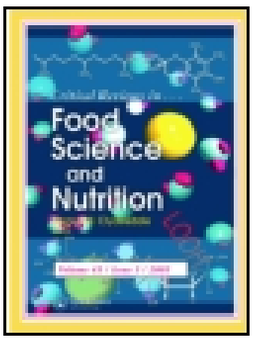

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REVIEW

Vitamin D – current stage of knowledge about analysis and supplementation

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ABSTRACT

Nowadays, topics related to the proper nutrition of the body, which requires a complex of compounds and supplementation of these ingredients have undoubtedly gained popularity, so it should come as no surprise that there is a widespread interest in vitamin D in science, medicine, analytics and nutrition. In the world of developing technologies, new directions of physiological action of this vitamin on the body are being discovered. Issues related to the demand for vitamin D in various populations and its sources in food, the appropriate form of supplementation, safety and toxicity are extremely important. The present manuscript focuses on the concise evaluation of key data in the field of vitamin D. Structure and physicochemical properties, demand and delivery trails, deficiency and its diagnosis, supplementation, interactions of vitamin D with supplements and drugs are discussed. Attention has also been paid to the methods of vitamin D analysis in various matrices, which allow for an accurate and precise quality assessment of dietary supplements, drugs and food products. The presented information allows deeper understanding of the mechanisms responsible for the development of many diseases in the context of vitamin D levels.

KEYWORDS

Vitamin D; supplementation; quantification; analytical methods

Introduction

As for the genesis of vitamin D, it is worth mentioning that it was discovered in 1922. This was done by Dr McCollum, who assigned letter "D" to the newly discovered compound, as it was the fourth vitamin discovered (Sajkowska-Kozielewicz and Paradowska 2016; Gruber 2015). What we call "vitamin D" is in fact a group of lipophilic substances, which include vitamin D_3 (cholecalciferol, calciol) synthesized in the skin of animals and humans, vitamin D_2 (ergocalciferol) found in plant products, vitamin D_1 in fish liver oil, and other compounds that are considered less important (D_4 – 22-dihydroergocalciferol, D_5– sitocalciferol) (Figure 1). The two main vitamins, D_3 and D_2, are formed under exposure to UV radiation; both after metabolism show biological activity in a living organism.

Looking at the chemical structure of these two most important forms (D_2 and D_3), it can be concluded that the difference lies only in the side chain, with vitamin D_2 having an additional methyl group (C22) and a double bond (C23-C24). Both compounds belong to the 9,10-secosteroids group, and due to the presence of a conjugated double bonds system, they absorb UV radiation (Jones and Makin 2000).

7-dehydrocholesterol (7DHC) is enzymatically converted into cholesterol by 7-dehydrocholesterol reductase (Kandutsch-Russell pathway). In addition, 7DHC is a precursor of vitamin D synthesis in the skin under exposure to UVB radiation in the wavelength range of 290–315 nm. As a result of the photochemical reaction, pre-vitamin D_3 is formed, which under the influence of thermal energy is isomerized to vitamin D_3 (Prabhu et al. 2016). A similar pathway is observed in the biosynthesis of the D_2 form, which differs in the starting compound – ergosterol, and the whole process takes place in plants and fungi (Franek and Napiórkowska 2009). Another interesting source of vitamins D_2, D_3 and their provitamins may be freshwater microalgae (Jäpelt and Jakobsen 2013), in which they are most probably synthesized by sun exposure

Both forms of vitamin D must be converted to the active metabolite in order to have a chance to elicit a physiological response in the body (Figure 2). This is done through double hydroxylation in the liver and kidneys. As a result, 25-hydroxycholecalciferol (calcifediol; 25(OH)D) is formed, which is a predominant metabolite found in blood, most frequently measured in serum. Non-renal conversion of 25(OH)D has been demonstrated in bone tissue, placenta, monocytes and granular tissue (Walicka et al. 2008; Prosser and Jones 2004). There are many diseases and physiological processes that display annual periodicities (Dopico et al. 2015). This can result in a deficiency of the active form of vitamin D. Both steps of hydroxylation, and the transport of vitamin D also depend on the presence of magnesium. In addition, vitamin D, which is activated in the body, can increase the absorption of magnesium in the intestines (Uwitonze and Razzaque 2018). Magnesium deficiency lowers the level of 25(OH)D, which results in impaired calcium and phosphate metabolism in the body. Similar changes apply to vitamin D_2 (Gruber 2015; Walicka et al. 2008).

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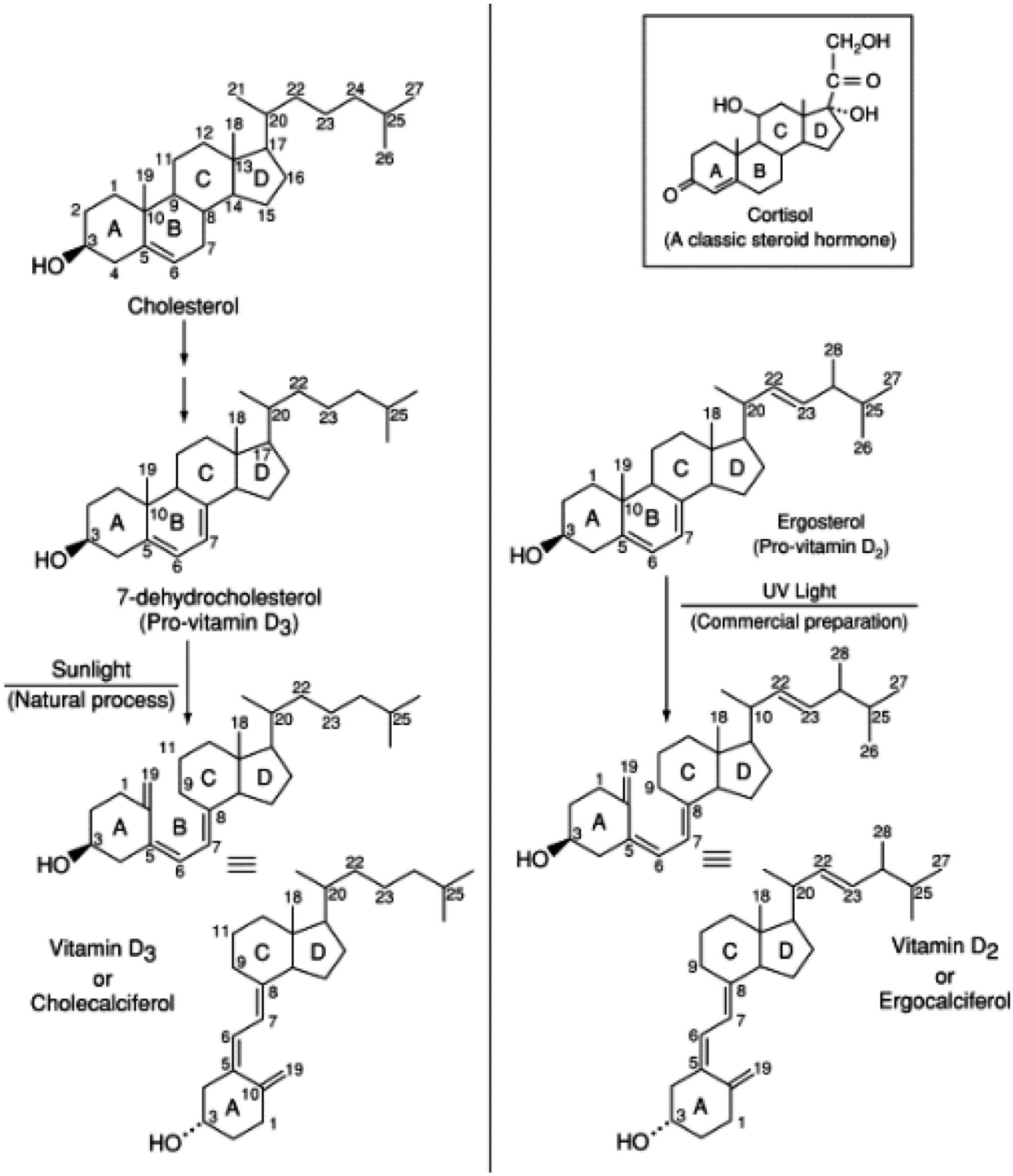

Figure 1. Structures of vitamin D_3, vitamin D_2 and their respective provitamins (ChE, 2019).

The degradation process of vitamin D metabolites involves attaching subsequent hydroxyl groups to the molecule, resulting in the formation of water-soluble compounds, that can be excreted by the kidneys (Lehmann and Meurer 2010; St-Arnaud and Naja 2011). One of such metabolites, 24,25(OH)2D, exhibited biological activity related to the bone tissue regeneration process (Cline 2012). Experimental data indicate that the ratio of 25(OH)D to 24,25(OH)2D may be a good parameter to assess the effectiveness of supplementation. Reference values for this parameter have been developed, which allow to assess the degree of vitamin D elimination from the human body. There are however no reference values for serum concentration of 24,25(OH)2D (Cashman et al. 2015). Unfortunately, not all organisms have the ability to synthesize vitamin D. For example, some domestic animals (dogs, cats) are dependent on exogenous vitamin D intake, and necessary vitamin concentration can be obtained from the diet (How,

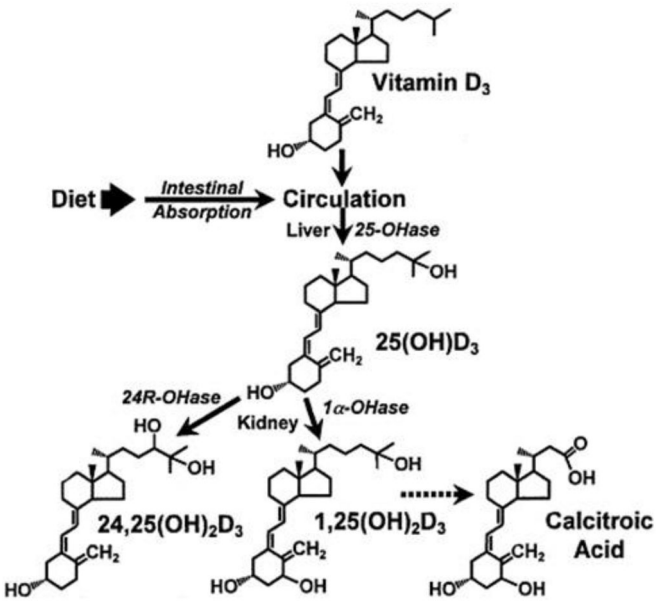

Figure 2. The metabolism of vitamin D_3 (NAP 1997).

Hazewinkel, and Mol 1994). Currently, most pet foods are fortified with vitamin D (Young and Backus 2016).

The importance of vitamin D

The physiological functions of the active form of vitamin D are usually related to their effect on the small intestine, bones and kidneys, what results in changes of calcium and phosphates blood levels. Numerous studies on the action of this vitamin at the molecular level have proved that it interferes with the proliferation and differentiation of cells, mainly in the skin, intestines and bone marrow (Jones and Makin 2000).

Vitamin D acts directly by binding with the nuclear vitamin D receptor (VDR; also known as NR1I1) and regulation of the transcription of genes coding proteins involved in various processes, such as calcium transport or cell division. Therefore, we can infer the pleiotropic activity of vitamin D (Sajkowska-Kozielewicz and Paradowska 2016). Vitamin D influences the calcium-phosphate balance and ensures proper bone mineralization and muscle function. Studies of postmenopausal women consuming magnesium and vitamin D have shown an increase in the level of osteocalcin, a protein that is a marker of bone turnover volume, and indicator of osteoblast activity (Aydın et al. 2010). Thus, vitamin D deficiency may result in osteoporosis, muscle weakness, osteomalacia, and in children: rickets, growth disorders and skeletal deformation. It is therefore of importance to secure proper level of vitamin D in pregnant women. Otherwise, there is a risk of abnormal bone development in the fetus, lower weight and disturbed growth (Grant and Holick 2005). The exact vitamin dose for pregnant women and infants must be determined individually by a medical doctor. After the end of the first year, the need for vitamin D in children generally decreases, however, pediatricians recommend its regular administration until the age of 3. This is the period of the body's fastest growth and demand for calcium, and without this duo the bones would be weak, fragile and prone to deformation and curvature.

A relationship between vitamin D deficiency and the occurrence of several acute or chronic diseases was also established, including preeclampsia, tooth decay in children, periodontitis, autoimmune diseases, cardiovascular diseases, cancer, diabetes and neurological disorders (Holick 2017; Fogacci et al. 2020). Incorrect management of vitamin D increases the risk of cancer, autoimmune diseases and mental problems (Selting et al. 2016), and a simultaneous magnesium deficiency promotes DNA damage, negatively affects blood cholesterol, blood pressure and calcium calcification, and increases the risk of atrial fibrillation and diabetes (by regulating blood glucose levels) (Kraus et al. 2014; Gow et al. 2011). There are studies which confirm an association between a low blood 25(OH)D level and the risk of type 2 diabetes (Pittas et al. 2019). An experiment made on older men (70–88 years of age) showed that vitamin D reduces the risk of diabetes (Baynes et al. 1997). Production, appropriate secretion and use of insulin in the body largely depends on this vitamin as well. Considering how many functions vitamin D performs, it is reasonable to conclude that it may also influence the course of the neoplastic process. Pro-apoptotic effect against tumor cells is postulated. Epidemiological studies show a relationship between vitamin D deficiency and an increased risk, especially of breast and prostate cancer (Osińska et al. 2017). The incidence of these types of cancer are much lower in regions with high sun exposure. Adequate vitamin D levels may translate into a greater chance of cancer survival (Kimmie 2015). However, further randomized trials are required for all of the above-mentioned directions of vitamin D action (Gruber 2015; Grant and Holick 2005; Kulie et al. 2009).

Vitamin D has two primary activities – anti-inflammatory and immune-regulating. In the early 20th century, patients with tuberculosis were admitted to sanatoriums, where they were subjected to heliotherapy (exposure to sunlight), what was believed to directly kills tuberculosis. Thus, vitamin D was unwittingly used to treat infections (Martineau et al. 2011, 2017). In turn, the people of the North have used cod liver oil (a source of vitamin D) for centuries to protect against infections. Subsequent studies have shown that the vitamin D receptor is located on immune cells – B, T lymphocytes and antigen presenting cells, all of which are able to synthesize its active metabolite. Research confirms the improvement in the functioning of the immune system and the beneficial effect of vitamin D in reducing the risk of inflammatory and cardiovascular diseases. Meta-analysis carried out by Zhang et al. indicates a lower incidence of colds or influenza in people supplemented with this vitamin (Zhang et al. 2019).

Some current studies aim to clarify the role of vitamin D in the pathogenesis of nervous system diseases such as dementia and depression (Parker, Brotchie, and Graham 2017). Another report indicated its role in controlling the gene responsible for the greater risk of developing multiple sclerosis (MS) (Ramagopalan et al. 2009). More and more scientific publications confirm the need for vitamin D

supplementation in people who suffer mental illness (Pittampalli et al. 2018), because its deficiency is associated with deterioration of information processing speed, decreased fluidity of expression, insomnia or susceptibility to stress (Wichniak, Wierzbicka, and Jernajczyk 2011).

It has also been noticed that vitamin D participates in the production of so-called natural antibiotics (cathelicidin and defensin) and regulates the function of T lymphocytes (Adams et al. 2009; Brehm et al. 2010; Uwitonze and Razzaque 2018). It can also be described as a precursor in the formation of antibacterial peptides that help control bacteria responsible for oral infections, including caries and gum disease, enamel and tooth underdevelopment, delayed tooth eruption and loss (Kopeć 2019).

Qualitative and quantitative analysis

The proven role of vitamin D in maintaining good health and the increasing need for its supplementation, have influenced the development of methodology and progress in the analysis of 25(OH)D, as well as other metabolites. Determination of 25(OH)D level has for some time now been a commonly used to assess vitamin D deficiency or intoxication, and to monitor disorders related to calcium intake, including metabolic bone disease. For this purpose, until the end of the 20th century, a laborious and time-consuming manual radiocompetitive method using tritium (^{3}H) was applied. In recent years many new methods have been introduced for the determination of vitamin D metabolites, including RIA (Radioimmunoassay), ELISA (Enzyme-linked immunosorbent assay), and chromatographic methods (HPLC, GC/MS). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is currently the reference method. However, the most commonly applied is chemiluminescent immunoassay (CLIA), which is used in automated analytical platforms. Determination of vitamin D in biological materials is quite difficult due to the high hydrophobicity of 25(OH)D (interference of matrix components), the presence of vitamin D_3 and D_2 derivatives or stereoisomers of some metabolites. The available literature describes many examples of the use of various analytical techniques for vitamin D quantification. In addition to the original research papers, there are also several reviews describing the problem of determination and interpretation of results (Rani 2014; Yin et al. 2019). It seems that the most popular are chromatographic techniques, spectrophotometry, immunoassays and electrophoretic tests.

Extraction issue

Extraction of vitamins from various materials such as dietary supplements, food or blood is a key step in the quantification of these substances, affecting the accuracy of the obtained result. The main problem is their relatively low content in these samples, and the possibility of degradation during the extraction process. The most common type of extraction is liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Very often, analyzed sample requires pretreatment and preparation prior to the main extraction process. Solid ingredients, e.g. food or tablets, should be crushed and homogenized. Liquid matrices, i.e. serum or milk, also require initial purification, e.g. precipitation of the protein with an organic solvent, like ethanol. In some situations, ultrasonication with ethanol is also performed to deport the sample (Yin et al. 2019). In the case of samples containing vitamin D, pre-saponification is often required in order to release the compound from its esters. This is usually done by treatment with potassium hydroxide in an aqueous or alcoholic medium at the temperature of about 60–100 °C for 20–45 min. To prevent thermal isomerization of vitamin D, this process should be carried out at room temperature with stirring. In addition, the stability of this vitamin is enhanced by addition of antioxidants, e.g. ascorbic acid, pyrogallol or hydroxytoluene with butanol (Perales et al. 2005).

In the LLE extraction appropriate solvents are selected, depending on whether saponification is performed. If so, the process starts with e.g. hexane, ethyl ether, pentane or a mixture thereof. When saponification is not needed, than mixtures of methylene chloride, methanol, hexane, chloroform and ether are most commonly used. It should be noted that the use of a given solvent provides a satisfactory extraction of one of the vitamin metabolites, not necessarily all, due to differences in polarity. If it is necessary to extract many metabolites, appropriately selected solvent mixtures are used, e.g. methanol-chloroform (2:1, v/v), ethyl acetate-cyclohexane (1:1, v/v) or hexane-isopropanol (1:2, v/v) (Jones and Makin 2000; Perales et al. 2005). During SPE extraction, vitamin D is transferred to special columns with adsorbent fillings. Two options are usually chosen. First, a C18 carbon chain modified silica gel column can be used with methanol as polar eluent; second, a polar Chromabond XTR column and hexane as solvent (Perales et al. 2005). Other adsorbents used are NH_2 modified silica and electrospun nanofibers (PTSPE) (Bartolucci et al. 2011; Chen et al. 2011).

One of less common methods is e.g. MSPE (magnetic solid phase extraction). In this case, substances adsorbed on the magnetic carrier are more efficiently and faster distributed by magnetic field (Yin et al. 2019). It was used to determine vitamins D_3, A and E in medicines with magnetic methylsilane nanoparticles (Momenbeik and Yazdani 2015). Another method is DLLME (dispersive liquid-liquid microextraction), which uses an aqueous solution with sample and two solvents: extraction solvents and second, dispersed in both phases. Thanks to shaking, the surface of contact is very large, and whole process is quick and efficient (Yin et al. 2019). Researchers in Spain used this extraction method for the first time in the analysis of vitamins D and K in vegetables and other food products. The dispersing phase was acetonitrile and the extraction phase was carbon tetrachloride (Viñas et al. 2013). However, in the SLE method (liquid-liquid extraction), samples are bonded with diatomaceous earth and then eluted with a appropriate solvent. This method was used to analyze vitamin D content by UPSFC-MS/MS in serum with methanol (Jenkinson et al. 2018).

Separation techniques

Thin layer chromatography is a method that separates the components in a sample between two phases: stationary and

mobile, according to the different affinities of compounds for both phases. It can be used to separate the components of the mixture, their identification and quantification. Using TLC, results can be obtained at relatively low costs and in a short time (Cimpoiu and Hosu 2007). According to the available literature, vitamin D can be quantitatively and qualitatively determined by TLC in various samples – dietary supplements, drugs, food or serum in the form of metabolites, as a single compound and in combination with other vitamins and lipid substances. One of the encountered difficulties results from possible degradation of vitamin D by environmental factors, i.e. oxygen (Hossu et al. 2009). The spots on the chromatogram are visualized by observation under UV light or by spraying the plate with reagents forming colored spots, e.g. concentrated perchloric acid (brown-orange) or sulfuric acid (orange) (Churacek and Gasparic 1978). An example of subsequent vitamin D determination can be found in the work published by German chemists (Janecke and Maass-Goebels 1960). They were able to separate vitamin D from mixtures with other sterols and their degradation products by TLC using silica gel G plates and different mobile phases, e.g. chloroform: hexane: ethyl acetate (9:1, v/v). As concerns simultaneous determination of vitamin D with other vitamins, such as A, D_2 and E, these were analyzed using the mobile phase: acetonitrile: benzene: chloroform (10:10:1, v/v) on the non-polar stationary phase. 10% antimony chloride was used for visualization (Cimpoiu and Hosu 2007). Individually, vitamin D_2 was determined on silica gel 60 F_{254}, using a mixture of hexane and ether (9:1, v/v) as the mobile phase (Hossu et al. 2009). Vitamin D_2 analysis on the same stationary phase was also performed using a mixture of cyclohexane and ethyl acetate as eluent. The method proved to be reliable with the ergocalciferol content in the sample from 0.05 to 3 μg (Sirec et al. 1978). High-performance thin layer chromatography (HPTLC) was also used in the analysis of the content of cholecalciferol in fish liver oil. For this purpose, a mobile phase consisting of chloroform and ether (9:1, v/v) and plates with silica gel 60 F_{254} were used, enabling visualization under UV light at 280 nm (Demchenko et al. 2011). Other scientists conducted the analysis using three developing systems (hexane-isopropanol (85:15, v/v), dichloromethane-isopropanol (9:1, v/v), chloroform-ethyl acetate (5:5, v/v)) and silica gel plates, which showed similar effectiveness of TLC and HPTLC methods for separation of vitamin D and its metabolites (Pyka 2005). 25-hydroxycholecalciferol (produced in the liver) in blood samples is the most commonly determined by immunochemical or HPLC methods. However, quantification of 1,25-dihydroxyvitamin D3 and other its metabolites in plasma was also performed by HPTLC. The analysis was developed on silica gel 60 plates with chloroform: ethanol: water (183:16:1, v/v/v) as the developing system, and the plates were observed under UV light (Justova, Wildtova, and Pacovsky 1984). By selecting the appropriate conditions, a mixture of many compounds was determined: derivatives of vitamins D, K and A on the stationary phase - silica gel impregnated with silver nitrate (Pyka 2005). A method of simultaneous determination of vitamin D_3 and K_1 in preparations acting as rodenticides, was also described. Separation was performed on silica gel plates in two developing systems: dichloromethane: methanol: acetic acid (45:4:1, v/v/v) and chloroform: methanol (97:3, v/v). Detection was made by observation under UV light (Opong-Mensah and Porter 1988).

High performance liquid chromatography (HPLC) is currently the most commonly used chromatographic method that allows the use of various detectors. Compared to classical liquid chromatography, it is characterized by a much higher resolution, which is achieved by reducing the diameter of the adsorbent grains in chromatographic column. This method is becoming the routine method for analyzing vitamin D and its metabolites (Tsuprykov et al. 2018). In 2010, the results of the analysis of vitamins D_2 and D_3 in medicinal preparations using HPLC, with their validation were published. The two forms of vitamins were separated at 40 °C in a Teknokroma C18 column, and the used eluent included acetonitrile and methanol (75:25, v/v). Separated vitamins were identified and analyzed using a diode array spectrophotometric detector (DAD) at 265 nm. There was no interference from different lipophilic vitamins (Al-Qadi, Battah, and Hadidi 2010). Vitamin D_3 was successively determined by HPLC using spectrophotometric detection and acetonitrile: water (99:1, v/v) mixture as the eluent, in order to separate it from degradation products, and to test stability in pharmaceutical formulations (Roskar and Temova 2016). Regarding the determination of vitamins D_2 and D_3 in food products, an HPLC method has been developed to separate and quantify these vitamins in milk. The best mobile phase was methanol, and the process temperature was 15 °C (Araujo-León et al. 2017). Also in milk, an independent, simultaneous determination of various vitamins and fat-soluble provitamins was carried out in one analyzed sample (Gomis, Fernández, and Gutiérrez Alvarez 2000). Vitamin D metabolites in the serum can also be determined by HPLC with UV detection. The separation was carried out in normal phase or by combination of normal and reversed phases or only in reversed phase. Previously, the use of reversed phases required prior purification of the sample in normal phase, and the determinations were too complex to be used routinely. A quick and easy-to-perform method for the separation and analysis of 25(OH)D_3 and 25(OH)D_2 is now known, using a LiChrospher 60RP column and an eluent consisting of methanol in water (760 mL/L). If prior to analysis the material is extracted by hexane, the recovery of metabolite will be better (Turpeinen, Hohenthal, and Stenman 2003).

Mass spectrometry (MS) is a method based on the initial ionization of compound that produces a mixture of ions that are then separated and sorted by masses. As a result, a mass spectrum is obtained, containing information on the molecular weight of the compound and the generated fragments. The coupling of MS with liquid chromatography (LC) increases the specificity of the method and allows to obtain the spectrum of a substance present in the sample in a very small amount. Vitamin D was analyzed by this method in a reversed phase system, which ensures easier

sample ionization and high reproducibility (Jakobsen and Japelt 2012). Currently, the technique of LC coupled with tandem mass spectrometry (LC-MS/MS) is applied, which is characterized by even more satisfactory parameters. Some examples described in the literature include the assay of vitamin D metabolites in blood, due to their low concentration in the analyte (Shah, Petroczi, and Naughton 2012). The LC-MS/MS method was also developed for the simultaneous determination of vitamin D in human serum (Abu Kassim, Shaw, and Hewavitharana 2018). Quantitation of 25(OH)D_2 and 25(OH)D_3 was made by combining LC-MS/MS with isotope dilution analysis, which gave the possibility of using a very small amount of sample and easy process automation (Maunsell, Wright, and Rainbow 2005). However, not until 2016, the methodology based on LC-MS/MS was used for the quantification of cholecalciferol and ergocalciferol (Riley 2016). A supercritical fluid chromatography and reverse phase LC-MS was also applied for the analysis of vitamin D in oily and fatty samples (Hamada et al. 2018).

Gas chromatography is of limited use due to the analytical requirements related to the volatility and high vapor pressure at the process temperature. This is due to the fact that the mobile phase is an inert gas (e.g. helium, nitrogen), and the stationary phases must have a low vapor pressure. During vitamin D analysis by GC, it is necessary to derivatize the sample in order to increase volatility and protect the compound against thermal decomposition. The best solution turned out to be the analysis after trimethylsilylation of hydroxyl groups, and the study with the production of cyclic boronates between adjacent hydroxyl groups [24,25(OH)$_2$] (Jones and Makin 2000).

Electrophoresis is a method that separates components based on their different mobility in an electric field. Capillary electrophoresis is a modification of traditional technique, with many variants of analysis. In the studies of vitamin D, micellar electrokinetic chromatography deserves special attention, because it enables the analysis of uncharged compounds through the use of surfactants, creating micelles above the critical micellar concentration. In this way vitamin D_2 was analyzed in the presence of vitamins A and E in pharmaceutical preparations (Liu, Jia, and Hu 2010). Also, simultaneous determination of lipophilic and lipophobic vitamins using capilary electochromatography, in which separation takes place under the influence of electronic flow of eluent through a capillary filled with an adsorbent was reported (Yamada, Kitagawa, and Ohtani 2013). The disadvantage of this method is that only spectrophotometric detector can be used (Yin et al. 2019).

Immunoassay

Techniques used to determine vitamin D and its metabolites also include immunological methods. They are mainly used to measure the concentration of metabolites in the blood to determine possible deficiencies. The assay is based on the formation of a complex of the tested compound with a specific antibody or the so-called vitamin D-binding protein. The mail problems encountered using this type of assays are the different affinities of the antibody to the vitamin D_2 and D_3 metabolites (i.e. they do not reflect total supply of vitamin to the body), and reduced accuracy (interference of inactive form of the vitamin and its epimers). Radioimmunoassay (RIA) is a modification of this method which uses radioactive marker ligands (most often I^{125}), however another problem arises, namely the need for special handling of radioactive products (Turpeinen, Hohenthal, and Stenman 2003; Hollis et al. 1993). Recently, the CLIA method (chemiluminescence immunoassay), which uses a marker emitting UV radiation, has become more and more popular (Atef 2018). In the ELISA method enzymes that catalyze reactions leading to formation of products in an amount proportional to the content of compound are used for labeling (Zerwekh 2008). Due to the speed and simplicity of these assays, immunological tests have found use in the routine evaluation of the content of vitamin D metabolites in serum.

UV-VIS spectrophotometry

It is a commonly known method based on measuring the absorption of radiation in the ultraviolet and/or visible range. As a result, an electron spectrum is obtained, which reflects a dependence of the intensity of absorbed radiation vs wavelength. Vitamin D_3 was determined spectrometrically at wavelength of 264 nm in capsules and tablets in the presence of other fat-soluble vitamins (Ashok and Kumar 2011). A method for the determination of vitamin D_3 was also developed, based on the formation of complex with molecular iodine, which has two absorption maxima at 292 and 360 nm (Sanchez Perez, Gallego Matilla, and Hernandez Mendez 1993). The analysis of vitamins D_2 and K_1 in the presence of rutin was also carried out, however, due to similar absorption range, it was necessary to initially separate these vitamins by TLC. Then, measurements were made for vitamin K_1 at wavelength of 248 nm, and for vitamin D_2 at 265 nm (Bączyk et al. 1981).

Electrochemical methods

Other methods employed for the determination of vitamin D, includeelectrochemical analysis using compound labeling technique with a special sensor. An example is the use of a specially designed compound: 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione, which reacted with 25(OH)D (Carlucci et al. 2013). The CYP 27B1 enzyme was also used, which was applied to the electrode and voltammetric measurement was performed (Ozbakir et al. 2016).

Vitamin D testing kits

Automated, ready-made tests are available to measure the 25(OH)D metabolite in blood to diagnose vitamin D deficiency. Quantification methods can be divided into chemical, such as LC-MS and HPLC, or binding assays, i.e. CBPA, RIA, CLIA and ELISA (Enko et al. 2015). These sets differ in price and reliability. The National Institute of Standards with the NIH in 2010 organized a vitamin D

quality control program, that compares the methodology of available tests with the standards set by NIST (NIST 2019).

In the US, the organization "The vitamin D Council," commends the cheapest tests for 25(OH)D. One can order "In-home test kit", put a drop of blood on the test and send it to the laboratory for analysis (Sturges and Canell 2018). "Better You", the dietary supplements and nutritions store of West Birmingham NHS and Sandwell offer the next home test. Samples are analyzed by LC-MS, and results are sent by e-mail within 10 days. Additionally, if the results are not satisfactory, the producer sends a supplementation plan, and give a discount code for "DLux vitamin D oral spray" (BetterYou 2019). Another test kit, developed by GrassrootsHealth, is available from the Dr. Mercoli Store in the US. The people who applied this test became part of a project to study vitamin D levels in the society. The results were sent by e-mail within 7–10 days (MRP 2019). The Cerascreen also offers a vitamin D test. After the instillation of blood, the test is sent to DST Diagnostic Systems & Technologies laboratory, and the results are available on the website after logging in. Dr John Douillard's on the Life Spa Store website proposes a very similar set (CeraScreen 2019).

There are several studies comparing the reliability of the commercially available tests. One of them compares the four available tests using various analytical methods, i.e. IDS-iSYS 25(OH)D from UK based on CLIA, its improved version IDS-iSYS 25(OH)D^s also from UK, ORGENTEC 25(OH)D_3/D_2 from Germany based on ELISA, and ClinMass® LC-MS/MS Complete Kit from Germany. The study showed that a similar deviation compared to LC-MS/MS was shown by IDS-iSYS 25(OH)D^s and ORGENTEC 25(OH)D_3/D_2, while the IDS-iSYS 25(OH)D test gave the lowest deviation. All tests were considered appropriate to measure the level of vitamin D metabolite (Enko et al. 2015). In 2016, a study was published comparing the LC-MS/MS method with the immunoassay technique represented by two tests: Siemens ADVIA Centaur Vitamin D Total and Roche Elecsys Vitamin D Total. The results showed that immunoassays give unsatisfactory results compared to LC-MS/MS, and show insufficient reactivity to vitamin D_2 metabolite, so they may give incorrect results (Li et al. 2016). Researchers in Sweden presented a comparison of three methods used in available tests for measuring vitamin D levels in the blood: HPLC-APCI-MS, RIA and CLIA. The results of 25(OH)D measurements in the blood of the twins showed very discrepant results. The highest values were obtained by HPLC-APCI-MS, and the lowest by CLIA. Based on the percentage of people with deficiencies, according to HPLC-APCI-MS it is 8%, while for CLIA it increases to 43% – this is a significant difference (Enko et al. 2015).

Demand and delivery trails

A proper level of vitamin D depends on several factors, including: the efficiency of the systems for the formation and delivery of vitamin to the body; sun access ensuring proper skin synthesis, providing the right amount of vitamin with food. When these processes are impaired, it may be necessary to supplement vitamin D level with dietary supplements. According to the Food and Nutrition Board, the recommended intake of vitamin D with food products is 600 IU/day, for children up to 12 months – 400 IU/day, and in group above the age of 70 years, this value increases to 800 IU/day (Cashman et al. 2016). Among the nutrients rich in this vitamin we find, of course cod liver oil, also fish (tuna, salmon, sardines, mackerel), eggs, milk and mushrooms (Figure 3) (Healthline 2019). Fortified food such as milk, yoghurt or grains are very popular in US (ODS 2019).

An important issue is vitamin D production aided by sunlight. According to the recommendations, from April to September (in Central Europe) people should spend at least 15 minutes a day in the sun between 10 am and 3 pm with bare arms, shins, face and neck (Rusińska et al. 2018). It might seem that if someone stays in the sun every day, the right dose of vitamin D is produced. However, the results of studies on people living in Hawaii who spend about 29 h a week in the sun showed that on average 51% of the respondents had too low content of the analyzed metabolite (Binkley et al. 2007). So it becomes clear that supplementation may be necessary in individual cases.

A separate issue is the demand for vitamin D in animals, which depends a lot on species, age and weight. Among livestock, the most sensitive to shortages is poultry and young pigs. In addition, pets that do not spend much time outdoors, and have abundant fur that make it difficult for the skin to have a direct contact with sunlight, are at risk. Generally, if the animal stays outdoors all the time, there is no risk of vitamin D deficiency, although winter becomes a problem. It is claimed that if an animal is carnivorous, it can be supplied with an adequate amount of this vitamin. Whereas, cats and dogs have a higher vitamin D requirement, due to the proven lower content of its precursor in the skin. In addition, this demand can be reduced by appropriate levels of calcium and phosphorus (in the ratio 1.2:1) in the body. The most common effect of vitamin D deficiency in young animals is rickets, and in adults it is osteomalacia, as in humans. Symptoms of these conditions include bone and chest deformities, enlarged joints, and bending of the hind legs (DSM 2019). In cows, reduced milk production and reproductive processes are observed. When vitamin D deficiency occurs during pregnancy, the calves are born weakened or stillborn. Sometimes a cow develops milk fever, when, due to the lack of vitamin D, the body is unable to supply enough calcium for milk production prior to parturition and lactation. The symptoms include circulatory depression, impaired consciousness and paresis. If the disease is not treated quickly, it can lead to coma and death (McDowell 2000).

Shortages and their effects

Considering all the important functions performed in our body by vitamin D, the negative effects of its deficiency are self-evident, and currently affect more and more people. Vitamin D deficit is mainly associated with insufficient exposure to sunlight, caused by unfavorable geographical

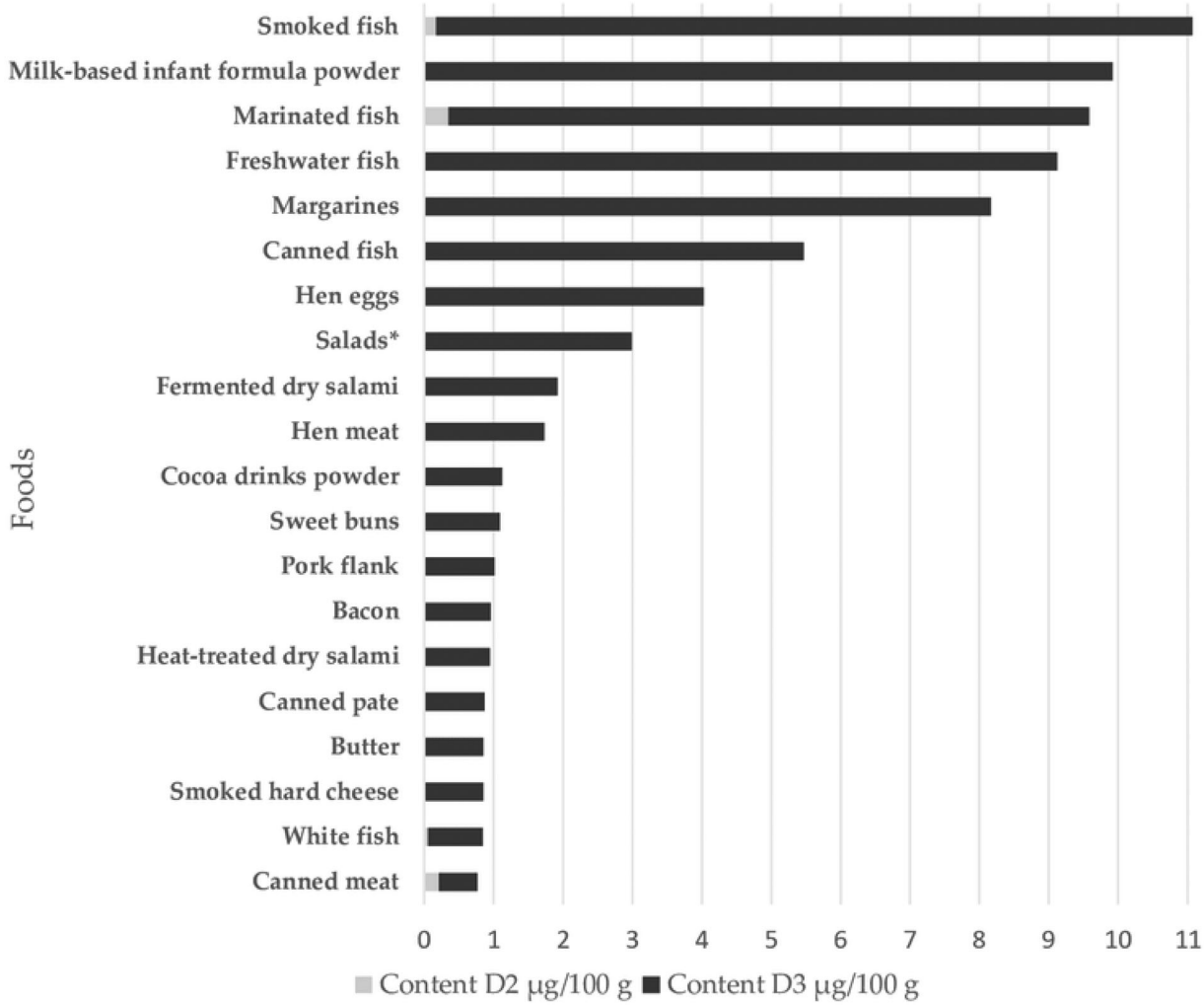

Figure 3. The content of vitamin D_3 and D_2 in different food products (Bischofova et al. 2018).

Table 1. Serum 25-hydroxyvitamin D [25(OH)D] concentrations and health (NAP 1997).

nmol/L	ng/mL	Health status
<30	<12	associated with vitamin D deficiency, leading to rickets in infants and children and osteomalacia in adults
30 to <50	12 to <20	generally considered inadequate for bone and overall health in healthy individuals
≥50	≥20	generally considered adequate for bone and overall health in healthy individuals
>125	>50	emerging evidence links potential adverse effects to such high levels, particularly >150 nmol/L (>60 ng/mL)

location, the use of creams with UV filters (in which case the efficiency of vitamin D synthesis decreases by up to 90%) or avoiding the sun for fear of the negative effects of radiation. Separate cases are people who suffer from renal or hepatic failure, which leads to abnormal vitamin D metabolism and does not allow for the formation of an active form of the compound. It should also be noted that there is an increased risk of vitamin D deficiency in people with impaired fat absorption, nursing mothers and people with dark skin or shielding the body from the sun, e.g. for religious reasons (Table 1) (ODS 2019). In the elderly, an increased risk of vitamin D deficiency is associated with altered metabolism, the coexistence of other diseases and reduced skin regeneration, therefore supplementation in this age group is always indicated (Grant and Holick 2005). The ability to synthesize vitamin D decreases and the risk of fractures increases with age, e.g. femoral neck (Rejnmark et al. 2012).

The results of the meta-analysis indicate that the frequency of vitamin D deficiency was higher in obese people, regardless of age or latitude (Pereira-Santos et al. 2015; ODS 2019), which is related to its accumulation in adipose tissue. Doctors and nutritionists emphasize the importance of vitamin D to aid weight loss, noting that when its deficiency is supplemented to an appropriate level, the process of losing unnecessary kilograms is faster.

To uniquely identify a deficiency of vitamin D, the level of the hepatic metabolite 25(OH)D is measured, which corresponds to the body's total supply of vitamin from various

Table 2. Recommended dietary allowances (RDAs) for vitamin D (NAP 1997).

Age	Male	Female	Pregnancy	Lactation
0–12 months	400 IU (10 µg)	400 IU (10 µg)		
1–13 years	600 IU (15 µg)	600 IU (15 µg)		
14–18 years	600 IU (15 µg)	600 IU (15 µg)	600 IU (15 µg)	600 IU (15 µg)
19–50 years	600 IU (15 µg)	600 IU (15 µg)	600 IU (15 µg)	600 IU (15 µg)
51–70 years	600 IU (15 µg)	600 IU (15 µg)		
>70 years	800 IU (20 µg)	800 IU (20 µg)		

sources, but not accumulated in tissues (Rusińska et al. 2018). The normal concentration of 25(OH)D metabolite is 30-50 ng/mL, below these values deficiency is recognized (Rani 2014). Measurement of the second metabolite 1,25(OH)2D is not recommended as it does not have a long enough half-life and may remain on its normal level ever during a deficiency period (ODS 2019).

In the case of animals, the optimal concentration of 25(OH)D is considered to be above 100 ng/mL. It has been observed that when the 25(OH)D concentration in dogs falls below the recommended values, the risk of developing cancer significantly increases (Selting et al. 2016). In addition, low vitamin D levels in dogs can be a risk factor for congestive heart disease (significantly lower 25(OH)D levels have been found in sick animals) (Kraus et al. 2014).

Vitamin D for children

Many children do not like foods that are a source of vitamin D (ie fish, oil, egg yolks), so it is worth introducing to their diet vitamin-enriched foods, e.g. cereals, milk or juices. It is difficult to get enough vitamin D from the sun, especially since most children spend a lot of time at home and school, while when they are outdoors, they protect skin to prevent melanoma and skin damage from too much sun exposure.

Children older than 1 year of age need 600 IU or more of vitamin D per day. Some children may need higher doses of vitamin D, especially those with health problems such as obesity, celiac disease, cystic fibrosis, bone fractures or pain, after bone surgery, or on medications that block vitamin D absorption. Since breast milk does not supply the baby with enough vitamin D, breastfed babies require vitamin D supplementation. They can receive 400 IU drops a day directly, or the mother can take 5000 IU a day (half the safety threshold) to fortify her milk with vitamin D. Most over-the-counter vitamins for children contain 600 IU of vitamin D, which is the recommended daily dose established by the US Food and Drug Administration for children 1 year and older (Kruse and Dubowy Kruse and Dubowy 2017). Also teenagers, even if they stop growing, still need vitamin D, especially to maintain a proper weight (Misra et al. 2008).

Vitamin D deficiency can develop due to malnutrition, malabsorption, enzyme-inducing drugs, and other causes. It can manifest itself among others as hypocalcemia during periods of increased growth rate (infancy and adolescence) (Lee, So, and Thackray 2013). Relationships between serum vitamin D content, lung function and asthma pathology in children with severe refractory asthma (STRA) have been investigated and have shown a correlation between vitamin D levels and the structure and functioning of the airways (Gupta et al. 2011). Osteopenia and osteoporosis are often seen in inflammatory bowel disease (IBD), and childhood vitamin D deficiency contributes to decreased bone acquisition. Studies have been carried out to monitor vitamin D levels in the treatment of IBD in Australian children, assessing clinical factors (e.g. location and severity) and the potential benefits of nutritional therapy (Levin et al. 2011). In a randomized trial, authors proved that vitamin D deficiency promoted the incidence of influenza A in school-age children (Gruber 2015). A comparative study of obese and healthy children in North Texas revealed widespread vitamin D deficiency in obese children and linked it to risk factors for type 2 diabetes (Olson et al. 2012). One of the most common childhood psychiatric disorders is attention deficit hyperactivity disorder (ADHD). Based on the findings of low vitamin D levels associated with neuropsychiatric diseases, the serum levels of this vitamin were examined in children with ADHD. The results showed that there was an association between lower 25(OH)D levels and ADHD in childhood and adolescence (Goksugur et al. 2014). A study of vitamin D levels in children with Hashimoto's thyroiditis showed vitamin D deficiency compared to the control group and inverse correlation with anti-thyroid peroxidase. It has been pointed out that low vitamin D levels may play a role in the autoimmune process in Hashimoto's thyroiditis in children (Çamurdan et al. 2012). There have also been studies on the relationship between vitamin D levels and dental caries in Canadian schoolchildren. Reduced serum 25(OH)D levels were significantly associated with lower household education, no twice-daily brushing, and annual dental visits (Schroth et al. 2016). It is known that some medications influence the action of vitamin D. Therefore, care should be taken not to cause a toxic effect (e.g. development of kidney stones), and possible supplementation should be directed by a pediatrician.

Supplementation

The symptoms of vitamin D deficiency are not specific. These include fatigue, poor mood, insomnia, headache, frequent infections (also in mouth and esophagus), excessive hair loss, pain in muscle, bone and joint. However, such ailments can arise from many other conditions. To make sure that the body has adequate vitamin D level, people should be checked for 25(OH)D metabolite level in serum. In the case of a deficiency, appropriate supplementation can be used after consulting a physician.

First of all, the need for supplementation and the need for treatment should be clearly distinguished. Dietary supplements can be used in the absence of clinical confirmation of the deficiency, only to supplement the appropriate diet. It is very important to remember to use only proven supplements, as many of them are of poor quality. Appropriate dosage depends on age, climatic zone, body weight, diet,

Table 3. Dosage methods of vitamin D_3.

Combination	Main application	Comments
D_3	prevention and treatment of osteoporosis; pleiotropic extrosseus action	vitamin D deficiencies are common in all age groups
D_3 & K_2	prevention and treatment of osteoporosis; bone mineralization and repair, formation of new blood vessels	caution in people with blood clotting problems
D_3 & K_2 & Ca	prevention and treatment of osteoporosis; D_3 increases the level of Ca in the body, and K_2 coordinates the processes of its absorption	only Ca does not raise its level in the bones, and the excess is deposited in the arteries
D_3 & Mg	prevention and treatment of osteoporosis; Mg supports the proper absorption of D_3 and contributes to its activation in the body	Mg deficiency (highly processed food)
D_3 & A	against toxic effects; slows down skin aging; stimulates skin proteins to regenerate	loss of elasticity, discoloration, irritation
D_3 & D_2	prevention and treatment of osteoporosis; during research	theoretically similar action, but D_2 has a shorted half-life and should not be combined with D_3

and the presence of risk factors, and may be modified by national societies (Table 2).

According to the 2012 guidelines for vitamin D supplementation, in Central Europe, the daily dose for healthy people aged 11–75 is 800–2000 IU/day (depending on the body weight). This value increases to 2000–4000 IU/day for people over 75 years of age (Płudowski et al. 2013). Interestingly, it has been proven that administering a cumulative weekly or monthly dose may have the same effect as taking the vitamin daily in single doses, and this greatly facilitates cooperation with the patient (Souberbielle et al. 2010). In case of diagnosed deficiencies, the administered dose depends on concentration of 25(OH)D and should be used for at least 3–6 months. Additionally, every 6–8 weeks a control test should be performed, and after improving the content, one should continue using vitamin D at doses consistent with the guidelines for supplementation in healthy people. Moreover, after completing regular supplementation, 25(OH)D level should be examined after 3 months (Płudowski et al. 2013).

The preparations used to supplement vitamin D deficiency may contain various forms of this vitamin, cholecalciferol (D_3) or ergocalciferol (D_2; on the American market) (Gruber 2015). The preparations are usually in the form of tablets or capsules, generally as an ingredient of cod liver oil (Table 3). In US and New Zealand very popular is food containing vitamin D, e.g. cheese, milk, yoghurt (Thomson and Cressey 2011). In the UK, an oral spray with vitamin D is available in doses 1000 and 3000 IU for adult or 400 IU for children. Vitamin D is absorbed very quickly from this product, what helps to avoid gastrointestinal interactions. Preparations containing vitamin D in doses of 400, 500, 800, 1000, 2000, 4000 IU, are also available in combination with other ingredients, e.g. calcium. Very often, preparations with vitamin D are supplemented with vitamin K, which is associated with the synergistic effect of both vitamins, e.g. on the skeletal, cardiovascular, immune systems and anti-cancer effects (Kidd 2010). The principle of combining the three components, i.e. vitamin D, K and calcium, has many supporters who believe that such a trio has a beneficial effect on bone physiology (Wawrzyniak et al. 2015; Anuszewska 2011). Some argue that vitamin D in large amounts can reduce the level of vitamin K in the body (Masterjohn 2007). There are also opinions about the difficulties in controlling the appropriate level of calcium during supplementation, and about the lower than expected effect of vitamin K (Kostecka 2017; Tripkovic et al. 2012). There is also the issue of simultaneous supplementation of vitamin D and A. Retinol has been found to be effective in protecting against the toxic effects of vitamin D, possibly by reducing the amount of protein that is gamma-carboxylated by vitamin K (Masterjohn 2007). It should be noted that vitamin D analogues and metabolites are not routinely used in supplementation in healthy people; calcidiol is used in the case of liver failure, and calcitriol, α-calcidol and paricalcytol in people with renal failure (Rusińska et al. 2018).

Theoretically, the source of vitamin D can be tanning salons using UVB radiation. However, it is not a recommended method due to its potential harmful effect on the skin. UV radiation can promote the DNA mutation in skin cells and initiate of carcinogenesis (Grant and Holick 2005; D'Orazio et al. 2013).

As previously mentioned, there are various forms of vitamin D, although the physiological compound in humans and animals is vitamin D_3. Its positive effect has been proven in randomized clinical trials (Tripkovic et al. 2012; (Rani 2014). Differences in the plasma half-lives of 25(OH)D_2 and 25(OH)D3 depending on DBP concentration and genotype were observed (Jones et al. 2014). Furthermore, ergocalciferol and its metabolites have a lower affinity for the transporter protein and have a shorter half-life. Although it is absorbed to a similar extent as cholecalciferol, but after a few days of supplementation, its concentration begins to drop rapidly, unlike vitamin D3, which remains at the same level for about 14 days (Alshahrani and Aljohani 2013). It was also observed that the metabolism of vitamin D_2 in contrast to D_3 is significantly reduced with age. Vitamin D_2 can degrade faster under the influence of temperature and moisture (Arnarson 2018). These two forms were combined in some pharmaceutical formulations, based on the assumed effectiveness of high doses of vitamin D_2 in prevention of rickets and treatment of osteomalacia, before studies comparing activity of vitamin D_2 and D_3. Currently, it is believed that ergocalciferol should not be added to such preparations (Tripkovic et al. 2012; Romagnoli et al. 2008).

In the case of animals, supplementation is in the form of food and feed enriched with vitamin D_3 in the form of powder, emulsions, oil solutions, as well as gelatin granules with vitamin with the highest confirmed stability is necessary. For dogs, The National Research Council reports that the optimal amount of cholecalciferol is 13.8 μg/kg of food for all breeds. According to the Association of American Feed Control Officials, the value is 500 IU/kg of food for dogs

and adult cats, while for young cats 750 IU/kg of food. Interestingly, in cats, rickets is most often the result of abnormal levels of phosphorus and calcium, not vitamin D deficiency. Sometimes, feed irradiation is used to increase the amount of available vitamin D. The source of vitamin D for animals is also sebum, obtained from the hair. The animals that feed on pastures get their own vitamin D_2, which is also found in hay. As for the activity of vitamin D_2 and D_3, a similar phenomenon is observed, with the exception of birds, poultry and a few less popular mammals, in which cholecalciferol is more active. However, this claim has not yet been properly confirmed (DSM 2019). The stability of vitamin D in fortified feed should be ensured by e.g. removing air, proper storage or addition of antioxidants. It has been shown that cholecalciferol is more stable than ergocalciferol in preparations in the presence of other minerals (McDowell 2000). As can be seen from the presented information, the growing awareness of control and possible supplementation with vitamin D gives real possibilities to improve human health and well-being. Unfortunately, in animals (especially dogs), vitamin D levels are not controlled. If there are large differences in the production and consumption of fortified feed, be careful, because dogs do not have the appropriate defense mechanisms (like human) to remove excess vitamin D from the body, and too high concentrations are toxic to them (Gow et al. 2011; Sharp, Selting, and Ringold 2015).

Toxicity

It should be remembered that vitamin D as a lipophilic compound has the ability to accumulate in adipose tissue. It is connected with the real risk of overdosage and, consequently, a toxic effect. That occurs when the amount of metabolite formed in the liver exceeds 200 ng/mL (500 nmol/L), therefore it is recommended to keep this value below 100 ng/mL (Lappe 2011). The occurrence of hypersensitivity manifested by excessive synthesis of calcitriol is also known. It is often caused by cancer, hyperparathyroidism or sarcoidosis (Alshahrani and Aljohani 2013). Generally, it is not possible to overdose on vitamin D due to the excess production in the skin during prolonged sun exposure, because vitamin degradation products are formed simultaneously. Likewise, it would be difficult to exceed the appropriate vitamin D levels from eating too much food containing this vitamin. However, overdosing can occur as a result of administration of supplements or/and medicines. The toxic effect is believed to occur with vitamin D supplementation at a dose above 10,000 IU/day, and in less sensitive people even over 40,000 IU/day. The symptoms of short-term toxicity include polyuria, weakness of muscle, palpitations, weight loss, and nausea. It has been proven that long-term intake of doses even lower than 10,000 IU/day can lead to many diseases, such as calcification of vessels due to increased calcium levels, kidney stones, pancreatic cancers and cardiovascular diseases (ODS 2019; Lappe 2011).

In animals, cholecalciferol is more toxic than ergocalciferol in too large amounts. It is believed that in many cases exceeding the recommended amount of vitamin D by 4–10 times with long-term use is still a relatively safe. The effects of overdose, as in humans, are calcifications, weight loss, disturbances in calcium and phosphate levels, in broilers it leads to heart failure, and in other birds to arrhythmias. Young dogs showed changes in the kidneys, growth disorders and physical activity. Due to clinical signs of toxicity, vomiting, polyuria and weakness may occur (DSM 2019).

Interactions

When preparations containing vitamin D are used, it is necessary consider possible interactions with other drugs or supplements. First, attention should be paid to medications that affect calcium levels in blood, which control vitamin D metabolism through negative feedback. Excessive storage of vitamin D in the body can lead to toxic effects and hypercalcemia (high levels of calcium in blood) that leads to calcification of the heart, kidneys, lungs and blood vessels. Such medications are, for example, glucocorticoids or thiazide diuretics. Also, agents that reduce fat absorption in the gastrointestinal tract – colesevelam or orlistat, change the level of vitamin D in blood. Substances that activate liver enzymes, such as antiepileptic drugs – phenobarbital or phenytoin, increase the formation of inactive metabolites (ODS 2019). Severe swelling of the legs and constipation may occur in patients in whom hypertension drugs are combined with calcium and vitamin D. Vitamin D cannot be used simultaneously with foods that contain a lot of fiber, such as cereal flakes.

A very important issue is the effect of magnesium on the level of vitamin D. This element is involved in the metabolism of vitamin D. In US National Health and Nutrition Examination Survey hypothesized that magnesium can increase the level of 25(OH)D in the body, which was later proven in subsequent research (Zittermann 2013; Deng et al. 2013). The consumption of magnesium reduces the likelihood of insufficient vitamin D levels in the body. Vitamin D can simultaneously stimulate the intestinal absorption of magnesium, a deficiency of which can result in tiredness, fatigue or problems with concentration. This is very important if someone also supplements magnesium and vitamin D at the same time, because it can lead to toxicity of this vitamin. Also one should note the importance of securing an adequate level of magnesium in preventing vitamin D deficiency.

Conclusions

Lifestyle changes in recent years have significantly reduced people's ability to synthesize vitamin D. Moreover, the ability of living organisms to produce the endogenous form of vitamin D does not extend to them all. At the same time, its administration, in some cases, may disrupt various metabolic pathways. The information presented in this review provides a deeper understanding of the mechanisms responsible for the development of many diseases related to vitamin D levels, which allows to reduce the risk of their occurrence. Adjusting the doses of vitamin D intake to the actual needs of humans and animals is a very important goal for health protection.

Disclosure statement

No potential conflict of interest was reported by the authors.

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