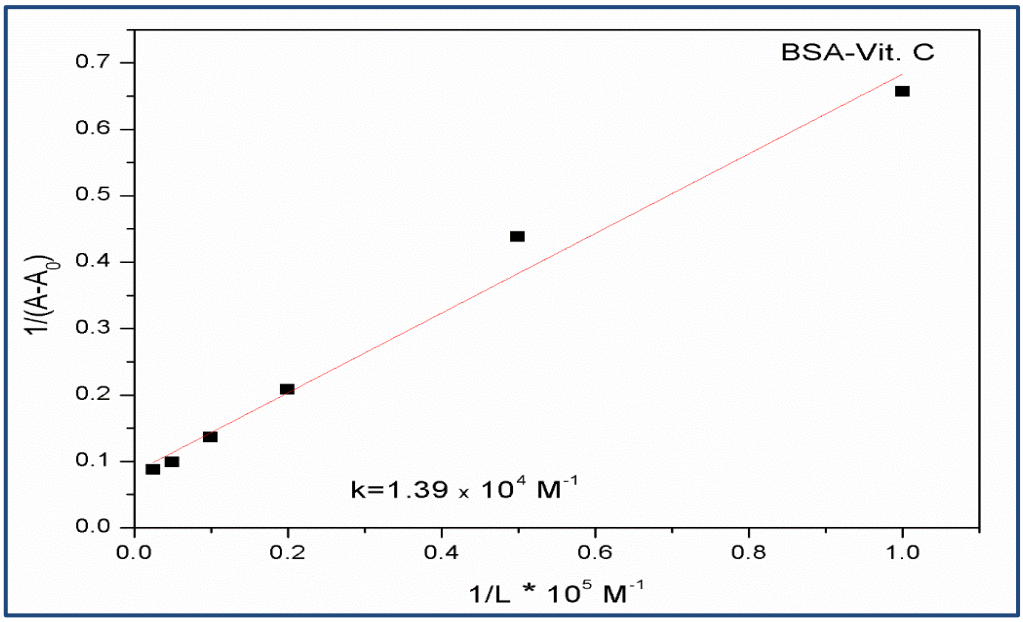

Figure 4.2B: The plot of $1/(A-A_0)$ vs. 1/L for BSA with different concentrations of vitamin C.

4.2. Fluorescence spectroscopic studies

Fluorescence spectroscopy is used to obtain excitation and emission spectrum and to measure the fluorescence intensity. From the measurement can provide the interaction of solvent molecule with fluorophores, conformational changes and finding the binding interaction [Lakowicz,2006].

In the fluorescence measurements, the BSA concentration was kept at 40mg.L^{-1}. The excitation wave length was set at 280 nm (excitation of Trp and Tyr), and the emission spectra were read at 400-650 nm at a scan rate of 100 nm.min^{-1}.

The emission fluorescence spectra of BSA in the presence of cobalamin (vitamin B_{12}) andascorbic acid (vitamin C) are shown in figure 4.3A and figure 4.3B, respectively. The maximum intensity for BSA in absence of ascorbic acid/cobalamin is observed at 440 nm. In figure4.3A show that the emission fluorescence intensity of BSA decrease with increasing of cobalamin (vitamin B_{12}) concentrations. However, in figure 4.3B show that the emission fluorescence intensity of BSA increases with increasing of ascorbic acid (vitamin C) concentrations.

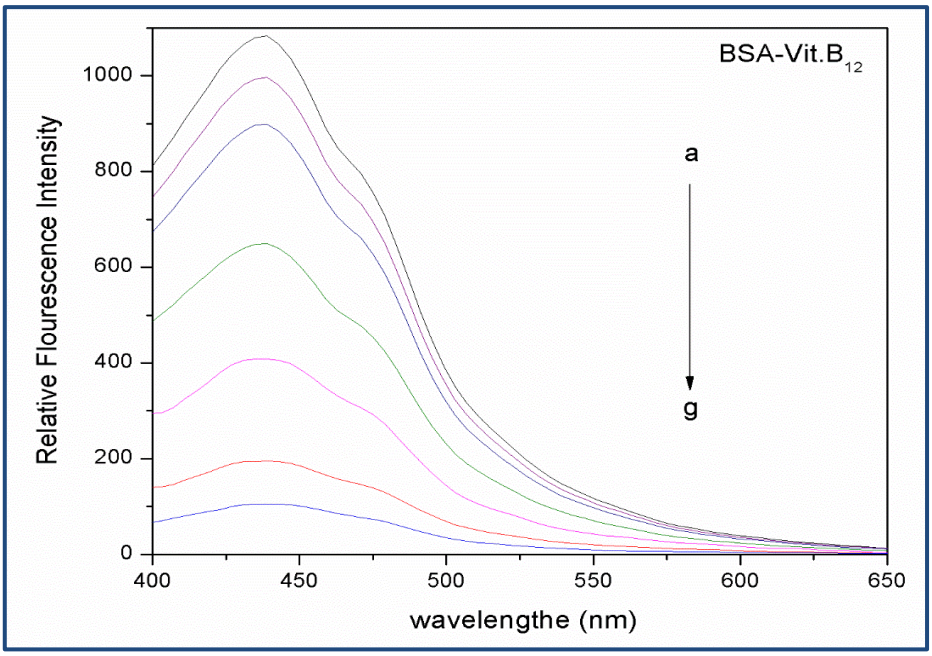

Figure 4.3A: Fluorescence emission spectra of BSA in the absence and presence of different concentrations of vitamin B_{12} (a= Free BSA, b=1mg/ml,c=2mg/ml, d=5mg/ml, e=10mg/ml, f=20mg/ml, g=40mg/ml).

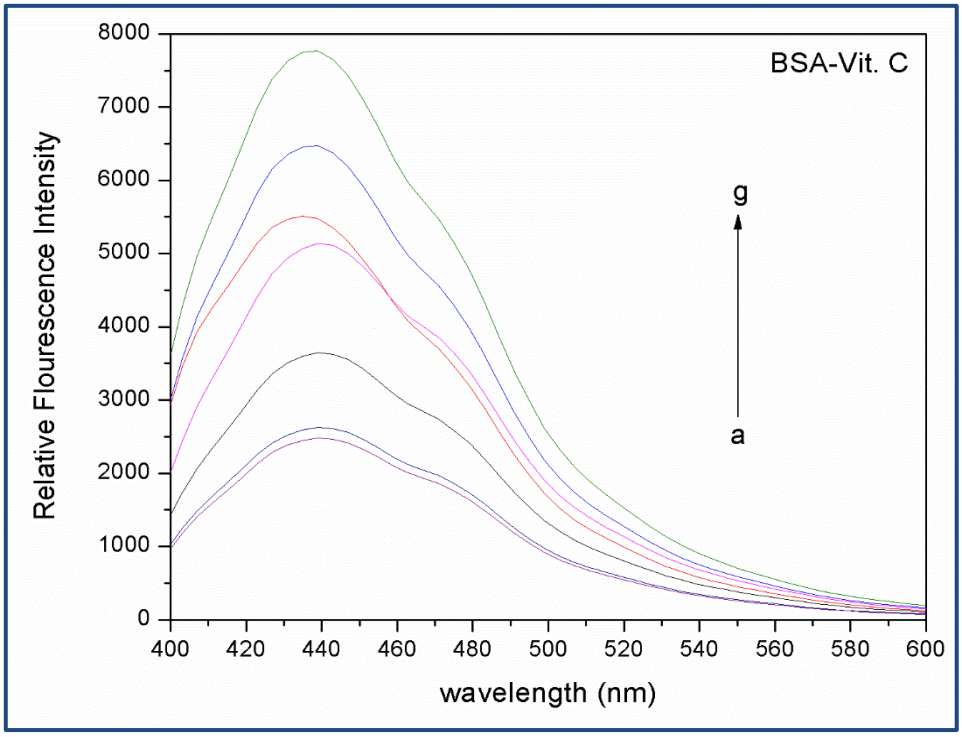

Figure 4.3B: Fluorescence emission spectra of BSA in the absence and presence of different concentrations of vitamin C (a= Free BSA, b=1mg/ml,c=2mg/ml, d=5mg/ml, e=10mg/ml, f=20mg/ml, g=40mg/ml).

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

The different mechanisms of quenching are usually classified as either dynamic quenching or static quenching. In the case of a "dynamic quenching", it is the diffusion-limited collision between the quencher and the fluorophore molecules that allows for energy transfer without radiation. The term "static quenching" implies the formation of ground-state non-fluorescent complex [Krimm et al., 1986].

Fluorescence quenching data are first analyzed with the Stern-Volmer equation:

$$\frac{\mathbf{F_0}}{\mathbf{F}} = \mathbf{1} + \mathbf{k_{sv}}[\mathbf{L}] = \mathbf{1} + \mathbf{k_q}\boldsymbol{\tau}_\mathbf{0}[\mathbf{L}] \qquad (4.3)$$

Where F_0 and F represent the steady-state fluorescence intensities in the absence and presence of quencher, respectively. $\boldsymbol{k_q}$ is the bimolecular quenching constant, $\boldsymbol{\tau_0}$ is the average lifetime of protein in the absence of quencher, and its value is 10^{-8}s, [L] is the concentration of the quencher, and $\boldsymbol{k_{sv}}$ is the Stern-Volmer quenching constant [samari et al., 2012]. Hence, this equation is applied to determine $\boldsymbol{k_{sv}}$ by linear regression of a plot of F_0/F against [L].

According to equation (4.3), linear Stern-Volmer plots for the BSA fluorescence quenching by ascorbic acid (vitamin C) and cobalamin (vitamin B_{12}) are shown in figure 4.4A and 4.4B, respectively.

In figure 4.4A and 4.4B shown that for ascorbic acid and cobalamin, the Stern-Volmer plot exhibits an upward curvature, concave toward the y axis, at high quencher concentration which represent a linear Stern-Volmer plot that means the only one mechanism (dynamic or static) of quenching occurs. However, positive deviation from the Stern-Volmer equation is frequently observed when the extent of quenching is large. The positive deviation may be an indication that this upward curvature indicates that the fluorophore can be quenched by either static and dynamic quenching processes or the presence of sphere of action [Lima et al., 2007].

The Stern-Volmer quenching constant $\boldsymbol{k_{sv}}$ and the bimolecular quenching constant $\boldsymbol{k_q}$derived from equation (4.3) is (4.78×10^3 L.mol^{-1} and 4.78×10^{11} L.mol^{-1}.s^{-1}, respectively) for vitamin C and (2.42×10^4 L.mol^{-1} and 2.42×10^{12} L.mlo^{-1}.s^{-1}, respectively) for vitamin B_{12}.

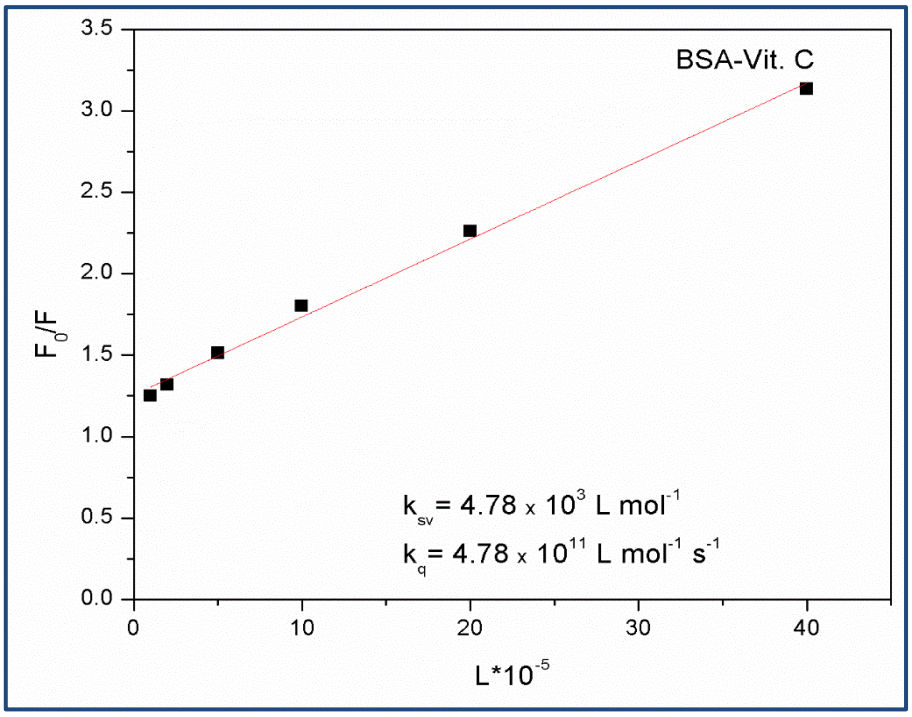

Figure 4.4A: The Stern-Volmer plot for vitamin C – BAS complex.

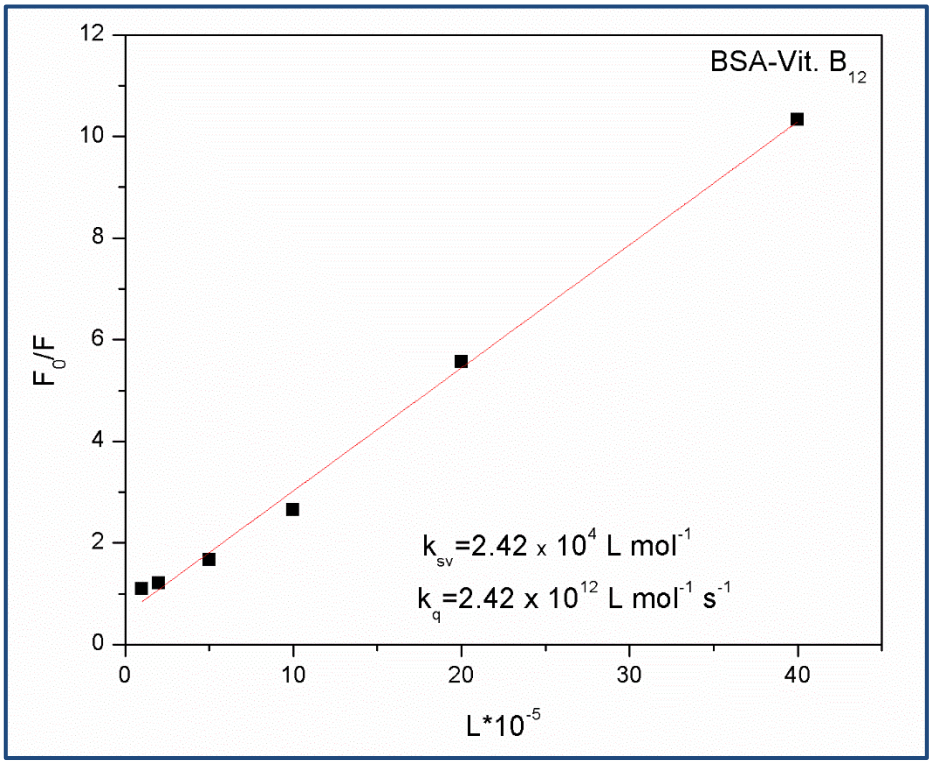

Figure 4.4B: The Stern-Volmer plot for vitamin B_{12} - BSA complex.

4.2.2 Determination of the binding constant using fluorescence spectrophotometer

The steady-state fluorescence spectra were applied to determine changes of BSA conformation generated by the binding interactions with vitamin C/vitamin B_{12}[Lakowicz,2006], thus the fluorescence data were analyzed considering the modified form of the classic Stern-Volmer relation(the Lehrer equation) for the static quenching process [Lehrer,1971].

$$\frac{F_0}{F_0 - F} = \frac{1}{\alpha\, k[L]} + \frac{1}{\alpha} \quad (4.4)$$

Where kdenotes the binding constant and α-the fraction of the accessible to the quencher fluorophore population. The value of binding constant k calculated from the intercept/ slop ratio from the plot of $(1/F_0\text{-}F)$ vs. $(1/L\times10^5)$ for vitamin C/ vitamin B_{12}-BSA complex.

The value of binding constant from figure 4.5A for vitamin C is equal 1.6×10^4 M^{-1} and the binding constant k from figure 4.5B is equal 1.44×10^4 M^{-1} for vitamin $B_{12,}$ the results of binding constant from fluorescence spectroscopy are almost equally the results of binding constant that taken from the UV-Vis spectroscopy.

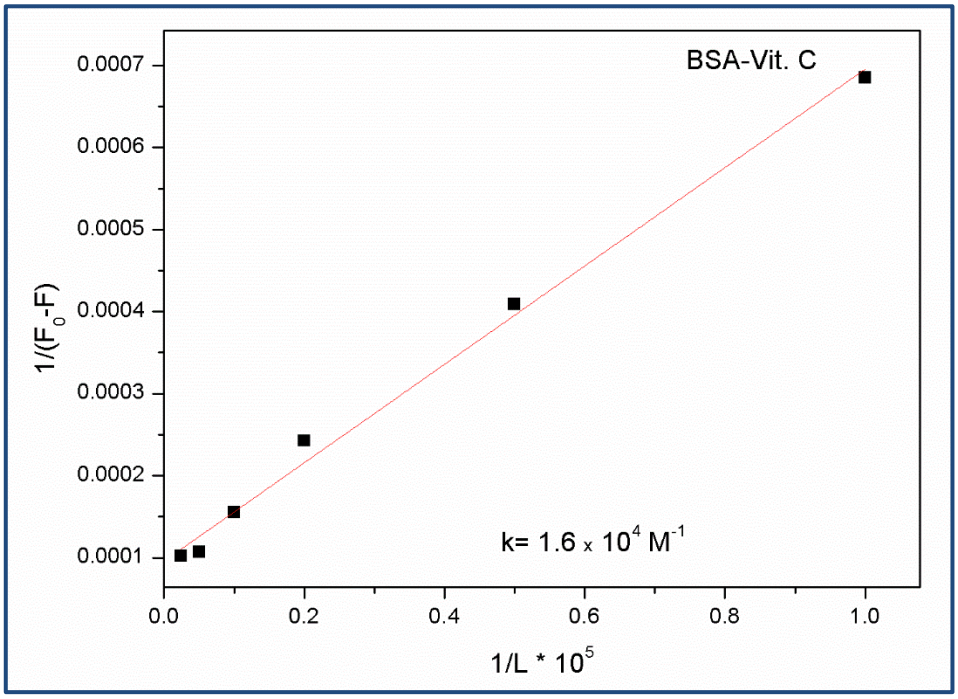

Figure 4.5A: The plot of $1/(F_0\text{-}F)$ vs. $1/[L*10^5]$ for vitamin C-BSA complex.

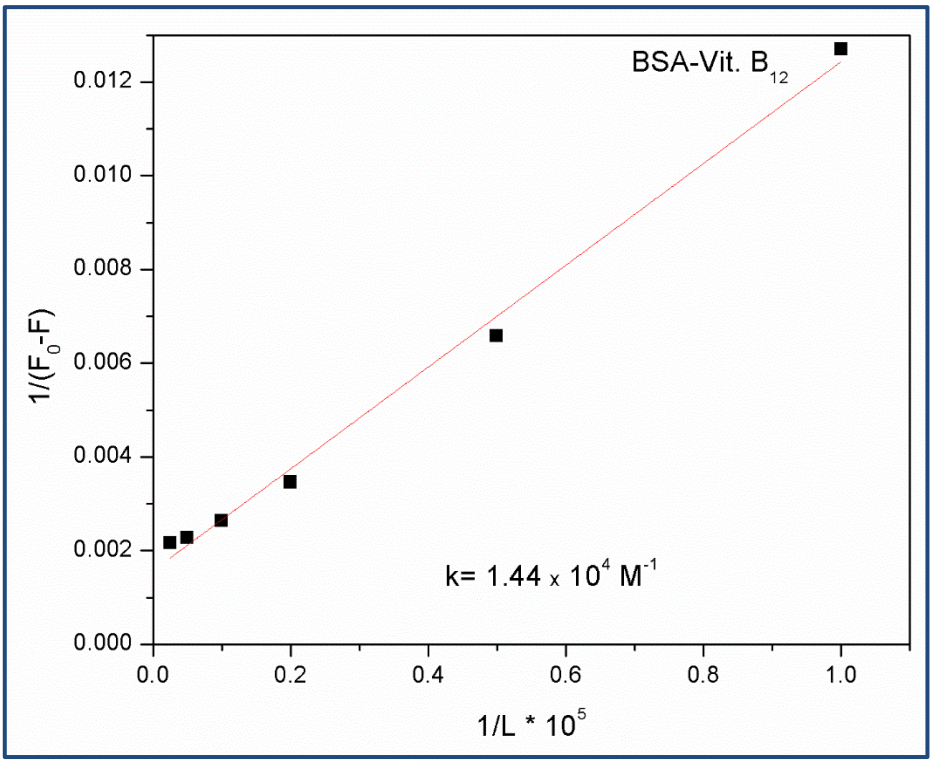

Figure 4.5B: The plot of $1/(F_0-F)$ vs. $1/[L*10^5]$ for vitamin B_{12}-BSA complex.

4.3 FT-IR spectroscopic studies

The FT-IR spectra can be used to directly analyze the effect of vitamin C/ vitamin B_{12} on secondary structure of BSA. FT-IR spectra of protein exhibit a number of amid bands, which represent different vibrations of the peptide moiety. Among the amide bands of the protein, the amide I band (1600-1700 cm-1, mainly C=O stretch), amide II band (1480-1600 cm^{-1}, C-N stretch coupled with N-H bending mode), and amide III band (1220-1330 cm^{-1}, C-N stretch coupled with N-H bending mode) which have a relationship with the secondary structure of the protein [Tian et al., 2004].

The FT-IR spectra of free BSA and the difference spectra after binding with vitamin C/vitamin B_{12} in phosphate buffer solution were recorded in the second derivative of them (figure 4.6A, 4.6B and 4.6C). the peak position of the amide I and amide II bands are observed at 1657 and 1546 cm^{-1} (vitamin C), and 1652 and 1547 cm^{-1} (vitamin B_{12})together with the changed in the peak shape and peak intensity upon the addition of vitamin C/ vitamin B_{12} to BSA. These results indicate that vitamin C/vitamin B_{12} interact with both the C=O and C-N groups in the protein polypeptides and result in the

rearrangement of the polypeptide carbonyl hydrogen-bonding network [Zhang et al., 2012].

The peak positions of amid I and II bands for free BSA are observed at 1653 and 1547 cm^{-1} in the second derivative of free BSA as showing in figure 4.6A. I observed increase in intensity of the amide I band in the presence of vitamin C/ vitamin B_{12} is due to the drug binding to protein C=O,C-N and N-H groups.

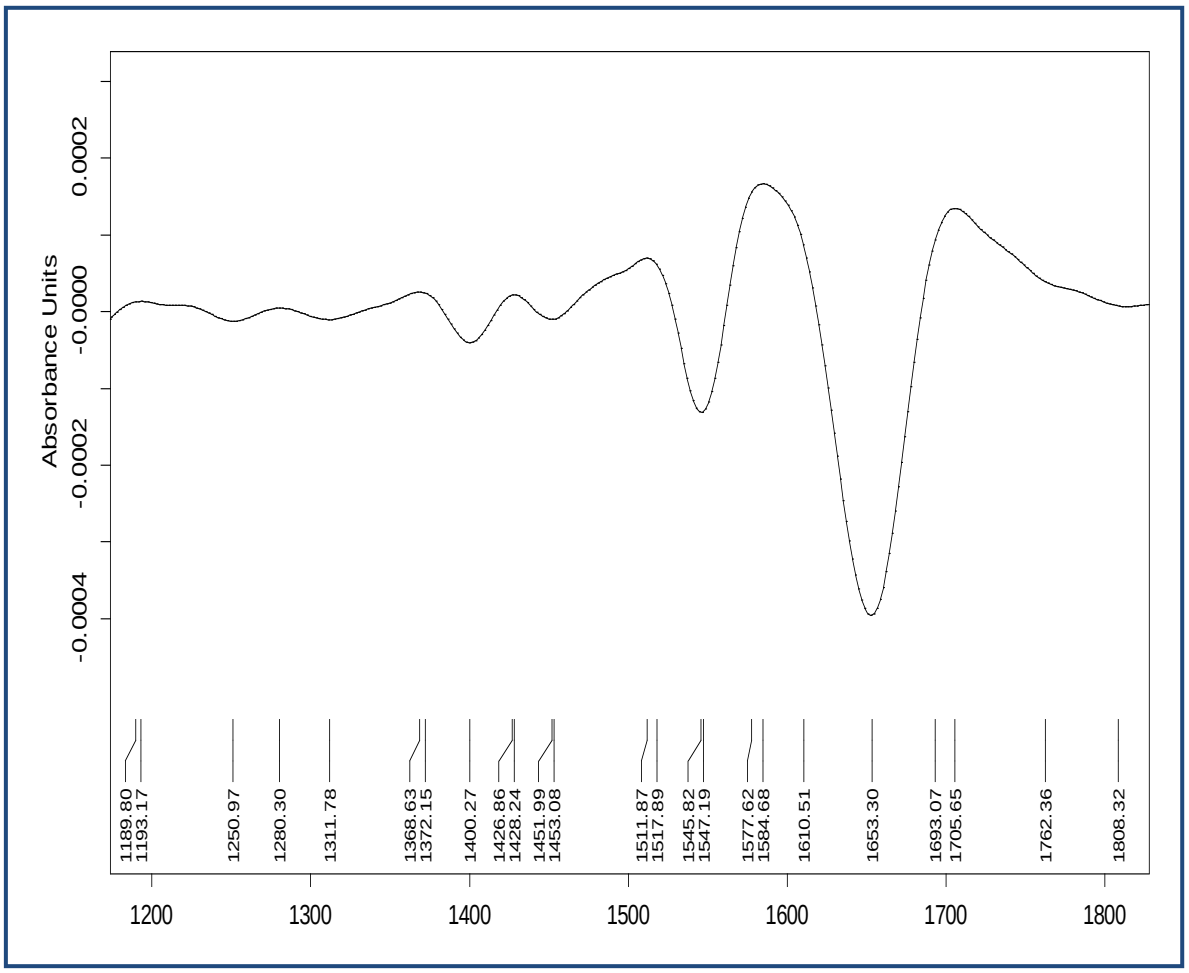

Figure4.6A: FT-IR spectra in the region of 1200-1800 cm^{-1} for free BSA.

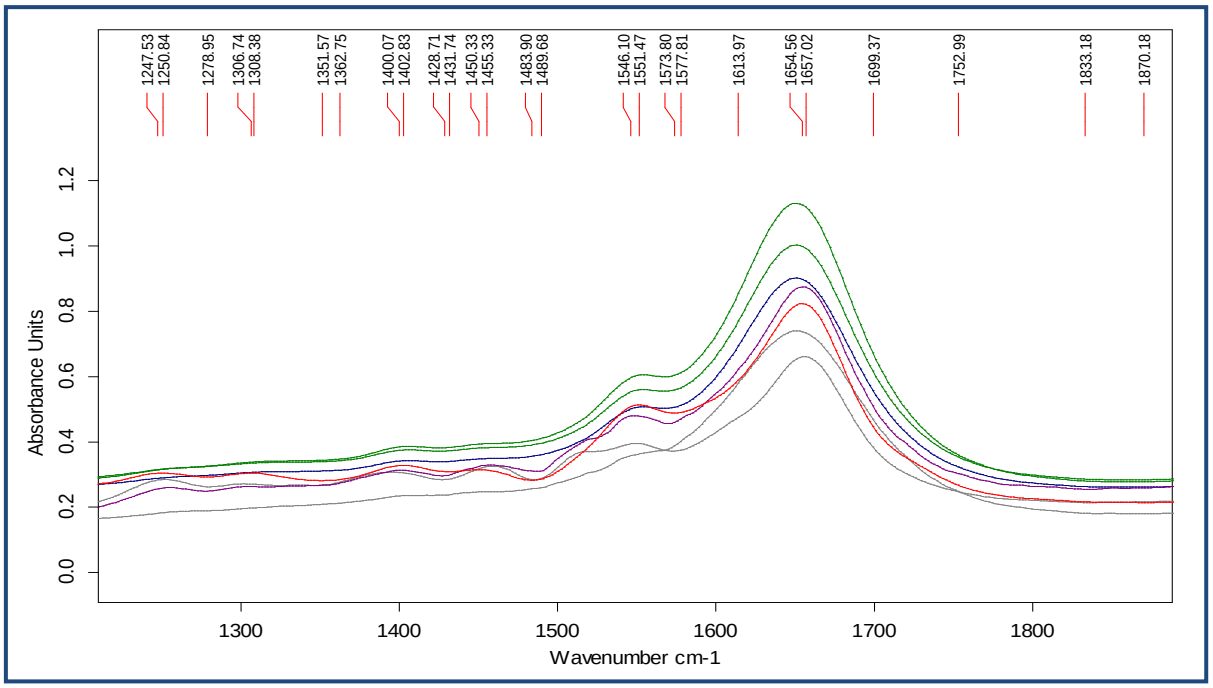

Figure 4.6B: FT-IR spectra in the region of 1200-1800 cm^{-1} for BSA-Vitamin C complex.

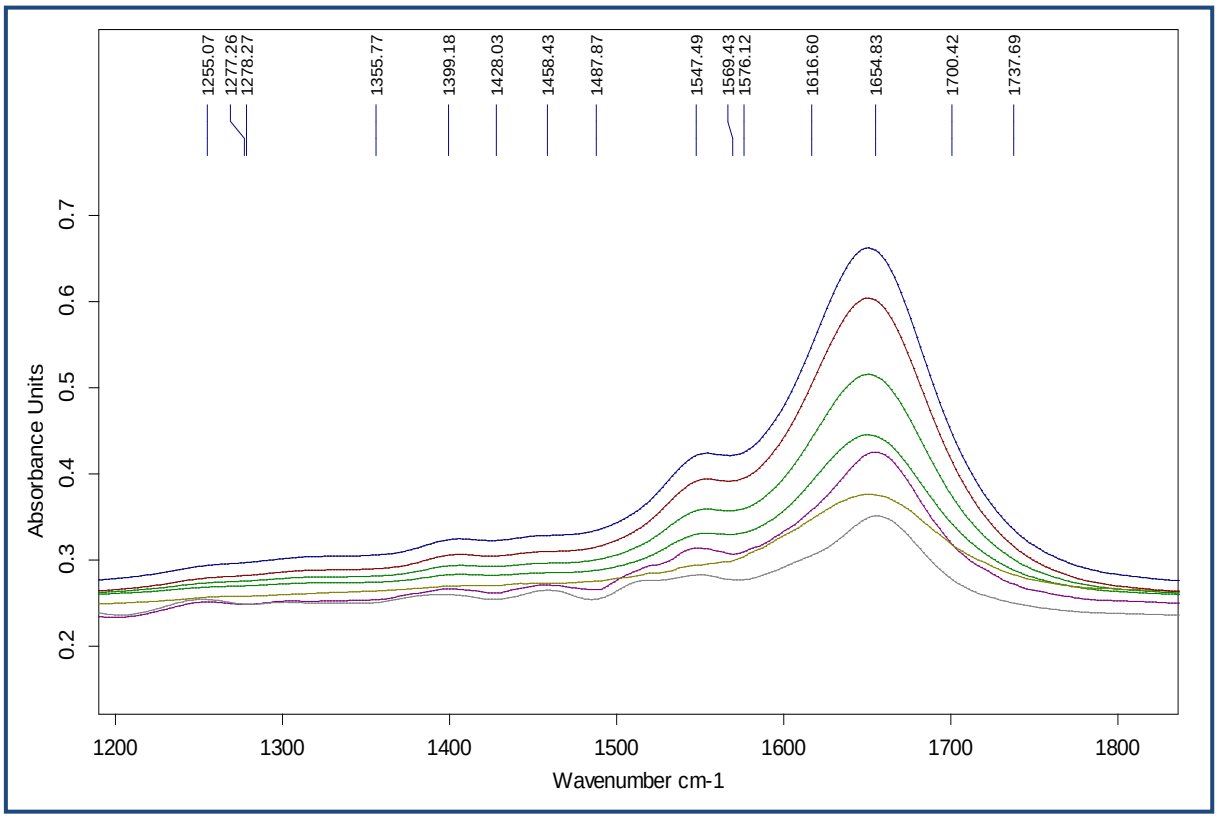

Figure 4.6C: FT-IR spectra in the region of 1200-1800 cm^{-1} for BSA-vitaminB_{12} complex.

Table 4.1: Band assignment in the absorbance spectra of BSA with different vitamin C concentrations for Amide I-III regions.

Band regions (cm^{-1})	BSA Free	BSA-Vitamin C 1mg.ml^{-1}	BSA-Vitamin C 2mg.ml^{-1}	BSA-Vitamin C 5mg.ml^{-1}	BSA-Vitamin C 10mg.ml^{-1}	BSA-Vitamin C 20mg.ml^{-1}	BSA-Vitamin C 40mg.ml^{-1}
Amid I (1600-1700)	1610	1609	1611	1610	1609	1611	1614
	1653	1657	1658	1656	1658	1659	1661
	1693	1699	1701	1698	1703	1704	1705
Amid II (1480-1600)	1589	1490	1491	1491	1490	1492	1493
	1545	1546	1545	1545	1546	1547	1547
	1573	1574	1573	1574	1576	1573	1578
Amid III (1220-1330)	1248	1248	1249	1248	1249	1249	1249
	1280	1279	1278	1280	1278	1277	1276
	1311	1309	1310	1311	1312	1312	1313

Table 4.1 represents the peak position of BSA with different vitamin C concentrations which given by the second derivative of it and showing by amide I, II and III.

For amide I, the peak position for the different spectra of the vitamin C-BSA complex was observed shifted ad iea from 1610 to 1614 cm^{-1}, 1653 to 1661 cm^{-1} and from 1693 to 1705 cm^{-1}as showing in Table 4.1.

For amide II, the peak position was shifted from 1589 to 1493 cm^{-1}, 1545 to 1547 cm^{-1} and from 1573 to 1578 cm^{-1} as showing in Table 4.1.

And for amide III, the peak position was observed a small shifted from 1248 to 1249 cm^{-1}, 1280 to 1276 cm^{-1} and from 1311 to 1313 cm^{-1}as showing in Table 4.1.

Table 4.2: Band assignment in the absorbance spectra of BSA with different vitamin B_{12} concentrations for amide I-III regions

Band regions (cm^{-1})	BSA Free	BSA-Vitamin B_{12} 1mg.ml^{-1}	BSA-Vitamin B_{12} 2mg.ml^{-1}	BSA-Vitamin B_{12} 5mg.ml^{-1}	BSA-Vitamin B_{12} 10mg.ml^{-1}	BSA-Vitamin B_{12} 20mg.ml^{-1}	BSA-Vitamin B_{12} 40mg.ml^{-1}
Amid I (1600-1700)	1610	1612	1609	1605	1604	1604	1603
	1653	1651	1650	1648	1646	1645	1642
	1693	1689	1689	1688	1686	1687	1684
Amid II (1480-1600)	1589	1587	1588	1586	1581	1584	1582
	1545	1542	1542	1539	1543	1538	1540
	1573	1597	1597	1596	1596	1597	1595
Amid III (1220-1330)	1250	1250	1249	1248	1248	1248	1246
	1309	1305	1305	1305	1302	1302	1303
	1311	1312	1311	1312	1312	1309	1309

Table 4.2 represents the peak position of BSA with different vitamin B_{12}concentrations which given by the second derivative of it and showing by amide I, II and III.

For amide I, the peak position for the different spectra of the vitamin B_{12}-BSA complex was observed shifted and increased from 1610 to 1603 cm^{-1}, 1653 to 1642 cm^{-1} and from 1693 to 1684 cm^{-1}as showing in Table 4.2.

For amide II, the peak position was shifted from 1589 to 1582 cm^{-1}, 1545 to 1540 cm^{-1} and from 1573 to 1595 cm^{-1} as showing in Table 4.2.

And for amide III, the peak position was observed a shifted from 1250 to 1246 cm^{-1}, 1309 to 1303 cm^{-1} and from 1311 to 1309 cm^{-1}as showing in Table 4.2.

From above, noticed that the change in the peak position indicates that the second structure of BSA change after interacted with vitamin C/ vitamin B_{12} drugs. From table 4.1 was noticed that the intensity increase in the difference spectra of the vitamin C-BSA for amide I, amide II and amide III bands, this happen due to drug binding to protein C=O,C-N and N-H groups.

From table 4.2 was noticed that the intensity decrease in the difference spectra of the vitamin B_{12}-BSA for amide I, amide II and amide III bands, this happens according to a major reduction of protein α-helical structure at these concentration.

Chapter Five:

Conclusions and Future Work

5.1 Conclusions

In this thesis, I present detailed analysis for vitamin C and vitamin B_{12} binding to BSA. In addition, to studied the secondary structure of BSA.

Data from UV-Vis experiments suggest that the intensity of the protein absorption peak increasing with increasing of vitamin C concentration and decreasing with increasing of vitamin B_{12} concentration due to the existence of aromatic amino acid in the components of protein molecule. From the data, I determined the value of the binding constant k for vitamin C and vitamin B_{12} binding with the bovine serum albumin (1.39×10^4 M^{-1} and 1.61×10^4 M^{-1}, respectively).

In fluorescence experiments, I determined from the Stern-Volmer plot, the Stern-Volmer quenching constant $\boldsymbol{k_{sv}}$ and the bimolecular quenching constant $\boldsymbol{k_q}$, it is (4.78×10^3 $L.mol^{-1}$ and 4.78×10^{11} $L.mol^{-1}.s^{-1}$, respectively) for vitamin C and (2.42×10^4 $L.mol^{-1}$, $2.42\times10^{12}L.mlo^{-1}.s^{-1}$, respectively) for vitamin B_{12}. In addition, determined the values of binding constant from the Lehrer equation for binding of vitamin C and vitamin B_{12} with BSA complex which are ($1.6\times10^4M^{-1}$ and 1.44×10^4 M^{-1}, respectively).

In FT-IR experiments observed that the secondary structure of BSA change after interacted with vitamin C and vitamin B_{12}, which means that vitamin C and vitamin B_{12} interacts with both the C=O and C-N groups in the protein polypeptides and result in a change of the secondary structure of BSA.

5.2 Future work

For future work, it can be interesting to investigate the interaction between BSA and the vitamins by using other spectroscopic techniques such as NMR spectroscopy or using isothermal titration calorimetry (ITC). In addition, we can add another type of vitamins, adding cholesterol or other types of lipids to study the effect of it in our body and blood.

Bibliography

[Guo et al., 2009] Guo X-J, Sun X-D, Xu S-K. *Spectroscopic investigation of the interaction between riboflavin and bovine serum albumin*. J Mol Struct.931:55–9.doi: 10.1371/journal.pone.0059106, (2009).

[Li et al., 2014] Xiangrong Li, ab Gongke Wang,a Dejun Chena and Yan Lu ,*Binding of ascorbic acid and α-tocopherol to bovine serum albumin: a comparative study,Mol. BioSyst.*, 10, 326, (2014).

[Ling, 2017] Ling, *Studies on the Interaction of Cefepime Hydrochloride with Bovine Serum Albumin by Fluorescence, Synchronous Fluorescence, Three- Dimensional Fluorescence and Circular Dichroism*, J Bioanal Biomed,9:107-113. doi: 10.4172/1948-593X.1000162, (2017).

[journal of Photoscience, 2004] *Spectroscopic studies on the binding of vitamins to bovine serum albumin and the influence of additives on binding,Journal of Photoscience*, 11 ,29-33, (2004).

[Zhang et al., 2012] Zhang K, Dong J, Cheng X, Bai W, Guo W, Wu L, Zuo L: *Association between vitamin C deficiency and dialysis modalities*. Nephrology (Carlton), 17(5):452-457,(2012).
[young et al., 2012]Yang HT, Lee M, Hong KS, Ovbiagele B, Saver JL.*Efficacy of folic acid supplementation in cardiovascular disease prevention: an updated meta-analysis of randomized controlled trials*. Eur J Intern Med;23:745-54,(2012).

[peters, 1998] Peters. T. *All about Albumin. Biochemistry, Genetics and Medical Applications*; Academic Press: San Diego, CA, (1996).

[Morrisett et al., 1974] JOEL D. MORRISETT,~ HENRY J. POWNALL, AND ANTONIO M. GOTTO, JR, *Bovine Serum Albumin STUDY OF THE FATTY ACID AND STEROID BINDING SITES USING SPIN-LABELED LIPIDS*, THY JOUBNAL OF BIOLOGICAL CHEMISTBY Vol. 260, No. 7, pp. 2487-2494,197s,(1974).

[kajiyama et al., 1998] kajiyama AM, Del Giacco EJ, Allen RH, Stabler SP, Lindenbaum J. *Effective treatment of cobalamin deficiency with oral cobalamin*. Blood;92:1191,(1998).

[Carpenter et al., 1986] Carpenter, K. J. ,*The History of Scurvy and Vitamin C*. Cambridge University Press, Cambridge, 288 pp., (1986).

[Wikipedia], https://en.wikipedia.org/wiki/Vitamin_B_{12}.

[Martirosyan, 2015] Danik M. Martirosyan, *Vitamin C: optimal dosages, supplementation and use in disease prevention*, Functional Foods in Health and Disease; 5(3): 89-107,(2015).

[Smith, 2008]Padraic Smith, *Vitamin B12 Deficiency: Causes, Evaluation and Treatment*, Fourth Year Physiology,(2008).

[Beatty et al., 2016] Beatty, Yeum, and K. J, *The intracellular vitamin A-binding proteins: An overview of their functions*. Nutr. Rev. 49, 1–12,(2016).

[Stabler, 2013] Sally P. Stabler, M.D., *Vitamin B12 Deficiency, clinical practice*, The new england journal of medicine,(2013).

[Jaffé et al., 1964] Jaffé, H. H., and M. Orchin, *Theory and Applications of Ultraviolet Spectroscopy*, John Wiley and Sons, New York, (1964).

[Parikh et al., 1974] Parikh, V. M. Reading, MA, (1974).

[Stern et al., 1971] Stern, E. S., and T. C. J. Timmons, *Electronic Absorption Spectroscopy in Organic Chemistry*, St. Martin's Press, New York, (1971).

[Scott, 1971] Scott, *Interpretation of the Ultraviolet Spectra of Natural Products*, Pergamon Press, New York, (1964).

[Silverstein et al., 2005] Silverstein, R. M., F. X. Webster, and D. J. Kiemle*Spectrometric Identification of Organic Compounds*, 7th ed., John Wiley and Sons, New York, (2005).

[Friedel et al., 1951] Friedel, R. A., and M. Orchin, *Ultraviolet Spectra of Aromatic Compounds*, John Wiley and Sons, New York, (1951).

[Graselli et al., 1975] Graselli, J. G., and W. M. Ritchey, eds., *Atlas of Spectral Data and Physical Constants*, CRC Press, Cleveland, OH,(1975).

[Harris et al., 1989] Harris, Daniel C., and Michael D. Bertolucci. *Symmetry and Spectroscopy: an Introduction to Vibrational and Electronic Spectroscopy*. New York: Dover Publications, (1989).

[Szymanski, 1980] Szymanski,*Interpreted Infrared Spectra*, Vols. 1–3, Plenum Press, New York, (1980).

[Colthrup et al., 1990] Colthrup, N. B., L. H. Daly, and S. E. Wiberley, *Introduction to Infrared and Raman Spectroscopy*, 3rd ed., Academic Press, New York, (1990).

[Nakanishi et al., 1998] Nakanishi, K., and P. H. Solomon, *Infrared Absorption Spectroscopy*, 2nd ed., Holden–Day, San Francisco, (1998).

[Macomber et al., 1998] Macomber, R. S*., A Complete Introduction to Modern NMR Spectroscopy*, John Wiley and Sons, New York, (1998).

[Nelson et al., 2003] Nelson, J. H., *Nuclear Magnetic Resonance Spectroscopy*, Prentice Hall, Upper Saddle River, NJ,(2003).

[Pople et al., 1959] Pople, J. A., W. C. Schneider, and H. J. Bernstein, *High Resolution Nuclear Magnetic Resonance*, McGraw–Hill, New York, (1959).

[Pretsch et al., 2000] Pretsch, E., P. Buhlmann, and C. Affolter, *Structure Determination of Organic Compounds*, 3rd ed., Springer-Verlag, Berlin, (2000).

[Roberts, 1959] Roberts, *Nuclear Magnetic Resonance: Applications to Organic Chemistry*, McGraw–Hill, New York, (1959).

[Roberts et al., 2000] Roberts, J. D., *ABCs of FT-NMR*, University Science Books, Sausolito, CA, (2000).

[Chatwal, 1979] G. R. Chatwal, S. K. Anand*Instrumental methods of chemical analysis*, Himalaya Publishing House (1979).

[Kittel, 1995] C. Kittel*Introduction to Solid State Physics* (7 th Ed.), John−Wiley and Sons (1995).

[Koney, 1969] Koney, *Fluorescence and phosphorescence of proteins and nucleic acids*, Plenum Press, New York (1967).

[Wehry, 1975] Wehry, *Modern fluorescence spectroscopy*, Plenum Press, New York (1976), Vol. 11459 pp.

[Miller, 1981] Miller, *Standardization & fluorescence spectrometry*, Chapman and Hall, (1981).

[Melhuish et al., 1981] Melhuish, W.H., Zander, *Nomenclature, symbols, units and their usage in spectrochemical analysis - VI molecular luminescence spectroscopy*, Pure App. Chem. 53, 1953, (1981).

[Kalsi, 1996] P.S. Kalsi, *Spectroscopy of Organic Compounds*, 2nd Edition, New Age International publishers, New Dehi, (1996).

[Stuart et al., 1997] Stuart, B. H., *Biological Applications of Infrared Spectroscopy*, ACOL Series, Wiley, Chichester, UK, (1997).

[Gremlich et al., 2000]Gremlich, H.-U.and Yan, B., *Infrared and Raman Spectroscopy of Biological Materials*, Marcel Dekker, New York,(2000).

[Weckhuysen, 2000]Weckhuysen, *in Spectroscopy of transition metal ions on surfaces*, Leuven University Press, (2000).

[Nano Drop 3300 Fluorospectrometer V2.7 user's Manual, 2008] *Nano Drop 3300 Fluorospectrometer V2.7 user's Manual*, (2008).

[Nano Drop 1000 Spectrophotometer V3.7, User's Manual, 2008] *Nano Drop 1000 Spectrophotometer V3.7 User's Manual*, (2008).

[Surewicz et al., 1993] Surewicz, W., Moscarello, M., Mantsch, H., J. Biol. Chem., 262, p8598, (1993).

[Griffiths et al., 2007] Griffith M, Mayer U, Capron A, *The TORMOZ gene encodes a nucleolar protein required for regulated division planes and embryo development in Arabidopsis*, Plant Cell:pp. 2246-63, (2007).

[OPUS Bruker manual, 2004] *OPUS Bruker manual*, (2004).

[Haris et al., 1999] Harris D, Myrick T, Rundle S. *The Arabidopsis Homolog Of Yeast TAP42 And Mammalian Alpha4 Binds To The Catalytic Subunit Of Protein Phosphatase 2A And Is Induced By Chilling. Plant Physiol*, (1999).

[Workman, 1998] Workman,*AppliedSpectroscopy:OpticalSpectrometers*.Academic Press.(1998).

[Kauppinnen et al., 1981] Kauppinen J, Moffatt D, Mantsch H, Cameron D, *Fourier Self-Deconvolution: A Method For Resolving Intrinsically Overlapped Bands.AppliedSpectroscopy*.pp. 271-276, .(1981).

[Goormaghtigh et al., 2006] Goormaghtigh E, Ruysschaert J, RaussensV,*Evaluation of the Information Content in Infrared Spectra for Protein Secondary Structure Determination*, Biophys J:pp. 2946-2957,(2006).

[chi and liu, 2011] Z.X. Chi and R. T, Liu, *Phenotypic characterization of the binding of tetracycline to human serum albumin*, biomacromolecules, (2011).

[Stephanos et al., 1996] Stephanos J, *Drug-protein interactions: two-site binding of heterocyclic ligands to a monomeric hemoglobin*, J InorgBiochem:pp. 155-69, (1996).

[Koltz et al., 1971] Klotz, M., andHunston,L., *Biochemistry*, 10, p3065,(1971).

[Ouameur et al., 2004] Ouameur, A., Mangier, E., Diamantoglou1,S., Carpentier,.,Biopolymers, 73, p503, (2004).

[Lakowicz, 2006] Lakowicz, J., *Principles of Fluorescence Spectroscopy*, 3rded, Springer Science and Business Media, US, (2006).

[Krimm et al., 1986] Krimm, S.,andBandekar, *Adv. Protein Chem.*, 38, 181-364, (1986).

[Samari et al., 2012] F.Samari, B.Hemateenejad, M.Shamsipur, M.Rashidi and H. Samouei, *Affinity of two novel five coordinated anticancer complex to human and bovine serum albumin:Aspectrocopic approach*, Inorg.Chem.,(2012).

[Lima et al., 2007] S. A. C. Lima, A. Cordeiro-da-Silva, B. D. Castro and P. Gameiro, *Sensitivity of P-glycoprotein tryptophan residues to benzodiazepines and ATP interaction,*Biophys. Chem., 125, 143–150,(2007)**.**

[Lehrer, 1971] S.S.Lehrer, Bichemistry10, 3254-3263, (1971).

[Tian et al., 2004] J. N. Tian, J. Q. Liu, W. Y. He, Z. D. Hu, X. J. Yao and X. G. Chen, *Probing the binding of scutellarin to human serum albumin by circular dichroism, fluorescence spectroscopy*, FTIR, and molecular modeling method, Biomacromolecules,1956 -1961,(2004).

[Zhang et al., 2012] G. W. Zhang, L. Wang and J. H. Pan, *Probing the binding of the flavonoid diosmetin to human serum albumin by multispectroscopic techniques*, J. Agric. Food Chem., 60, 2721–2729, (2012).

دراسة التفاعل بين بروتين الالبومين البقري مع فيتامين C وفيتامين B_{12} باستخدام تقنيات اجهزة التحليل الطيفي.

إعداد: إسراء ابراهيم محمود ابو سرور

إشراف:د.حسين سمامرة

ملخص:

تم في هذا البحث دراسة تفاعل وارتباط بروتين الالبومين البقري **BSA** مع فيتامين **C** وفيتامين**B_{12}** من خلال ثلاث طرق ربط مختلفة, تم دراسة تفاعلهم وارتباطهم من خلال استخدام جهاز مطياف الاشعة فوق البنفسجية والاشعة المرئية , جهاز فوريير لتحويل طيف الاشعة تحت الحمراء و جهاز المطياف الضوئي.

أظهرت البيانات المأخوذة من جهاز مطياف الاشعة فوق البنفسجية والمرئية, إلى ان شدة امتصاص البروتين تزداد كلما زاد تركيز فيتامين **C** وتقل كلما زاد تركيز فيتامين **B_{12}**. تم استخدام النتائج لإيجاد ثابت الربط بين بروتين الالبومين البقري وفيتامين **C** وفيتامين **B_{12}**, **$1.61\times10^{4}\ M^{-1}$**, **($1.39\times10^{4}\ M^{-1}$**على التوالي).

في تجربة جهاز المطياف الضوئي, تم ايجاد ثابت شترن فولمر للتبريد بالاضافة الى ثابت التبريد ثنائي الجزئ لفيتامين (**$4.78\times10^{11}\ L.mol^{-1}.s^{-1}$**, **$4.78\times10^{3}\ L.mol^{-1}$** **C**, على التوالي) ولفيتامين**B_{12}** **$2.42\times10^{4}\ L.mol^{-1}$** (و **$2.42\times10^{12}\ L.mlo^{-1}.s^{-1}$**, على التوالي). بالاضافة إلى إيجاد ثابت الربط باستخدام معادلة ليرر بين فيتامين **C** و فيتامين **B12** مع خليط بروتين الالبومين البقري **$1.6\times10^{4}\ M^{-1}$**(و **$1.44\times10^{4}\ M^{-1}$**, على التوالي).

في تجربة فوريير لتحويل طيف الاشعة تحت الحمراء, تم ملاحظة بأن البنية الثانية لبروتين الالبومين البقري يحدث عليه تغيير بعد ان يتفاعل مع فيتامين**C** و فيتامين **B_{12}**, وهذا يعني بأن كلاً من فيتامين **C**وفيتامين **B_{12}** قد تفاعلا وارتباطا مع مجموعة **C=O**و**C−N** التي يتكون منها البوليبتيد الخاص بالبرويتن, مما يؤدي إلى تغيير التركيبة الثانية لبروتين الالبومين البقري **BSA**.