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RESEARCH ARTICLE

STUDY THE INTERACTION OF HYDROPHOBIC VITAMINS (VITAMIN E AND VITAMIN D) WITH HSA USING SPECTROSCOPIC TECHNIQUES

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ABSTRACT

The interaction of hydrophobic vitamins (vitamin E and vitamin D) with human serum albumin(HSA) at physiological (pH 6.9- 7.4) has been studied using UV-VIS spectrometer, and an FT-IR spectroscopy. The interaction of hydrophobic vitamins (vitamin E and vitamin D) with HSA has been investigated by using UV-absorption, and Fourier transforms infrared (FT-IR) spectroscopy. The binding constants of vitamin E and vitamin D have been determined by UV-absorption. The values of the binding constants are calculated at room temperature: ($1.21\times10^{2}M^{-1}$) and ($6.8\times10^{1}M^{-1}$) for vitamin E- HSA and vitamin D- HSA mixtures, respectively. FT-IR spectroscopy with Fourier self-deconvolution technique and second derivative resolution enhancement procedures were applied in the analysis of the amide I, amid II, and amid III regions to determine the protein secondary structure and hydrophobic vitamins binding mechanisms. All peaks positions in the three amide regions (amid I, amide II and amide III) have been assigned and any changes due to concentration changes have been investigated. The FTIR spectra measurements indicate a change in the intensity of absorption bands due to change in the concentrations in drugs. In addition a larger intensity decrease in the absorption band of α-helix relative to that of β-sheets has been observed. This variation in intensity is related indirectly to the formation of H-bonding in the complex molecules, which accounts for the different intrinsic propensities of α-helix and β-sheets.

INTRODUCTION

Serum albumins are the most abundant proteins in plasma. One of the most biological functions of albumins is their ability to carry drugs as well as endogenous and exogenous substances. Serum albumins, the major soluble protein constituents in the blood stream, bind and transport several exogenous and endogenous molecules like fatty acids, nutrients, steroids, and a large list of drugs. The formed complexes are involved in transport and regulatory processes. Since most of the administered drugs bind extensively and reversibly to serum albumins and drugs are transported mainly as complexes with proteins, the biological activities of the drug such as the overall distribution, metabolism, and efficacy in the body are correlated with their affinities towards serum albumins. Therefore, protein-drug interaction has an enormous biological interest and has been found to play a significant role in pharmacology [1]. Among the serum albumins, human serum albumin (HSA) is an appropriate protein model for studying the interaction between serum albumins and drugs because of its medically important, unusual ligand-binding properties, low cost, availability, and structural homology. Vitamin D is currently undergoing investigation in connection with the structure and function of the brain, since vitamin D or certain of its analogues are of interest in the prevention of various aspects of neurodegenerative or neuroimmune diseases [2]. Vitamin D protects the neurones of the hippocampus [3], and modulates the transport of glucose to the brain [4]. Its role in models of multiple sclerosis is under study as a result of recent positive findings, in particular concerning the duration and intensity of crises [5]. Vitamin D is a precursor of a renal steroid hormone, 1, 25-dihydroxyvitamin D [6]. This precursor, however, cannot be provided by enzymatic synthesis. Its supply depends on ultraviolet (UV) irradiation of the skin or absorption from the diet. Since few natural foodstuffs contain much vitamin D, observers consider the endogenous, cutaneous production of cholecalciferol (D3) to be the physiological mechanism of precursor supply [7]. Figure 1 show structure of cholecalciferol (D3). The major function of vitamin D is absorption of calcium and phosphorus, calcification of bones. In rickets, there is reduced calcification of the growing ends (epiphyses) of bones. Vitamin D and its metabolites are transported in the circulation by vitamin D-binding protein and the complex enters the cell together with megalin and cubilin, recently characterized carrier proteins. Vitamin D exerts its actions in a variety of cell types by binding to the nuclear vitamin D receptor (VDR), which shares its structure with many other nuclear steroid hormone , such as the glucocorticoid, thyroid hormone and estrogen receptors [8]. Vitamin E protect especially against ageing, in particular the brain, notably in association with

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selenium. However, alpha-tocopherol also plays a role at the level of cognitive functions [9]. In fact, what is known as vitamin E is in reality a mixture of numerous substances: tocotrienols and tocopherols (alpha, bêta, gamma, delta); moreover, alpha tocopherol has 3 asymmetric carbons, hence 3 different molecules. Each of these molecules is classically ascribed a coefficient of vitamin activity determined by various methods, either in vivo or in vitro. In terms of nutrition, only alpha-d-tocopherol - and not gamma-tocopherol - is bioavailable and integrated in biological membranes, including those in the brain [10]. The eventual specific roles of gamma-tocopherol are currently investigated. Nutritional vitamin E deficiency alters brain fatty acid profile [11]. Experimental vitamin E deficiency induces retinal abnormalities [12]. In animal, early vitamin E supplementation in young but not aged mice reduces Abeta levels and amiloid deposition in a transgenic model of Alzheimer disease [13].

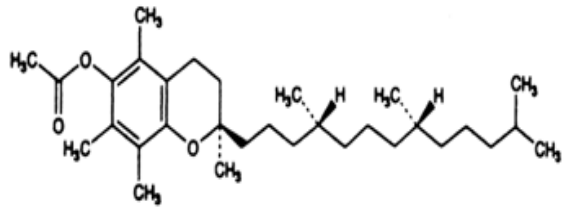

Figure 1. Chemical Structure of vitamin E.

High doses of vitamin E are proposed in the treatment of disorders of the central nervous system in the aged [14,15] and using antioxidant vitamin supplements (including vitamin E) reduces risk of Alzheimer disease [15]. The risk of dementia is significantly increased for the lowest vitamin E concentration compared to the highest one (OR=2.54) [16]. Vitamin E may slow functional deterioration leading to nursing home placement [17]. In general, at the molecular level, the tocopherols are extremely important. They have numerous roles, in particular they neutralise the active and toxic forms of oxygen and scavenge free radicals. That is to say, they protect unsaturated fatty acids against peroxidation, and thus contribute to maintaining the integrity and stability of cellular structures in the brain. They act in lipid phase at a very low concentration (about one molecule per two thousand fatty acid molecules) and take part in a vast complex and interactive protective system, in cooperation with beta-carotene, vitamin A, vitamin C, and various enzymes that function with selenium, copper, zinc, and manganese. A distinct clinical syndrome (with paralysis) of combined vitamin E and vitamin C deficiency have been described in guinea pigs [18]. Interestingly, alternative nonantioxidant functions of vitamin E have been proposed and in particular of a "gene regulator" [19].

Tocopherols are found in very large amounts in certain vegetable oils, and also in eggs, a little in meat and tripes, without forgetting vegetables such as cereal germs and green vegetables (salads, cabbage, and spinach). Vitamin E was discovered by Evans and Bishop as a fat soluble factor necessary for normal reproduction in rats [20]. Vitamin E is a powerful antioxidant which plays an essential role in protecting cell membranes and plasma lipoproteins from free radical damage [21]. Its deficiency has been associated with various chronic disorders such as atherosclerosis, ischemic heart disease, and immune deficiency, different types of cancer, and neurological syndromes that possess a strong oxidative stress component and can be successfully treated with dietary vitamin E supplementation [22]. There are eight naturally occurring homologues of vitamin E in edible plant oils, vegetables and fruits, with α- and γ-tocopherol being the most common in the Western diet. Figure 2 shows the chemical structure of vitamin E. Due to its hydrophobicity and primary location in the plasma membrane, vitamin E requires special carrier/transport mechanisms in the aqueous environment of plasma; no specific protein has been described so far for vitamin E. 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 [23].

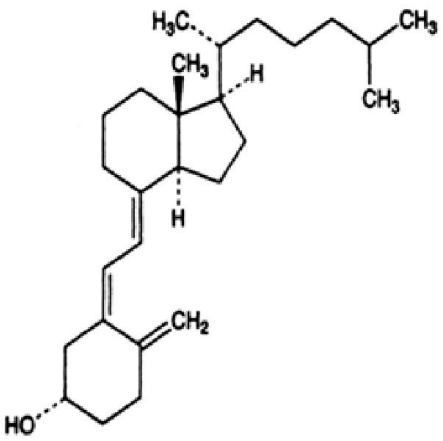

Figure 2. Chemical Structure of cholecalciferol (D3)

The primary pharmacokinetics function of HSA is participating in absorption, distribution, metabolism and excretion of drugs. Drugs distribution is mainly controlled by HSA, because most drugs travel in plasma and reach the target tissues by binding to 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 [24]. Recently, Hisaeda and co-workers studied the interactions of Vitamin B12 and its hydrophobic ester derivatives with HSA [25]. However, the binding affinity and the change of the conformation of human serum albumin when interacting with B12 have not yet been systematically investigated, and multiple drug binding sites have been reported for HSA by several researchers [26]. Two vitamins have been investigated which are: vitamin E and vitamin D, since they are easy in preparing samples and they are an available in our laboratory. In this work, the interaction of hydrophobic vitamins with Human serum albumin (HSA) protein will be investigated by using FT-IT spectroscopy, and UV-spectrophotometer. 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. Our studies is limited to the mid- infrared range, 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. One of the advantages of infrared spectroscopy is that it can be used with proteins that are either in solution or in thin films. In addition, there is a growing body of literature on the use of infrared to follow reaction kinetics and ligand binding in proteins, as well as a number of infrared studies on protein dynamics. Other spectroscopy techniques are usually used in studying the interaction of hydrophobic and proteins, fluorescence and UV spectroscopy are commonly used because of their high sensitivity, rapidity and ease of implementation.

MATERIALS AND METHODS

HSA (fatty acid free), vitamin E (α-Tocopherol), and vitamin D (Cholecalciferol) were purchased from Sigma Aldrich chemical company and used without further purifications. The data were collected using samples in the form of thin films for FT-IR measurements and liquid form for UV-VIS.

Preparation of stock solutions

Preparations of the thin film samples required three stock solutions as described below:

HSA was dissolved in 25% ethanol in phosphate buffer Saline and at physiological (pH 6.9- 7.4), to a concentration of (80mg/ml), and used at final concentration of (40 mg/ml) in the final vitamin- HSA solution.

Vitamin E with molecular weight of (430.71 $g.mol^{-1}$), was dissolved in 25% ethanol in phosphate buffer Saline and, then the solution was placed in ultrasonic water path (SIBATA AU-3T) for two days to ensure that all the amount of vitamin E was completely dissolved.

Vitamin D with molecular weight (384.64$g.mol^{-1}$), was dissolved in 25% ethanol in phosphate buffer Saline and, then the solution was placed in ultrasonic water path (SIBATA AU-3T) for two days to ensure that all the amount of vitamin E was completely dissolved.

HSA concentration was fixed at 40 $mg.ml^{-1}$ in all samples. However, the concentration of vitamin E in the final HSA-vitamin E solutions was decreased such that the molecular ratios (HSA: vitamin E) are 1:20, 1:10, 1:5, 1:2, and 1:1. All samples were made by mixing equal volume from HSA to equal volume from different concentration of vitamin E.

HSA concentration was fixed at 40 $mg.ml^{-1}$ in all samples. However, the concentration of vitamin D in the final HSA-vitamin D solutions was decreased such that the molecular ratios (HSA: vitamin D) are 1:20, 1:10, 1:5, 1:2, and 1:1. All samples were made by mixing equal volume from HSA to equal volume from different concentration of vitamin D. 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 E 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 25^0c. The same procedure was followed for HSA-vitamin D films preparation.

UV-absorption spectroscopy

The absorption spectrum of a measurements were performed by a Nano-Drop ND-1000 Fluor spectrometer at 25°C. The excitation had been done at the wavelength of 210 nm and the maximum emission wavelength is at 280 nm. The excitation source comes from one of three solid-state light emitting diodes (LED's). A 2048-element CCD array detector covering 220–750 nm, is connected by an optical fiber to the optical measurement surface. The UV absorbance intensity of HSA increased with the increasing of vitamin E or vitamin D concentration as shown in figure 3, figure 4 respectively . In addition, the binding of the vitamins to HSA resulted in a slight shift of the HSA absorption spectrum. These results clearly indicated that an interaction and some complex formation occurred between HSA and the two vitamins separately, and also indicated that the peptide strands of protein molecules extended more upon the addition of vitamin E or vitamin D to HSA [27,23]. It is evident from the spectra of the pure vitamins the little or no absorption effect which supports that the resulted peaks are due to the interaction between the vitamins and HSA. The emission spectra were recorded for free HSA (40 mg/ml) and for its complexes with vitamins E and D solutions with different concentrations of HSA: Vitamins E and D. The solution of vitamins and HSA were incubated for 1 h (at 20°C) before spectroscopic measurements were taken.

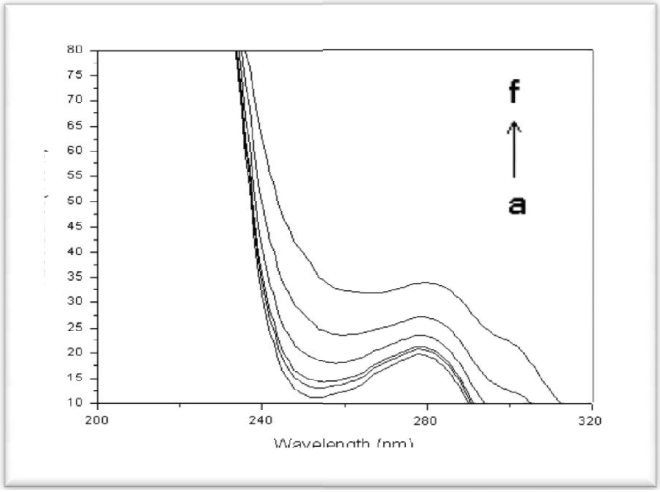

Figure 3. UV-Absorbance spectra of HSA with different molar ratios of vitamin E, HSA: vitamin E (a=1:0, b=1:1, c=1:2, d=1:5, e=1:10, f=1:20), no UV absorption for vitamin E.

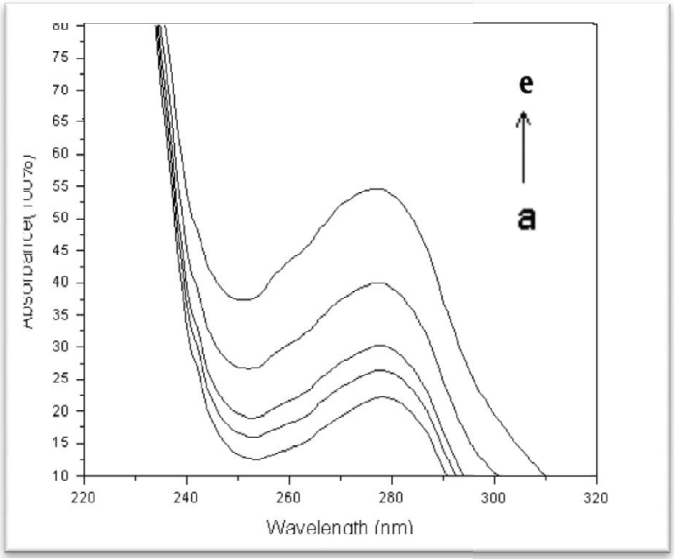

Figure 4. UV-Absorbance spectra of HSA with different molar ratios of vitamin D, HSA: vitamin D (a=1:0, b=1:1, c=1:2, d=1:5, f= e =1:10). no UV absorption for vitamin D.

FT-IR Spectroscopy Experimental Procedures

The FT-IR measurements were obtained on a Bruker IFS 66/S spectrophotometer equipped with a liquid nitrogen-cooled MCT detector and a KBr beam splitter. The spectrometer was continuously purged with dry air during the measurements. The absorption spectra were obtained in the wave number range of 400–4000 cm^{-1}. A spectrum was taken as an average of 60 scans to increase the signal to noise ratio, and the spectral resolution was at 4 cm^{-1}. The aperture used in this study was 8 mm, since we found that this aperture gives best signal to noise ratio. Baseline correction, normalization and peak areas calculations were performed for all the spectra by OPUS software. The peak positions were determined using the second derivative of the spectra. The infrared spectra of HSA, vitamin E–HSA complexes, and vitamin D – HSA mixtures, 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 E / vitamin D solution) – (protein solution)} were generated using the featureless region of the protein solution 1800-2200 cm^{-1} as an internal standard [28]. The accuracy of this subtraction method is tested using several control samples with the same protein or drug concentrations, which resulted into a lat base line formation. The obtained spectral differences were used here, to investigate the nature of the drug–HSA interaction. We had also used ELAB 12/05 thermo system to directly and simultaneously determine the thermo-dependent structural changes of drug-protein complexes.

RESULTS AND DISCUSSION

Analysis of UV-absorption spectroscopy of HSA by Vitamins E and D

The vitamin E or vitamin D - HSA complexes binding constants were determined using UV-VIS spectrophotometer results according to published method [29,30,31], by assuming that there is only one type of interaction between vitamin E or vitamin D and HSA in aqueous solution, which leads to establish Equations. (1) and (2) as follows:

HSA + vitamin E ↔ vitamin E: HAS .....(1)
HSA + vitamin D ↔ vitamin D: HSA

K = [vitamin E: HSA]/ [vitamin E][HSA] .....(2)
K = [vitamin D: HSA]/ [vitamin D][HSA]

The absorption data were treated using linear double reciprocal plots based on the following equation [32]:

$$\frac{1}{A-A_0}=\frac{1}{A_\infty-A_0}+\frac{1}{K[A_\infty-A_0]}X\frac{1}{L} \quad \text{.....(3)}$$

where A_0 corresponds to the initial absorption of protein at 280 nm in the absence of ligand , A_∞ is the final absorption of the ligated protein, and A is the recorded absorption at different vitamin E or vitamin D concentrations (L). The double reciprocal plot of $1/(A- A_0)$ vs. $1/L$ is linear as shown in figures 5 and 6, the binding constant (K) can be estimated from the ratio of the intercept to the slope to be ($1.21\times10^{2}M^{-1}$) and ($6.8\times10^{1}M^{-1}$) for vitamin E- HSA and vitamin D-HSA complexes, respectively.

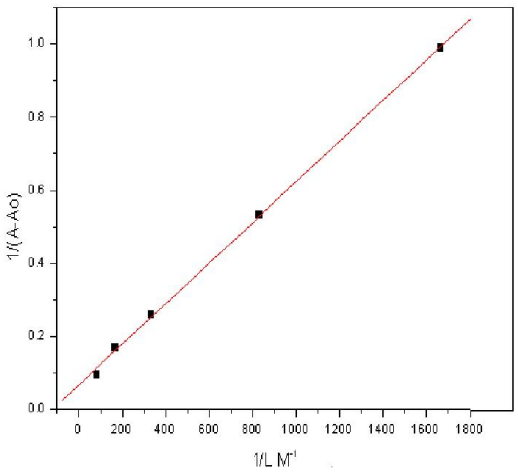

Figure 5. Plot of 1/(A-Ao) vs. 1/L for HSA with different concentrations of vitamin E.

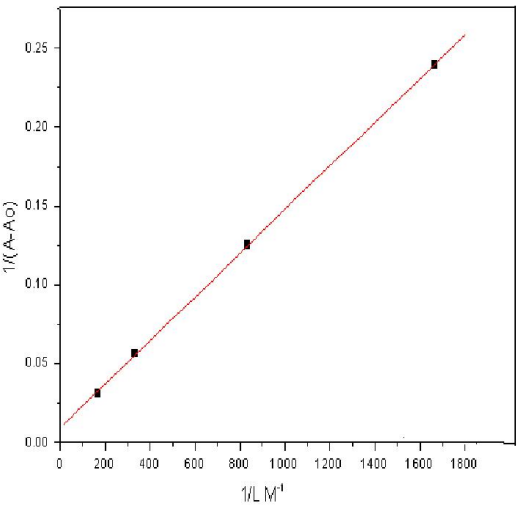

Figure 6. Plot of 1/(A-Ao) vs. 1/L for HSA with different concentrations of vitamin D

Table 1. Band assignment in the absorbance spectra of HSA with different vitamin E molecular ratios for amide I, amide II, and amide III region

Bands	HSA Free	HSA- V.E	HSA- V.E	HSA- V.E	HSA- V.E	HSA- V.E
		1:01	1:02	1:05	1:10	1:20
Amide I	1618	1610	1610	1610	1610	1611
(1600-1700)	1632	1629	1628	1628	1628	1628
			1643	1642	1642	1642
	1657	1658	1659	1659	1659	1660
	1680	1678	1678	1678	1677	1677
	1695	1694	1693	1693	1693	1693
Amide II	1496	1497	1498	1497	1497	1497
(1480-1600)	1517	1515	1515	1515	1515	1514
		1532	1532	1532	1532	1532
	1544	1549	1549	1549	1549	1549
	1577	1579	1579	1579	1579	1580
	1594	1594	1594	1594	1594	1594
Amide III	1231	1231	1231	1231	1231	1231
(1220-1330)	1244	1245	1246	1245	1245	1246
	1267	1265	1264	1264	1263	1263
		1278	1279	1280	1281	1281
	1295	1295	1295	1295	1295	1295
	1310	1309	1309	1309	1309	1309

Table 2. Band assignment in the absorbance spectra of HSA with different vitamin D molecularratios for amide I, amide II, and amide III region

Bands	HSA Free	HSA- V.D	HSA- V.D	HSA- V.D	HSA- V.D	HSA- V.D
		1:01	1:02	1:05	1:10	1:20
Amide I	1618	1612	1611	1612	1611	1612
(1600-1700)	1632	1632	1629	1628	1628	1627
		1645	1645	1646	1643	1643
	1657	1658	1658	1658	1658	1659
	1680	1679	1678	1678	1678	1677
	1695	1694	1694	1694	1694	1693
Amide II	1496	1497	1498	1498	1498	1498
(1480-1600)	1517	1515	1515	1515	1515	1515
		1532	1532	1532	1532	1532
	1544	1550	1549	1549	1549	1549
	1576	1578	1579	1579	1580	1580
	1594	1594	1594	1594	1594	1594
Amide III	1231	1232	1232	1232	1232	1232
(1220-1330)	1244	1245	1245	1245	1245	1245
	1267	1266	1266	1266	1266	1265
		1278	1279	1279	1279	1279
	1295	1295	1295	1295	1295	1295
	1310	1309	1309	1309	1309	1309

The values obtained is indicative of a weak vitamin E or vitamin D protein interaction with respect to the other vitamins-HSA complexes with binding constants in the range of 10^5 and 10^6 M^{-1} [33]. The reason for the low stability of the vitamin E or vitamin D-HSA complexes can be attributed to the presence of mainly hydrogen bonding interaction between protein and the vitamin E or vitamin D polar groups or an indirect vitamin - protein interaction through water molecules [34]. Similar weak interactions were observed in *cis* Pt(NH3)2–HSA and taxol–HSA complexes [35,36].

FT-IR Spectroscopy

FT-IR spectroscopy is a good technique for the study of hydrogen bonding [37], and has been identified as one of the few techniques that is established in the determination of protein secondary structure at different physiological systems [38,28,39]. FT-IR spectroscopy provides information about the secondary structure content of proteins, unlike X-ray crystallography and NMR spectroscopy which provide information about the tertiary structure [40]. FTIR spectroscopy works by shining infrared radiation on a sample and seeing which wavelengths of radiation in the infrared region of the spectrum are absorbed by the sample. Each compound has a characteristic set of absorption bands in its infrared spectrum. Characteristic bands found in the infrared spectra of proteins and polypeptides include the Amide I and Amide II. [41,42]. Amide I band ranging from 1700 to 1600 cm^{-1} and arises principally from the C=O stretching [43], and has been widely accepted to be used [44]. 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}.And 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. [45,38]. The spectra of second derivative of HSA free, the spectra of vitamin E- HSA mixtures with different percentages of vitamin E, and the spectra of vitamin D -HSA mixtures with different percentages of vitamin D are shown in figure 7 and figure 8, respectively. As shown from the figures, the major spectral absorbance of amide I band at 1657 cm^{-1} (mainly C=O stretch), and amide II band at 1543 cm^{-1} (C-N stretching coupled with N-H bending modes). The reduction in the intensity of three amid bands is related to

vitamin E- HSA or vitamin D- HSA interactions [23]. In table 1 and table 2 the peak positions of HSA with different ratios of vitamin E or vitamin D are listed, respectively. The changes of these peak positions and peak shapes demonstrated the secondary structure of the HSA had been changed by the interaction of vitamin E or vitamin D. The minor changes in peak positions can be attributed to the effect of the newly imposed H-bonding between the vitamin molecules and the protein. It is suggested that, the shift to a higher frequency for the major peak in Amide I region (1657 to 1660 cm^{-1}) for HSA- vitamin E system, and (1657 to 1659 cm^{-1}) for HSA-vitamin D system came as a result of stabilization by hydrogen bonding by having the C-N bond assuming partial double bond character due to a flow of electrons from the C=O to the C-N bond [46,47,23]. There was no major spectral shifting for the protein amide I band at 1656 cm^{-1} and amide II band at 1544 cm^{-1}, upon vitamin E interaction [48] as shown in the top of the two curves of figure 9.

The bottom five curves shows the difference spectra [(protein solution + vitamin E solution)-(protein solution)] were obtained in order monitor the intensity variations of these vibrations. At low vitamin E concentration, a strong negative feature at 1656 cm^{-1} and other one at 1543 cm^{-1} were observed in the difference spectra of vitamin E – HSA mixtures with a little shift and became stronger as vitamin E ratios was increased, and are attributed to decrease in the intensity of the amide I band at 1656 cm^{-1} and amide II band at 1544 cm^{-1}, as a result of vitamin E interaction(H-bonding) with the protein C=O and C-N groups [35 ,48,23]. The band at 1518 cm^{-1} of the free HSA related to the tyrosine amino acid side chain vibration [46,49,50], exhibited no spectral changes upon vitamin E mixture.

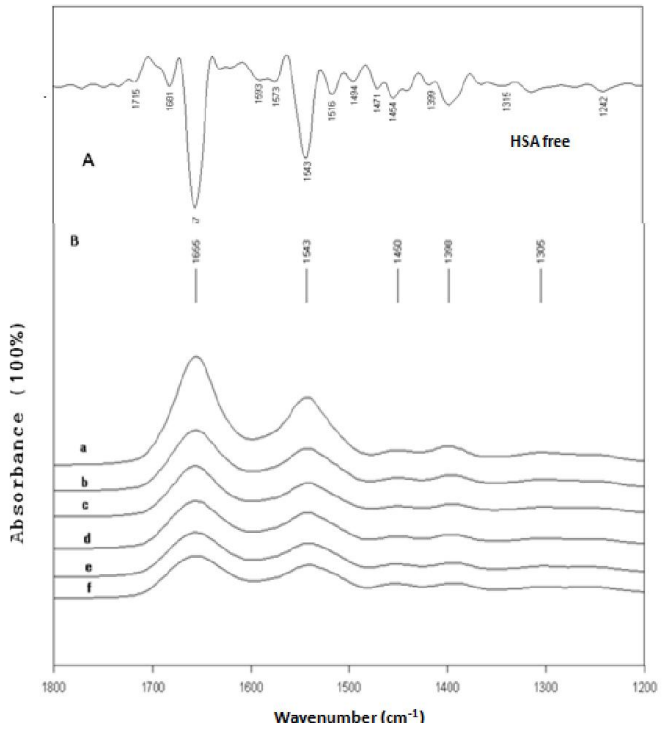

Figure 7. The spectra of HSA free (second derivative) And B: (a, b, c, d, e, f) vitamin E -HSA with ratios (0:1, 1:1, 2:1, 5:1, 10:1, 20:1), respectively

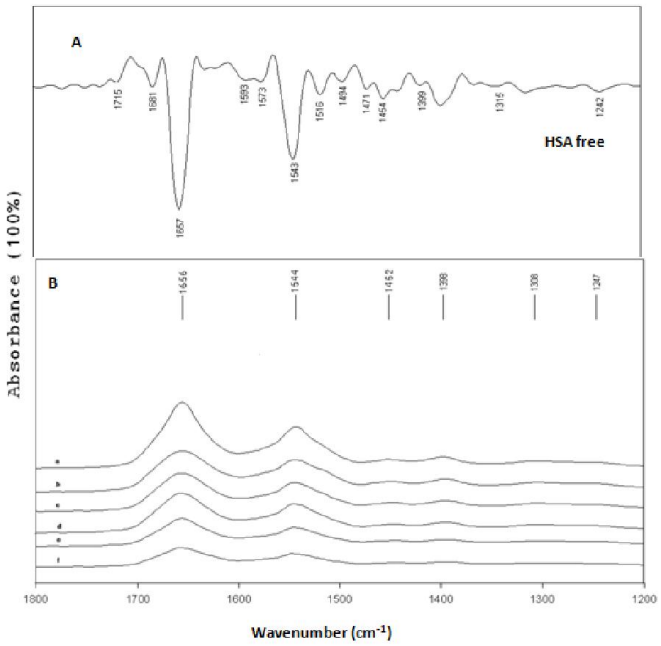

Figure 8. The spectra of HSA free (second derivative) And B: (a, b, c, d, e, f) vitamin D -HSA with ratios (0:1, 1:1, 2:1, 5:1, 10:1, 20:1), respectively

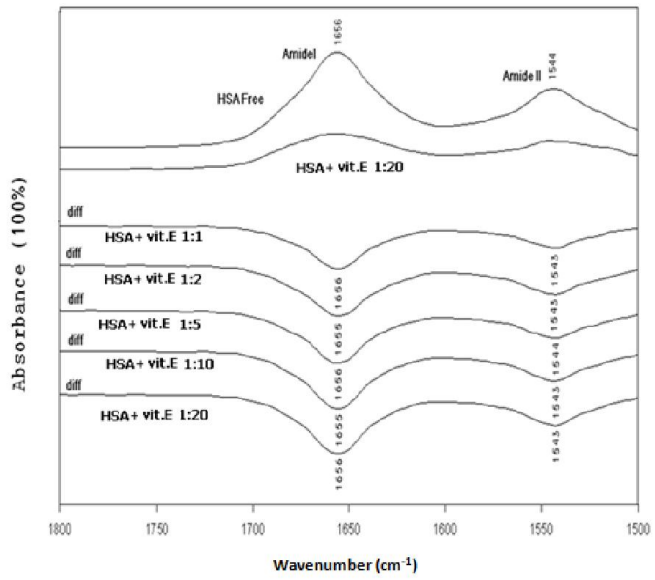

Figure 9. FTIR spectra (top two curves) and difference spectra [(protein solution+ vitamin E solution)-(protein solution)] (bottom five curves) of the free human serum albumin (HSA) and its vitamin E complexes in aqueous solution

It should be that the decrease in the intensity of the amide I band at 1656 cm^{-1} is also due to the reduction of the protein α-helix structure, upon vitamin E interaction, which is quantitatively determined and will be discussed further on [35,24]. There was no major spectral shifting for the protein amide I band at 1656 cm^{-1} and amide II band at 1544 cm^{-1}, upon vitamin D interaction [48] as shown in the top of the two curves of figure 10. The bottom five curves shows the difference spectra [(protein solution + vitamin D solution)-(protein solution)] were obtained in order monitor the intensity variations of these vibrations.

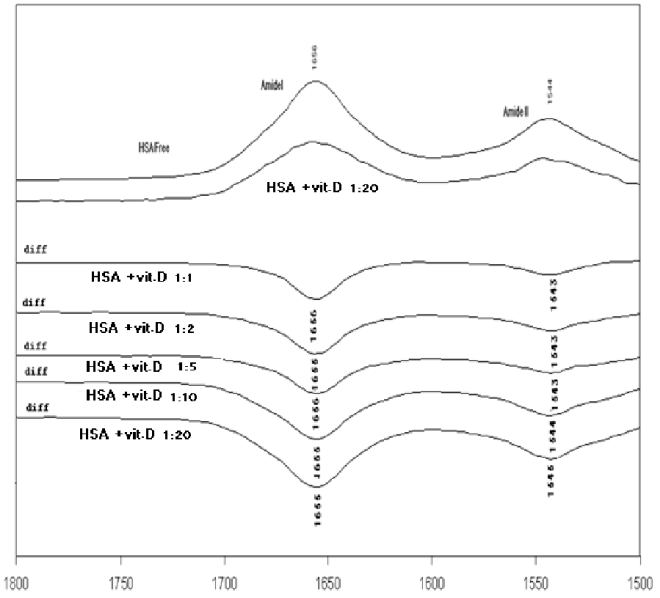

Figure 10. FTIR spectra (top two curves) and difference spectra [(protein solution+ vitamin D solution)-(protein solution)] (bottom five curves) of the free human serum albumin (HSA) and its vitamin D complexes in aqueous solution

At low vitamin D concentration, a strong negative feature at 1656 cm^{-1} and other one at 1544 cm^{-1} were observed in the difference spectra of vitamin D – HSA mixtures with a little shift and became stronger as vitamin D ratios was increased, and are attributed to decrease in the intensity of the amide I band at 1656 cm^{-1} and amide II band at 1544 cm^{-1}, as a result of vitamin D interaction(H-bonding) with the protein C=O and C-N groups [35,48,23]. The band at 1518 cm^{-1} of the free HSA related to the tyrosine amino acid side chain vibration [46,49,50], exhibited no spectral changes upon vitamin E mixture. It should be that the decrease in the intensity of the amide I band at 1656 cm^{-1} is also due to the reduction of the protein α-helix structure, upon vitamin E interaction, which is quantitatively determined and will be discussed further on [35,24]. It is important to note that although the major spectral changes observed are associate with the polypeptide amide I and amide II bands, with a big vitamin molecule, protein interaction cannot be limited with the polypeptide C=O and C-N donor and thus, other types of complexation with amino acid residues cannot be excluded. However, based on our infrared results a lone, we cannot locate the presence of other types of vitamins- protein interactions [35].

The Determination of the secondary structure of HSA and its vitamin E or vitamin D complexes were carried out on the basis of the procedure described by Byler and Susi [51]. In this work a quantitative analysis of the protein secondary structure for the free HSA, vitamin E–HSA, and vitamin D-HSA complexes in dehydrated films are determined from the shape of Amide I, II and III bands. Baseline correction was carried out in the range of (1700–1600 cm^{-1}), (1600-1480 cm^{-1}), and (1330–1220 cm^{-1}) to get amide I, II, and III bands. Then Fourier self-deconvolution and second derivative were applied to these three ranges respectively to increase spectral resolution and therefore to estimate the number, position and the area of each component bands. Based on these parameters curve-fitting process was carried out by Opus software (version 5.5) to obtain the best Lorentzian-shaped curves that fit the original HSA spectrum.

Table 3. Secondary structure determination for the free HSA and its vitamin E mixture for amide I , amide II , amide III

2 nd Structure	HSA Free	HSA-V.E	HSA-V.E	HSA-V.E	HSA-V.E	HSA-V.E
	(%)	1:1 (%)	1:2 (%)	1:5 (%)	1:10 (%)	1:20(%)
			Amide I			
β- sheets (cm^{-2}) (1610-1624)	15	18	15	19	20	23
Random (cm^{-2}) (1625-1640)	11	15	14	16	15	16
α- hilex (cm^{-2}) (1646-1671)	50	42	42	37	35	33
Turn (cm^{-2}) (1672-1687)	13	11	12	12	12	11
Anti β- sheets (cm^{-2}) (1689-1700)	11	14	17	16	18	17
			Amide II			
β- sheets (cm^{-2}) (1488-1504)	23	28	27	28	30	30
Random (cm^{-2}) (1508-1523)	11	13	14	14	13	14
α- hilex (cm^{-2}) (1528-1560)	45	37	34	33	32	31
Turn (cm^{-2}) (1562-1585)	9	8	9	8	7	6
Anti β- sheets (cm^{-2}) (1585-1598)	12	14	16	17	18	19
			Amide III			
β- sheets (cm^{-2}) (1220-1256)	32	37	36	37	39	39
Random (cm^{-2}) (1257-1285)	11	15	14	14	13	14
Turn (cm^{-2}) (1287-1301)	12	11	11	10	10	9
α- hilex (cm^{-2}) (1302-1329)	45	37	39	39	38	38

Table 4. Secondary structure determination for the free HSA and its vitamin D mixture for amide I , amide II , amide III

2 nd Structure	HSA Free	HSA-V.D	HSA-V.D	HSA-V.D	HSA-V.D	HSA-V.D
	(%)	1:1 (%)	1:2 (%)	1:5 (%)	1:10 (%)	1:20(%)
			Amide I			
β- sheets (cm^{-2}) (1610-1624)	15	16	19	20	22	25
Random (cm^{-2}) (1625-1640)	11	13	13	14	15	15
α- hilex (cm^{-2}) (1646-1671)	50	47	43	40	35	32
Turn (cm^{-2}) (1672-1687)	13	11	11	10	11	10
Anti β- sheets (cm^{-2}) (1689-1700)	11	13	14	16	17	18
			Amide II			
β- sheets (cm^{-2}) (1488-1504)	23	26	27	29	30	32
Random (cm^{-2}) (1508-1523)	11	11	12	12	13	14
α- hilex (cm^{-2}) (1528-1560)	45	40	37	37	33	31
Turn (cm^{-2}) (1562-1585)	9	8	9	8	8	7
Anti β- sheets (cm^{-2}) (1585-1598)	12	15	15	14	16	16
			Amide III			
β- sheets (cm^{-2}) (1220-1256)	32	35	34	37	37	38
Random (cm^{-2}) (1257-1285)	11	12	14	12	13	13
Turn (cm^{-2}) (1287-1301)	12	10	11	9	10	10
α- hilex (cm^{-2}) (1302-1329)	45	43	41	42	40	39

The individual bands are identified with its representative secondary structure, and the content of each secondary structure of HSA is calculated by area of their respective component bands. The procedure was in general carried out considering only components detected by second derivatives and the half widths at half height (HWHH)for the component peaks are kept around $5cm^{-1}$ [24]. The component bands of amide I were attributed according to the well-established assignment criterion [52,53]. Amide I band ranging from 1610 to $1700cm^{-1}$ generally assigned as follows 1610–1624 cm^{-1} are generally represented to β-sheet, 1625–1640 cm^{-1} to random coil, 1646–1671 cm^{-1} to α-helix, 1672–1787 cm^{-1} to turn structure, and 1689-$1700cm^{-1}$ to β-ant parallel. For amide II ranging from 1480 to $1600cm^{-1}$, the absorption band assigned in the following order: 1488–1504 cm^{-1} to β-sheet, 1508–1523 cm^{-1} to random coil, 1528–1560 cm^{-1} to α-helix, 1562–1585 cm^{-1} to turn structure, and 1585-$1598cm^{-1}$ to β-ant parallel.

And for amide III ranging from 1220 to $1330cm^{-1}$ have been assigned as follows: 1220–1256 cm^{-1} to β-sheet, 1257–1285 cm^{-1} to random coil, 1287–1301 cm^{-1} to turn structure, and 1302–1329 cm^{-1} to α-helix. Most investigations have concentrated on Amide I band assuming higher sensitivity to the change of protein secondary structure [54]. However, it has been reported that amide II and amide III bands have high information content and could be used for prediction of proteins secondary structure [55,56,52,37]. Based on the above assignments, the percentages of each secondary structure of HSA were calculated from the integrated areas of the component bands in Amide I, II, and respectively. Where the area of all the component bands assigned to a given conformation is then summed and divided by the total area.

The obtained number is taken as the proportion of the polypeptide chain in that conformation. The Secondary structure determination for the free HSA and its vitamin E or vitamin D mixture with different vitamin concentrations are given in (table 3 and table 4). The second derivative resolution enhancement and curve – fitted Amide I and secondary structure determinations of the free human serum albumin (A, B) and its vitamin E or vitamin D mixture (C, D) with the highest concentrations in dehydrated films are shown in the figure 11 and figure 12.

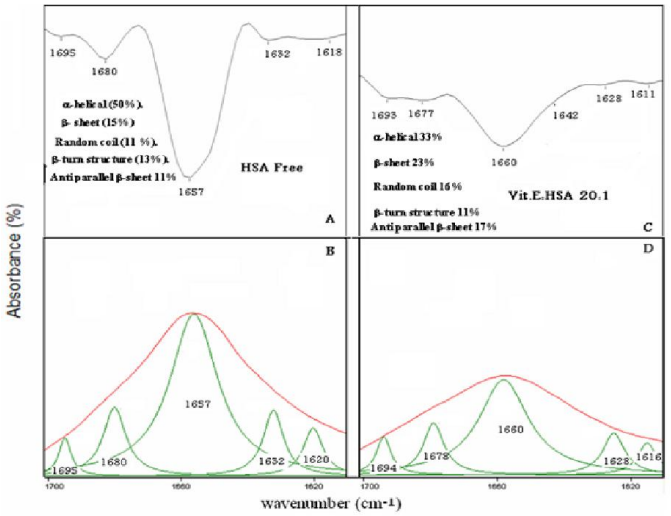

Figure 11. Second-derivative enhancement and curve-fitted Amide I region (1600-1700 cm^{-1}) and secondary structure determination of the free human serum albumin (A and B) and its vitamin E mixture(C and D) with 20:vitamin E : HSA ratios

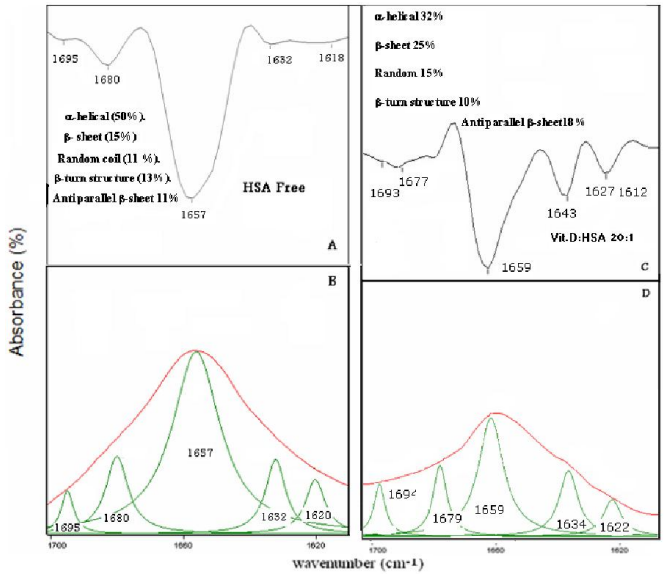

Figure 12. Second-derivative enhancement and curve-fitted Amide I region (1600-1700 cm^{-1}) and secondary structure determination of the free human serum albumin (A and B) and its vitamin D mixture(C and D) with 20:vitamin D : HSA ratios

It is generally accepted that infrared spectra of proteins in films and in solution may display distinct differences, but these differences are due to the presence or absence of the water or buffer molecules that imprint their mark on the spectra. It has been shown that the structural information content is of the same quality in films and in solution with an (error of < 1%) for both systems [31]. The reduction of α-helix intensity percentage in favor of the increase of β-sheets percentage are believed to be due to the unfolding of the protein in the presence of vitamin E or vitamin D as a result of the formation of H bonding between HSA and the hydrophobic vitamins . The newly formed H-bonding result in the C–N bond assuming partial double bond character due to a flow of electrons from the C=O to the C–N bond which decreases the intensity of the original vibrations [46,47]. It seems that the H-bonding affects more of the original bonding in α- helix than in β-sheets depending on the accessibility of the solvent and on propensities of α- helix and β-sheets of the HSA [57].

The hydrogen bonds in α-helix are formed inside the helix and parallel to the helix axis, while for β-sheet the hydrogen bonds take position in the planes of β-sheets as the preferred orientations especially in the anti-parallel sheets, so the restrictions on the formation of hydrogen bonds in β-sheet relative to the case in α helix explains the larger effect on reducing the intensity percentage of α-helix to that of β-sheet [24,58,59]. Similar conformational transitions from an α-helix to β-sheet structure were observed for the protein unfolding upon protonation and heat denaturation [60,39,58,57]. These results indicate that vitamin E and vitamin D interact with HSA through C=O and C-N groups in the HSA polypeptides. The vitamin E or vitamin D–HSA mixture caused the rearrangement of the polypeptide carbonyl hydrogen bonding network and finally the reduction of the protein α-helical structure. The binding of vitamin E and vitamin D to HSA has been investigated by UV-absorption spectroscopy and by FTIR spectroscopy. From the UV-absorption study we determined values for the binding constant for vitamin E -HSA and vitamin D-HSA complexes. The results indicate that Vitamin E interaction with HSA is similar to the interaction of vitamin D to HSA [61], and the affinity of vitamins to HSA is low [62]; which is obviously seen in our results with a low binding constants we got between vitamin E or vitamin D interaction with HSA. Relatively vitamin E interaction with HSA was stronger than vitamin D with HSA interaction. This may be due to the chemical structure of vitamin E and vitamin D; there are more side interaction in presence of vitamin E to interact with HSA. While in the presence of vitamin D there is less side interaction, this is line with the reported results that vitamin E binds to HSA in sub domains IIA and IIIA [61], and vitamin D have one binding site located in domain II [63].

Conclusion

The interaction between hydrophobic vitamins and human serum albumin(HSA) is great interest and important from pharmaceutical point of view. In this work, the interaction of vitamin E and vitamin D with HSA was investigated by means of UV-VIS spectrophotometer, and FT-IR spectroscopy. The experimental results indicates a low binding affinity between vitamin E or vitamin D with HSA. Analysis of FT-IR spectrum indicated that increasing the concentration of vitamin E or Vitamin D lead to the unfolding of protein, decreasing the percentage of the α-helical structure in favor of β-sheet structure. Beside that it can be inferred that the binding forces which are involved in the binding process includes hydrophobic interactions. The newly formed H-bonding result in the C–N bond assuming partial double bond character due to a flow of electrons from the C=O to the C–N bond which decreases the intensity of the original vibrations. The binding study of vitamin E or vitamin D with HSA is of great importance in pharmacy, pharmacology and biochemistry. This research can supply some important information to clinical research and provide the theoretical basis for the new vitamins designing. Therefore, this research need further studies to be a useful guide for synthesis of efficient vitamin E and vitamin D such as the determinations of binding sites, binding location, and thermodynamic parameters (enthalpy ,free energy ,entropy) at different temperatures to deduce the type of the acting force for the binding reaction between vitamin E or vitamin D with HSA. Furthermore, it is needed to investigate the effect of ions on the binding constants, because the existence of metal ions 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.

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REFERENCES

1. V. M. Rosenoer, M. Oratz, and M. A. Rothschild, *Albumin: Structure, Function, and Uses*, Pergamon Press, Oxford, UK, 1977.
2. Garcion E., Wion-Barbot N., Montero-Menei C.N., Berger F. and Wion D. New clues about vitamin D

functions in the nervous system. Trends Endocrinol. Metab. 2002; 13: 100-105.
3. Langub M.C., Herman J.P., Malluche H.H., Koszewski N.J. Evidence of functional vitamin D receptors in rat hippocampus. Neuroscience 2001; 104: 49-56.
4. Stio M., Lunghi B., Lantomasi T., Vincenzini M.T. and Treves C. Effect of vitamin D deficiency and 1,25-dihydroxyvitamin D3 on metabolism and D-glucose transport in rat cerebral cortex. J. Neurosci. Res. 1993; 35: 559-566.
5. Zittermann A. Vitamin D in preventive medicine: are we ignoring the evidence? Br. J. Nutr. 2003; 89: 552-572.
6. Haddad, J., Matsuoka, L., Hollis, B., Hu, Y., (1993 The American Society for Clinical Investigation, Inc, V 91, 2552-2555.
7. Fraser, D., (1983). The physiological economy of vitamin D. Lancet, I,969-972.
8. Lane K Christenson, and Luigi Devoto, Cholesterol transport and steroidogenesis by the corpus luteum, Reproductive Biology and Endocrinology 2003, 1:90 doi:10.1186/1477-7827-1-90.
9. Schmidt R., Hayn M., Reinhart B., Roob G., Schmidt H., Schumacher M., Watzinger N. and Launer L.J. Plasma anti-oxydants and cognitive performances in midle-aged and older adults : results of the Austrian Stroke Prevention Study. J. Am. Geriatr. Soc. 1998; 46 : 1407-1410.
10. Clement M., Dinh L. and Bourre J.M. Uptake of dietary RRR-alpha and RRRgamma-tocophérol by nervous tissues, liver and muscle in vitamin-E-deficient rats. Biochim. Biophys. Acta 1995; 1256 : 175-180.
11. Clement M. and Bourre J.M. Alteration of brain and liver microsomal polyunsaturated fatty acids following dietary vitamin E deficiency. Neurosci. Lett. 1993; 164 : 163-166.
12. Goss-Sampson M.A., Kriss T. and Muller D.P. Retinal abnormalities in experimental vitamin E deficiency. Free Radic. Biol. Med. 1998; 25 : 457-462.
13. Sung S., Yao Y., Uryu K., Yang H., Lee V.M., Trojanowski J.Q. and Pratico D. Early vitamin E supplementation in young but not aged mice reduces Abeta levels and amyloid deposition in a transgenic model of Alzheimer's disease. FASEB J. 2004; 18: 323-325.
14. Vatassery G.T., Bauer T. and Dysken M. High doses of vitamin E in the treatment of disorders of the central nervous system in the aged. Am. J. Clin. Nutr. 1999; 70 : 793-801.
15. Zandi P.P., Anthony J.C., Khachaturian A.S., Stone S.V., Gustafson D., Tschanz J.T., Norton M.C., Welsh-Bohmer K.A. and Breitner J.C. Reduced risk of Alzheimer disease in users of antioxidant vitamin supplements: the Cache County Study. Arch. Neurol. 2004; 61 : 82-88.
16. Larrieu S., Letenneur L., Helmer C., Dartigues J.F. and Barberger-Gateau P. Nutritional factors and risk of incident dementia in the PAQUID longitudinal cohort. J. Nutr. Health Aging 2004; 8 : 150-154.
17. Grundman M. Vitamin E and Alzheimer disease: the basis for additional clinical trials. Am. J. Clin. Nutr. 2000; 71 : 630S-636S.
18. Hill K.E., Montine T.J., Motley A.K., Li X., May J.M. and Burk R.F. Combined deficiency of vitamins E and C causes paralysis and death in guinea pigs. Am. J. Clin. Nutr. 2003; 77 : 1484-1488.
19. Azzi A., Gysin R., Kempna P., Ricciarelli R., Villacorta L., Visarius T. and Zingg J.M. The role of alpha-tocopherol in preventing disease: from epidemiology to molecular events. Mol. Aspects Med. 2003; 24 : 325-336.
20. Jerkovic, L., Voegele, A., Chwatal, S., Kronenberg, F., (2005). Journal of Proteome Research , 4, 889-899 889.
21. Mann, J. and Truswell, S. (2002): Essentials of Human Nutrition. 2nd ed, Oxford University Press, New York.
22. Beharka, A., Redican, S., Leka, L., Meydani, S., (1997). Methods Enzymol, 282, 247-263.
23. Abu Tair, M., Ghithan s, J., Darwish , M., Abu-hadid, M., (2010). Journal of Applied BiologicalSciences 5 (13): 35-47.
24. Darwish, S., Abu sharkh, S., Abu Teir, M., Makharza, S., Abu-hadid, M., (2010). Journal of Molecular Structure, 963, p122.
25. Hisaeda, Y., Masuko, T., Hanashima, E., (2006). Sci. Technol. Adv. Mater. 7, 655–661.
26. Na Hou, H., Zu-De Qi a, B., Ling-Liao, F., Zhang, Y.,(2008). Journal of Pharmaceutical and Biomedical Analysis, 47, 134–139.
27. Peng, L., Minboa, H., Fang, C., Xi, L., Chaocan, Z., (2008). Protein & Peptide Letters, 15, p360.
28. Surewicz, W., Moscarello, M., Mantsch, H., (1987). J. Biol. Chem., 262, p8598.
29. Stephanos, J., and Inorg, J., (1996). Biochemistry, 62, p155.
30. Klotz, M., and Hunston, L., (1971). Biochemistry, 10, p3065.
31. Ahmed Quamour, A., *et al*. (2004). Biopolymers, 73, p503.
32. Lakowicz, J., (2006): Principles of Fluorescence Spectroscopy, 3rd ed, Springer Science+Business Media, USA.
33. Kragh-Hansen, U., (1981). Pharmacol. Rev., 33, p17.
34. Sulkowaska, A., (2002), J. Mol. Strurct., 614, p227.
35. Purcell, M., Neault J., Tajmir-Riahi, H., (2000). Biochimica et Biophysica Acta, 1478, p61.
36. Neault, J.F., and Tajmir-Riahi, H.A.(1998). Biochimica et Biophysica Acta, 1384, p153.
37. Li, Y., He, W., Dong, Y., Sheng, F., Hu, Z., (2006). Bioorganic & Medicinal Chemistry, 14, p1431.
38. Sirotkin, V., Zinatullin, A., Solomonov, B., Faizullin, D., Fedotov, V., (2001). Biochimica et Biophysica Acta, 1547, p359.
39. Arrondo, J., and Muga, A., (1993). Prog. Biophys. Mol. Biol., 59, p23.
40. Kendrew, J., Bodo, G., Dintzis, H., Parrish, R., Wyckoff, H., Philips, D., (1958). Nature 18, 662-666.
41. KONG, J., (2007). Acta Biochimica et Biophysica Sinica, 39(8): 549–559.
42. Byler, M., and Susi, H., (1986). Biopolymers, 25, p469.
43. Vandenbussche, G., Clercx, A., Curstedt, T., Johansson, J., Jornvall, H., Ruysschaert, J., (1992). European Journal of Biochemistry, 203, p201.
44. Workman, J., (1998): Applied Spectroscopy: Optical Spectrometers, Academic Press, San Diego.

45. Goormaghtigh, E., Cabiaux, V., Ruysschaert, J., (1990). European Journal of Biochemistry, 193, p409.
46. Chirgadze, Y., Fedorov, O., Trushina, N., (1975). Biopolymers 14, p679.
47. Jackson, M., and Mantsch, H., (1991). J. Chem., 69, p1639.
48. Krimm, S., and Bandekar, J., (1986). Adv. Protein Chem., 38, 181-364.
49. Matsuura, H., Hasegawa, K., Miyazawa, T., (1986). Spectrochim. Acta 42A , 1181-1192.
50. Olinger, J., Hill, D., Jakobsen, R., Brody, R., (1986). Biochim. Biophys. Acta 869, 89-98.
51. Byler, M., and Susi, H., (1986). Biopolymers, 25, p469.
52. Jiang, M., Xie, M., Zheng, D., Liu, Y., Li, X., Chen, X., (2004). Journal of Molecular Structure, 692, p71.
53. Ivanov, A. *et al*. (1994). Journal of Applied Spectroscopy, 60, p305.
54. Vass, E., Holly, S., Majer, Zs., Samu, J., Laczko, I., Hollosi, M., (1997). Journal of Molecular Structure, 47, p408.
55. Oberg, K., Ruysschaert, J., Goormaghtigh, E., (2004). Eur. J. Biochem., 271, p2937.
56. Xie, M., and Liu, Y., (2003). Chem. J. Chin. Univ., 24, p226.
57. Parker, S., (1983): Applications of infrared, raman, and resonance raman spectroscopy in biochemistry, Plenum Press, New York.
58. Holzbaur, I., English, A., Ismail, A., (1996). Biochemistry 35, p5488.
59. Zhang, W., Li, A., (1999): Medical Chemistry, Higher Education Press.
60. Beauchemin, R., *et al*. (2007). Biomacromolecules 8, p3177.
61. Ha, J., Ha, C., Chaob, J., Petersena, C., Theriault, A., Bhagavana, N., (2003). Biochimica et Biophysica Acta, 1640, p119.
62. L. K. Amundsen, and H. Siren, Determination of association constants between steroid compounds and albumins by partial-filling ACE, ELECTROPHORESIS, Volume 28, Issue 20, pages 3737–3744, No. 20 October 2007.
63. Kragh-Hansen, U., (1981). Pharmacol. Rev., 33, p17.

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