

Vitamin D Level in Patients with Bone Diseases and Dental Caries

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Abstract
VITAMIN D, it is a steroid (steroids are group of substances derived from perhydrocyclopentano phenanthrene nucleus) prohormone with antirachitic properties, also known as cholecalciferol, calciferol. calcipotriol ,ergocalciferol, and vitamin D**2.** HPLC is a new come in the field of liquid chromatography. It is probably the single most used analytical technique today. HPLC system is unique among laboratory instruments because it can be assembled using components from different manufacturers and suppliers. A wide variety of tissues and cells, including the brain, gonads, breast, skin, mononuclear cells, and activated B and T lymphocytes, possess VDR. The first insight into biological action of (1,25 (OH)2 D) in non calcemic tissues was the observation that promyelocytic leukemic cells with a VDR transformed into mature, biochemical functioning macrophages after being treated with (1 , 25 (OH)2 D)(35) During 5 months (March, April, May, June and , August) 60 blood samples (29 males and 31 females) were collected from healthy volunteers and simultaneously 92 blood samples (33 males and 59 females) were collected from patients attending to AL – Hakims General Hospital, AL- Zahra Hospital , and some other private clinic in AL- Najaf city. All of the patients are from Al- Najaf city, Iraqi people, they lived in this society with poor to middle life style and the income of these families approximately at the same.The study primarily was intended to adapt a method for measuring vitamin D levels in serum samples in order to make reliable evaluation of this vitamin in Iraqi population (Al-Najaf governorate). The HPLC method was the method of choice in this field .The reverse phase HPLC method has a very high sensitivity and needs only a very small amount of sample without any complicated pretreatment for the used serum which may be a major source of errors. The mean of vitamin D levels in both sexes and in different seasons was 19.21ng / ml the other related statistical parameters and serum calcium level.
Keywords: Vitamin D, Calcium, HPLC, Bone disease, Rickets, Seasonal change, Osteomalasia, Osteoporosis, Dental caries, Postmenopausal.

Introduction:

1-High Performance Liquid Chromatography (HPLC).

HPLC is a new come in the field of liquid chromatography. It is probably the single most used analytical technique today (1). HPLC system is unique among laboratory instruments because it can be assembled using components from different manufacturers and suppliers. Although many systems are sold as complete packages, greater number are assembled by bench level and customized for specific needs (2) HPLC is especially suited to analysis of variety of compounds for example , thermally labile compounds can be analyzed by HPLC at ambient temperatures and highly polar or in volatile compounds can be analyzed too . Sample treatment is often minimal, since aqueous solutions can be used in HPLC. Preparative HPLC has also found an important use in the isolation and purification of various compounds(3) The main components of HPLC can be summarized as follow (4) .

A- Pumps. (May be up to 3 units).
B- Injector
C- Column.
D- Detector.
E- Fraction collector
F- System-controller.
G- Controller as computer system.

Accessory Equipment:
1- Two-position rotary valves FCP
2- Solenoid valve unit
3-Helium degasser
4- Syringe unit

1- 2- VITAMIN D.

It is a steroid (steroids are group of substances derived from perhydrocyclopentano phenanthrene nucleus) prohormone with antirachitic properties, also known as cholecalciferol, calciferol, calcipotriol ,ergocalciferol,and (vitamin D**2)**(7).

1-2-1-The Role of Vitamin D.

The most important roles of vitamin D are:

A- The maintaining of normal calcium levels in blood, which accomplishes it by increasing absorption of calcium from food and reducing urinary calcium loss. Both effects keep calcium in the body and therefore spare the calcium that stored in bones (7). When necessary, Vitamin D mediates the transport of calcium from the bone in to the blood stream, which does not benefit bones

B- Although the overall effect of vitamin D on the bone is complicated, vitamin D is necessary for healthy bones and teeth (7)

C- The relations between calcium absorption and vitamin D are similar to that of a locked door and key. Vitamin D is the key that unlocks the door and allows calcium to leave the intestine and enter the blood stream (8)

D- Vitamin D also works in the kidneys to help resort calcium that otherwise would be excreted (8).

E- Vitamin D is maintaining serum calcium and phosphorus levels within the normal range to sustain a wide variety of metabolic and physiological functions, including maintaining bone health and normal neuromuscular function (9)

F-Adequate vitamin D is required to regulate calcium absorption in times of increased requirement (growth, pregnancy) and when dietary calcium intake is low (8)

G-Vitamin D also appears to have other roles within the body, such as enhancing immune function (10), blood cell formation, also helps cells to "differentiate" a process that may reduce the risk of cancer (11). From animal and human studies, researchers have hypothesized that vitamin D may protect people from multiple sclerosis (7), auto immune arthritis, and juvenile diabetes (11).

1-2-2- Sources of Vitamin D

Vitamin D comes from food, but sun light is essential to convert it to active form. Naturally occurring vitamin D is rare in foods. Vitamin D is used to refer to either vitamin D**2** or vitamin D**3**, both of which can be converted to active vitamin D metabolites (12). Vitamin D**2** originates from the yeast and plant sterol ergosterol, whereas vitamin D**3** originates from 7-DHC (7- Dehydrocholesterol) in animals (12). The major structural difference between vitamin D**2** and vitamin D**3** is in the side chain. Unlike vitamin D**3**, vitamin D**2** has a double bond between carbon number 22and 23 and a methyl group at carbon number 24 (12). The major natural sources of vitamin D are fatty fish like mackerel salmon and fish oils, including cod and tuna liver oils. The major dietary sources of vitamin D are foods that fortified with vitamin D

1-2-3- Fortification of Foods.

Fortification with vitamin D for different foods in USA may be mandatory (specific foods must contain a certain level of vitamin D), or voluntary (manufacturers are permitted to add vitamins to foods, but are not obliged to do so).There are many manufacturers choose to fortify foods with vitamin D depending on the amount of food that taken by the population

1-2-4-Photosynthesis of Vitamin D3 in Human.

7- Dehydrocholesterol (7- DHC, Previtamin D3), the immediate precursor of cholesterol, is present in the viable epidermis and dermis. During exposure to sun light,7-DHC (Dehydrocholesterol) absorbs sun light (Ultraviolet radiation, UVB), with wavelength of about 290 and 315 nm. This process causes transformation of 7-DHC to Previtamin D**3** as in (12). After previtamin D**3** is formed, it undergoes an internal transformation of its double bonds that is stimulated by the body's temperature to form vitamin D3 over a period of a few hours. As a previtamin D3 is converted to vitamin D3, its three-dimensional structure changes, facilitating vitamin D3 translocation from the skin cells into blood stream. In the blood stream, the vitamin is bond to a specific vitamin D-binding protein commonly known as an Alpha-globulin (12).

1-2-4-1- Sun light mediated regulation of vitamin D3 synthesis in the skin.

It is not possible to make an intoxicating amount of vitamin D3 in the skin, due to prolonged exposure to sun light. The reason is that once previtamin D**3** is photosynthesized in the skin, it can either be converted to vitamin D3 or may be degraded in to biologically inert photoproducts known as lumisterol and tachysterol (12)

1-2-4-2- Effect of melanin pigmentation on the cutaneous production of previtamin D3.

Loomis in 1967 (16) popularized that the melanin pigmentation in humans evolved to protect people who lived at or near the equator from producing excessive, intoxicating amount of vitamin D**3**.The results also speculated that as peoples migrated north or south of the equator, they lost their skin pigmentation in order to promote an adequate amount of vitamin D3 in their skin to protect their bones from developing rickets and osteomalacia. Melanin is an excellent sun screen that absorbs the ultraviolet radiation from sun light. Therefore, melanin competes with 7- DHC in the skin for the UVB photons. As a result, increased skin pigmentation decreases the production of previtamin D**3** in the skin.

1-2-4-3-Environmental effects on the production of vitamin D3.

Sunlight is the energy source of most life forces, which affected the human health. Time of the day, season of the year, and latitude have dramatic effects on the amount of solar UVB radiation that reaches the earth surface. In winter, the UVB photons that produce vitamin D pass through the ozone layer at an oblique angle and are absorbed by the ozone in great numbers. More UVB photons are able to penetrate the ozone layer in the spring, summer, and full months because the sun is directly overhead (18).

1-2-4-4- Effect of aging on the cutaneous production of vitamin D3.

Aging affects many different metabolic processes. Therefore, it is not surprising that aging also decreases the ability of human skin to produce vitamin D**3**. Aging decreases the concentration of 7-DHC in the epidermis and thereby reduces skin production of vitamin D**3** by approximately 75% in the age of 70 years if compared with younger adults (19).

1-2-4-5- Effect of sunscreen use and cloth on the cutaneous production of vitamin D3.

The chronic excessive exposure to sunlight can increase the risk of skin cancer and cause photo aging of the skin. This has led to general recommendation, depending on people races that to avoid sunburn, they should always wear a sunscreen before going out doors (20).

1-2-5- Vitamin D metabolism and biosynthesis.

Vitamin D2 and vitamin D3 that are used to fortify foods are ingested, mixed with other lipids, taken up by entrocytes, and incorporated into chylomicrons. Chylomicrons are released by the entrocytes and enter the lymphatic system, which drain into the venous blood stream. Ultimately, this vitamin (in chylomicrons remnants) reaches the liver, where it is hydroxylated and again enters the circulation bound to vitamin D-binding protein (21). Vitamin D3 that is synthesized in the skin enters the circulation and then bound to the vitamin D-binding protein. Both vitamin D2 and vitamin D3 in the circulation are taken up by the liver and metabolized to 25-hydroxy vitamin D (25 (OH)D) and to 1,25-dihydroxy vitamin D (1,25 (OH)2 D)(21). The first step of hydroxylation is conversion of vitamin D to 25(OH) D. This hydroxylation is catalyzed by specific microsomal and mitochondrial enzyme, designated as vitamin D–25 hydroxylase (25(OH)ase)(23). 25-Hydroxy vitamin D is transported in blood tightly bound by DBP. The half-life of circulating (25(OH) D) is 2to3 weeks. At physiological concentration (10-50 ng /ml), (25(OH) D) is biologically inactive. The active form of vitamin D is (1, 25(OH) 2D), which is produced by the 25hydroxyvitamin D-1-hydroxylase (2). This enzyme is a mitochondrial cytochrome P mixed-function oxidase requiring both NADPH and molecular oxygen for its activity. The kidneys are the principle site (if not the only physiologically significant site) for the synthesis of this enzyme except during pregnancy, when the placenta produces (1,25(OH)2 D)(23) .

1-2-5-1- Regulation of Vitamin D in Blood.

Circulating concentration of 1, 25 (OH)2 D are tightly regulated primarily by PTH and phosphate levels (24,25,26,27,28) . PTH increases (1,25(OH) 2D) concentration by increasing its synthesis by induction of the enzyme 1hydroxylase. Phosphate restriction and hypophosphatemia also increase (1, 25(OH)2 D) by inducting 1 – hydroxylase . Whereas phosphate supplementation and hyperphosphatemia have the opposite effect . Hypocalcaemia increases (1, 25(OH)2 D) indirectly by stimulating the secretion of PTH(27) .

1-2-5-2- Transport of vitamin D in blood.

Vitamin D and its metabolites that are produced in skin or absorbed in the intestine from diet circulating in the blood in association with vitamin D-binding protein, a specific, high- affinity transport protein also known as group-specific component (25,26,28). The structure of this protein has been determined in human. Vitamin D binding protein contains 458 amino acid residues, with a molecular weight of 51.335 kDa as deduced from its amino acid sequences. DBP is in great excess, with fewer than 5% of binding sites normally occupied. Only 0.03% of (25(OH) D) and 0.4% of (1, 25(OH)2 D) is normally free in plasma. DBP concentrations are increased in pregnancy and with estrogen therapy and decreased in nephrotic syndrome. The lesser amounts of vitamin D and its metabolites are associated with albumin, the more hydrophobic parent compound also associated with lipoprotein (24,25,26,27,28) .

1.2.6- Biological functions of 1, 25-dihydroxy vitamin D.

The major biological functions of vitamin D that are essential for the normal development of teeth, help to maintain blood levels of calcium and phosphate, help to regulate the heart through calcium absorption, help to build more bone mass and prevent bone loss, maintain good health and vitality , necessary for calcium and phosphorus utilization and absorption from the small intestine, prevent and cure infantile rickets, promote strong teeth, and protect against muscle weakness(27), also to maintain calcium homeostasis in order to maintain cellular metabolic processes and neuromuscular functions. The principal biological function of (1,25(OH)2 D) is to increase the efficiency of intestine to absorb calcium. (1,25(OH)2 D)directly affects the entry of calcium through the plasma membrane of the intestinal absorptive cell, thereby enhancing the movement of calcium through membrane of the entrocytes into the circulation(9).

1.2.7- Molecular biology of vitamin D.

Vitamin D is lipophilic, as its active form, therefore, the mechanism of action of (1,25 (OH)2 D) is similar to the action of the small hydrophobic hormones that act via nuclear receptors, such as retinoic acid, thyroid hormones, estrogen, and glucocorticoids All target tissues for vitamin D contain a nuclear receptor for (1,25(OH)2 D), known as vitamin D receptors, (VDR), the VDR recognizes (1,25(OH)2 D) 1000 times more than that recognizes (25(OH) D)

1.2.8-Pharmacological functions of 1, 25 (OH)2 D in non-calcemic tissues.

A wide variety of tissues and cells, including the brain, gonads, breast, skin, mononuclear cells, and activated B and T lymphocytes, possess VDR. The first insight into biological action of (1,25 (OH)2 D) in non calcemic tissues was the observation that promyelocytic leukemic cells with a VDR transformed into mature, biochemically functioning macrophages after being treated with (1 , 25 (OH)2 D)(35) .

1-2-9- Vitamin D in Bone Health.

Vitamin D plays a critical role in mineralization of the bone, and bone disorders result from inadequate circulating levels of the biologically active metabolite of vitamin D. Vitamin D acts to maintain the plasma calcium and phosphate concentrations so that skeletal mineralization occurs normally (21). Vitamin D deficiency exists when the concentration of (25 (OH) D) in the blood serum occurs at 12ng/ml or less. The normal concentration of 25(OH) vitamin D in the blood serum is 25-50ng/ ml(39). When vitamin D deficiency continues for many months in growing children, the disease commonly referred to as rickets will occur. A prolonged deficiency of vitamin D in adults results in osteomalacia. Both diseases involve defects in bones. Vitamin D deficiency, as well as rickets and osteomalacia, tends to occur in persons who do not get enough sunlight and who fail to eat food that are rich in vitamin D (40) .

1-2-10- Vitamin D deficiency and dental problems.

Teeth are valued not least for their contribution to appearance and social acceptability. The costs of treatment of oral diseases, though, are high. The most important oral diseases are defects in the structure and appearance of teeth, dental caries, dental erosion, enamel hypoplasia, periodontal disease, and oral cancer(42). There is a growing realization of the detrimental impact of dental impairment on food choice, nutrient intake and nutritional status(42). Dental caries causes infection and pain and is, thus, of medical, social and economic importance. It is very much related and affected by diet(42). The important role of vitamin D and plasma calcium levels in the etiology and prevention of enamel hypoplasia was illustrated by the results of randomized clinical trial (49). Especially in pregnant women at the twelfth week of pregnancy, mothers, who received the supplement, and their infants, had higher plasma concentration of calcium. Nutritional deficiencies also clearly cause defects in enamel formation, probably through a variety of mechanisms. It would seem that calcium plasma level is important, while hypocalcaemia is a clear risk factor(50). .

1-2-11- Vitamin D deficiency and rickets.

The most bone disease that affects children due to vitamin D deficiency is rickets. It causes progressive softening and weakening of the bones' structure. There is a loss of calcium and phosphate from the bones, which eventually causes destruction of the supportive matrix. It is most likely to occur during periods of rapid growth, when the body demands high levels of calcium and phosphate (22) .Rickets may be seen in young children (6 to24 months) old and is uncommon in new borns. Rickets that caused by dietary lack of the minerals is rare in developed countries because calcium and phosphate are present in milk and green vegetables (14) .

1-2-12-Vitamin D Deficiency and Osteoporosis.

Osteoporosis is a very significant metabolic bone disease, specially in elderly people, they have a mineralization defect in the skeleton that results in poor mineralization of the collagen matrix (osteoid).This mineralization defect can lead to increased risk of skeletal fracture & dental defect as increased of dental caries (41). As a consequence of vitamin D deficiency, the efficiency of intestinal calcium absorption decreases from the usual 30% or 50% to no more than 15%. This result in a decrease in the ionized calcium concentration in the blood, which signals the calcium sensor in the parathyroid glands to increase the production & secretion of parathyroid hormone (PTH) (12) .PTH, in turn, tries to conserve calcium in kidney by increasing renal tubular reabsorbtion of calcium. With vitamin D, PTH helps to mobilize monocyte like stem cells to become active bone calcium resorbing multinucleated grant cells known as osteoclasts, which can cause erosion of the skeleton causing or exacerbating osteoporosis.

1-2-13- Vitamin D deficiency and post- menopausal osteoporosis.

Vitamin D plays a good role in the preventing fractures of the axial and appendicular skeleton in elderly women with postmenopausal osteoporosis. Postmenopausal osteoporosis, is gradual loss of bone mass, it is a complex chronic, multifactorial process, and apparently, an inevitable accompaniment to normal ageing. Both sexes are affected, but the women are mainly affected by the disease (54). Elderly people are more prone to develop a vitamin D deficiency, which may cause osteomalacia, exacerbated osteoporosis, and increases the risk of fractures. Vitamin D deficiency is a "silent epidemic" because it is fairly wide spread but, usually goes untreated and undetected.

1-2-14- Vitamin D Toxicity.

Vitamin D toxicity is inevitably the result of over dosing of vitamin D supplements. Excessive exposure to sunlight does not lead to over production of vitamin D (40). An excess of vitamin D increases the absorption of calcium which leads to enhance calcium level in the blood. This causes calcium precipitation in the soft tissues and produce calcium stones. Excess calcium levels also cause calcification or hardening of the blood vessels, thus endangering the arteries of the heart and lungs which may ultimately results in death

1-2-15- Optimal level of vitamin D.

Perhaps the most important reason to keep vitamin D levels above 40 ng /ml is naturalistic. Therefore, the normal human vitamin D level is about 40 ng /ml (61). So that the proposed optimal levels of vitamin D are between 40- 65 ng /ml, these levels are high enough to reduce the risk of the disease (61). Vitamin D level should never be below 32 ng/ml. Any levels below 20 ng / ml are considered serious deficiency state and will increase risk of breast and prostate cancer and autoimmune diseases like rheumatoid arthritis and also dental problems. The above values are the newest ones from the clinical research (59).

1-3-Calcium.

Is the fifth most common element and most prevalent cation found in the body? An average human body contains approximately 1kg of calcium. It is found in three compartments, skeleton, soft tissues and extracellular fluid (60, 61). The skeleton contains 99% of the body's calcium. Predominantly as extra cellular crystals of unknown structure with a composition approaching that of hydroxyapatite (Ca10 (PO4)6 (OH)2). Soft tissues and extracellular fluid contain about 1% of the body's calcium (62). In blood, virtually all of the calcium is in the plasma, which has a mean normal calcium content of approximately 9.5 mg/dl. Calcium exists in three physiochemical states in plasma, of which approximately 50% is free or ionized, 40% is bounded to plasma proteins, and 10% is complexed with small anions (63). Free or ionized calcium fraction is the biologically active form. Its concentration in plasma is tightly regulated by the calcium regulating hormones. PTH and 1,25-dihydroxy vitamin D.

1-3-1-Dietary calcium and health.

The inadequate dietary calcium has been associated with a number of common chronic medical disorders, which include osteoporosis, osteoarthritis, cardiovascular disease (hypertension and stroke), diabetes mellitus, dyslipidemias, hypertensive disorders of pregnancy, obesity and cancer. Previously it was thought that there is no relation between dietary calcium and medical disorders. Recent findings, indicates that there is a relation between calcium intake and medical disorders. The identification and isolation of cell surface calcium receptor has provide a theoretical link between changes in extracellular calcium concentration and cell function (68).

1-3-2-Calcium requirements through Life.

Calcium is a major component of mineralized tissues and is required for normal growth and skeletal development. Optimal calcium intake is important, to maximize the peak of adult bone mass, its maintenance, and to minimize bone loss among the elderly (69). All these will reduce the risk of osteoporosis in the society. Calcium requirements vary throughout an individual's life time.

1-3-3- Pathological aspect in calcium absorption.

Calcium absorption is central to the body's calcium and bone homeostasis and accounts for more of the variance on balance than does calcium intake. The absorption process comprises saturable, active and passive, diffusion component (70).

1-3-4-Calcium absorption in postmenopausal.

It is known that estrogen therapy improves calcium malabsorption induced by surgical or natural menopause, but the mechanisms involved are still under debate. It was reported previously that calcium absorption raises on postmenopausal estrogen replacement. It has been suggested that estrogen may have direct positive effect on the intestinal mucosa cells (72).

1-3-5-Calcium regulation.

The calcium regulating hormones function like a fire bridge when little calcium is being consumed, they are not activated that much, which is good, no fire. When too much calcium is consumed, the calcium hormones are very active, stimulating the absorption of calcium into the bones, and subsequently deportation and excretion. The more this processing is accelerated, the more bones are eroded. So after calcium is absorbed, calcitonin or thyrocalcitonin, inhibits deportation of calcium from the bones, while the calcium automatically keeps pouring in. Parathyroid hormone [PTH], stimulates uptake of calcium into the bone, (73) and therefore osteoblast apoptosis,(74) and deportation of calcium from the bones (75) and inhibition of calcium excretion, generally increasing a low blood calcium level. Logically, elevated PTH level accelerates ageing of the bones, low level of PTH prevent bone loss (76). PTH also stimulates secretion of calcitroil (1, 25 dihydroxycholecalciferol) vitamin D. Estrogens are multifunctional hormones, and one of their functions involves the bones. The calcium hormones mentioned above induce circulation of calcium from blood into the bones and vice versa. Estrogens are the brakes on this system to minimize erosion. Calcium is absorbed into the bones due to osteoblasts, which increase free phosphate level in the bones, which causes the passive influx of calcium to restore the calcium – phosphate ratio. Estrogen does not stimulate osteoblasts, (82) but even inhibits osteoblast activity (83) and therefore, inhibits calcium influx in bones (84) and also inhibits deportation of calcium from the bones.

The Aims of the work.

1- The search was a biochemistry academic search which was primary one in Najaf city by a new technique which was called high performance liquid chromatography (HPLC).
2- To estimate vitamin D levels in normal control group in some Iraqi population in Najaf city.
3- To estimate vitamin D levels in some patients affected by dental caries and some bone diseases such as rickets and osteoporosis.
4- To compute the differences of serum vitamin D levels in relationships with dental caries and some of bone diseases and healthy subjects.
5- To determine the sensitivity of measurement of vitamin D in sera by modified current method of HPLC technique.

EXPERIMENTAL PATIENTS & METHODS:
EXPERIMENTAL
2-1- Patients and Controls.
During 5 months (March, April, May, June and , August) 60 blood samples (29 males and 31 females) were collected from healthy volunteers and simultaneously 92 blood samples (33 males and 59 females) were collected from patients attending to AL – Hakims General Hospital, AL- Zahra Hospital , and some other private clinic in AL- Najaf city. All of the patients are from Al- Najaf city ,Iraqi people, they lived in this society with poor to middle life style and the income of these families approximately at the same. The selected cases were:
A- Dental Caries: Fourty two samples of venous blood were obtained from patients attended at 9:0 o'clock in the morning (13 males and 29 females), ages range between (6-30) years.
B- Rickets: 14 patients (9 males and 5 females) with rickets, they are diagnosed by the x-ray, ages range between (1- 5) years. They had not taken any treatment.
C- Osteoporosis: Thirty blood samples were obtained (11 males and 19 females) from patients attended to the outpatient clinics .
D-Postmenopausal: This group consist of (6) females ages range (50-70) years.

2-2-Materials and Methods:
2-2-1-Instruments and Apparatus.
A- High Performance Liquid Chromatography (HPLC): This apparatus was assembled from different units they are:
1- Pump of liquid chromatography LC-10AT vp Shimaduz Company, Japan.
2- UV-Vis Detector SPD-10AVvp: Shimaduz Company, Japan.
3- System Controller SCL-10Avp: Shimaduz Company, Japan.
4-HPLC-column: MRC-ODS type, Japan.
Accessory Equipment:
1-Flow controller valve, FCV-10AL vp: Shimaduz Company, Japan.
2-Reservoir Tray: Shimaduz Company, Japan.
B-UV-Vis Spectrophotometer, UV-1650 PC, Shimadzu Company, Japan.
C-Vortex Super-Mixer: Lab Line instruments,Inc. Made in U.S.A.
D-Centrifuge Janetzki T5 Dor-7123 Engelsdorf/ Lab G.D.R.
E-Incubator Memmert, Germany.
2-2-2-Chemicals.
A-Acetonitrile for HPLC: From BDH-UK .
B-Methanol Analytical Reagent: Gain Land Chemical Company, U.K.
C-Propan-2-ol: BDH Laboratory Supplies, B H 15 UK.
D- Calcium-Kit: The kit was contained many reagents, purchased from
Biomerieux R Sa Marcy-I Etoil / France.
2-2-3-Blood Pretreatment.
Blood was drawn from the vein, usually from the inside of the elbow or the back of the hand in children. After withdrawing 5 ml of venous blood by a disposable sterilized syringe (Tyco Health care), the blood transferred to plastic (Afma- dispo) anticoagulant free tube, then left for several minutes to clot and centrifuged by the centrifuge for 15 minutes with 1600 xg. Then 3 ml of separated serum was isolated. One milliter was used from this serum to determine 25- hydroxy vitamin D concentration.
2-2-4-Determination of UV Spectrum of Vitamin D.
The computerized shimadzu spectrophotometer was used to determine the most appropriate wave lengths that can be used as spectral parameters for vitamin D determination in the photometric detector in the HPLC. The spectrum wave lengths were scanned for vitamin D absorption peaks in the range between 190 nm and 400nm, which was related to UV visible region (fig. 2-4). Different concentrations of vitamin D standard solutions were

used (25, 50, 100, 200, 100 µg /dl). The selected wave length for vitamin D determination in this study, which was practically the most appropriate one ranges between 256nm - 262nm .

2-2-5-The extraction of 25 – hydroxy vitamin D : The 25- hydroxy vitamin D was extracted from serum by the polar solvent acetonitrile which has been effectively used to free vitamin D metabolites by deproteinization or denaturation of DBP(vitamin D binding protein) (87,88) . One ml of serum to 1 ml of acetonitrile was added , mixed by vortex for 5 minutes and centrifuged for 15 minutes at (1600xg) .

2-2-6-Preparation of HPLC for Direct Measurements.

The HPLC separation works with an isocratic method which mean that the chromatography can be carried out with single pump, at 35◦C with reversible phase column was applied . Chromatograms are detected by the UV-detector. The retention time of 25- hydroxy vitamin D was 30 minutes for each run.

The Conditions for Vitamin D measurement were as follow .

A-Mobile Phase:100 % Acetonitrile .

B-Flow Rate: 1 ml / min .

C- Wave Length : 256nm- 262nm .

D- Column shim pack: MRC- ODS [Octadecylslyle] .

E- Temperature : Room temperature or 35 ◦C.

2-2-7-Vitamin D Measurement by HPLC

1-The HPLC was operated according to the previous parameter illustrated, in section (2-2-6):

2-2-8-Determination of Calcium .

Total calcium in sera was determined by a colorimetric method. Without deproteinization calcium ion reacts with the methylthymol blue indicator [MTB] in an alkaline medium.

Ca + MTB----- Ca-MTB complex . The color intensity of the Ca-MTB complex, was measured at 612 nm, and it was proportional to the quantity of calcium present in the sample.

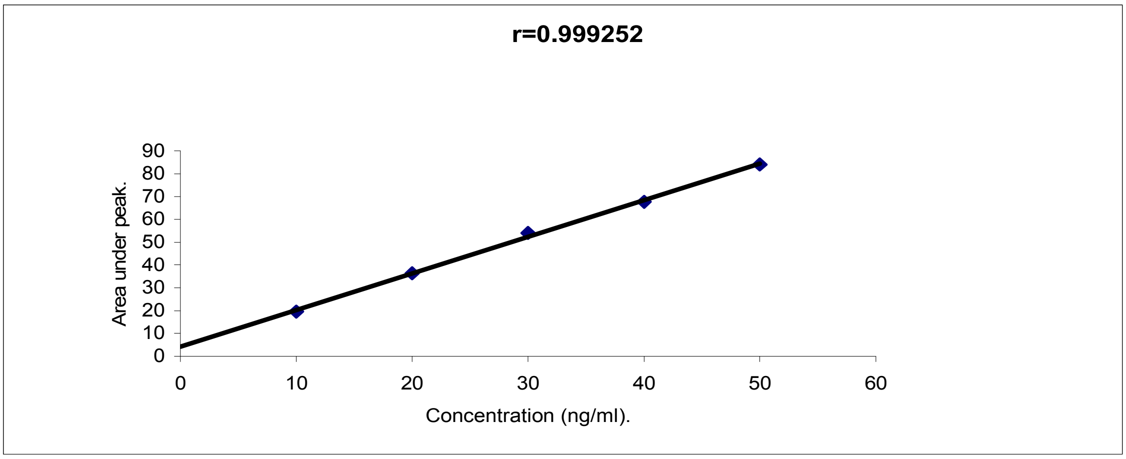

Figure 2-5 Standard curve for vitamin D determination by HPLC
(Area under peak µVx 10000 and concentration ng/ml)

Results and Discussion

3-1-Determination of Vitamin D Levels in Sera.

The study primarily was intended to adapt a method for measuring vitamin D levels in serum samples in order to make reliable evaluation of this vitamin in Iraqi population (Al-Najaf governorate).The HPLC method was the method of choice in this field .The reverse phase HPLC method has a very high sensitivity and needs only a very small amount of sample without any complicated pretreatment.

3-2-Vitamin D Levels in Healthy Individuals:

3-2-1: Normal Value of Vitamin D in Iraqi Population.

The mean of vitamin D levels in both sexes and in different seasons was 19.21ng / ml the other related statistical parameters and serum calcium level are shown in table 3-2. This value in fact can be relatively considered as sub-normal value.

Holick. in 2000 (92) reported that the normal range of serum 25- hydroxy vitamin D level in human is 25-56 ng/ml in both sex.

A recent study from Harvard in USA in June 2004 found that 24% of healthy adolescents had 25-hydroxy vitamin D levels less than 35ng/ ml (86). The slightly decrement in vitamin D level in healthy Iraqi population may be attributed to the malnutrition and unavailability of animal sources nutrition or insufficiency of such types of diet in Iraqi people food due to the previously imposed embargo and low income of these people, where the diet is mainly composed of carbohydrate and fiber components. The vegetarian diet of a major sector of Iraqi people adversely affects 25-hydroxyvitamin D status(56).

Table 3- 2: Vitamin D Level in Sera of Healthy Males and Females.

Group Type		**Vitamin D** ng/ml				**Calcium** mg/dl		
Sex	Age yrs	No.	X	SD	CV%	X	SD	CV%
Male	3-65	29	24.56	4.52	22.47	10.87	2.22	20.45
Female	1-65	31	14.37	1.86	12.93	10.72	2.22	20.70
Total	1-65	60	19.21	5.51	33.91	10.79	2.19	20.32

It is important to mention that low content of vitamin D in the whole meal may contribute in decreasing serum vitamin D levels in Iraqi people, which is related to decrease in socioeconomic or other causes like the genetic factor which is likely to be involved in this phenomenon.

3.2.2- Seasonal Variation in Serum Vitamin D Levels.

The results shown in table 3 reveals, through using statistical function test, that there is a highly significant ($p<0.01$) difference between the two means of 25-hydroxy vitamin D in summer and winter.

Table 3- 3: Vitamin D Level in Serum during Seasonal Change.

Type Group	Summer (March, June, August)				Winter (May, April)				CS
	Vitamin D			Calcium	Vitamin D			Calcium	
	No.	X±SD ng /ml	CV%	X±SD mg/dl	No.	X±SD ng/ml	CV%	X±SD mg/dl	
Male	10	29.06±3.27	11.25	10.67±1.92	9	19.56±1.86	9.20	10.09±2.53	HS
Female	10	15.80±1.73	11	10.96±2.65	11	13.06±0.52	3.99	10.01±2.03	HS
Total	20	22.43±4.57	20.37	10.82±2.26	20	15.99±2.12	13.25	10.05±2.20	HS

The comparison was made in female groups in summer and winter seasons. The statistical analysis for the two means reveals that there is a high significant difference between them which indicates that the seasonal variation in vitamin D is evident in healthy Iraqi population.

3.2.3- Difference between Males and Females in Serum Vitamin D Levels.

The statistical comparison of serum 25-hydroxy vitamin D levels between males and females reveal that there is a high significant difference ($p<0.01$). between the two sexes. This result is shown in table 3-4 below.

Table 3- 4: Vitamin D Levels in Male and Female.

Male (3-65) yrs				**Female (1-65) yrs**				**CS**
Vitamin D			**Calcium**	**Vitamin D**			**Calcium**	
No.	X±SD ng/ml	CV%	X±SD mg/dl	No.	X±SD ng/ml	CV%	X±SD mg/dl	
29	24.56±5.52	22.40	10.87±2.22	31	14.37±1.86	12.90	10.72±2.22	HS

Male levels are higher than females in control group. Generally there is 42.2% decline in mean serum of vitamin D level of female if compared with male. This finding was in accordance with the results that reported by many other investigators(86).

3-2-4- Vitamin D Level in Serum According to Age Group.

Table 3-5 shows the statistical comparison of 25- hydroxy vitamin D levels between males of control group in age (1-30) years and the age (31-65). years reveals significant differences ($P<0.01$). The same significant results were obtained from the female group levels are the same thing in the same aged subjects reveals a significant difference ($p<0.01$).

Table 3-5: serum vitamin D levels according to Age Group

Group Type	**(1-30) year**				**(31-65) year**				**CS**
	Vitamin D			**Calcium**	**Vitamin D**			**Calcium**	
	No.	X±SD ng/ml	CV%	X±SD mg/dl	No.	X±SD ng/ml	CV%	X±SDmg/dl	
Male	14	23.62±5.37	22.77	10.85±2.36	15	20.19±5.02	20.66	10.12±2.53	S *
Female	18	14.06±1.74	12.37	10.55±2.21	13	12.22±1.02	8.34	10.03±2.40	S *
Total	32	19.21±6.51	33.91	10.79±2.19	28	15.87±5.12	32.22	10.00±2.35	S *

3-3-Vitamin D Levels in Diverse Diseases:

3-3-1-Serum Vitamin D and Dental Caries.

Statistical comparison of the means of serum 25-hydroxy vitamin D levels in patients with dental caries or teeth decay, and control subjects in table 3- 6 indicates the highly significant difference (p.<0.01) between them. The mean serum 25-hydroxy vitamin D in patients is lower than that of controls with 59.49%, this was shown in table 6 below, with other details.

Table 3-6: Serum Vitamin D Levels in Teeth Decay and Healthy.

Group Type	Patient Group				Control Group				CS
	Vitamin D			Calcium	Vitamin D			Calcium	
	N0.	X±SD ng/ml	CV%	X±SD mg/dl	No.	X±SD ng/ml	CV%	X±SD mg/dl	
Male	13	5.63±0.12	2.23	8.54±1.67	19	24.56±5.52	22.48	10.87±2.22	HS
Female	29	6.11±0.42	6.87	8.40±1.20	21	14.37±1.86	12.95	10.72±2.22	HS
Total	42	5.96±0.34	5.70	8.96±1.82	40	19.21±6.51	33.91	10.79±2.19	HS

The results in table 3-6 are specific to dental decay, since this malady is the most important oral disease and it is mostly a nutritional disease. It shouldn't be forgotten, though, that the mouth has been termed" the mirror of the body" with many nutrition deficiency disease, e.g. vitamin D, Iron and vitamin B complex, giving oral signs and symptoms.

3.3.2- Serum Vitamin D Level and Rickets.

Table 3- 7 shows that there is a highly significant decrement (P<0.01) in mean serum 25-hydroxyvitamin D level in patients with rickets if compared with normal age matched individuals.

Table 3-7: Serum Vitamin D Level in Rickets and Controls.

Type Group	Rickets Group				Control Group				CS
	Vitamin D			Calcium	Vitamin D			Calcium	
	No.	X±SD ng/ml	CV%	X±SD mg/dl	No.	X±SD ng/ml	CV%	X±SD mg/dl	
Male	9	5.24±1.07	20.41	8.83±1.48	10	20.56±4.45	21.64	10.87±2.22	HS
Female	5	4.84±0.95	19.62	8.18±1.36	5	14.37±1.86	12.95	10.72±2.22	HS
Total	14	5.10±1.02	20	8.77±1.46	15	17.44±5.51	31.59	10.79±2.19	HS

The result reveals that the changes which happen in the calcium levels in rickets group is important, it is in accordance with the decrement of vitamin D level in this group, this fact indicates the parallel relationship between vitamin D level and calcium ion concentration in plasma, and shed a light on its role in preventing rickets in young children.

3.3.3- Serum Vitamin D Levels and Osteoporosis.

Table 3- 8 shows that there is a highly significant decrement (P<0.01) in mean serum 25- hydroxy vitamin D levels in patients with osteoporosis in old men and women if compared with age matched control group of healthy volunteers, there is 35.2% decrement in serum vitamin D levels in those patients, other statistical values are included in table 3-8.

Table 3-8: Serum Vitamin D Levels in Osteoporotic Patients and Controls.

Group Type	Osteoporotic Patient				Control Group				C S
	Vitamin D			Calcium	Vitamin D			Calcium	
	No.	X± SD ng/ml	CV%	X±SD mg/dl	No.	X±SD ng/ml	CV%	X±SD mg/dl	
Male	11	4.99±0.98	19.63	8.77±1.80	15	24.56±5.52	22.48	10.87±2.22	HS
Female	19	5.57±0.93	16.69	8.46±1.98	21	14.37±1.86	12.95	10.72±2.22	HS
Total	30	5.36±0.94	17.53	8.69±1.85	36	19.21±6.51	33.91	10.79±2.19	HS

3.3.4- Serum Vitamin D Levels in Postmenopausal

The table below shows the mean value and the range of serum vitamin D concentrations of postmenopausal osteoporotic and control subjects. They are only female groups and there is a high significant decrement (P<0.01) in vitamin D levels in postmenopausal if compared with mean values of younger women .

Table 3- 9: Serum Vitamin D Levels in Postmenopausal Women and Younger Women.

Postmenopausal Group				Younger Women Group				CS
Vitamin D			Calcium	Vitamin D			Calcium	
No.	X±SD ng/ml	CV%	X±SD mg/dl	No.	X±SD ng/ml	CV%	X±SD mg/dl	
6	6.52±0.89	13.65	7.98±1.04	8	14.06±1.74	12.37	10.55±2.21	HS

In the presence of osteoporosis, vitamin D insufficiency can amplify bone loss and thus enhance fracture risk. It follows that, at any age, but especially in the elderly, and adequate intake of both calcium and vitamin D is important for the preservation of bone mass and prevention of osteoporosis .

Conclusions

The most important conclusions from the current work are:-

1- Vitamin D can be measured successfully by the HPLC technique with high accuracy and precision with the current modified method.

2- The normal values of vitamin D levels in the controls group in Al- Najaf city were in general lower than that reported by other investigators in western countries or USA.

3- The level of vitamin D is extremely affected by the seasonal variation in Iraqi and that may be related to the amount of light reaches the earth during summer and winter terms in our region.

4- Children with rickets are generally affected by the decrement of vitamin D levels, which affects intestinal calcium absorption . The number of children with rickets is increased in winter season and this appears clearly at the end of April.

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