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Study The Interaction Of Hydrophilic Vitamins (vitamin C and vitamin B12) With HSA Using Spectroscopic Techniques

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Dedication

I dedicate this thesis to all members of my lovely family who supported me throughout my life and encouraged me to achieve my goals , to my true friends who always supported me , to my teachers who made all they had to make me better and better, to my homeland Palestine , to the great martyrs and prisoners, the symbol of sacrifice to the scientists .

Thank you for all the unconditional love, guidance, and support that you have always given me, helping me to succeed and instilling in me the confidence that I am capable .

Mohammad AymanMohammadAbuallan

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: 

Mohammad Ayman Mohammad Abuallan

Date: 15/ 03/2018

Acknowledgement

At first and last my great commendation and thanks to Allah, who gave me the ability to accomplish this work, and put in my way such a nice supporting people I would like to thank all people who deserve my gratitude and made this work possible and enjoyable experience for me , My great thanks to my supervisor Dr. Husain Alsamamra for the support he gave me during the research and for providing me the ability to work. He has been an excellent teacher and a brilliant guidance for his wisdom, patience, encouragement, positive criticism, sage advice and assistance in bringing this work to completion. You are a teacher whom I will not forget all my life and believed in me , I sincerely thank you, My great thanks also go to all teachers in the physics department who always helping as a son , thank you all .

Mohammad AymanAbuallan

Abstract

Vitamin C is an important regulatory for iron uptake while vitamin B_{12} is essential for proper functioning of folic acid. 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 vitamin C and B_{12} with human serum albumin was investigated by using fluorescence spectroscopy and UV absorption spectrum. From spectral analysis, both vitamins 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=1. 28×10^{4} M^{-1} for HSA-Vitamin C and k=2.21×10^{4} M^{-1} for HSA-vitamin B_{12}. In additionthe Stern-Volmer constant is calculated at room temperature for both vitamin C and B_{12}. FT-IR spectroscopy was used to determine the protein secondary structure. The observed spectral changes indicate an increase of intensity for HSA-vitamin C interaction and a reduction of intensity for HSA-vitamin B_{12} interaction.While in the difference spectra of vitamin B_{12}-HSA complexes, intensity decreases as the concentration of vitamin B_{12} increases for amide I. 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
E	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
$\grave{W}_e$	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
$E(z,v)$	amplitude of the wave (travelling in the z-direction) incident on the beam splitter
$E_0(v)$	maximum amplitude of the beam at z=0
z_1	distance travelled by the beams when they recombine
z_2	Another distance travelled by the beams when they recombine
$I_R(x)$	the flux associated at path difference x
$I_R(0)$	flux associated with waves at zero arm displacement
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

Infrared (IR) spectroscopyis a technique based on the vibrations of the atoms of a molecule. An infrared spectrum is commonly obtained by passing infrared radiation through a sample and determining what fraction of the incident radiation is absorbed 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 (Banwell, 1972).

IR spectroscopy is an absorption method in the wavelength region of 1 to 100 μm in that extends the region of the visible light to longer wavelengths and smaller frequencies/energies. The energy of infrared light is not sufficient to induce transitions of valence electrons. Instead, infrared radiation excites vibrational and rotational motions in molecules. Except for the differences in the energy transfer from the radiation to the molecule, the principles of IR spectroscopy are the same as those of visible ultra violet (VIS-UV) spectroscopy or other spectroscopic techniques (Wilson, 1995).

Atoms can move relative to each other causing the bond to vary in length, this causes bond stretching, or one atom can move out of plane relative to the other, causing bond bending. Resonance frequencies for linear molecules 3N-5 while they are 3N-6 for nonlinear molecule some of which will interact with incident infrared radiation and N is the degree of freedom(Gordon, 2011).

Serum albumin, often referred to as blood albumin, is a type of globular proteinfound in vertebrate's blood. Human serum albumin is encoded by the Albumin gene (ALB gene). Other mammalian forms, such as bovine serum, are chemically similar (Hawkins, 1982).

1.2 Proteins

Proteins are complex macromolecules made up of successive amino acids that are covalently bonded together in a head-to-tail arrangement through substituted amide linkages called peptide bonds.

The 20 amino acids that make up the building blocks of proteins differ in the structure of their R groups, which 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) the difference only on the side chain labeled R in the figure 1.1(Rosenberg, 2005).

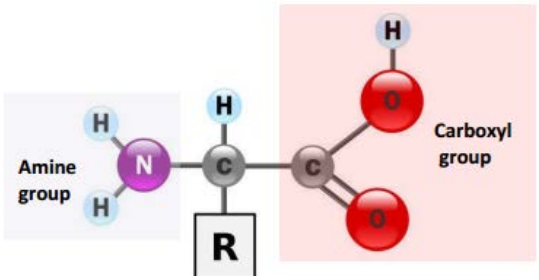

Figure 1.1: general structure of all amino acids (Nelson, 2005).

A protein molecule is made from a long chain of these amino acids, each linked to its neighbor through a covalent peptide bond. Proteins are therefore also known as polypeptides. Each type of protein has a unique sequence of amino acids as in figure 1.2, exactly the same from one molecule to the next. Many thousands of different proteins are known, each with its own particular amino acid sequence(Alberts et al. 2002).

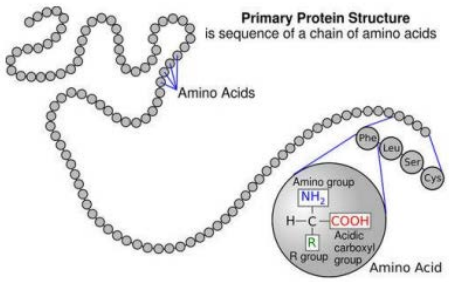

Figure 1.2 :polypeptide (a chain made up of many linked amino acids)(Smith et al. 2005).

1.2.1 Protein structure

There are four levels of protein structure which determine protein function these structure are primary, secondary, tertiary, and quaternary (Stabler, 2013) .

1.2.2 Primary structure

This structure is the simplest level of protein structure , it is simply the sequence of amino acids in a polypeptide chain as shown in figure 1.3 , and its unique for each protein (Richardson, 1981).

Figure 1.3 : Primary Structure of protein (Smith et al. 2005).

1.2.3 Secondary structure

Local folded structures that form within a polypeptide due to interactions between the carbonyl of one amino acid and the amino hydrogen of another ,the most common types of secondary structures are the (alpha helix) α-helix and the (beta pleated sheet) β-pleated sheet as shown in figures 1.4 and 1.5 respectively , both structures are held in shape by hydrogen bonds (Smith et al. 2005).

In an α-helix, the carbonyl (C=O) of one amino acid is hydrogen bonded to the amino (N-H) of another amino acid in the down chain ,and The R groups of the amino acids stick outward from the α-helix, (Richardson, 1981)

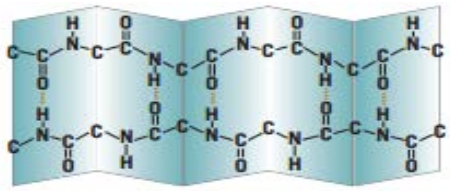

Figure 1.4 : Beta Sheet (Smith, 2005).

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 extend 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), or antiparallel, pointing in opposite directions (meaning that the N-terminus of one strand is positioned next to the C-terminus of the other (Nelson, 2005).

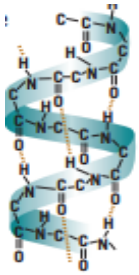

Figure 1.5 : Apha helix (Smith, 2005).

1.2.4 Tertiary Structure

The three dimensional space resulted from the different secondary structure elements interactions between the R groups of the amino acids that make up the protein ,these interaction include hydrogen bonding, ionic bonding, dipole-dipole interactions, and London dispersion forces, and its produce a linear or spherical structure as in figure 1.6(Gideon Greenspan, 2003).

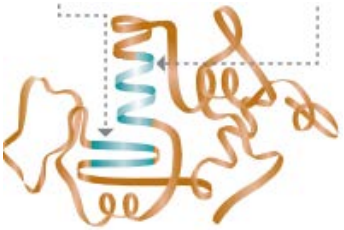

Figure 1.6 : Tertiary Structure (Smith et al. 2005).

1.2.5 Quaternary Structure

Many proteins are made up of a single polypeptide chain and have only three levels of structure (the ones we've just discussed). 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, such as hemoglobin and Deoxyribonucleic acid(DNA) polymerase (Gregory and Dagmar, 2004). Figure 1.8 shows the Fourier transformation infra red(FTIR) spectrum for a protein and the bands.

Figure 1. 7 : Quaternary Structure (Smith et al. 2005).

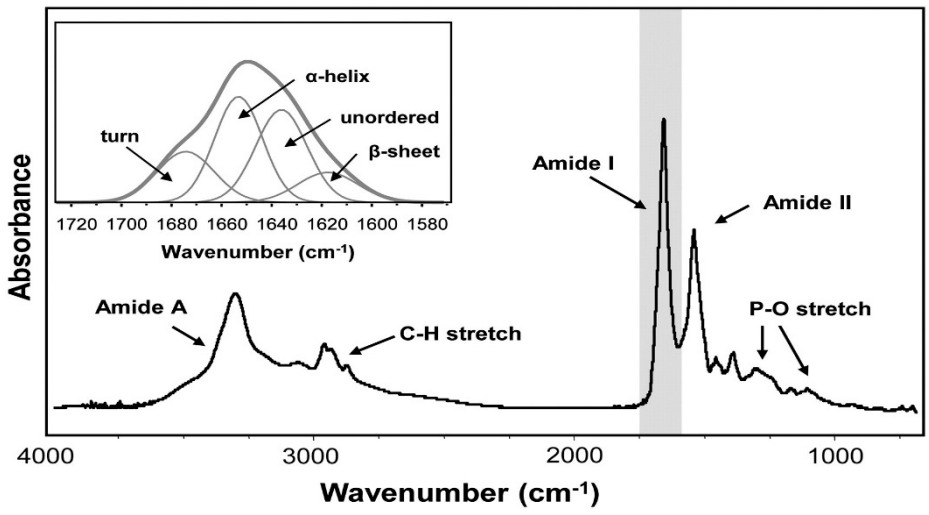

Figure 1.8: FTIR spectrum of a typical protein illustrating the Amide I and Amide II bands (Lisa et al. 2013).

1.3 Human Serum Albumin

Human serum albumin (HSA) is the most abundant protein in blood plasma and 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. HSA serves as the major soluble protein constituent of the circulatory system, it contributes to colloid osmotic blood pressure, 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).

The most important physiological role for the protein is to bring such solutes into blood stream and then deliver them to the target organs, as well as to maintain the potential of hydrogen(pH) and osmotic pressure of plasma (Abu Teir et al. 2014).

HSA concentration in blood plasma is 40 mg/ml.And the three dimensional structure of HSA was determined through x-ray crystallographic measurements (Carter et al. 1989).

HSA is composed of a single polypeptide chain of 585 amino acids as shown in figure 1.9 and contains35 cysteines, which form 17 disulfide bridges, the thiol group of the unpaired cysteine at the amino acid position 34 plays 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^{+},)also a single tryptophan at amino acid position 214 plays important role in providing a valuable research tool for protein chemists and biochemists, and also in Many spectroscopic and fluorescence studies because of the single tryptophan residue ,including molar concentration determination, fluorescence quenching studies, fluorescence lifetime studies, also HSA contains many cysteine residues and charged amino acids, and it has small numbers of glycine, methionine (JiSook, 2004).

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 et al. 2012).

HSA consists of three homologous domains in a heart-shaped conformation and named each domain as domain I, II, and III as shown in figure 1.10 , and each domain is known to be made up by two separate helical subdomains (named A and B), connected by random coil, these subdomain A and B consists of 10 alpha helixes and alpha helix (1 - 6)forms subdomain A and helixes (7 - 10) forms subdomain B (Peters et al. 1985).

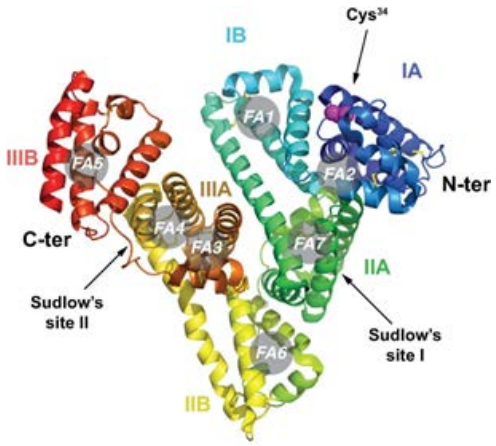

Figure 1.10 : Molecular structure of HSA. Crystal structure of HSA with an indication of its subdomains (IA, IB, IIA, IIB, IIIA an IIIB), of the N and C termini (Bhattacharya et al. 2000).

The N terminal end attaches Cu^{+2} and Ni^{+2} ions. The two high affinity fatty acid attachment sites are in Domain III. Other fatty acid sites are located in the center of Domains I and II. The presence of fatty acid significantly enhances the 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) (Rothschild et al. 1988).

Aromatic and heterocyclic ligands were found to bind within two hydrophobic pockets in sub-domains 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).

Therefore, it is often used as a probe to investigate the binding properties of drugs with HSA. It has been 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).

Multiple drug binding sites have been reported for HSA by several researchers (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 concentrations of free drugs in plasma, whereas weak binding can lead to a short lifetime or poor distribution. Its remarkable capacity to bind a variety of drugs results in its prevailing role in drug pharmacokinetics and pharmacodynamics (Kandagal et al. 2007).

1.4 VITAMINS

Vitamins are organic molecules required in very small quantities in the diet for health, growth, and survival (Truswell, 2003).

Most Vitamins are used for the synthesis of coenzymes, complex organic molecules that assist enzymes in catalyzing biochemical reactions, so the absence of a vitamin from the diet or an inadequate intake results in characteristic deficiency signs and symptoms reflect an inability of cells to carry out certain reactions. The Vitamins are divided into two classes, water-soluble Vitamins and fat-soluble vitamins. This classification has little relationship to their function but is related to the absorption and transport of fat-soluble Vitamins with lipids. The fat soluble Vitamins are A, D, E, and K and the water-soluble Vitamins are C (ascorbic acid) and the family of B Vitamins (Mann and Truswell, 2002).

1.4.1 Vitamin C

Ascorbic acid (Vitamin C) is a water-soluble micronutrient required for multiple biological functions. Ascorbic acid is a cofactor for several enzymes participating in the post translational hydroxylation of collagen, in the biosynthesis of carnitine, in the conversion of the neurotransmitter dopamine to norepinephrine, in peptide amidation and in tyrosine metabolism. In addition, Vitamin C is an important regulator of iron uptake, It reduces ferric Fe^{+3} to ferrous Fe^{+2} ions, thus promoting dietary non-haem iron absorption from the gastrointestinal tract, and stabilizes iron-binding proteins (Aysun, 2009).

Vitamin C is a crucial component to human health. This particular Vitamin is water soluble (causing quick elimination and preventing storage) and cannot be synthesized by humans; therefore, it is essential that Vitamin C be incorporated into our diet (Gallie, 2013).

The particular mechanism that prevents synthesis is the absence of gulonolactone oxidase (GLO) (Mandlet al. 2009),which is necessary to catalyze the enzyme L-gulono-1,4-lactone oxidase, the final step in the biosynthetic pathway of Vitamin C (Chatterjee, 1973).

There are numerous reasons why Vitamin C is important to our health, but many involve its aspect as essential factor in the synthesis of collagen, carnitine and norepinephrine (Doll and Ricou, 2013).

The importance of Vitamin C stems from its powerful antioxidant capacity. The term antioxidant has been defined as, “any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate” (Halliwell et al.1995).

In a general sense, Vitamin C acts as a cofactor and reduces certain enzymes by providing them with electrons, due to its chemical structure (Padayatty and Levine, 2001).

Those enzymes can react with biomolecules known as lipids, proteins and DNA, and cause harm. In order to help prevent that, Vitamin C reduces oxygen species when lipid peroxidation is formed, reduces radical inhibitors in protein oxidation, and prevents nitrosamine formation to reduce DNA damage (Padayatty et al. 2003).

The two main components of Vitamin C are ascorbate and dehydroascorbic acid (DHA) (Mandl et al. 2009). The transport of ascorbate through the human body involves two sodium-dependent Vitamin C transporters (SVCT): SVCT1 and SVCT2 (Li et al.2007).

The empirical formula is $C_6H_8O_6$, and the molecular weight is 176.13 $g.mol^{-1}$ (Krishnasarmapathy , 2010). And chemical structure of Vitamin C is shown in figure 1.11.

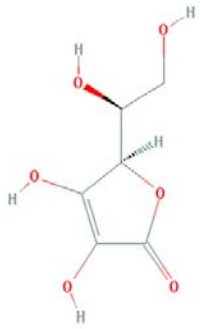

Figure 1.11: Ascorbic acid structure(National Center for Biotechnology Information ,https://pubchem.ncbi.nlm.nih.gov/compound/ascorbic_acid#section=2D-Structure).

1.4.2 Vitamin B_{12}

Vitamin B_{12} is a water-soluble Vitamin naturally present in some foods, added to others as a dietary supplement. Vitamin B_{12} exists in several forms and contains mineral cobalt, compound with vitamin B_{12} activity are collectively called cobalamins, vitamin B_{12} is required for proper red blood cell formation, neurological function and DNA synthesis. Vitamin B_{12} functions as a cofactor for methioine synthase and L-Methylmalonyl- CoA mutase(VirendraGoyal, 2015).

Vitamin B_{12} exists in many forms in the body, two of which are biologically active coenzymes: methylcobalamin and adenosylcobalamin. Methylcobalamin is a coenzyme with methionine synthase, a key enzyme in the folic acid dependent synthesis of pyrimidine's and purines. Adenosylcobalamin is involved in the enzymatic degradation of fatty acids by methylmalonyl-CoA mutase. These enzymes are needed for normal function of bone marrow and the central nervous system (Stabler, 2013).

Vitamin B_{12} was discovered because of its relationship to the disease pernicious anemia (PA). PA was a fatal illness before the 1920s. But this changed after Whipple suggested raw liver as a treatment. He found that ingesting large amounts of liver seemed to cure anemia from blood loss (Whipple et al. 1920).

Minot and Murphy (1926) then described the dramatic recovery of 45 patients suffering from PA after they consumed a special diet of lightly cooked liver.

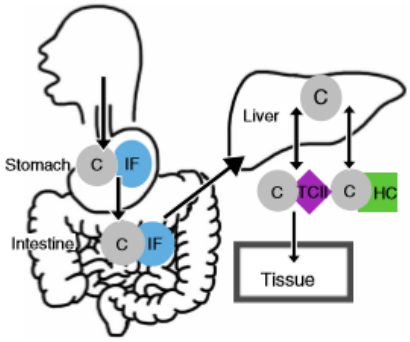

Figure 1.12 : Cobalamin's complex metabolism (Brescoll and Daveluy , 2015).

When cobalamin is ingested from food sources as shown in figure 1.12, it is first released from the proteins in food by pepsin in the stomach. It then binds to haptocorrin, which is found in saliva and protects cobalamin from the acidic environment of the stomach. Haptocorrin is then degraded in the duodenum by digestive proteases and the free cobalamin binds to intrinsic factor. Intrinsic factor is needed for cobalamin to be absorbed by receptors on the enterocytes in the terminal ileum and to protect it from catabolism by intestinal bacteria. Cobalamin is then transported into the portal circulation and taken up by the liver. The majority of cobalamin in circulation is bound to haptocorrin, with a smaller percentage bound to transcobalamin II, and a negligible amount of circulating free cobalamin(Ermens et al. 2003).

It is taken up into tissues, mainly the liver, via transcobalamin receptors, which are located on endothelial cells and not hepatocytes themselves. The liver stores enough cobalamin to last several years before symptoms of deficiency present (Kozyraki and Cases, 2013).

The elucidation of the X-ray structure of Vitamin B_{12} was finally accomplished by Dorothy Hodgkin (Hodgkin et al. 1979) .The empirical formula is $C_{63}H_{88}CoN_{14}O_{14}P$, and the molecular weight is 1355.4 $g.mol^{-1}$ (National Institute of Advanced Industrial Science and Technology, the website : http://sdbs.db.aist.go.jp/sdbs/cgi-bin/direct_frame_disp.cgi?sdbsno=2057).The chemical structure of vitamin B_{12} is shown in figure 1.13.

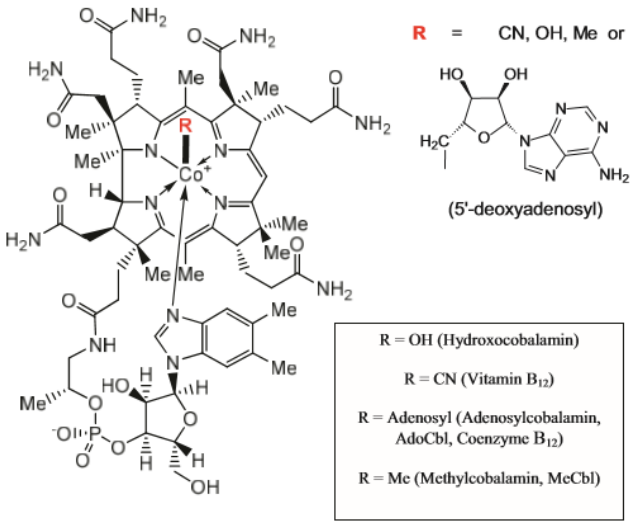

Figure 1.13 : The chemical structure of vitamin B_{12} and derivatives (Woodward, 1973).

Vitamin B_{12} is essential for rapid DNA production during cell division. This is especially important in tissues such as the bone marrow, where cells are dividing rapidly. Where there is a lack ofVitamin B_{12}, the synthesis of DNA can be disrupted and abnormal cells called megaloblasts are produced during red blood cell formation. This can result in megaloblasticanaemia (Chanarin, 1979 ; Anthony, 1991)

VitaminB_{12} deficiency could also lead to damage in the nervous system by interrupting the maintenance of myelin. Despite the extensive animal-model research of B_{12} deficiency for more than 30 years, the specific role of Vitamin B_{12} in maintaining normal myelination remains poorly understood.9 Failure to convert methylmalonyl CoA into succinyl CoA results in a high level of methymalonic acid, which is a destabiliser of myelin (Metz, 1992 ; Pant et al. 1968)

Mammals are not able to synthesis cobalamins, so they must be supplied from the diet.Vitamin B_{12} can be found primarily in meat, fish, eggs and dairy products. The daily requirement of Vitamin B_{12} for the human body is low 1-2 μg (Chanarin, 1979).

1.5 Recent studies

Xiangrong Li et al. (2013), investigated the interaction between the three antioxidants and HSA by several spectroscopic techniques. Experimental results proved that the three antioxidants quench the fluorescence of HSA through a static (proanthocyanidins) or static-dynamic combined quenching mechanism (ascorbic acid and α-tocopherol).

FereshtehShiri et al. (2017), investigated the competitive binding of isoxsuprine hydrochloride with HSA in the absence and presence of folic acid and ascorbic acid using different spectroscopic probes and molecular docking studies. The results of fluorescence suggested that isoxsuprine alone or in the presence of ascorbic acid can bind to HSA and quench the fluorescence of HSA with static mechanism but For HSA–folic acid–isoxsuprine system, dynamic type of quenching mechanisms is involved .

Xiangrong Li et al. (2014), investigated the ability of bovine serum albumin (BSA) to scavenge α, α-diphenyl-β-picrylhydrazyl(DPPH) radical using UV-VIS absorption spectra. The result shows that the antioxidant activity of BSA against DPPH radical is similar to glutathione and the value of IC50 is $5.153\times10^{-5}molL^{-1}$.

Xiangrong Li et al. (2014), investigated the Binding of ascorbic acid (water-soluble antioxidant) and α-tocopherol (lipid-soluble antioxidant) to BSA using isothermal titration calorimetry (ITC), in combination with fluorescence spectroscopy, UV-VIS absorption spectroscopy and FT-IRspectroscopy.

Hou et al. (2008), investigated the binding reaction between Vitamin B_{12} and HAS by fluorescence quenching, UV-VIS absorption and circular dichroism spectroscopy. On the basis of the results of fluorescence quenching the mechanism of the interaction of B_{12} with HSA has been found to be a dynamic quenching procedure .

Wang et al. (2005), studied the interaction between The HSA and vitamin B_6 using fluorescence technique the results indicate that astatic quenching was occurred .

Mina MemarpoorYazdi and HanieMahaki (2012) examined the interaction of Riboflavin and l-Argininewith HSA using different spectroscopic techniques .

Alsamamra et al.(2017), studied the interaction of the Testosterone with BSA , Absorption SpectroscopyusingUV-VIS absorption spectrophotometry and fluorescence spectroscopy to determine thebinding constant .

1.6 Research statement

Infrared spectroscopy provides measurements of molecular vibrations due to the specific absorption of infrared radiation by chemical bonds. It is known that 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 the analysis of this single band allows elucidation of conformational changes with high sensitivity (Darwish et al. 2010 ; Alsamamra et al. 2018).

This work will be limited to the mid-range infrared, which covers 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. 2010 ; Alsamamra et al. 2017).

Other spectroscopic techniques are usually used in studying the interaction of drugs and proteins, fluorescence and UV-VIS spectroscopy are commonly used because of their high sensitivity, rapidity and ease of implementation. (Wybranowski et al. 2008; Li et al. 2008; Li et al. 2006).

As mentioned above, all these techniques will be used to study the interaction between HSA and vitamin C as well as with vitamin B_{12}.

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

Chapter two : Theoretical Background

2.1 introduction

In this chapter, I will talk about the spectroscopic techniques being used for my thesis and the physical principle for each one , In particular about Fourier transformation infrared spectroscopy and ultra-violet spectroscopy and florescence spectroscopy .

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 as shown in figure 2.1 , EMW propagate as a sine or cosine waves at the speed of light in vacuum (Stuart, 2004). The electromagnetic spectrum consists of radio waves, microwaves, infrared radiation, visible light, ultraviolet radiation, X-rays and gamma rays as shown in figure 2.2.

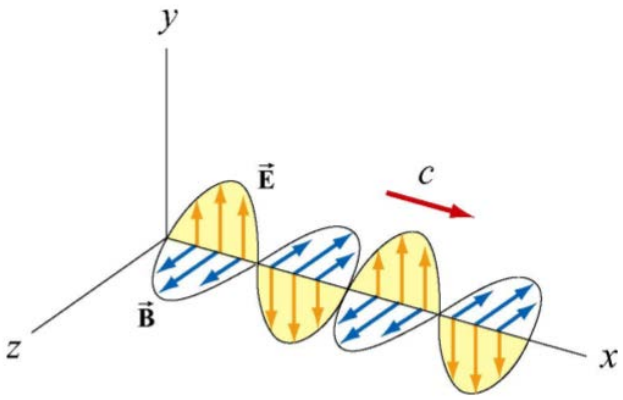

Figure 2.1: Plane electromagnetic wave propagating (Sharma, 2007).

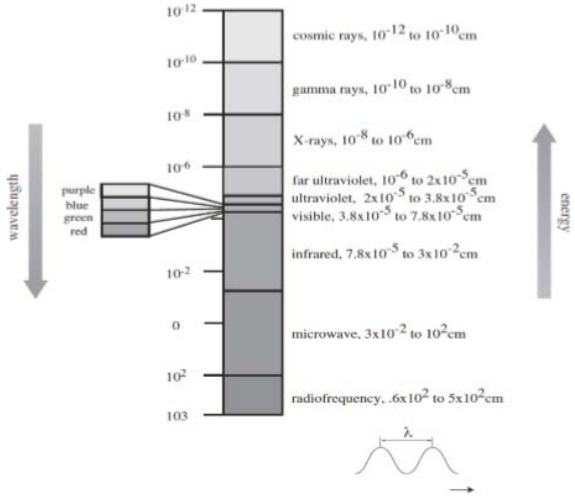

Figure 2.2: Electromagnetic spectrum (Shernan, 2014).

In vacuum, IR radiation has a wavelength between ≈ (780 nm - 1 mm) , spanning three orders of magnitude and can be divided in Near, Mid and Far IR. Our work utilizes region of Mid-IR from 4000-400 cm^{-1} where vibrational and rotational bands are observed, and the UV-VIS region from 10-800 nm (Hollas, 2004).

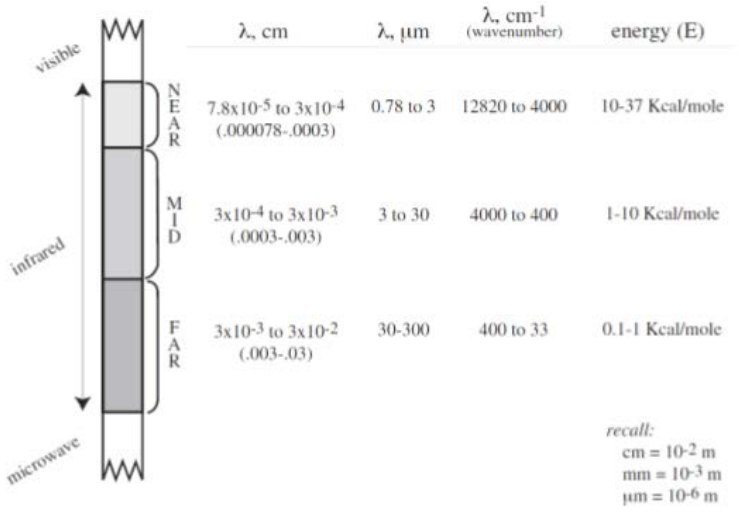

Figure 2.3: The IR region of electromagnetic spectrum (Shernan, 2014).

Thw wavelength λis inversely proportional to frequency$v$ and it is governed by the relation :

$$\lambda = \frac{c}{v} \tag{2.1}$$

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

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

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

Where E is the energy and$h$ is Planck's constant and$v$ is frequencyand λ is wavelength (Pavis et al. 2008;Yadav 2005; Williams 1976 ; Ball 2001).

The 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:

$$E_{total} = E_{ele} + E_{vib} + E_{rot} + \cdots \quad (2.3)$$

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, this occur 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. It is clear 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.

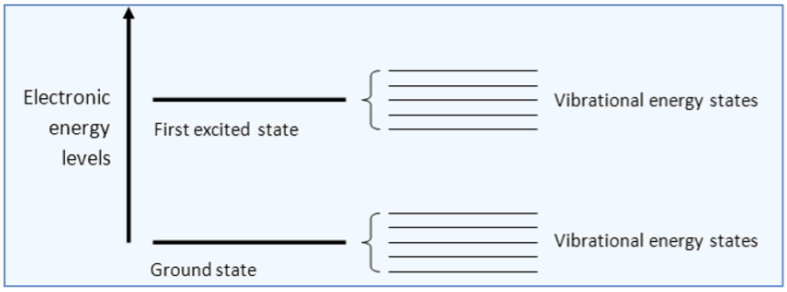

Figure 2.4: A schematic representation of the quantized electronic and vibrational energy levels of a molecule.

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

$$\frac{I}{I_0} = 10^{-\varepsilon Cl} \quad (2.4)$$

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 :

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

where A is called Absorbance (Hollas, 2004 ; Schulman, 1977)

2.2.1 Infrared (IR) Spectroscopy

An infrared spectrum is commonly obtained by passing infrared radiation through a sample and determining what fraction of the incident radiation is absorbed 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 (ν= 400-4000 cm^{-1}) studies the changes in the vibrational and rotation movements of the 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 et al. 2006).

A molecule can only absorb radiation when the incoming infrared radiation is of the same frequency as one of the fundamental modes of vibration of the molecule. This means that the vibrational motion of a small part of the molecule is increased while the rest of the molecule is left unaffected , vibrations can involve 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 spans a section of the electromagnetic spectrum having wavenumbers from roughly 13,000 to 10 cm^{-1}, or wavelengths from 0.78 to about 1000 μm. It is bound by the red end of the visible region at high frequencies and the microwave region at low frequencies,

The IR region is commonly divided into three smaller areas: near IR, mid IR and Far-IR. Mid-IR between 4000 and 400 cm^{-1} is the most frequently used region and it is what we have used in this study (Shernan, 2014).IR spectroscopy is one of the oldest and well established experimental and analytical techniques available to today's scientists (Settle, 1997).

Simply, it is absorption measurement of different IR frequencies by a sample positioned in the path of an IR beam. The main goal of IR spectroscopic analysis is to determine the chemical functional groups in the sample. Different functional groups absorb characteristic frequencies of IR radiation. Using various sampling accessories, IR spectrometers can accept a wide range of sample types such as gases, liquids and solids. Thus, IR spectroscopy is an important and popular 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 corresponds to a frequency of vibration of a part of a sample molecule. As the starting point for introducing the concept of harmonic vibrations, it is instructive to consider molecules as an array of point masses that are connected with each other by massless springs representing the intra-molecular interactions between the atoms (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 et al. 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:

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

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 that the circular frequency of the harmonic vibration increases when rigidity of the spring (or the strength of the bond) increases but decreases with increasing masses of the atoms (spheres).

2.2.2 Normal modes of vibration

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 zdirections, corresponding to three degrees of freedom. Thus, a molecule of N atoms has in total 3N degrees of freedomas shown in table 2.1 , but not all of them correspond to vibrational degrees of freedom (Settle, 1997).

It can be seen easily that a nonlinear molecule (where the atoms are not located in straight line) has three rotational degrees of freedom, whereas there are only two for a linear molecule. 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 et al. 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 correspond 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 from a change in bond angle between two atoms or movement of a group of atoms, relative to the reminder of the molecule (Mirabeela, 1998).

The frequency of normal modes 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).

Many of the group frequencies vary over a wide range 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 do not obey exactly the simple harmonic motion, real bonds do not follow hooks law they are not so elastic. If for example a bond stretches of 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 on 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).

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

Where :

δ: constant for a particular molecule.

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:

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

W_e: is an oscillating frequency .

$\grave{W}_e$: is the oscillation frequency in wave number.

X_e: is the corresponding an-harmonicity constant which is positive and small for bond stretching (≈+0.01) . this means that the vibration levels crowded more closely with increasing n (Banwell, 1972).

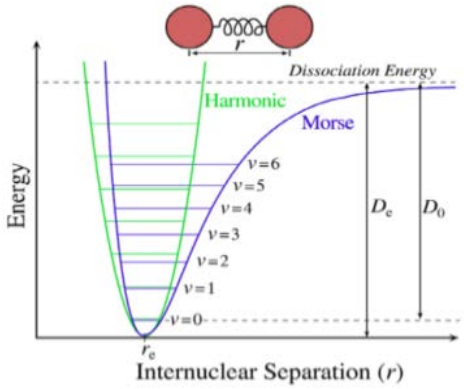

Figure 2.5: potential energy of a diatomic molecule as a function of atomic displacement (inter-nuclear separation) during vibration. The Morse potential (blue) and harmonic oscillator potential (green) (Settle, 1997).

2.3 FT-IR Spectroscopy

FT-IR spectroscopy is a measurement of wavelength and intensity of the absorption of IR radiation by a sample (Settle, 1997).

The old ways of spectroscopy were heavily dependent on dispersion elements such as prisms or gratings so the different components of light (λ, ν) are 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).

Fourier transform spectrometers have greatly extended the capabilities of IR spectroscopy 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

Principle of IR absorption by molecules is at the very heart of IR spectroscopy. Absorption is the process by which the energy of a photon is taken up by the matter.

There are several types of physical processes that could lie behind absorption, depending on the quantum energy of the particular frequency of electromagnetic(EM) radiation for example : ionization and electronic transitions (Settle, 1997).

In case of energy absorption, molecules are excited to a higher energy states including IR absorption.

IR radiation does not have enough energy to induce electronic transitions as seen with UV-VIS . It corresponds to energy changes on the order of 8 to 40 KJ/mole (Shernan, 2014).

Absorption of IR is restricted to excite vibrational and rotational states of a molecule. Its energy range corresponds to the range encompassing the stretching and bending vibrational frequencies of the bonds in the most covalent molecules (Settle, 1997).

If the frequency of the radiation matches the vibrational frequency of the molecule then the radiation will be absorbed, causing a change in the amplitude of molecular vibration. However not all bonds in a molecule are capable of absorption infrared energy only if 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 fingerprint of a sample with absorption peaks which corresponds to the frequencies of vibrations between bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum (Settle, 1997).

Therefore, IR spectroscopy can result in a positive identification (qualitative analysis) of every 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

IR absorption information is generally presented in the 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 whereas absorbance ranges from infinity to zero (Shernan, 2014).

2.3.3 Theory of FT-IR spectroscopy

FT-IR is the preferred method of IR spectroscopy. The resulting spectrum after IR passes through the sample represents the molecular absorption and transmission creating a molecular fingerprint of the sample (Thermo Nicolet, 2001).

FT-IR spectrometer basically depends on a simple optical device called an interferometer. As shown in (Fig. 2.6), the interferometer requires two mirrors, an infrared light source, an infrared detector and a beam splitter.

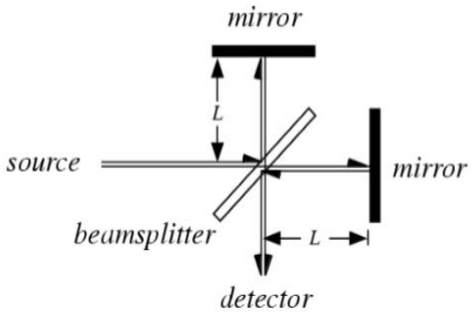

Figure 2.6: The Michelson interferometer (Viji, 2006).

The beam-splitter is the heart of the interferometer. Essentially 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 et al. 2008).

The two mirrors reflect both beams back to the beam-splitter where each of the two beams is again half reflected and half transmitted. Two output beams result: one travels to the detector as the other travels to the source (Viji, 2006).

When the two beams return to 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 computer to convert the space domain into wave number domain (Viji, 2006).

The basic integral equation used in FT-IR spectroscopy can be obtained from the definition of Fourier integral theorem. The basic equation used in the case of Michelson interferometer can be derived as follows:

Let the amplitude of the wave (travelling in the z-direction) incident on the beam splitter be given as

$$E(z,v) = E_0(v)e^{i(\omega t - 2\pi v_z)}\,dv \tag{2.17}$$

where :

$E_0(v)$is the maximum amplitude of the beam at z=0. (Shernan, 2014).

The amplitude of the beam is divided at the beam splitter and two beams are produced. Let $z_1$and z_2 be the distances travelled by the beams when they recombine. Each beam undergoes one reflection from the beam splitter and one transmission through the beam splitter. If r and t are the reflection and transmission coefficients, respectively, of the beam splitter, then the amplitude of the recombined wave E_R is

$$E_R[z_1, z_2, v]dv = rtE_0(v)[e^{i(\omega t - 2\pi v_{z1})} + e^{i(\omega t - 2\pi v_{z2})}]dv \tag{2.18}$$

By definition, the intensity after recombination of the beams for the fixed spectral range dv is given as

$$\begin{aligned} I[z_1, z_2, v]dv &= E_R(z_1, z_2, v)E_R^*(z_1, z_2, v)dv \\ &= 2E_0^2(v)|rt|^2[1 + cos2(z_2 - z_1)v]dv \end{aligned} \tag{2.19}$$

And the total intensity at any path difference $x = (z_2 - z_1)$for the whole spectral range is obtained by integrating equation (2.19) as

$$I_R(x) = 2|rt|^2 \int_0^\infty E_0^2(v)dv + 2|rt|^2 \int_0^\infty E_0^2(v)\cos(2\pi vx)\,dv \tag{2.20}$$

Fourier cosine transform of equation (2.20) converts intensity into spectrum

$$E_0^2(v) = (1/\pi|rt|^2) \int_0^\infty \left[I_R(x) - \frac{1}{2}I_R(0)\right]\cos(2\pi vx)\,dx \tag{2.21}$$

In the above equation, $I_R(0)$represents the flux associated with waves at zero arm displacement where the waves for all frequencies interact coherently. Thus, $I_R(0)$is the flux associated with coherent interference and $I_R(x)$ is the flux associated at path difference x.

The spectrum $S(v)$which is proportional to $E_0^2(v)$ can be given from equation

$$S(v) \propto E_0^2(v) \tag{2.22}$$

$$E_0^2(v) = constant \times \int_0^\infty \left[I_R(x) - \frac{1}{2} I_R(0)\right] \cos(2\pi v x)\, dx \tag{2.23}$$

The interferogram is Fourier transformed with the help of computer to convert the space domain into the wave number domain (Cooper, 2004).

The optical system in an FTIR spectrometer is very simple (Fig. 2.7): the interferometer requires two mirrors, an infrared light source, an infrared detector, and a beam splitter.

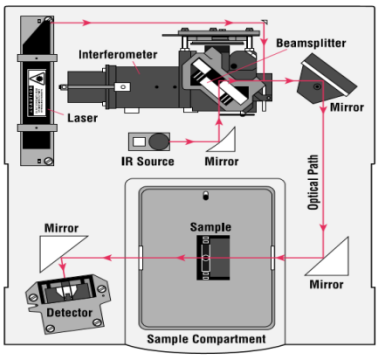

Figure 2.7: A Simple Spectrometer Layout (Thermo Nicolet, 2001).

An infrared source is polychromatic, emitting light over a broad range of frequencies. Each frequency still yields a unique cosine signal: the resulting interferogram represents the sum of all cosine waves generated by each individual infrared frequency. However, only at the point where both mirrors are equal distances from the beamsplitter does complete constructive interference occursimultaneous constructive interference of all wavelengths. At any other point, only some wavelengths interfere constructively while others do not. The Fourier transform resolves the frequency and intensity of each cosine wave in the interferogram. In other words, this algorithm converts the measured .intensityversus mirror-displacement signal (the interferogram) into a plot of intensity versus frequency (a spectrum) (Hsieh et al. 2008).

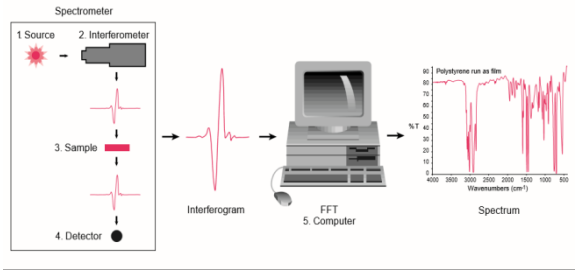

Figure 2.8: FT-IR spectrometer layout and basic components (chemwiki.ucdavis.edu).

2.3.4 Amide bands

There are nine infrared absorption bands for a polypeptide or a protein which are: 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 (1700-1600 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).

Other amide vibrational bands are very complex depending on the details of the force field, the nature of side chains, and hydrogen bonding. So it is 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 &Yu 2007, Smith 2011).

Designation	Approximate frequency	Descreption
Amide A	3300	NH streching
Amide B	3100	NH streching
Amide Ⅰ	1600-1690	C=O streching
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 (1700-1600 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 more than water (H_2O) since water absorbs strongly in the spectral region that overlap with amide I band and therefore it can affect the spectra, on the other hand D_2O has relatively low absorbance in the region between 1400-1800 cm^{-1}(BaiandNussinov, 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: Deconvoluted 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) studies the changes in electronic energy levels within the molecule arising due to transfer of electrons from π- or non-bonding orbitals. It commonly provides the knowledge about π-electron systems, conjugated unsaturation, aromatic compounds and conjugated non-bonding electron systems etc(Subodh et al.2006).

This absorption spectroscopy uses electromagnetic radiations between 190nm to 800 nm and is divided into the ultraviolet (UV 190-400 nm) and visible (VIS 400-800 nm) regions. Since the absorption of ultraviolet or visible radiation by a molecule leads transition among electronic energy levels of the molecule. It is also often called as electronic spectroscopy.

Energy absorbed in the UV regions produces changes in the electronic energy of the 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).

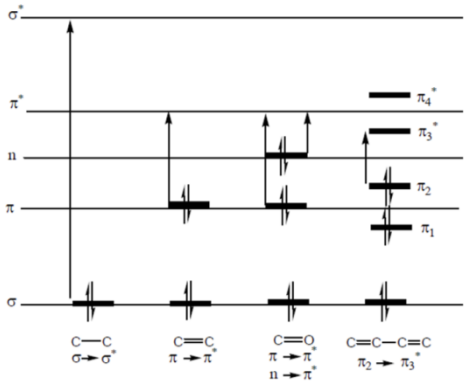

Figure 2.9: Relative energies of orbitals most commonly involved in electronic spectroscopy of organic molecules (Subodh Kumar, 2006)

For most molecules, the lowest energy occupied molecular orbitals are σ orbitals, which correspond to σ bonds. So the possible electronic transitions are:

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

The energy of radiation being absorbed during excitation of electrons from ground state to excited state primarily 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).

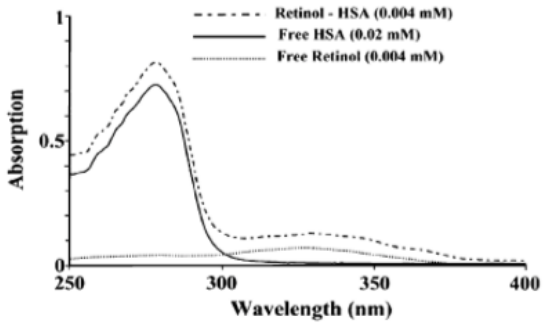

Figure 2.10: UV-absorption spectra of free HSA (0.02 mM), free retinol (0.004 mM) and their protein complexes (Subodh Kumar, 2006).

The wavelength of the radiation that will be absorbed by organic molecule is determined by 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 is shown in (Fig. 2.11), as a rule the transition occur 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 ($n \to \pi^*$and $\pi \to \pi^*$) can be achieved with radiation available in the range 200-650 nm which corresponds to UV-vis region (Yadav, 2005 ;Kalsi, 2004 ;Raaman, 2006) .

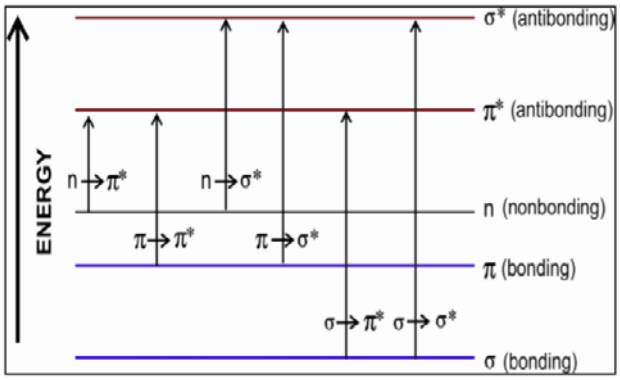

Figure 2.11: Generalized molecular orbital energy level diagram and possible transitions for organic compounds (Yadav, 2005;Raaman, 2006).

The $\pi \rightarrow \pi^*$ transitions are generally intense while $n \rightarrow \pi^*$ transitions are weak, so only molecules that have π bonds and atoms with non bonding electrons absorb light in the range 200-700nm and it is called chromophores. A list of some 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).

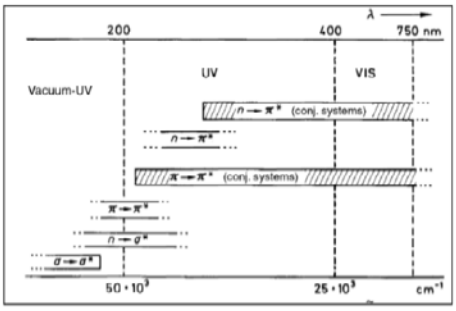

Figure 2.12: Absorption ranges for various electronic transitions (Yadav, 2005).

Table 2.4: Absorption characteristics of some common chromophoric groups (Kalsi, 2004).

chromophore	λ_{max}	Type of transition
>C=C<	177	$\pi \rightarrow \pi^*$
-C=C-	178	$\pi \rightarrow \pi^*$
>C=O	186	$n \rightarrow \sigma^*$
	280	$n \rightarrow \pi^*$
	180	$n \rightarrow \sigma^*$
	293	$n \rightarrow \pi^*$
-COOH	204	$n \rightarrow \pi^*$
$-CONH_2$	214	$n \rightarrow \pi^*$
-N=N-	339	$n \rightarrow \pi^*$
$-NO_2$	280	$n \rightarrow \pi^*$
-N=O	300	$n \rightarrow \pi^*$
	665	$n \rightarrow \pi^*$

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

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

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. From the value obtained we can determine if the binding between HSA and Vitamin is weak or strong by comparing it to the complex constants for strongly bound ligand-protein complexes vary within the range 10^6 to 10^8 M^{-1} (Hornaback, 2006).

A spectrometer is an instrument that measures the intensity of a light beam as a function of wavelength. 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 consist of a number of fundamental components: Light Sources (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 typically a photodiode or a charge-coupled device(CCD). Photodiodes are used with monochromators, which filter the light so that only light of a single wavelength reaches 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 (Nikita, 2015).

The absorption of UV light by proteins has been analyzed in detail and proposed as a structural probe from the very early days 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).

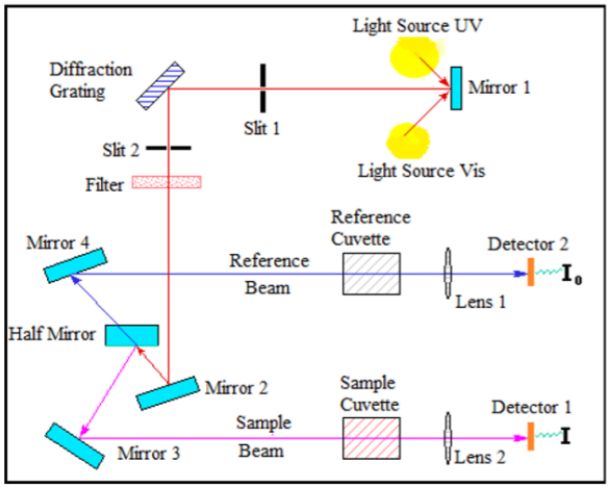

Figure 2.13: Schematic diagram of UV-VIS−NIR Spectrophotometer (Nikita , 2015)

The light source is a monochromator; the light is split into two equal intensity beams by a half mirrored device before it reaches the sample. One beam, the sample beam, passes through a small transparent container (cuvette) containing a solution of the compound being studied in a transparent solvent. The other beam, the reference, 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. Quartz or fused silica cuvettes are required for spectroscopy in the UV-Vis−NIR region. 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 an screen. The intensities of these light beams are then measured by electronic detectors and compared (Nikita, 2015).

2.5 Fluorescence

Luminescence is emission of light by a substance not resulting from heat; it is thus a form of cold body radiation. Luminescence is formally divided into two categories fluorescence and phosphorescence depending on the nature of the excited states (Kossi et al. 2007).

Fluorescence and phosphorescence are photon emission processes that occur during molecular relaxation from electronic excited states. Fluorescence is that process in which the emission of light by a substance that has absorbed light or other electromagnetic radiation (Johnson and Spence, 2010).

A fluorophore is a molecule that is capable of fluorescing. In its ground state, the fluorophore molecule is in a relatively low energy, stable configuration, and it does not fluoresce. Fluorophre is unstable at high energy configurations, so it eventually adopts the lowest-energy excited state, which is semi-stable (Kossi et al. 2007).

The process responsible for the fluorescence of fluorescent probes and other fluorophores is illustrated by the simple electronic-state diagram (Jablonski diagram) shown in Figure (2.14) below

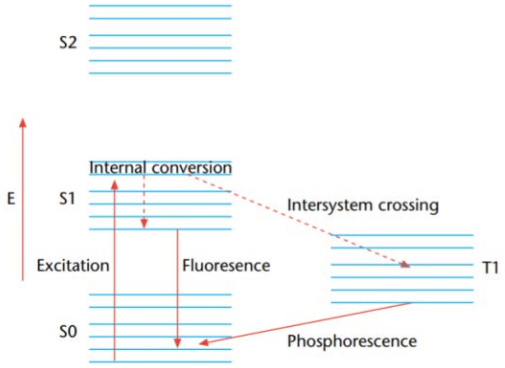

Figure 2.14: The Jablonski diagram of fluorophore excitation (Dong and So , 2002).

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).

Fluorescence is a spectrochemical method of analysis where the molecules of the analyte are excited by irradiation at a certain wavelength and emit radiation of a different wavelength. The emission spectrum provides information for both qualitative and quantitative analysis (Kossi et al. 2007).

When light of an appropriate wavelength is absorbed by a molecule, the electronic state of the molecule changes from the ground state to one of many vibrational levels in one of the excited electronic states. The excited electronic state is usually the first excited singlet state. Once the molecule is in this excited state, relaxation can occur via several processes. Fluorescence is one of these processes and results in the emission of light (Johnson and Spence, 2010).Corresponds to the relaxation of the molecule from the singlet excited state to the singlet ground state with emission of light. Fluorescence has short lifetime (~10^{-8} sec) so that 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 fluorescence lifetime and quantum yield are the most important characteristics of a fluorophore. Quantum yield is 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, the excited state exists for a finite time (typically 1^{-10} nanoseconds). During this time; the fluorophore undergoes conformational changes

and is also subject to a multitude of possible interactions with its molecular environment. These processes have two important consequences. First, the energy of S_2 is partially dissipated, yielding a relaxed singlet excited state S_1 from which fluorescence emission originates. Second, 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. Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower, and therefore of longer wavelength, than 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).

Fluorophores are divided into two main classes. First intrinsic fluorophores are those that occur naturally, such as aromatic amino acids. Secondly extrinsic 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 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.

Decrease of fluorescence intensity by interaction of the excited state of the fluorophores with its surroundings is known as quenching and is relatively rare. Quenching can occur in several mechanisms, collisional quenching occurs when exited state fluorophores are deactivated upon contact with some other molecule in solution, which is called 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 biomolecular quenching constant,$\tau_0$is the unquenched lifetime, and$[l]$is the quencher concentration. The quenching constant indicates the sensitivity of the fluorophores to the quencher. There are a wide variety of molecules that acts as a collisional quencher such as oxygen, halogen, amines, and electrondeficient molecules like acrylamide (Turro, 1991).

Quenching mechanism differ according to fluorophores quencher pair, for instance quenching of indole by acrylamide occur as a result of electron transfer from indole to acrylamide, while 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 occur 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

Upon production of excited states by promotion of an electron into a higher orbital the direction of spin of the electrons is preserved, since most of molecules have an even number of electrons they will be arranged in pairs. However, it is possible for the spin of the promoted electron to be reversed so that it is no longer paired and the molecule has two independent electrons of the same spin in different orbitals (Lakowicz, 2006).

Quantum theory predicts that such a molecule can exist in three forms that differs slightly, but normally indistinguishable in their energy, and the molecule is said to exist in a triplet state. The indirect process of conversion from the excited state which is produced by absorption energy from singlet state to a triplet state is known as intersystem crossing (Fig. 2.13) and can occur in many substances 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).

Direct transition from the ground state, usually singlet state, for a molecule with an even number of electrons, to an excited triplet state is theoretically forbidden, which means that the reverse transition from triplet to ground state will be difficult. Thus, 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, so phosphorescence can be defined as the transitions from the triplet state to the ground state (Sharma, 2007 ;Lakowicz, 2006).

In absorption spectroscopy, though the mechanism of absorption of energy is different in the ultraviolet, infrared and nuclear magnetic resonance regions, the fundamental process is the absorption of a discrete amount of energy.

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, if there exists within the molecule an energy transition of magnitude$E = hv$ Although almost all parts of electromagnetic spectrum are used for understanding the matter, in organic chemistry we are mainly concerned with energy absorption from only ultraviolet and visible, infrared, microwave and radiofrequency regions (Subodh et al. 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 (NanoDrop ND-1000), and Bruker IFS 66/S FT-IR. Section four presents the experimental procedure in details.

3.2 Samples and materials

HSA, Vitamin C, and Vitamin B_{12}were purchasedfrom 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 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- HSA solution.

3.2.2 Preparation of vitamin C stock solution

Vitamin C with molecular weight 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.

3.2.3 Preparation of vitamin B12 stock solution

Vitamin B_{12} with molecular weight (1355.4 $g.mol^{-1}$), 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 B_{12} was completely dissolved.

3.2.4 HSA-Vitamin C samples

The final concentrations of HSA-Vitamin C solutions were prepared by mixing equal volume from HSA to equal volume from different concentration of Vitamin C.

HSAconcentration 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 $mg.ml^{-1}$).

3.2.5 HSA-vitamin B_{12}samples

The final concentrations of HSA-Vitamin B_{12} solutions were prepared by mixing equal volume from HSA to equal volume from different concentration of Vitamin B_{12}. HSA 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 $mg.ml^{-1}$).

3.2.6 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 HSA-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 HSA-Vitamin B_{12} films preparation.

3.3 Instruments

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 spectrophtometer 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) µlsample 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 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:

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

Thus, the measured light intensity of both the sample and of the blank are required to calculate 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.

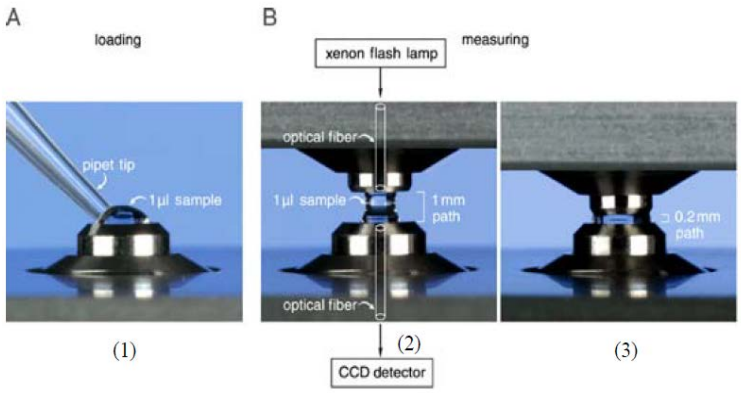

Figure 3.1 : main steps for using the sample UV-VIS spectrometer (NanoDrop 1000 Spectrophotometer V3.7, User's Manual, 2008).

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 Fluorospectrometerwas “balnked”.

A (10)µlsample 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 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.

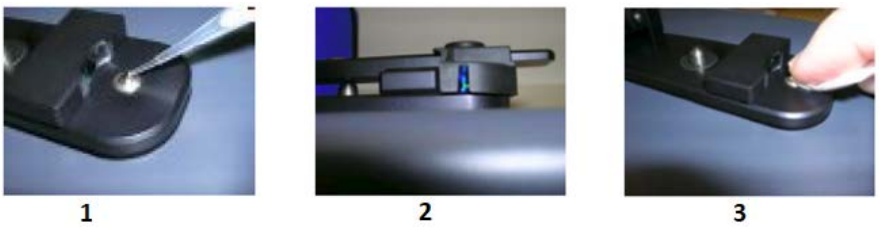

Figure 3.2: Main steps for using the sample fluorescence spectrometer (NanoDrop 3300 Fluorospectrometer).

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 byOptic User (OPUS)software. The peak positions were determined using the second derivative of the spectra.

The infrared spectra of HSA, vitamin-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 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).

3.4.4 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,

Secondderivative procedure have 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 (Jackson et al. 1981). Both second derivative and deconvolution procedures have been successfully applied in the qualitative study of a large number of protein (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 andNuclear 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 : Results 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 (Vitamin C and Vitamin B_{12}). 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 268 nm for Vitamin C and at 278 nm for Vitamin B_{12}. The absorption spectra of different concentrations of Vitamin C (Fig.4.1.a) and Vitamin B_{12} (Fig.4.1.b) with HSA showed an increase of the intensity as the Vitamin C concentration increases; this is due to major ligand protein interaction at protein surface which does not limit the mobility of ligand around HSA molecule while the intensity deceases with increasing Vitamin B_{12} concentration which originates from the existence of aromatic amino acids being the components of a protein molecule as well as its complicated molecular structure.

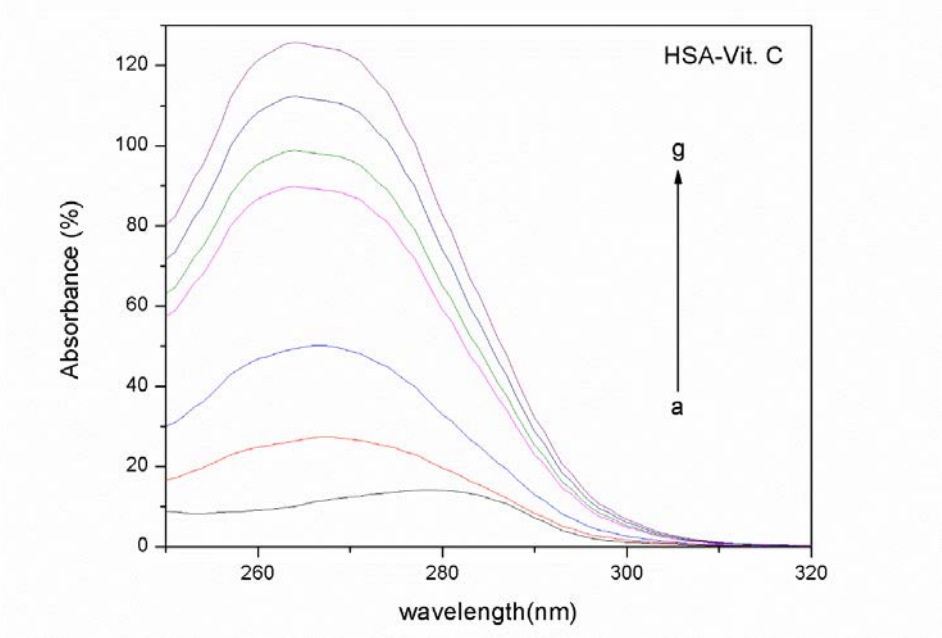

Figure 4.1.a : UV-absorbance spectra of HSA with different concentrations of Vitamin C
(a=freeHSA,b=1mg/ml,c=2mg/ml,d=5mg/ml,e=10mg/ml,f=20mg/ml,g=40mg/ml)

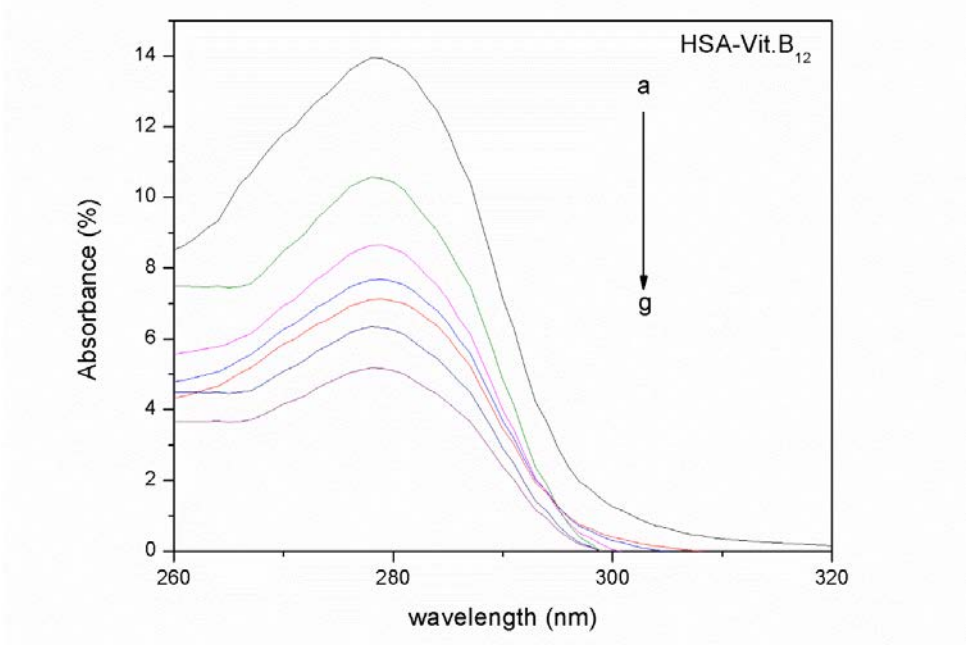

Figure 4.1.b :UV-absorbance spectra of HSA with different concentrations of Vitamin B_{12}(a=freeHSA,b=1mg/ml,c=2mg/ml,d=5mg/ml,e=10mg/ml,f=20mg/ml,g=40mg/ml)

This result support that the peak shifts between free HSA solution and Vitamin C-HSA complexes are due to the interaction between Vitamin C and HSA. Repeated measurements were done for all the samples showing consistent results and no significant differences were observed, and the same thing for Vitamin B_{12}.

4.1.1 Binding constant

Vitamin-HSA 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 vitaminand HSA in aqueous solution, which leads to establish equation as follows:

$$K = [\text{ vitamin:HSA }] / [\text{ vitamin }][\text{ HSA }] \quad (4.1)$$

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

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

where A_0corresponds to the initial absorption of protein at 280 nm in the absence of ligand , $A_\infty$is the final absorption of the ligated protein, and A is the recorded absorption at different concentrations (L).

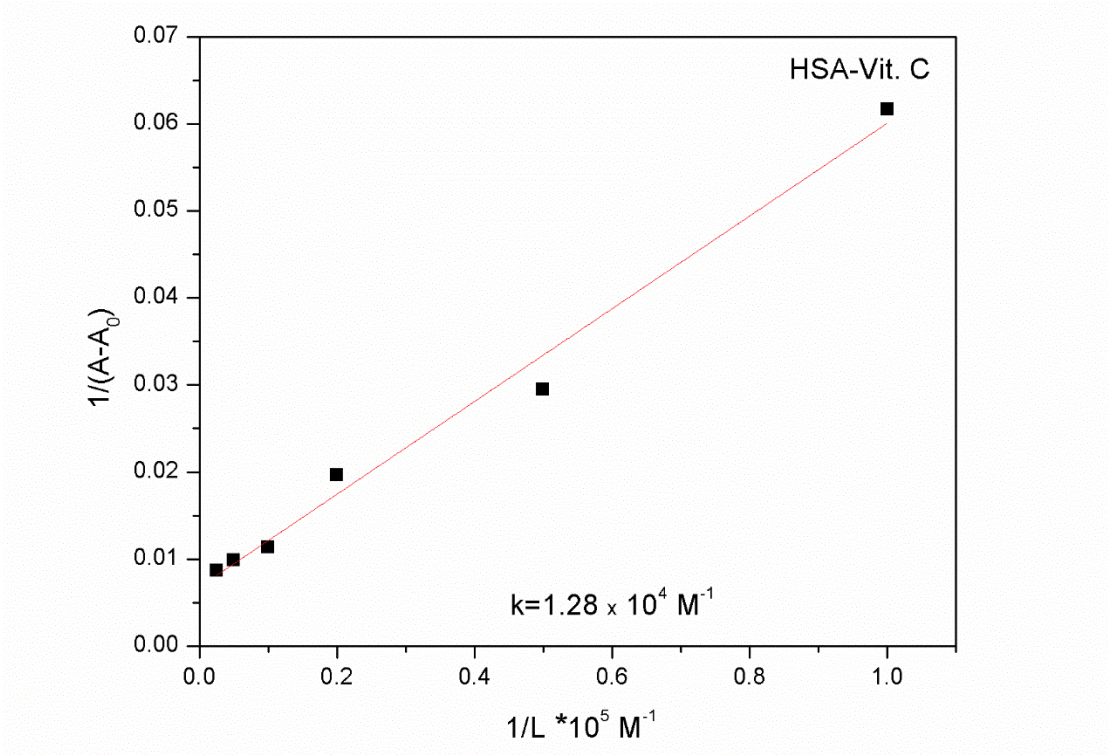

Figure 4.2.a :The plot of $1/(A-A_0)$ vs. 1/L for HSA with different concentrations of Vitamin C.

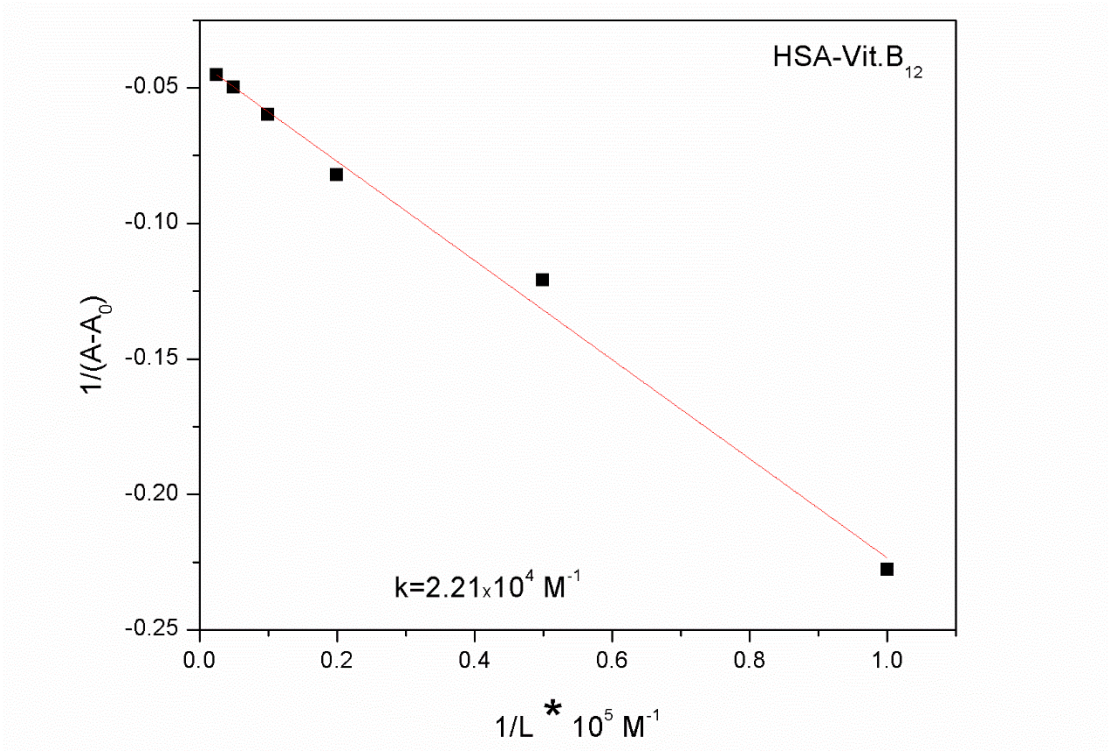

Figure 4.2.b :The plot of $1/(A-A_0)$ vs. 1/L for HSA with different concentrations of Vitamin B_{12}.

Fig.4.2.a and Fig.4.2.b represents the double reciprocal plots of $1/(A- A_0)$ vs. 1/L for HSA-Vitamin C and HSA-Vitamin B_{12} 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 Vitamin C ($K= 1.28x10^4\ M^{-1}$) and Vitamin B_{12} ($K= 2.21x10^4\ M^{-1}$) 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 or an indirect vitamin-HSA interaction through water molecules (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 460 nm (Fig.4.3.a) and the intensity decreases as the Vitamin C concentration increased while the peak maximum appears to be at 440 nm (Fig.4.3.b) and the fluorescence intensity decreased regularly with increasing of Vitamin B_{12} concentration. The peak positions shows little or no change at all.

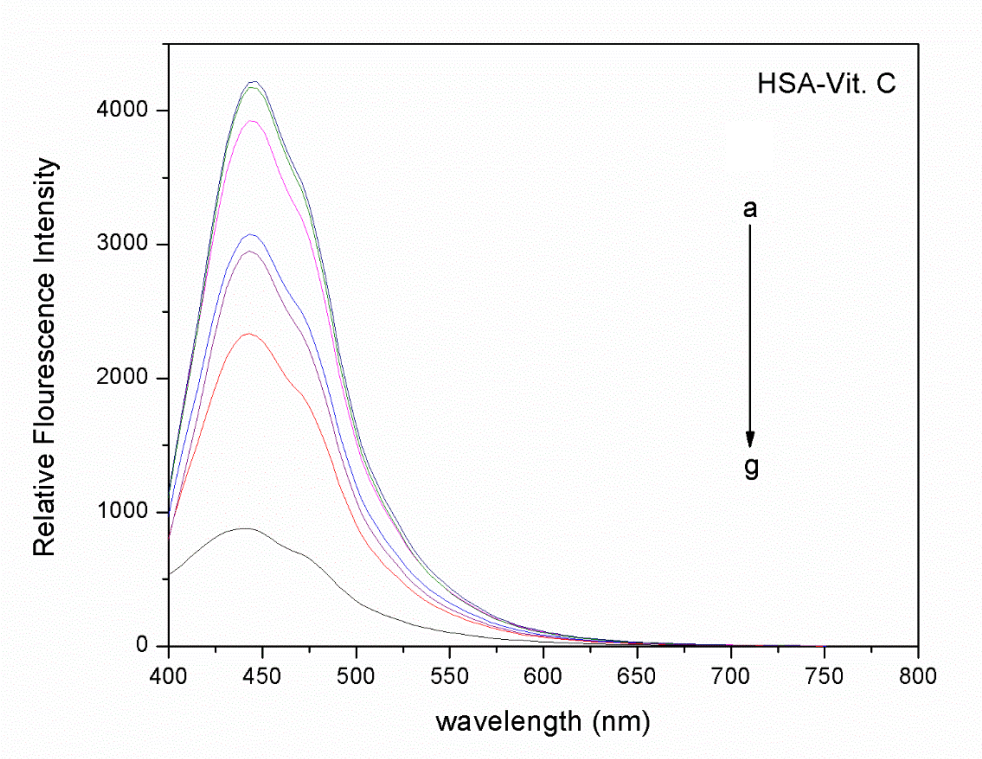

Figure 4.3.a:Fluorescence emission spectra of HSA in the absence and presence of Vitamin C in these concentrations (a=free HSA,b=1mg/ml, c=2mg/ml, d=5mg/ml, e=10mg/ml,f=20mg/ml,g=40mg/ml)

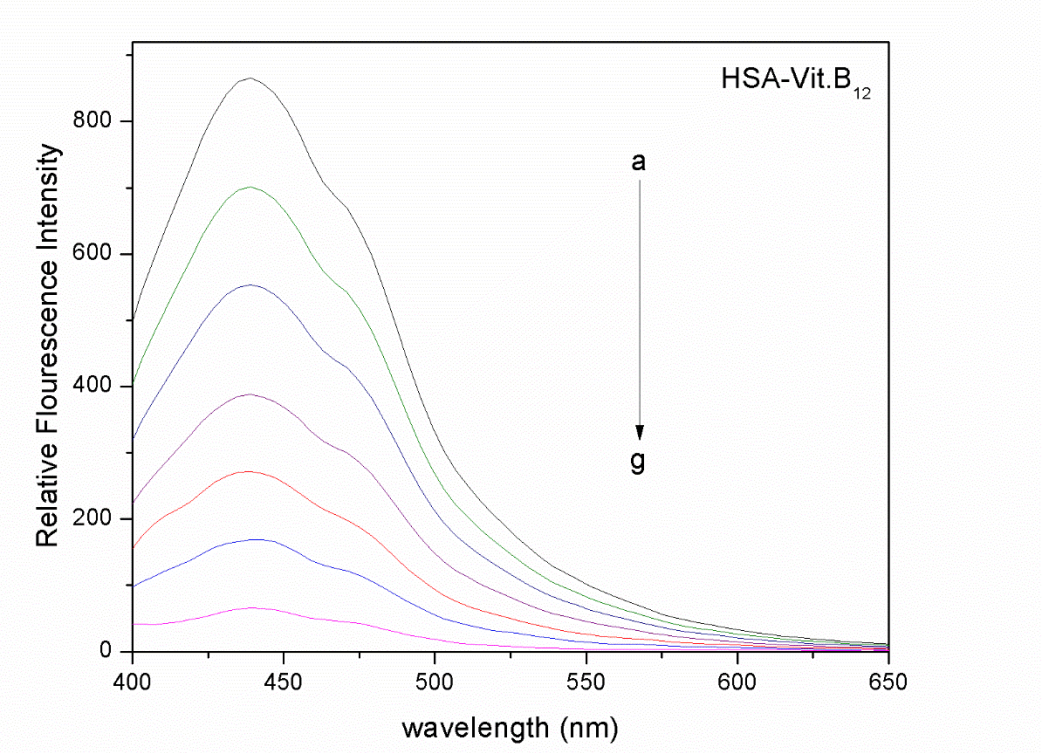

Figure 4.3.b: Fluorescence emission spectra of HSA in the absence and presence of Vitamin B12 in these concentrations (a=free HSA,b=1mg/ml,c=2mg/ml,d=5mg/ml,e=10mg/ml,f=20mg/ml,g=40mg/ml).

4.2.1 Stern-Volmer quenching constants (k_{sv}) and the quenching rate constant of the 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 et al. 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 intensities in the absence and presence of quencher (Vitamins C and B_{12}); 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}(Byler et al. 1986); $[L]$ is the concentration of quencher, k_{sv} is Stern-Volmer fluorescence quenching constant.

Linear curves were plotted according to the Stern-Volmer equation as shown in figure 4.4.a and in figure 4.4.b.

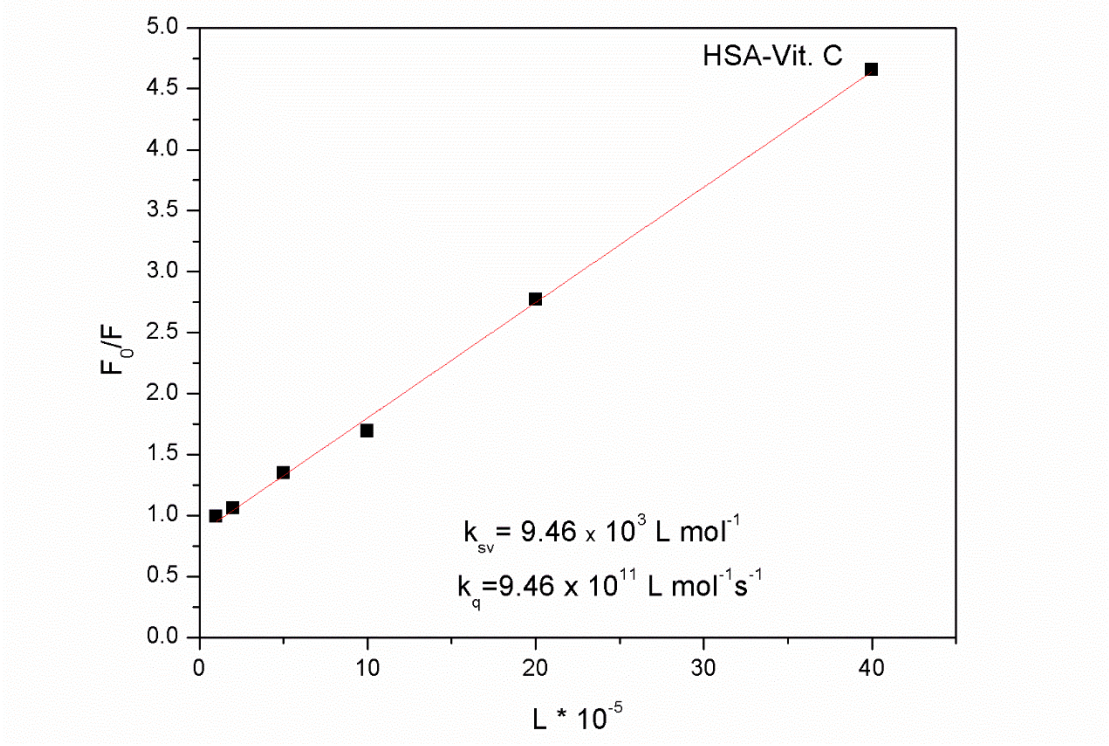

Figure 4.4.a : The Stern-Volmer plot for Vitamin C -HSA complex.

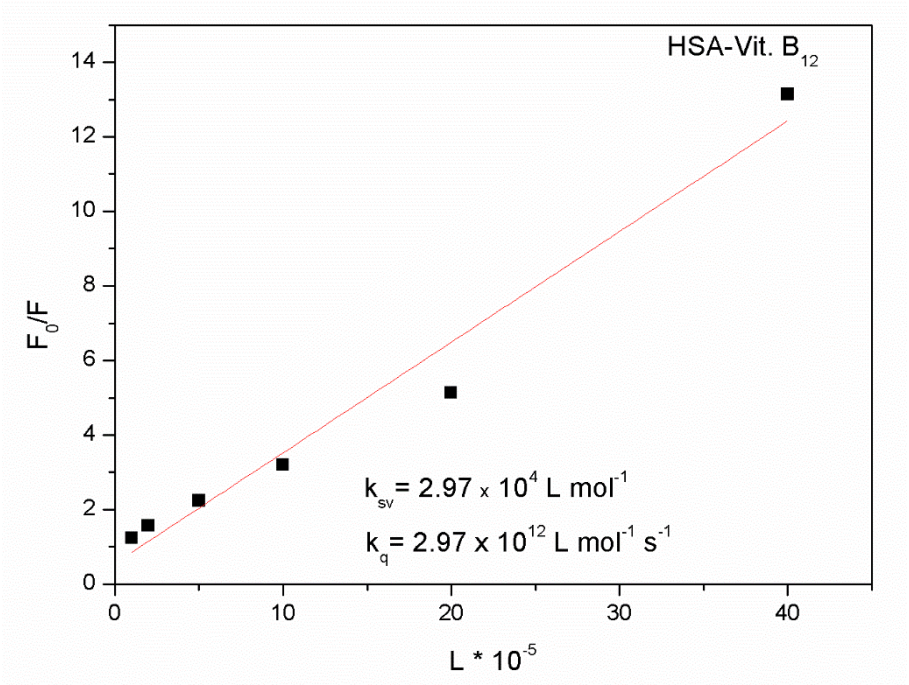

Figure 4.4.b :The Stern-Volmer plot for Vitamin B12 - HSA complex.

Fig.4.4.a and Fig.4.4.b represents the Stern-Volmer plot of HSA fluorescence intensities of Vitamin C and Vitamin B_{12} respectively. The curves are linear, suggesting the existence of a single type of quenching (dynamic or static) and/or a single binding site for both vitamins in the HSA neighborhood , The Stern-Volmer fluorescence quenching constant determined applying Eq. (4.2) are equal to (9.46×10^3, 2.97×10^4) L mol^{-1} for Vitamins C and B_{12} respectively , The Stern-Volmer quenching constant k_{sv} was obtained by the slope of the curves obtained in figure 4.4.a and 4.4.b , both values are much lower than other k_{sv} values for the similar systems signalized earlier in literature (Bialokoz 2017 ;Bhattacharya et al. 2000 ;Bai et al. 2008). The calculated k_{sv}values are much too large to be caused by collisional quenching in water (two-three orders of magnitude larger), as well as the values of k_q, which are equal to (9.46×10^{11}, 2.97×10^{12}) L mol^{-1} s^{-1} for vitamins C and B_{12}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 (Cheng et al). These values confirms clearly the existence of static (diffusion- independent) mechanism of fluorescence quenching (Bendich et al. 1986;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, et al., 1994)

$$\frac{1}{F_0 - F} = \frac{1}{F_0 K L} + \frac{1}{F_0} \qquad (4.3)$$

Where K is the binding constant of Vitamin C with HSA, and can be calculated by plotting $\frac{1}{F_0 - F}$ vs $\frac{1}{L}$, see figure 4.5.a .

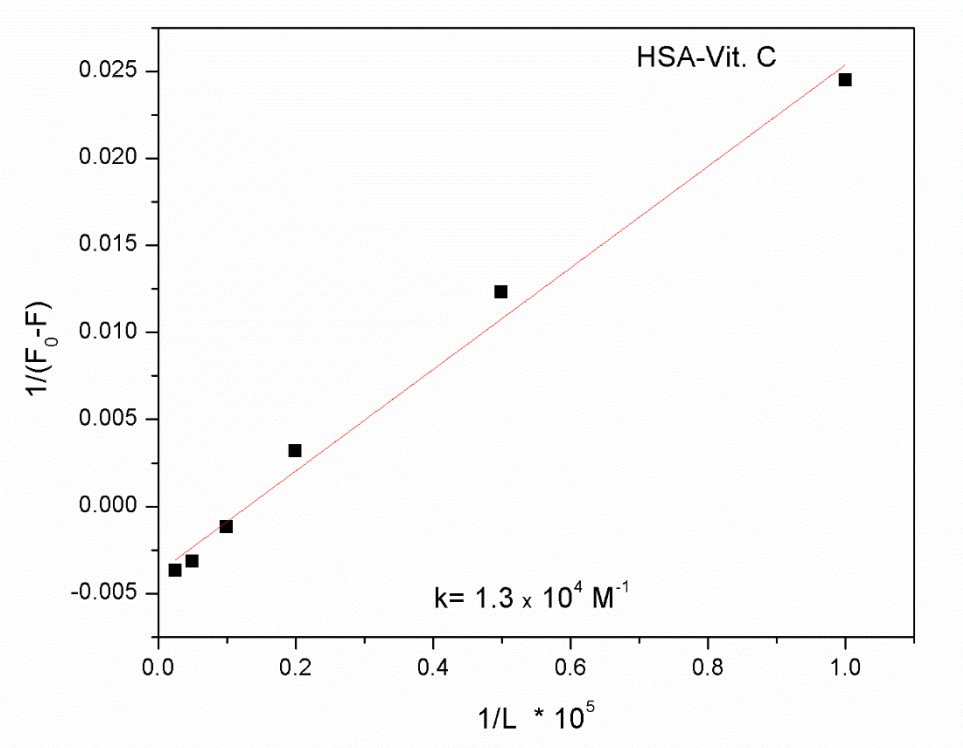

Figure 4.5.a: The plot of $1/(F_0\text{-}F)$ vs $1/[L*10^5]$ for Vitamin C-HSA complex.

Where K is the binding constant of Vitamin C with HSA, and can be calculated by plotting $\frac{1}{F_0 - F}$ vs $\frac{1}{L}$, see figure 4.5.b .

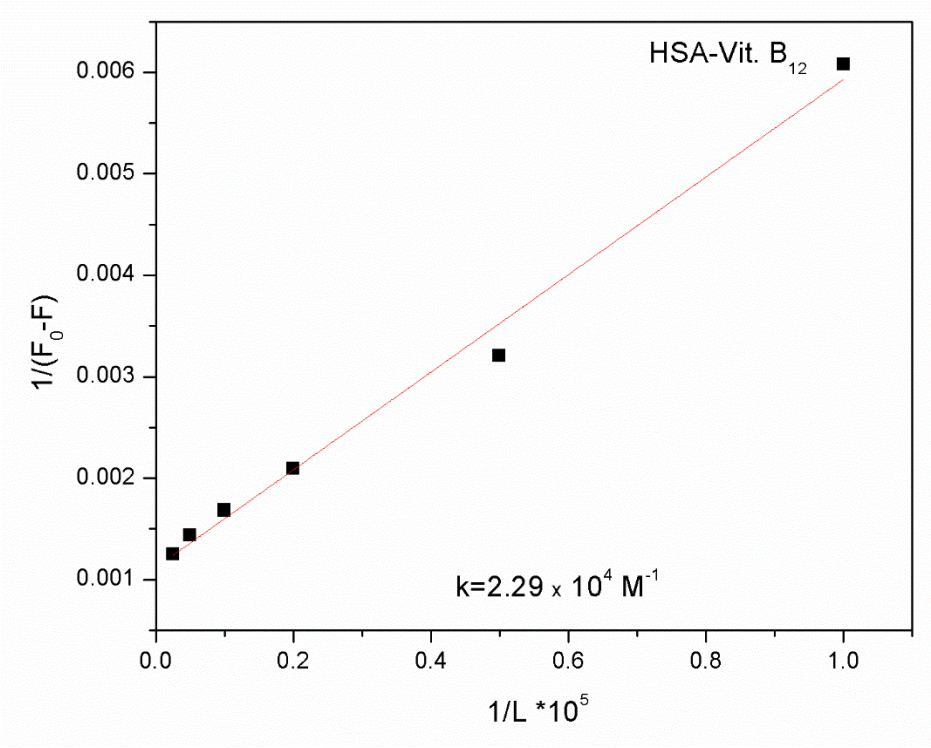

Figure 4.5.b :The plot of $1/(F_0\text{-}F)$ vs $1/[L*10^5]$ for Vitamin B12-HSA complex.

To determine the binding constants of HSA- vitamins system, a plot of $\frac{1}{F_0-F}$ vs $\frac{1}{L}$ for different vitamins concentrations are made and shown in Fig.4.5.a and Fig.4.5.b for Vitamins C and B_{12} respectively. The plots are linear and have a slope of $\frac{1}{F_0 K}$ and intercept $\frac{1}{F_0}$ according to eq. (4.3). The values of K which equal the ratio of the intercept to the slope were found to be (1.3×10^4 M^{-1}, 2.29×10^4 M^{-1}), which agrees well with the values obtained earlier by UV spectroscopy and supports 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 (Sirotkin et al., 2001; 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 (Ganim et al., 2006).

The modes most widely used in protein structural studies are amide I, amide II and amide III. Amide I band ranging from 1700 to 1600 cm^{-1} and arises principally from the C=O stretching (Vandenbussche G et al., 1992), has been widely accepted to be used (Workman et al., 1998). The amide II band is primarily N-H bending with a contribution from C-N stretching vibrations; amide II ranging from 1600 to 1480 cm^{-1} while amide III band ranging from 1330 to 1220 cm^{-1} which is due to the C-N stretching mode coupled to the in-plane N-H bending mode (Arrondo et al., 1993; Jackson et al., 1991).

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 1656 and 1544 cm^{-1}. Figures (4.7.a & 4.7.b) respectively shows the spectrum of HSA-Vitamin C and HSA-Vitamin B_{12} complexes with different concentrations.

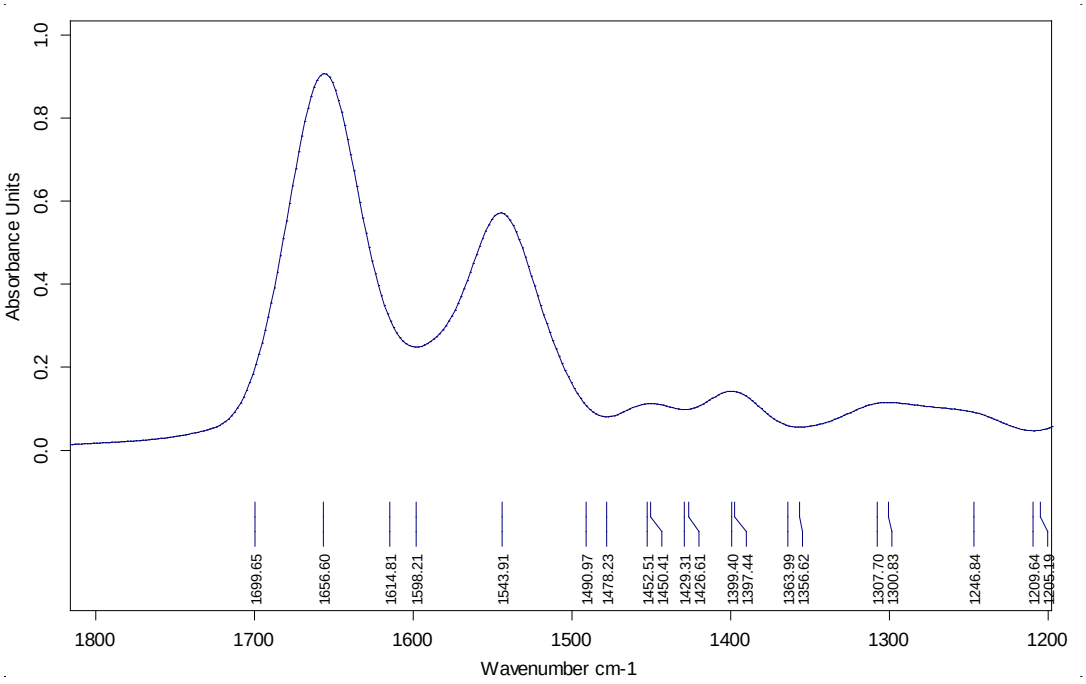

Figure 4.6 : Second derivative of free HSA.

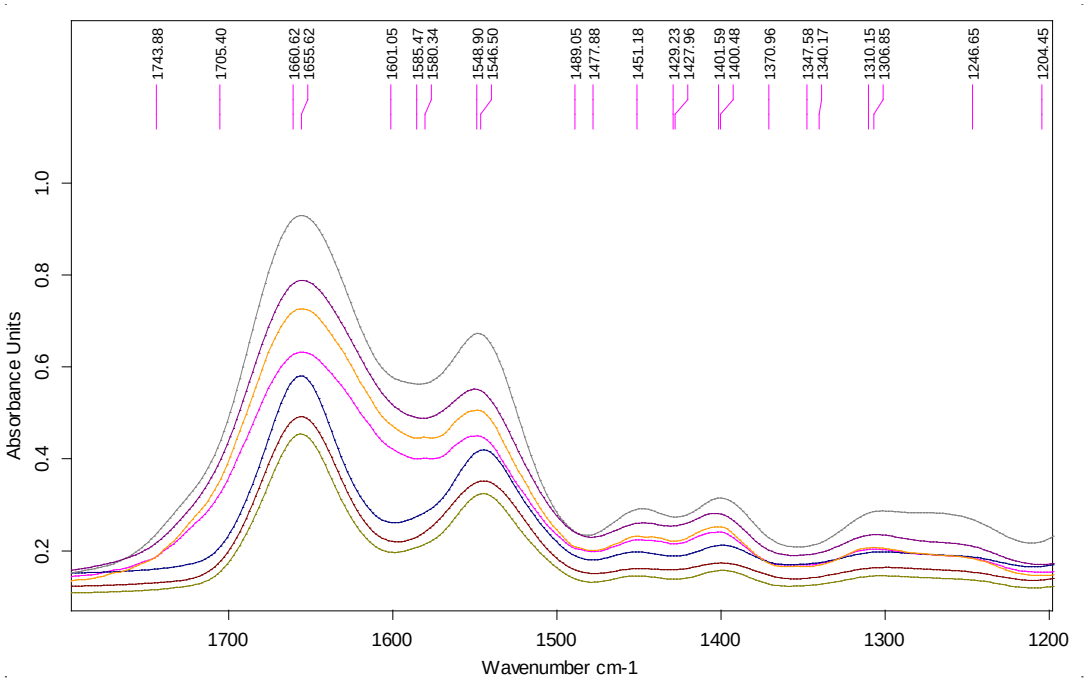

Figure 4.7.a : Different spectra of HSA and its complexes with different Vitamin C concentrations in the region 1800-1200 cm^{-1}.

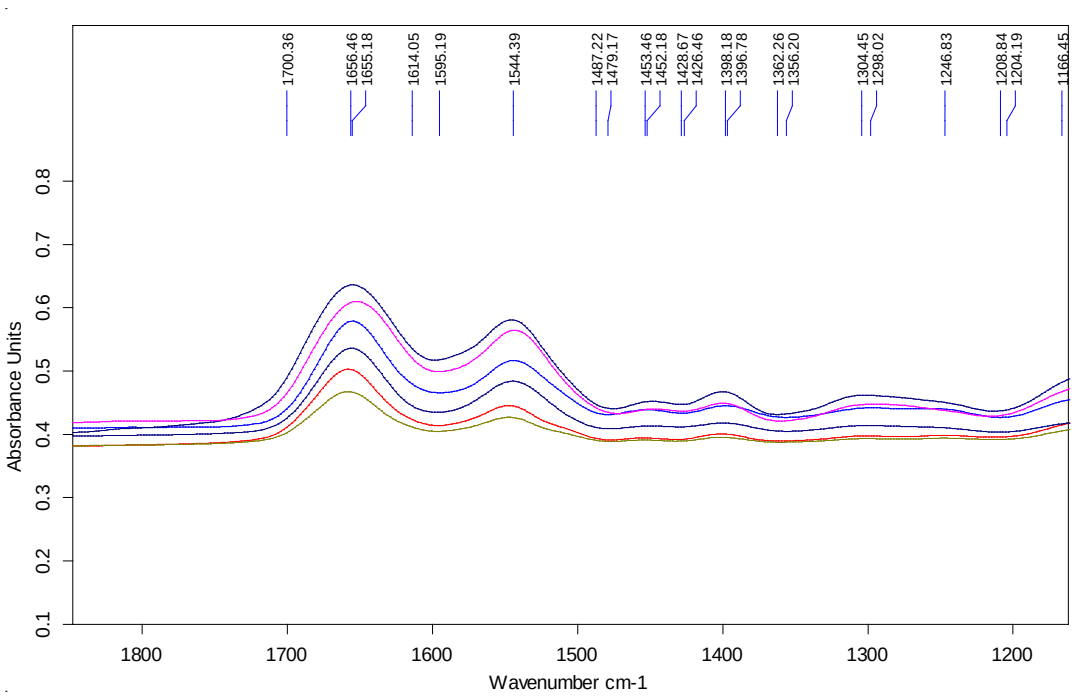

Figure 4.7.b : Different spectra of HSA and its complexes with different Vitamin B_{12} concentrations in the region 1800-1200 cm^{-1}.

The peak positions of HSA with different concentrations of Vitamins C and B_{12} are listed in tables 4.1 & 4.2 respectively. It is clearly that the amide bands of HSA infrared spectrum are shifted in two different manner.

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

Band regions (cm^{-1})	HSA Free	HSA-Vitamin C 1 $mg.ml^{-1}$	HSA-Vitamin C 2 $mg.ml^{-1}$	HSA-Vitamin C 5 $mg.ml^{-1}$	HSA-Vitamin C 10 $mg.ml^{-1}$	HSA-Vitamin C 20 $mg.ml^{-1}$	HSA-Vitamin C 40 $mg.ml^{-1}$
Amid I (1600-1700)	1658	1658	1658	1655	1654	1651	1650
	1620	1615	1598	1614	1597	1620	1607
	1598	1598	1597	1596	1595	1593	1591
Amid II (1480-1600)	1547	1546	1546	1546	1545	1544	1544
	1489	1489	1487	1485	1484	1484	1482
	1479	1478	1477	1477	1476	1473	1473
	1455	1452	1452	1451	1451	1451	1449
	1429	1427	1427	1426	1426	1426	1426
	1400	1399	1399	1398	1398	1398	1397
	1368	1368	1367	1366	1366	1366	1362
	1363	1361	1361	1358	1358	1356	1355
Amid III (1220-1330)	1310	1310	1309	1308	1308	1306	1304
	1248	1247	1247	1246	1246	1245	1244
	1216	1214	1214	1210	1209	1206	1203

Table 4.1: Band assignment in the absorbance spectra of HSA with different Vitamin B_{12} concentrations for Amid I-III regions

Band regions (cm^{-1})	HSA Free	HSA-Vitamin B_{12} 1 $mg.ml^{-1}$	HSA-Vitamin B_{12} 2 $mg.ml^{-1}$	HSA-Vitamin B_{12} 5 $mg.ml^{-1}$	HSA-Vitamin B_{12} 10 $mg.ml^{-1}$	HSA-Vitamin B_{12} 20 $mg.ml^{-1}$	HSA-Vitamin B_{12} 40 $mg.ml^{-1}$
	1704	1705	1705	1706	1710	1710	1712
Amid I (1600-1700)	1656	1656	1656	1658	1658	1659	1661
	1601	1606	1608	1615	1616	1624	1617
Amid II (1480-1600)	1569	1569	1571	1573	1576	1578	1580
	1543	1544	1544	1544	1545	1545	1546
	1478	1478	1481	1481	1484	1485	1490
	1451	1451	1451	1451	1451	1453	1457
	1398	1400	1399	1400	1401	1400	1415
Amid III (1220-1330)	1302	1307	1310	1309	1310	1311	1312
	1246	1247	1248	1247	1247	1250	1256
	1204	1209	1210	1212	1214	1216	1220

An intensity increase in the difference spectra of the Vitamin C-HSA complexes was observed for the protein amide I band at 1656 cm^{-1} and amide II at 1544 cm^{-1}. Positive features are located in the difference spectra for amide I and II bands at 1660 cm^{-1} and 1548 cm^{-1} (Vitamin C). These positive features are related to increase in intensity of the amide I and II bands upon drug-complexation due to drug binding to protein C=O, C-N and N-H groups. Additional evidence comes from the decrement of the peak positions as the concentration of Vitamin C increases as shown in table 4.1

In the difference spectra of Vitamin B_{12}-HSA complexes, the intensity decreases as the concentration of Vitamin B_{12} increases for amide I band at 1656 cm^{-1}, and amid II band at 1544 cm^{-1}.

Table 4.2. Provides an increment of the peak positions as the concentration of vitamin B_{12} increases; this results in stabilization by hydrogen bonding by having the C-N bond assuming partial double character due to a flow of electrons from the C=O to the C-N.

In summary, the binding of Vitamin C and Vitamin B_{12} to HSA has been investigated by UV-absorption spectroscopy, fluorescence spectroscopy and by FT-IR spectroscopy. The binding constant values indicate a relatively weak binding of both vitamins with HSA and the quenching constant indicate that the intrinsic fluorescence of HSA was quenched by both vitamins through static quenching mechanism. Analysis of FT-IR spectra reveals that HSA-Vitamin C interaction induces intensity reduction and HSA-Vitamin B_{12} interaction induces intensity reduction.

Chapter five : Conclusion

The interaction between hydrophilic vitamins and human serum albumin(HSA), it is great interest and important from pharmaceutical point of view. In my work the interaction of vitamin C and vitamin $B_{12}$with HSA was investigated by means of UV-VIS spectrophotometer, and FT-IR spectroscopy. Binding constant forvitamin C andvitamin $B_{12}$with HSA were determined by using UV-absorption spectroscopy to be K= (1. 28×10^4 M^{-1}), and (2.21×10^4 M^{-1})respectively, The binding constant values indicate a relatively weak binding of both vitamins with HSA and the quenching constant indicate that the intrinsic fluorescence of HSA was quenched by both vitamins through static quenching mechanism.

Analysis of FT-IR spectrum indicated that that HSA-vitamin C interaction induces intensity reduction and HSA-vitamin B_{12} interaction induces intensity reduction.

The binding study of vitamin C or vitamin B12 with HSA is of great importance in pharmacy, pharmacology and biochemistry. This research can supply some important information to medical research and provide the theoretical basis for studying the interactions of vitamins.

Therefore, this research need further studies to be a useful guide for synthesis of efficient drugs that interact with HSAsuch 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 drugsand 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 ascircular 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 vitamin , and also make thermodynamic investigations

andelectrochemical investigation for the interaction of vitamin with HSA ,and study the interaction with more protein transporters .

We can investigate such interaction between HSA and other important vitamins and drugs , and investigate the interaction with other protein transporters in the human , we can study such interaction by making computer’s simulation which help us to understanding the interaction more ,

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دراسة تفاعل الفيتامينات المحبة للماء (فيتامين ج و فيتامين ب 12) مع مصل البيومين البشري باستخدام التقنيات المطيافية

اعداد : محمد ايمن محمد ابو علان

اشراف : د.حسين السمامرة

الملخص:

فيتامين ج عامل منظم مهم لامتصاص الحديد و فيتامين ب 12 ضروري ليؤدي حمض الفوليك وظيفته ، البيومين المصل البشري هو البروتين الذائب الرئيسي وعنصر اساسي في الجهاز الدوراني وله الكثير من الوظائف الفسيلوجية تشمل نقل الكثير من المركبات الكيميائية ، في هذا العمل سيتم فحص التفاعل بين فيتامين ج و فياتمين ب 12 مع البيومين المصل البشري وذلك باستخدام تقنيات التحليل الطيفي الفلوري وايضا التحليل الطيفي للاشعة الفوق البنفسجية ، ومن خلال التحليل الطيفي كلا الفيتامينين اظهر قدرة عالية على اخماد الطيفي الفلوري لالبيومين المصل البشري من خلال اجراء الاخماد الاستاتيكي ، ثابت الربط المتوقع لكل من فيتامين ج و البيومين المصل البشري كان ث=($1.28\times10^{4}\ M^{-1}$) واما ثابت الربط لفيتامين ب 12 والبيومين المصل البشري فكان ث=($2.21\times10^{4}\ M^{-1}$) ، وقد تم حساب ثابت شتيرن فولمر عند درجة حرارة الغرفة لكل من فيتامين ج وفيتامين ب 12 ، اما التحليل الطيفي باستخدام تقنية تحويل فورييه للاشعة تحت الحمراء فتم استعمالها في تحديد بنية البروتين الثانوية ، التغيرات في الطيف الملاحظة اشارت لزيادة في التفاعل بين كل من فيتامين ج والبيومين المصل البشري مع زيادة التركيز في حين اظهرت اختزال في التفاعل بين كل من فيتامين ب 12 والبيومين المصل البشري ، فالشدة تتناقص عندما يزداد تركيز فيتامين ب 12 في منطقة 1 ، وهذا الاختلاف في الشدة يتعلق بطريقة غير مباشرة لتكون روابط هيدروجينية في الجزيء المعقد .