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Thesis Approval

Study of the Interactions of Vitamins E and D with HSA using UV-VIS and FT-IR Spectrometers

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Abstract

The distribution and metabolism of many biologically active compounds in the body whether vitamins or drugs or natural products are correlated with their affinities toward serum albumin. Thus, the study of the interactions of such compounds with albumin is of imperative and fundamental importance. In this study the interactions of hydrophobic vitamins (E and D) with human serum albumin at physiological pH has been studied using UV-VIS spectrophotometer, and FT-IR spectrometer. The present work deals with the interactions of hydrophobic vitamins (E and D) with HSA using UV-absorption, and Fourier transforms infrared (FT-IR) spectroscopy. The binding constants of Vit.E and Vit.D have been determined by UV-absorption. The values of the binding constants with HSA are calculated at room temperature to be: ($1.21\times10^{2}M^{-1}$) and ($6.8\times10^{1}M^{-1}$) for vitamins (E and D) respectively. FT-IR spectroscopy with Fourier self- deconvolution technique and second derivative resolution enhancement procedures were applied in the analysis of the amide I,II, and 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 determined and any changes due to concentration changes of the vitamins have been investigated. The FT-IR spectra measurements indicate a change in the intensity of absorption bands due to change in the concentrations of the two vitamins. 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.

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1. Introduction

1.1 Food

Our food contains a number of food constituents to meet the requirements of our body. These constituents must be digested to be utilized by our body. The process by which organisms obtain and utilize food for their growth, development and maintenance is called nutrition and the chemicals present in the food are called nutrients. On the other hand, digestion is the breaking down of complex constituents of food by enzymes into simpler soluble forms that can be absorbed and utilized by the cells of the body. There are six nutrients that are considered essential to life: Carbohydrates, Lipids, Nucleic acids-DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), Proteins, Minerals, and vitamins (Mann and Truswell, 2002).

Carbohydrates are substances having the empirical formula $C_x(H_2O)_y$, e.g $C_6(H_2O)_6$=glucose . The basic building block of carbohydrates is a monosaccharide. Digestion converts the larger carbohydrates to monosaccharide's, which can be absorbed into the bloodstream. They provide the largest single source of energy in the diet. Lipids are a group of compounds that dissolve in organic solvents such as petrol or chloroform, but are usually insoluble in water. Most lipids are either, oils which are liquid at room temperature or fats which are solid at room temperature. A molecule of fat consists of three fatty acids, bonded to a glycerol. They yield more energy when oxidized, so they can be major sources of energy. Nucleotides are the monomeric building block of the nucleic acids, DNA and RNA. Each nucleotide consists of a heterocyclic nitrogenous base, a sugar, and phosphate. DNA contains the purine bases adenine (A) and guanine (G) and the pyramiding bases cytosine (C) and thymine (T). RNA contains A,

G, and C, but it has uracil (U) instead of thymine. In DNA, the sugar is deoxyribose, whereas in RNA it is ribose. DNA and RNA serve as the genetic material for cells. Proteins are composed of amino acids that are joined to form linear chains. They are basis of many animal body structures such as muscles, skin, and hair. They also form the enzymes which catalyze chemical reactions throughout the body. Minerals are chemical elements required by living organisms. They are divided into electrolytes which are ions and minerals, such as Sodium (Na^+), potassium (K^+), and chloride (Cl^-). They establish ion gradients across membranes, maintain water balance, and neutralize positive and negative charges on proteins and other molecules. (Mann and Truswell, 2002; Smith et al., 2005).

1.2 Vitamins

Vitamins are 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 absorbed along with ingested dietary fats by the small intestines. They cannot be absorbed unless they are ingested with some fat. Water-soluble vitamins, by contrast, are absorbed along with water in the gastrointestinal tract and dissolve in the body fluids. 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; Smith et al., 2005).

1.2.1 Water-soluble vitamins:

Water soluble vitamins are hydrophilic; they are divided into B-complex vitamins and C-vitamin. Table 1.1 represents water soluble vitamins.

Table 1.1: water soluble vitamins (Mann and Truswell, 2002)

Vitamin	Function	Results of Deficiency	Sources
THIAMIN (B-1)	Coenzyme in carbohydrate metabolism; normal function of heart, nerves, and muscle.	anorexia, constipation, peripheral neuritis	meat, whole grains, nuts, peas.
RIBOFLAVIN (B-2)	growth and vigor; coenzyme in protein and energy metabolism	cracks at corners of mouth, glossitis, eye irritation and sensitivity to light, skin eruptions	Milk, liver, enriched cereals.
NIACIN (nicotinic acid)	Coenzyme in energy production; normal growth, health of skin, normal activity of stomach, and nervous system	Pellagra; weakness, lack of energy, and loss of appetite.	Meat, peanuts, enriched grains.
PYRIDOXINE (B-3)	Coenzyme in amino acid metabolism; protein synthesis	anemia, convulsions	Wheat, corn, meat, liver.
PANTOTHENIC ACID	Coenzyme in formation of fat, cholesterol.	Unlikely because of widespread occurrence and intestinal baceria synthesis	Liver, green leafy vegetables, asparagus, eggs
Folic Acid B-12	Growth and development of red blood cells	Pernicious anemia (B-12 is a necessary extrinsic factor that combines with intrinsic factor of gastric secretions for absorption)	Liver, meats, milk, eggs, cheese.
VITAMIN C (ascorbic acid)	Intercellular cement substance; firm capillary walls and collagen formation; helps prepare iron for absorption and release to tissues for red blood cell formation.	Scurvy, sore gums, hemorrhages, especially around bones,	Citrus fruits, tomatoes, cabbage, potatoes, melons.

1.2.2 Fat-soluble vitamins:

Hydrophobic vitamins are fat soluble vitamins that are transported in human plasma and extravascular fluids by carrier proteins (Jorkovic et al., 2005).Vitamin A is Necessary for the vision cycle process - adaption to light and dark; tissue growth, especially skin and mucous membranes; toxic in large amounts. Deficiency causes loss of vision, defects in bone growth, defects in reproduction, and lowered resistance to disease and infection. The most abundant forms which are found in use in the body are retinol, retinal, and retinoic acid. These forms of vitamin A will bind easily to other compounds. Transported forms commonly found in the corporal circulation are retinol bound to a binding protein and esters such as retinyl palmitate and retinyl acetate. Esterification makes vitamin A more stable. For that reason, synthetic or animal sources of vitamin A typically are in the form of esters. Figure 1.1 shows Structure of vitamin A palmitate. Naturally occurring vitamin A can be found in fish oils, milk, and liver (N'soukpo e ′-Kossi et al., 2007).

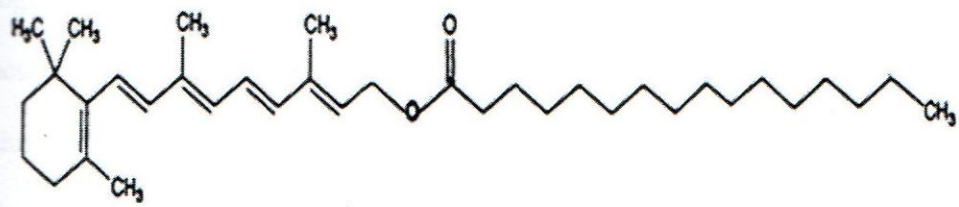

Figure 1.1 Structure of vitamin A palmitate(after Truswell, 2003).

The name 'vitamin K' was proposed by Henrik Dam of Denmark in 1935. K was the next letter of the alphabet not already used for a vitamin at that time. It is also the first letter of the German word *Koagulation*, which refers to its best known function. There are three forms of vitamin K; vitamin K_1, vitamin K_2 , vitamin K_3. In vitamin K deficiency there is a bleeding disorder, characterized by low plasma prothrombin activity (hypoprothrombinaemia). Vitamin K deficiency can occur in obstetric pediatric practice, in surgical patients and in medical

patients. Vitamin K_1 is present in dark-green leaves eaten as food. Other good sources are some vegetable oils. Small amounts are present in beef liver, apples and green tea, and there is some vitamin K2, in cheese (Pedersen-Bjergaard et al., 1999).

Vitamin D is a precursor of a renal steroid hormone, 1, 25-dihydroxyvitamin D (Hadad et al., 1993). 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 (Fraser, 1983). Figure 1.2 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 (Christensen et al., 2003).

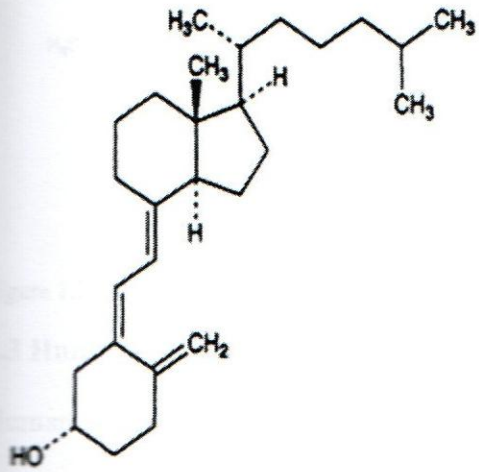

Figure 1.2 Structure cholecalciferol (D3) (after Truswell, 2003).

Vitamin E was discovered by Evans and Bishop as a fat soluble factor necessary for normal reproduction in rats (Jorkovic et al, 2005). Vitamin E is a powerful antioxidant which plays an essential role in protecting cell membranes and plasma lipoproteins from free radical damage (Mann and Truswell, 2002). 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 (Beharka, et al., 1997). 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 diet. The figure 1.3 show 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.

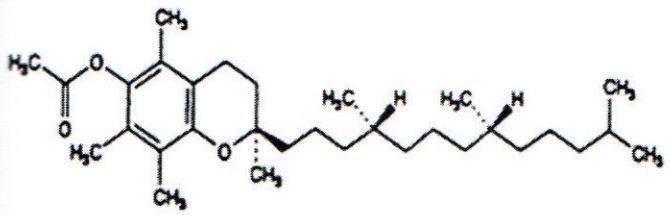

Figure 1.3 Structure of vitamin E acetate (after Truswell, 2003).

1.3 Human serum albumin (HSA)

Human serum albumin (HSA) is the most abundant protein in human 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., 2010). It is a globular protein consisting of a single peptide chain of 585 amino acids. This protein composed of three structurally similar domains (labeled as I, II, III). Each containing two sub domains (A & B) having six and four α-helices, respectively. See Figure (1.4).

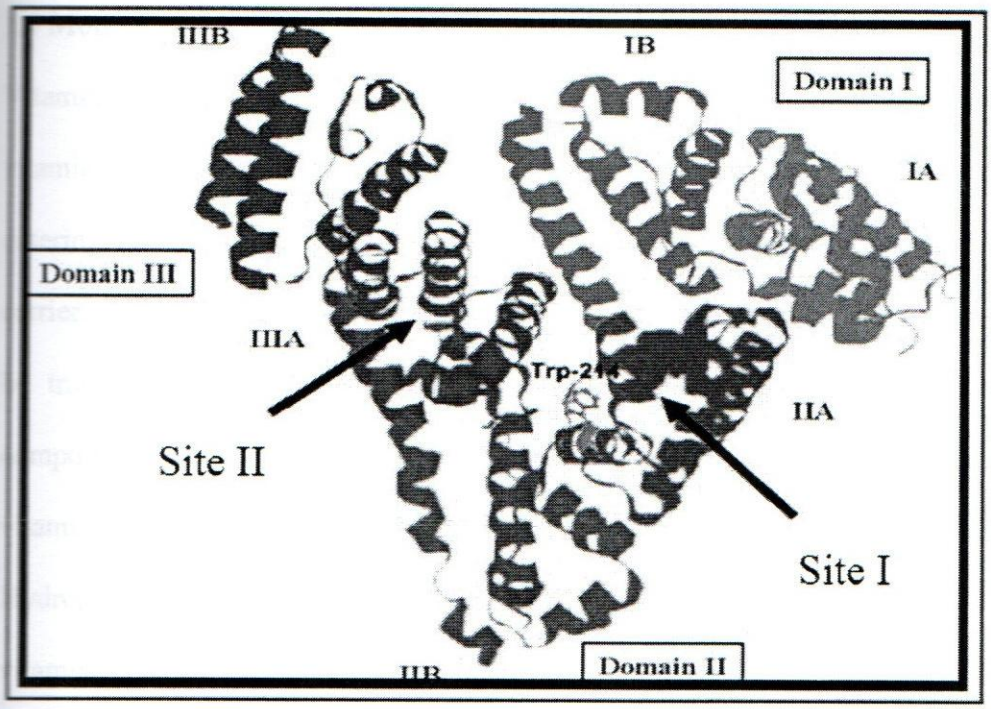

Figure 1.4 Chemical structure of human serum albumin (after Abou-Zied, 2009).

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 (Kragh-Hansen et al., 1981). Hisaeda and co-workers studied the interactions of Vitamin B12 and its hydrophobic ester derivatives with HSA (Hisaeda et al., 2006). However, the binding affinity and the change of the conformation of human serum albumin when interacting with B12 have not yet been systematically investigated. Also multiple drug binding sites have been reported for HSA by several researchers (Na Hou et al., 2008).

الملخص

إن توزيع عمليات الأيض لكثير من العناصر البيولوجية في الجسم سواء كانت أمصال أو منتجات طبيعية، مرتبطة بقدرة هذه العناصر على الارتباط مع مصل بروتين الدم البشريالبومان (HSA). إنّ دراسة ارتباط مثل هذه الجزيئات مع بروتين الدم البشري له أهمية أساسيةفي هذه الدراسة, حيث تم دراسة تأثير الفيتامينات (Eو D) على بروتين الدم البشري عند درجة الحموضة الفسيولوجية (7.3)، باستخدام جهاز مطياف الأشعة فوق البنفسجية (UV- VIS Spectrophotometer) وجهاز مطياف تحويل فورييز للأشعة تحت الحمراء (FT-IR). أظهرت النتائج أن شدة امتصاص الاشعة فوق البنفسجية لبروتين الدم تزداد بزيادة نسب فيتامين E أو فيتامين Dمع ثبات تركيز البروتين. ومن طيف الاشعة فوق البنفسجية تم حساب ثابت الترابط (Binding Constant) والقيم التي قيست هي ($1.21\times10^{2}M^{-1}$) للفيتامين E و ($6.8\times10^{1}M^{-1}$)للفيتامين D.باستخدام جهاز مطياف تحويل فورييز للأشعة تحت الحمراء مع تطبيق تقنية (Fourier Self-deconvolution) و (Second Derivative resolution) بالاضافة لتقنية (Curve fitting) تم تحليل مناطق أميد1 و أميد2 و أميد3 في بروتين الدم البشري لتحديد بنية البروتين في المنسوب الثاني (Secondary Structure) والية ارتباط الفيتامينات مع بروتين الدم، لقد لوحظ أنه مع زيادة تراكيز فيتامين E أو فيتامين D إلى بروتين الدم فإن شدة حزم الامتصاص IR (Absorption bands) تقل. أيضا جميع مواقع القمم (Peaks) تم تحديدها عند عدة نسب من الفيتامين E أو الفيتامين D بالمقارنة مع بروتين الدم البشري. إضافة إلى ذلك أظهر الطيف المقاس بواسطة جهاز مطياف تحويل فورييز للأشعة تحت الحمراء أن البنية الثانوية لبروتين الدم البشري، تغيرت نتيجة إضافة نسب من الفيتامين E أو الفيتامين D، الذي ظهر في انخفاض في شدة حزم الامتصاص الممثلة ل (α-helix) بالمقارنة مع حزم الامتصاص الممثلة ل (β- sheets)، هذا التباين و الاختلاف في الشدة يرجع الى تكوين الروابط الهيدروجينية (H-bonding) في الجزيئات المعقدة، وهو ما يفسر الميول الذاتي لكل من (α-helix)و (β- sheets) لتكوين هذه الروابط.

5. Conclusions and future work

The interaction of Vitamin E with HSA is similar to the interaction of vitamin D with HSA(Ha, et al., 2003), and the affinity of vitamins to HSA is low (Amundsen etal., 2007); which is obviously seen in our results with a low binding constants was get 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 pre nce of vitamin D there is less side interaction, this is in line with the reported results that vitamin E binds to HSA in sub domains IIA and IIIA (Ha et al., 2003), and vitamin D have one binding site located in domain II (Kragh-Hansen, U.1981).The interactions between hydrophobic vitamins and human serum albumin (HSA), it is of great interest and important from pharmaceutical point of view. In my work the interaction of vitamin E and vitamin D with HSA was investigated by means of UV-VIS spectrophotometer, and FT-IR spectroscopy. I have determined the parameters for binding of vitamin E or vitamin D with HSA. Were Vit.E-HSA and Vit.D- HSA binding constants by using UV-absorption spectroscopy are estimated to be K= $(1.21\times10^{2}M^{-1})$, and $(6.8\times10^{1}M^{-1})$respectively, The experimental results indicates a low binding affinity between Vit.E or Vit.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 Vit.E or Vit.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 Vit.E and Vit.D vitamins 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 Vit.E or Vit.D and 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 (Kragh-Hansen, U.1981).