Can we accurately measure the concentration of clinically relevant vitamin D metabolites in the circulation? The problems and their consequences

Czy umiemy wiarygodnie mierzyć stężenie klinicznie ważnych metabolitów witaminy D? Problemy i ich konsekwencje

Zbigniew Bartoszewicz[1, 2], Agnieszka Kondracka[1, 3], Radosław Jaźwiec[4], Michał Popow[1], Michał Dadlez[4], Tomasz Bednarczuk[1, 2]

[1]*Department of Endocrinology, Medical University of Warsaw, Poland*
[2]*Endocrinology Unit, Department of Human Epigenetics, Mossakowski Medical Research Centre, Polish Academy of Sciences, Poland*
[3]*Department of Neuropeptides, Mossakowski Medical Research Centre, Polish Academy of Sciences, Poland*
[4]*Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Poland*

Abstract

Increased interest in vitamin D measurements in clinical studies has contributed to the development in recent years of several new immunochemical assays (manual and for automatic analyzers). New methods, including HPLC (high performance liquid chromatography), and LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry) have also been introduced into routine diagnostic laboratories. Because of the variety of assays and methods used, the question arises which one is the most accurate for the measurement of vitamin D metabolites concentration. In this review, we summarise the advantages and disadvantages of these methods, describe the complexity of vitamin D metabolites pattern in the circulation, and discuss the problem of accurate measuring its concentration. **(Endokrynol Pol 2013; 64 (3): 238–245)**

Key words: *vitamin D, calcidiol, calcitriol, immunoassays, LC-MS/MS*

Streszczenie

Wzrost zainteresowania oznaczeniami stężenia witaminy D przyczynił się do opracowania w ostatnich latach nowych testów immunochemicznych (manualnych oraz do automatycznych analizatorów). Do laboratoriów diagnostycznych wprowadza się również techniki dotychczas niestosowane w rutynowych oznaczeniach witaminy D, takie jak HPLC (wysokosprawna chromatografia cieczowa) oraz LC-MS/MS (tandemowa spektrometria mas sprzężona z wysokosprawną chromatografią cieczową). W związku z różnorodnością testów i metod pojawia się pytanie o ich wiarygodność. W niniejszej pracy przeglądowej opisano występujące w krwiobiegu metabolity witaminy D, przedstawiamy zalety i wady stosowanych metod oznaczania ich stężenia oraz omawiamy czynniki mające wpływ na wiarygodność wyników tych oznaczeń. **(Endokrynol Pol 2013; 64 (3): 238–245)**

Słowa kluczowe: *witamina D, kalcydiol, kalcytriol, testy immunochemiczne, LC-MS/MS*

Introduction

The high interest in vitamin D due to the important, complex, pleiotropic role it plays in the human body and the consequences of its insufficiency for our health have resulted in a dramatic increase in vitamin D metabolite concentration measurements [1–4]. It is believed that vitamin D status in humans is best reflected by the concentration of its 25-hydroxylated isoforms (25(OH) D, calcidiol) which are the most frequently assessed vitamin D metabolites, whereas the measurement of 1,25-dihydroxylated forms (1,25(OH)$_2$D, calcitriol) are of limited use [5, 6]. A variety of vitamin D metabolites appear in the circulation, and the different assays and methods used for its determination affect the accuracy

of the results obtained. This issue, of great importance for clinical practice, we would like to discuss in this article.

The variety of vitamin D metabolites in the circulation

Vitamin D occurs in nature in two chemical forms: vitamin D$_2$ and D$_3$ which differ in a double bond between carbon atoms 22 and 23 and a methyl group at carbon atom 24 (Fig. 1). In humans, the most common form is vitamin D$_3$, mainly synthesised during skin exposure to UV rays of sunlight, which constitute up to 90% of the total vitamin D body pool [7, 8]. Casual UVB irradiance in summer can generate 1,000–2,000 IU/day of vitamin

✉ Zbigniew Bartoszewicz Ph.D., Endocrinology Laboratory, Department of Endocrinology, Medical University of Warsaw, Banacha 1a, 02–097 Warsaw, tel.: +48 22 599 17 53, fax: +48 22 599 19 75, e-mail: zbigniew.bartoszewicz@wum.edu.pl
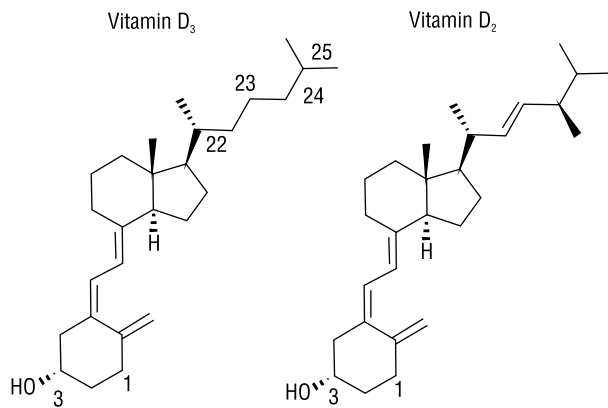
Figure 1. *Chemical structure of vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol) with marked carbon atoms undergoing hydroxylation. Carbon atom C-3 is a potential centre for epimerisation*

Rycina 1. *Struktura chemiczna witaminy D3 (cholekalcyferol) i D2 (ergokalcyferol) z zaznaczonymi atomami węgla podlegającymi hydroksylacji. Atom węgla C-3 stanowi potencjalne centrum epimeryzacji*

D_3 [8]. It can be also supplied with food of animal origin (mostly wild, oily fish) [9]. Vitamin D_2 is synthesised by plants, yeast and mushrooms and delivered with diet [10, 11]. Both D_2 and D_3 can be administered to patients as a drug in a case of vitamin D insufficiency. In people ingesting a high amount of D_2, the ratio of D_3 to D_2 can be substantially different from the average found in the population.

Vitamin D produced or taken with the diet is transported to the liver, and its excess is stored in fat tissue and muscle. The storage of vitamin D_3 in fat tissue considerably extends its half-life to as much as two months, whereas in plasma it lasts 2–3 days (Table I). The plasma half-life of vitamin D_2 is shorter because of its low affinity to specific transporting protein VDBP (vitamin D binding protein) protecting bound fraction against metabolism and excretion [12]. To achieve biological activity, vitamin D_2 and D_3 have to be hydroxylated, first in the liver to 25-hydroxylated metabolites 25(OH)D_2 and 25(OH)D_3 (calcidiol), then in the kidney (1α-hydroxylation) into vitamin D most biologically active calcitriol: 1,25(OH)$_2$D$_2$ and 1,25(OH)$_2$D$_3$ (Fig. 2) [6, 7, 13]. Synthesis of calcitriol is controlled in the kidney by a negative feedback mechanism protecting against excessive hydroxylation at the carbon atom C-1. High level of calcitriol inhibits the synthesis of PTH (parathyroid hormone) on genetic level. A diminished concentration of PTH decreases 1α-hydroxylase activity, and in that way synthesis of calcitriol. In addition, reabsorption of phosphates increased by calcitriol causes an enhanced release of FGF-23 (fibroblast growth factor 23) which

activates 24-hydroxylase, leading to the synthesis of 24,25(OH)$_2$D in the kidney — the first step of inactivation and removal of vitamin D from the circulation (Fig. 2). Although 24,25(OH)$_2$D is usually considered as an inactive metabolite, it plays an important role in the maturation and growth of cartilage [14, 15]. In the next step, water-soluble calcitroic acid is produced and excreted in bile. The vitamin D metabolites hydroxylated at C-23 or C-26 are also found in blood [13].

The variety of vitamin D metabolites in the circulation is further increased by two natural processes: epimerisation and lactonisation. Epimerisation of vitamin D metabolites is the process of enzymatic conversion around the asymmetric carbon atom, which does not change molecular weight and atoms sequence, the only difference being stereo configuration. Carbon atom C-3 is a potential epimerisation centre for 25(OH)D, 1,25(OH)$_2$D and other vitamin D metabolites. The C-3β-hydroxyl vitamin D metabolites that are synthesised in nature are converted into the corresponding C-3α-hydroxyl epimers (C3-epimers) by specific epimerases located in the cytosolic fraction of the liver and locally in cells (e.g. keratocytes, macrophages). Epimerisation influences the chemical and biological activity of vitamin D metabolites [16, 17]. Both α and β C3-epimers of vitamin D can be further hydroxylated in the kidney [18]. Another structural change of vitamin D is lactonisation with the formation of additional ring structure between C-23 and C-26 [19, 20]. The side chain modified lactones act as partial agonists of vitamin D receptor (VDR) and may modulate its activity [21].

Because of high hydrophobicity, the vitamin D metabolites are poorly soluble in aqueous media, and similarly to the thyroid and steroid hormones, are transported in blood in bound form – mostly to specific carrier protein VDBP and loosely bound to albumin (Table I). Free 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ forms in blood account for about 0.03–0.4% and 0.4%–2% of the total metabolite respectively. The level of unbound 1,25(OH)$_2$D is well correlated with its biological activity, which suggests that the free hormone hypothesis may apply to the vitamin D system [12]. However, methods permitting the direct measurement of free fractions of 25(OH)D or 1,25(OH)$_2$D for diagnostic purposes are unknown.

Binding to the blood components ensures the vitamin D metabolites high stability in serum and plasma [22]. They are stable even for several days when kept at room or increased temperature, and are unaffected either by light facilities or multiple freeze-thaw cycles [23], but caution has to be paid when vitamin D is released from VDBP and separated from blood because such samples are light- and temperature-sensitive [24, 25]. This applies to some vitamin D standards and
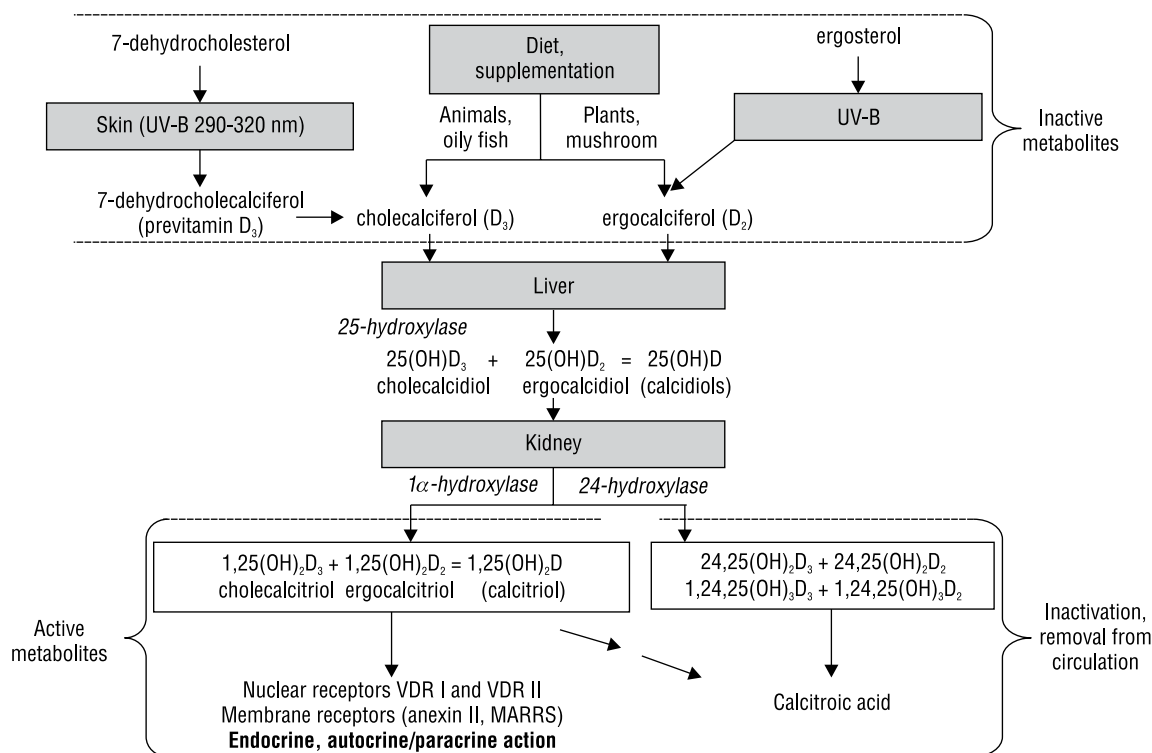
Figure 2. *Synthesis and metabolism of vitamin D*
Rycina 2. *Synteza i metabolizm witaminy D*

Table I. *Characteristics of vitamin D_3 and its metabolites*
Tabela I. *Charakterystyka witaminy D_3 i jej metabolitów*

Parameters	D_3	$25(OH)D_3$	$1,25(OH)_2D_3$
Concentration in plasma	0.4–2.8 ng/mL	8–56 ng/mL (winter up to 24 ng/mL)	16–65 pg/mL
Half life in plasma	36–78 hr	15–25 days	3.5–21 hr
Affinity to Calcitroic acid		$K_a = 500$–700 uM^{-1}	$K_a = 40$ uM^{-1}
Affinity to albumin		$K_a = 0.6$ uM^{-1}	$K_a = 0.054$ uM^{-1}
Bound to VDBP (%)		85–99%	60–85%
Bound to albumin (%)		13–15%	38%
Free fraction (%)		0.03–0.4%	0.4–2%
Affinity to VDR		$K_a = 0,6$ uM^{-1}	$K_a = 10^4$–10^5 uM^{-1}

samples extracted for HPLC and LC-MS/MS analysis which should be kept in amber tubes or dark glass vials at –70°C.

Clinical usefulness of vitamin D metabolite measurements

Providing the optimal vitamin D supply is essential for human health [1, 7, 26]. The role of vitamin D in the body is multidirectional [1, 27]. The active metabolite 1,25(OH)$_2$D is directly involved in the absorption of calcium and phosphate which is important to ensure proper bone mineralisation. There are studies indicating that serum level of 25(OH)D below 20 ng/mL is associated with an increased risk of cardiovascular disease, some cancers and autoimmune diseases [28, 29]. Recent reports on the non-classic mechanisms of vitamin D action have indicated its physiological role in the regulation of innate and acquired immunity [30–32]. Vitamin D stimulates the differentiation of monocyte

Table II. *Frequently used cut-points for vitamin D status [3, 11, 34]*

Tabela II. *Wartości odcięcia używane najczęściej dla oceny statusu witaminy D [3, 11, 34]*

Classification	25(OH)D ng/mL*	25(OH)D nmol/L*
Toxic level	> 100	> 250
Sufficiency or optimal level	30–80 in adults	75–200 in adults
	20–60 in children	50–150 in children
Insufficiency	20–30 in adults	50–75 in adults
	10–20 in children	25–50 in children
Deficiency	10–20 in adults	25–50 in adults
Severe deficiency	< 10	< 25

*Conversion factors for 25(OH)D: nmol/L x 0.40 = ng/mL (ng/mL = ug/L); ng/mL x 2.50 = nmol/L

precursors to more mature phagocytic macrophages and, by binding to the toll-like receptor (TLR2), the main receptor recognising pathogens, can stimulate the expression of the antibacterial protein cathelicidin. The stimulatory effect of vitamin D in the response to infection or inflammation has been demonstrated in many diseases such as tuberculosis, multiple sclerosis, Crohn's disease, type 1 diabetes, *E. coli* infection of the liver, malaria and typhoid [31].

The above examples unequivocally show the importance of optimal vitamin D supply which can be achieved by exposure to the sun, proper diet or supplementation with pharmaceutical preparations [33, 34]. Vitamin D status greatly depends on factors such as food habits, genetic factors, climatic conditions determining the skin synthesis and the cultural habits of exposing the body to sunlight (especially in women) [35, 36]. According to international and Polish recommendations, the optimal serum level of 25(OH)D is above 30 ng/mL for adults and 20 ng/mL for children (Table II) [6, 11, 33, 34]. Population studies clearly show that vitamin D insufficiency is a global epidemic, as well as being widespread in the Polish population [33, 34].

Choosing the best metabolite for assessing the body storage of vitamin D

Vitamin D status in the human body is best reflected by the total concentration of its 25-monohydroxylated isoforms: $25(OH)D_3$ and $25(OH)D_2$ [5, 6, 37]. The determination of the 25(OH)D is of great importance since it detects both a shortage of vitamin D and its overdosing in a case of poorly controlled supplementation [3, 26, 40].

Although $1,25(OH)_2D$ is the active form of hormone, its short half-life, significant dependence on renal failure, and low concentration in plasma (more than 1,000 times lower compared to $25(OH)_2D$) bring about technical challenges and then the limited usefulness of its measurement [12]. This parameter is not useful in vitamin D deficiency moni-

toring because, even in severe vitamin D deficiency, this parameter may still remain in the reference range. However, monitoring of $1,25(OH)_2D$ can be useful in rare cases of renal and hepatic failure and suspected vitamin D resistance or acquired and inherited disorders of $1,25(OH)_2D$ metabolism [37]. The concentration of vitamin D_3 and D_2 in plasma is diet- and supplementation-dependent, and highly affected by skin synthesis [12]. Attempts to determine the D_3 level in human fat tissue have also been described [38]. A higher concentration of D_3 in adipose tissue has been found in obese compared to normal weight people, although this analysis has no practical value [39].

Clinical reasons for vitamin D measurement

Vitamin D measurements are useful in the following clinical situations [1–6, 32, 41–43]:

Osteoporosis. Vitamin D is the only accessible hormone affecting the expression of calbindin in the gut which is responsible for the active absorption of calcium from the lumen of jejunum. The reduction of muscle strength and increased risk of falls with bone fracture is directly related to a vitamin D deficiency.

Disorders of malabsorption. Disorders of the absorption such as Crohn's disease, coeliac disease or gut amyloidosis (involving the proximal part of the jejunum) lead to impaired absorption of calcium and chylomicrons containing vitamin D_3.

Pregnancy. The metabolism of pregnant woman is related to the development of the foetus. Placental secretion of PTHrP (PTH-related protein) causes the transport of calcium ions against the natural gradient from mother to child. The increased demand for calcium increases absorption of this ion in the digestive tract. This adaptation requires the proper supply of vitamin D. Moreover, the correct concentration of vitamin D during pregnancy independently of other factors reduces the risk of diabetes and preeclampsia [44–46].

SZKOLENIE PODYPLOMOWE

Calcium and phosphate balance. It is now generally accepted that 25(OH)D should be measured in patients with primary hyperparathyroidism; balanced metabolism of vitamin D lowers the level of PTH and reduces the risk of 'hungry bones syndrome' after surgical treatment of primary hyperparathyroidism. An indication to measure calcitriol is primarily a differential diagnosis of hypercalcaemia due to the ability of certain granulomas to perform hydroxylation of vitamin D at C-1.

Renal failure. Renal failure leads to lowering of $1,25(OH)_2D$ due to impaired 1α-hydroxylation.

Rickets and osteomalacia. Testing of vitamin D status is used in differential diagnosis of rickets and osteomalacia (deficiency of vitamin D, the resistance of the receptor for vitamin D, hypophosphataemic rickets). Low calcitriol can be observed in vitamin D-dependent rickets type I (inborn reduction of 25(OH)D to $1,25(OH)_2D$ conversion), whereas its high level accompanies D-dependent rickets type II (inborn defect of $1,25(OH)_2D$ recognition by target tissue).

Monitoring of vitamin D supplementation, especially when applying vitamin D_2. Due to the moderate affinity to VDBP, $25(OH)D_2$ has a shorter half-life in serum than $25(OH)D_3$ and consequently may be less biologically active. Monitoring the supplementation can be crucial in patients with intestinal malabsorption, severe hepatic failure or who are at higher risk of vitamin D deficiency (e.g. very young and elderly people).

Other diseases. Low 25(OH)D concentration may be associated with cardiovascular events, hypertension, metabolic syndrome, high blood sugar and other chronic diseases. Optimal vitamin D level improves the outcome in critically ill patients. There is no justification for the determination of vitamin D in order to determine the risk of any cancer, despite statistical data showing the influence of vitamin D on improving immunological response and reducing the risk of cancer.

Overview of methods used to measure the vitamin D metabolites concentration

Many methods are used to measure vitamin D metabolites concentration in serum or plasma. The most popular are immunoassays, but due to unsatisfactory results of inter-laboratory quality control programmes, new methods including HPLC, GC-MS and LC-MS//MS are being introduced into routine diagnostic laboratories [47–55].

Immunoassays
The measurement of 25(OH)D concentration. Various formats of vitamin D immunoassays for the measurement of 25(OH)D concentration are available, including direct immunoassays and preceded by extraction immunoas-

says. All of them encounter methodological problems. Firstly, there are two forms of 25(OH)D: $25(OH)D_3$ and $25(OH)D_2$ which differ in their chemical structure (Fig. 1) as well as in their affinity to antibodies used in the assay (Table III). In consequence, the total concentration of 25(OH)D measured in a patient's serum is highly dependent on the $25(OH)D_3$ to $25(OH)D_2$ ratio and the source of antibody used for immunoassay construction [49–51]. Furthermore, in some assays VDBP is used in exchange for recognising antibody [56]. These are probably the most important reasons why it is difficult to compare results obtained by various immunoassays. Especially in people taking D_2 supplementation or being on a diet with relatively high vitamin D_2 doses (e.g. a vegetarian diet), the total 25(OH)D concentration may differ when measured with different immunoassays.

Another problem is the ability of vitamin D immunoassays to recognise the C-3 epimers of 25(OH)D. This is not a major problem for most adult human samples, where C-3 epimers are present in small amounts. According to Lensmeyer et al. [57], in 92% of adult samples tested by the most suitable LC-MS/MS method, the concentration of 3-epi-$25(OH)D_3$ is 3.0 ng/mL or lower, although the authors described some patients where 3-epi-$25(OH)D_3$ accounted for 25% of the total 25(OH)D level. In adults, the 3-epi-$25(OH)D_3$ concentration is not correlated with age [58] but usually the higher the concentration of $25(OH)D_3$, the higher amount of epimer is observed [57]. C-3 epimerisation is a problem of infants and mothers in the final stages of pregnancy [58, 59]. Total 3-epi-25(OH)D in infants ranged from 5 ng/mL to 93 ng/mL and contributed to 8.7–61.1% of total 25(OH)D concentrations [59]. Separate issues are reagents used to block the effect of steroid hormones and cross-reactions with compounds structurally related to 25(OH)D including D_2, D_3, $1,25(OH)_2D_2$, $1,25(OH)_2D_3$, $24,25(OH)_2D$, their lactons and C-3 epimers, which in individual patients can lead to false vitamin D results.

The problems described above make it difficult to reliably measure the 25(OH)D concentration by immunochemical methods and to establish the precise boundaries of vitamin D deficiency. As a consequence, the interassay differences have an impact on clinical decision making [52, 53]. For example, out of 494 patients examined, 52% were classified as deficient patients by DiaSorin Liaison immunoassay, but only 36% when the concentration of 25(OH)D was measured by IDS RIA test [52]. The significant disparity of 25(OH)D results obtained by different immunoassays has also been observed in participants of international quality programmes [51, 55, 56]. The Vitamin D External Quality Assessment Scheme (DEQAS) organised in Europe by Dr. Carter has compared the accuracy of 25(OH)D methods over more than two decades. The mean deviation

Table III. *Cross-reactions (%) of vitamin D$_2$ and D$_3$ metabolites in diagnostic immunoassays used for 25(OH)D measurement. Data from manufacturer's instructions. The cross-reactions with 1,25(OH)$_2$D$_2$ and 1,25(OH)$_2$D$_3$ were not considered, because of their irrelevant concentration compared to other vitamin D metabolites*

Tabela III. *Reakcje krzyżowe (%) metabolitów witaminy D$_2$ i D$_3$ w testach immunochemicznych używanych do diagnostycznych pomiarów stężenia 25(OH)D. Nie uwzględniono reakcji krzyżowych dla 1,25(OH)$_2$D$_2$ i 1,25(OH)$_2$D$_3$, ponieważ ich stężenie jest nieistotne w stosunku do innych metabolitów witaminy D*

Manufacturer (automatic system)	Roche Diagnostics (Elecsys, Cobas)	Roche Diagnostics (Elecsys, Cobas)	IDS (iSYS)	DiaSorin (Liaison)	Siemens (Advia, Centaur)	Abbott (Architect)	DiaSource	DiaSource	DiaSource	Immunodiagnostic	Immunodiagnostic
Test name	Vitamin D Total	Vitamin D$_3$ (25-OH) (previous version)	25-OH Vitamin D	25-OH-vit D Total	Vitamin D Total	25-OH Vitamin D	25OH-vit D Total ELISA	25OH-vit D Total RIA-CT	25OH-vit D3 RIA-CT	25-OH Vitamin D direct ELISA	25-OH Vitamin D EIA
25(OH)D$_3$	98	100	100	100	97.4	105	100	100	100	100	100
25(OH)D$_2$	81	< 10	75	100	106.2		83	95	< 0.3	67.8	100
C3-epi-25(OH)D$_3$	93			0	1	2.7	< 0.2				
24,25(OH)$_2$D$_3$	121	< 20	> 100			112	> 100	> 100	< 0.8	> 100	100
25,26(OH)$_2$D$_3$							>100				
D$_3$	5	< 1	< 0.01	1.1%	0.3	0.3	< 0.2	< 0.3	< 0.03		<1
D$_2$	6	< 1	< 0.03	1.4%	0.5	0.1	< 0.2	< 0.3	< 0.03	0.3	<1

(% bias) from the target value (all laboratory trimmed mean) for immunoassays has significantly improved over the years, from about 35% to 20%. [51, 54, 56] but still the results of 25(OH)D concentration should always be interpreted together with other laboratory results (concentration of ionised calcium, calcium excretion, concentration of phosphates and PTH) and the clinical picture of the patient.

The measurement of 1,25 (OH)$_2$D concentration. Although the concentration of 1,25(OH)$_2$D in blood is more than three orders of magnitude lower compared to 25(OH)$_2$D, in some clinical cases the measurement of 1,25(OH)$_2$D concentration can be useful. Unfortunately manual, laborious immunoassays are the only method applicable so far. All these methods are based on the preliminary separation of 1,25(OH)$_2$D by the use of columns with VDBP. After the release of bound vitamin D, the immunoassays with antibodies specific to 1,25(OH)$_2$D are used. The problem of disparate recognition of 1,25(OH)$_2$D$_2$ and 1,25(OH)$_2$D$_3$ by different antibodies which affects the final result of the assay is the same as in the case of 25(OH)D measurements.

LC-MS/MS

Due to the high complexity of instrumental setup and high requirements for specialised personnel in the diagnostic laboratory, LC-MS/MS has been primarily used as a reference method, although great advances in equipment automation, increases in staff qualifications and the widespread use of mass spectrometry methods in other areas have led to increased interest in LC-MS//MS methods in routine medical diagnostics, as well as an attractive alternative to immunoassays in general [38, 48, 50, 53, 57–62].

Description of the method. The specificity of the LC-MS/MS methods is based on differences in molecular masses of analysed substances and their characteristic fragments. Additionally, prior to MS analysis, all compounds are separated by liquid chromatography and distinguished by retention time. In order to provide better precision of quantification, stable isotope-labelled compounds are used as internal standards. For routine quantitative analysis of clinical samples, the most commonly used instrumental setup is high or ultra performance liquid chromatography (HPLC or UPLC) coupled with a triple quadrupole

SZKOLENIE PODYPLOMOWE

mass spectrometer [62]. The proper choice of ionisation method is of great importance for the best sensitivity and reproducibility of quantitative determinations. Mass spectrometers analyse ions in high vacuum which requires evaporation and ionisation of compounds of interest. There are three main methods of ionisation commonly used in LC-MS/MS. First and most popular is ESI (electrospray ionisation). Its advantages are: simplicity of operation, very broad dynamic range (up to four orders of magnitude), and general availability. This mode allows the analysis of compounds of high molecular masses (even above 30 kilodaltons) such as polypeptides and proteins. For the determination of non-polar compounds like vitamin D metabolites and some steroid hormones, better results can be achieved by two other modes of ionisation: APCI (Atmospheric Pressure Chemical Ionisation) and APPI (Atmospheric Pressure Photo Ionisation). In those modes, ionisation takes place in the gas phase, which limits analysis to relatively lower molecular masses (usually below 1 kilodalton). The most commonly used mode of operation in quantitative MS is called MRM (Multi Reaction Monitoring). In the MRM mode, a spectrometer using first quadrupole mass filter selects ions with the same mass to charge ratio (m/z) as compound of interest (parent ion). In the second quadrupole, these ions undergo decomposition induced in a controlled manner through collision with neutral gas molecules. Third quadrupole allows to pass only ion of fragments characteristic to analyte of interest (daughter ions). The monitoring of the two specific for a given compound ion pairs (parent ion – daughter ion) which is called 'ion transmission' is considered sufficient for an unambiguous verification of the identity of the analyte and enables quantitative analysis based solely on mass spectrometry data. Of all modes of operation used in mass spectrometry, MRM provides the best sensitivity and reproducibility of quantitative determinations, while ensuring the specificity unattainable by using spectrophotometric or fluorumetric detectors.

Determination of 25(OH)D. The measurement of 25(OH)D in the blood by LC-MS/MS seems to be particularly valuable because it enables not only un-ambiguous quantification of total serum 25(OH)D but also the identification of unusual or biologically less active metabolites, such as 3-epi-25(OH)D$_3$ and precise relationships between the various isoforms and their metabolites in the course of a single analysis of the same sample [57–59]. This method allows the avoidance of errors related to cross-reaction of antibodies with other vitamin D metabolites that occur in immunochemical methods and the diversity of results due to individual recovery of vitamin D metabolites and internal standards used in HPLC methods [60–62].

Other vitamin D metabolites. Although the sensitivity of LC-MS/MS is sufficient enough for the determination of 1,25(OH)$_2$D metabolites, such a method is not developed yet. Attempts are being made to determine vitamin D$_2$ and D$_3$ in tissues, particularly in adipose tissue [38,63] but these methods have not yet been introduced into routine laboratory practice.

HPLC and GC-MS

High performance liquid chromatography (HPLC) and gas chromatography coupled with mass spectrometry (GC-MS) are techniques used to determine vitamin D metabolites in research projects rather than in routine medical diagnostics. HPLC requires time-consuming initial separation including liquid-liquid extraction with organic solvents and solid phase extraction (SPE) for cleaning of the sample. A lot of efforts have been made to minimise the time-consuming operations and to develop on line sample clean-up [64]. However, all purification steps prior to vitamin D determination can lead to false results due to different individual recovery of the vitamin D isoforms and internal standards [62]. To correctly assess the concentration of various vitamin D metabolites by HPLC, their precise separation from other structurally related analytes on chromatography columns is necessary. This is not always possible, as well as the full separation of α and β C3-epimers [65–66]. Although GC-MS is a very sensitive method, it requires an additional step using harmful chemicals to convert the vitamin D metabolites into volatile derivatives. The yield of this reaction is dependent on the chemical structure of the compound and may be not equal for all vitamin D metabolites [67]. The basic problem, which is not completely resolved in either HPLC or GC-MS, is the accurate standardisation of the method.

Conclusions

Total 25(OH)D is the most commonly measured and most useful vitamin D metabolite in clinical practice. The unreliability of the currently used immunochemical methods causes that LC-MS/MS from the reference, becomes more frequently used routine method. The international quality programmes and internal quality-assurance systems improve the accurate measurement of vitamin D metabolites, but it is still important that laboratories and the medical community are aware of the limitations of the methods applied.

References

1. Holick ME. Vitamin D: evolutionary, physiological and health perspectives. Curr Drug Targets 2011; 12: 4–18.
2. Adams JS, Hewison M. Update in vitamin D. J Clin Endocrinol Metab 2010; 95: 471–478.

3. Rosner CJ. Vitamin D insufficiency. N Engl J Med 2011; 364: 248–254.
4. Hanley DA, Cranney A, Jones G et al. Vitamin in adult health and disease: a review and guideline statement from osteoporosis Canada. CMAJ 2010; 182: E610–E618.
5. Hollis BW. Assessment of circulating 25(OH)D and 1,25(OH)2D: emergence as clinically important diagnostic tools. Nutr Rev 2007; 65: S87–S90.
6. Hollis BW. Assessment of vitamin D status and definition of normal circulating range of 25-hydroxyvitamin D. Curr Opin Endocrinol Diabetes Obes 2008; 15: 489–494.
7. Holick MF. Vitamin D: a D-lightful health perspective. Nutr Rev 2008; 66: S182–S194.
8. Grant WB. The health benefits of solar irradiance and vitamin D and the consequences of their deprivation. In: Holick MF (ed.). Vitamin D — Physiology, Molecular Biology and Clinical Applications. Springer, New Jersey 2010; 745–764.
9. Office of Dietary Supplements, NIH. Dietary Supplement Fact Sheet: Vitamin D. 2008; http://ods.od.nih.gov/factsheets/vitamind-Health-Professional/
10. Matilla PH, Piironen VI, Uusi-Rauva EJ et al. Vitamin D Contents in edible mushrooms. J Agric Food Chem 1994; 42: 2449–2453.
11. Holick MF. Vitamin D and health: evolution, biological functions, and recommended dietary intakes for vitamin D. In: MF Holick (ed.). Nutrition and Health: Vitamin D. Springer Science+Business Media 2010: 3–34.
12. Vieth R. The pharmacology of vitamin D, including fortification strategies. In: Feldman F, Glorieux (eds.). Vitamin D, Second Edition. Elsevier, San Diego 2005; 995–1015.
13. Prosser DE, Jones G. Enzymes involved in the activation and inactivation of vitamin D. Trends Biochem Sci 2004; 29: 664–673.
14. St-Arnaud R, Glorieux FH. 24,25-dihydroxyvitamin D — active metabolite or inactive catabolite? Endocrinology 1988; 139: 3371–3374.
15. Dean DD, Boyan BD, Schwart Z et al. Effect of 1alpha,25-dihydroxyvitamin D3 and 24R,25-dihydroxyvitamin D3 on metalloproteinase activity and cell maturation in growth plate cartilage in vivo. Endocrine 2001; 14: 311–323.
16. Higashi T, Ogasawara A, Shimada K. Investigation of C-3 epimerization mechanism of 24,25-dihydroxyvitamin D3 in rat using liquid chromatography/mass spectrometry. Anal Sci 2000; 16: 477–482.
17. Masuda S, Kamao M, Schroeder NJ et al. Characterization of 3-epi-1a,25-dihydroxyvitamin D_3 involved in 1a,25-dihydroxyvitamin D_3 pathway in cultered cell lines. Biol Pharm Bull 2000; 23: 133–139.
18. Kamao M, Tatematsu S, Hatakeyama S et al. C-3 epimerization of vitamin D3 metabolites and further metabolism of C-3 epimers. J Biol Chem 2004; 279: 15897–15907.
19. Eguchi T, Takatsuto S, Ishiguro M et al. Synthesis and determination of configuration of natural 25-hydroxyvitamin D3 26,23-lactone. Proc Natl Acad Sci 1981; 78: 6579–6583.
20. Tanaka Y, Wichmann JK, Paaren HE, Schnoes HK, DeLuca HF. Role of kidney tissue in the production of 25-hydroxyvitamin D_3-26,23-lactone and 1a,25-dihydroxyvitamin D_3-26,23-lactone. Proc Natl Acad Sci 1980; 77: 6411–6414.
21. Inaba Y, Yamamoto K, Yoshimoto N et al. Vitamin D3 derivatives with adamantane or lactone ring side chains are cell type-selective vitamin D receptor modulators. Mol Pharmacol 2007; 71: 1298–12311.
22. Wielders JPM, Wijnberg FA. Preanalytical stability of 25(OH) — vitamin D_3 in human blood or serum at room temperature: solid as a rock. Clin Chem 2009; 55: 1584–1585.
23. Antoniucci DM, Black DM, Sellmeyer DE. Serum 25-hydroxyvitamin D is unaffected by multiple freeze-thaw cycles. Clin Chem 2005; 51: 258–260.
24. Zerwekh JE. The measurement of vitamin D analytical aspects. Ann Clin Biochem 2004; 42: 272–281.
25. Hollis BW. Measuring 25-hydroxyvitamin D in a clinical environment: challenges and needs. Am J Clin Nutr 2008; 88 (Suppl.): 507–510S.
26. Thacher TD, Clarke BL. Vitamin D insufficiency. Mayo Clin Proc 2011; 86: 50–60.
27. Plum LA, DeLuca HF. Vitamin D, disease and therapeutic opportunities. Nat Rev Drug Discov 2010; 9: 941–955.
28. Kuryłowicz A, Bednarczuk T, Nauman J. Wpływ niedoboru witaminy D na rozwój nowotworów i chorób autoimmunologicznych. Endokrynol Pol 2007; 58: 140–152.
29. Zeeb H, Greiner R. The role of vitamin D in cancer prevention. Dtsch Arztebl Int 2010; 107: 638–643.
30. van Etten E, Steffen K, Gysemans C et al. Regulation of vitamin D homeostasis: implications for the immune system. Nutr Rev 2008; 66: S125–S134.
31. Hawison M. Vitamin D and the immune system: new perspectives on an old theme. Endocrinol Metab Clin North Am 2010; 39: 365–379.
32. Souberbielle J-C, Body J-J, Lappe JM et al. Vitamin D and musculoskeletal health, cardiovascular disease, autoimmunity and cancer: recommendation for clinical use. Autoimmun Rev 2010; 8: 709–715.
33. Holick MF, Binkley NC, Bischoff-Ferrari HA et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab 2011; 96: 1911–1930.
34. Marcinkowska-Suchowierska E, Walicka M, Tałataj M et al. Vitamin D supplementation in adults — guidelines. Endokrynol Pol 2010; 61: 723–729.
35. Holick MF. Enviromental factors that influence the cutaneous production of vitamin D. Am J Clin Nutr 1995; 61: 638S–645S.
36. Tsiaras WG, Weinstock MA. Factors influencing vitamin D status. Acta Derm Venereol 2011; 91: 115–124.
37. Wootton A. Improving the measurement of 25-hydroxyvitamin D. Clin Biochem Rev 2005; 26: 33–36.
38. Blum M, Dolnikowski G, Seyoum E et al. Witamin D3 in fat tissue. Endocrine 2008; 33: 90–94.
39. Lin E, Armstrong-Moore D, Liang Z et al. Contribution of adipose tissue to plasma 25-hydroxyvitamin D concentrations during weight loss following gastric bypass surgery. Obesity 2011; 19: 588–594.
40. Jones G. Pharmacokinetics of vitamin D toxicity. Am J Clin Nutr 2008; 88: 582S–596S.
41. Vannell J, Hollis BW. Use of vitamin D in clinical practice. Altern Med Rev 2008; 13: 6–20.
42. Krasowski MD. Pathology consultation on vitamin D testing. Am J Clin Pathol 2011; 136: 507–514.
43. Yetley EA, Pfeiffer CM, Chleicher RL et al. NHANES monitoring of serum 25-hydroxyvitamin D: a roundtable summary. J Nutr 2010; 2030S–2045S.
44. Hollis BW, Wagner CL. Nutritional vitamin D status during pregnancy: reason for concern. CMAJ 2006; 174: 1287–1290.
45. Barrett H, McElduff A. Vitamin D and pregnancy: an old problem revisited. Best Pract Res Clin Endocrinol Metab 2010; 24: 527–539.
46. Grayson R, Hewison M. Vitamin D and human pregnancy. Fetal Matern Med Rev 2011; 22: 67–90.
47. Carter GD. 25-hydroxyvitamin D assays: The quest for accuracy. Clin Chem 2009; 55: 1300–1302.
48. El-Khoury JM, Reineks EZ, Wang S. Progress of liquid chromatography-mass spectrometry in measurement of vitamin D metabolites and analogues. Clin Biochem 2011; 44: 66–76.
49. Castro MDL, Fernandez-Romero JM, Ortiz-Boyer F et al. Determination of vitamin D_3 metabolites: state-of-the-art and trends. J Pharm Biomed Anal 1999; 20: 1–17.
50. Wallace AM, Gibson S, Hunty A et al. Measurement of 25-hydroxyvitamin D in the clinical laboratory: current procedures, performance characteristics and limitations. Steroids 2010; 75: 477–488.
51. Carter GD, Carter CR, Gunter E et al. Measurement of vitamin D metabolites an international perspective on methodology and clinical interpretation. J Steroid Biochem Mol Biol 2004; 467–471.
52. Barake M, Dauer RT, Salti I et al. 25-hydroxyvitamin D assay variation and impact an clinical decision making. J Clin Endocrinol Metab 2012; 97: 835–843.
53. Farrell CJ, Martin S, McWhinney B et al. State-of-the-art vitamin D assays: a comparison of automated immunoassays with liquid chromatography–tandem mass spectrometry methods. Clin Chem 2012; 58: 531–542.
54. Carter GD. 25-hydroxyvitamin D: a difficult analyte. Clin Chem 2012; 58: 486–488.
55. Singh RJ. Are clinical laboratories prepared for accurate testing of 25-hydroxy vitamin D? Clin Chem 2008; 54: 221–223.
56. Carter GD. Accuracy of 25-hydroxyvitamin D assays: confronting the issues. Curr Drug Targets 2012; 12: 19–28.
57. Lensmeyer G, Poquette M, Wiebe D et al. The C-3 epimer of 25-hydroxyvitamin D3 is present in adult serum. J Clin Endocrinol Metab 2012; 97: 163–168.
58. Strathmann FG, Sadikova K, Laha TJ et al. 3-epi-25 hydroxyvitamin D concentrations are not correlated with age in a cohort of infants and adults. Clin Chim Acta 2012; 413: 203–208.
59. Singh JR, Taylor LR, Reddy S et al. C-3 epimers can account for a significant proportion of total circulating 25-hydroxyvitamin D in infants, complicating accurate measurement and interpretation of vitamin D status. J Clin Endocrinol Metab 2006; 91: 3055–3061.
60. Jiwan J-LH, Wallemacq, Herent M-F. HPLC-high resolution mass spectrometry in clinical laboratory? Clin Biochem 2011; 44: 136–147.
61. Adamec J, Jannasch A, Huang J et al. Development and optimization of an LC-MS/MS-based method for simultaneous quantification of vitamin D_2, vitamin D_3, 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3. J Sep Sci 2011; 34: 11–20.
62. Ding S, Schoenmakers I, Jones K et al. Quantitative determination of vitamin D metabolites in plasma using UHPLC-MS/MS. Anal Bioanal Chem 2010; 398: 779–789.
63. Pramyothin P, Biancuzzo RM, Lu Z et al. Vitamin D in adipose tissue and serum 25-Hydroxyvitamin D after Roux-en-Y gastric bypass. Obesity 2011; 19: 2228–2234.
64. Brunetto MR, Obandoa MA, Gallignani M et al. HPLC determination of Vitamin D3 and its metabolite in human plasma with on-line sample cleanup. 2004; Talanta 64: 1364–1370.
65. Hymoller L, Jensen SK. Vitamin D analysis in plasma by high performance liquid chromatography (HPLC) with C(30) reversed phase column and UV detection-easy and acetonitrile-free. J Chromatog 2011; 1218: 1835–1841.
66. Jakobsen J, Bysted A, Andersen R et al. Vitamin D status assessed by a validated HPLC method: within and between variation in subjects supplemented with vitamin D3. Scand J Clin Lab Invest. 2009; 69: 190–197.
67. Coldwell RD, Porteous CE, Trafford DJ, Malkin HL. Gas chromatography-mass spectrometry and the measurement of vitamin D metabolites in human serum or plasma. Steroids 1987; 49: 155–196.

SZKOLENIE PODYPLOMOWE