



Revisión

Current trends in the analytical determination of vitamin D *Tendencias actuales en la determinación analítica de vitamina D*

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Abstract

Key words:

Liquid chromatography-tandem Mass Spectrometry (LC-MS/MS). Vitamin D. Immunoassay. Chromatography. Standardization.

Vitamin D is a micronutrient that plays a large role in bone disease, and researchers are now discovering that it also does so in non-skeletal disease, thus making high-quality analytical determination necessary. To make this determination, a series of immunochemical and physical methods are used.

These methods present a series of different ways of handling samples as well as different methodologies that bring a series of advantages and limitations based on the scope of work in which the vitamin D analysis methodology is applied.

Although the Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) is the gold standard method of analytical vitamin D determination, and is the only one to offer a more complete and accurate view of all metabolites of this vitamin, it is necessary to standardize all the analysis methodologies that allow accurate, reliable and quality analytical determination, since it is essential to obtain results that can reliably be extrapolated to the population, and that can be decisive in assessing a large number of pathologies.

Resumen

Palabras clave:

Cromatografía líquida-espectrometría de masas en tándem (LC-MS/MS). Vitamina D. Inmunoensayo. Cromatografía. Estandarización.

La vitamina D es un micronutriente que ejerce un gran papel en enfermedades óseas y actualmente se está descubriendo que también lo hace en enfermedades no óseas, por lo que una determinación analítica de calidad es necesaria.

Para realizar esta determinación se emplean una serie de métodos inmunoquímicos y físicos, los cuales van a presentar una serie de tratamientos diferentes de las muestras, así como diferentes metodologías que van a traer una serie de ventajas y limitaciones conforme al ámbito de trabajo en que se aplique la metodología de análisis de la vitamina D.

A pesar de que la cromatografía líquida-espectrometría de masas en tándem (LC-MS/MS) es el método gold standard de determinación analítica de la vitamina D, y de que es el que ofrece una visión más completa y precisa de todos los metabolitos de esta vitamina, es necesaria una estandarización de todas las metodologías de análisis que permitan una determinación analítica precisa, fiable y de calidad, ya que es imprescindible obtener unos resultados que sean extrapolables con fiabilidad a la población y que puedan ser determinantes para valorar un gran número de patologías.

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INTRODUCTION

Vitamin D is a fat-soluble vitamin of steroid nature (1). The denomination vitamin D refers to hormonal precursors characterized from the chemical perspective as open-ring steroids. Due to their great importance in the metabolic processes, two compounds are denominated: vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol), which can be contributed by the diet. Vitamin D₂ is present in plants, fungi and yeasts. Vitamin D₃, on the other hand, comes from animal products such as blue fish, eggs, and milk, but it is also formed in an endogenous process that begins with the photochemical transformation of 7-dehydrocholesterol upon exposing the skin to a narrow margin (295-300 nm) of ultraviolet B (UVB) radiation from the sun (2). This produces the appearance of previtamin D₃, which is subsequently isomerized to form vitamin D₃. Vitamins D₂ and D₃ are inactive and are mobilized in the blood bound to specific proteins, namely transcalfiferin and vitamin D-binding protein (DBP) (3). In the liver, they are hydroxylated to form 25-hydroxy-vitamin D [25(OH)D], which is the species that is found in the greatest proportion in the blood. A subsequent hydroxylation that mainly takes place in the renal tubules gives rise to 1,25-dihydroxy-vitamin D [1,25(OH)₂D], which is the form of vitamin D that has metabolic activity. The inactivation pathways of vitamin D include an oxidative pathway, through which the compounds [25(OH)D] and [1,25(OH)₂D] create various oxidized derivatives, including both 26,23-lactone and calcitric acid. Another pathway that inactivates vitamin D is epimerization followed by oxidation in C-24 (4).

The quantification of vitamin D found in biological samples presents recognized difficulties (5). This circumstance coincides with an increase in studies to evaluate the content of vitamin D (6,7) occurring over the last decade, which has been attributed to two factors: on the one hand, the prevalence of serious deficiencies of this vitamin in poor countries and, on the other, an increase in the use of vitamin D as a general marker of health status and because of its relationship with various pathologies (8). Traditionally, it was thought that the diseases associated with vitamin D deficiency were bone-related, as there is a widely documented causal association in scientific evidence between low vitamin D status and risk of developing rickets, osteomalacia and osteoporosis. Thus, this vitamin is an essential factor in bone metabolism and calcium homeostasis (9,10). However, new research in this field that is just beginning is focusing on the vitamin's role in the development or accentuation of non-skeletal diseases such as autoimmune disease, cardiovascular disease, infectious disease, as well as some types of cancers (10-16). Although it has been pointed out that vitamin D's involvement in these pathologies has been, in many cases, the result of extrapolation in epidemiological studies that may lack sufficient reliability (17), the idea that vitamin D supplementation could contribute to the treatment of the above-mentioned diseases has been maintained, and there has consequently been a remarkable increase in tests to determine the metabolites of [25(OH)D], which, although not the biologically active form of the vitamin, has served as a marker of vitamin D status in the blood in recent years (18). However, its measurement is only recommended in cases of bone diseases such

as osteomalacia and osteoporosis, people with impairments in the absorption of fat produced by illnesses such as inflammatory bowel disease, cystic fibrosis, celiac disease, and bypasses; or by medications that interfere with vitamin D such as medicines that favor bone resorption or those that interfere with vitamin D metabolism (8,19). Given the scarce scientific literature that focuses on the influence of vitamin D in these non-skeletal diseases, the trends in recent years aim to clarify the role of vitamin D in these diseases, thus opening a wide range of research lines that can help clarify the vitamin's role in the etiopathogenesis of said diseases (1).

Serum concentrations of vitamin D do not depend on homeostatic control, but rather on lifestyle and environmental factors; and bodily values essentially come from two sources: on the one hand, the cutaneous synthesis of vitamin D, which is the result of solar radiation, and on the other, intake through diet. This intake is very poor since, in addition to being present in few foods in significant quantities, the intake of these foods is not usually widespread throughout the global population (20). In addition, a series of socio-cultural factors will intervene in these serum concentrations (21).

However, despite the need for a high-quality analytical determination of vitamin D and a greater control of vitamin D concentrations in the body, the number of analytical determinations of vitamin D has increased exponentially in the last decade. Such a rapid increase is due to a growing interest in the study of those mechanisms of action in which vitamin D intervenes. In addition, supplementation with high doses of vitamin D has increased in recent years, both on doctors' advice and through self-medication. Upon evaluating the tests that have been performed, we currently find that while many tests are justified, such as those done when suffering a traumatic fall that involves bone fracture or in pathologies such as osteoporosis, the number of tests that are done in an unjustified and inappropriate way has increased in those people who do not really need them. This includes those who complain of tiredness, fatigue, self-administration of vitamin D supplements, etc., in addition to tests which are not ordered by a specialist rather than the family doctor. These unjustified and inappropriate tests have increased considerably, thus increasing extra costs. Therefore, in those populations where there is doubt as to whether or not to make an analytical determination of vitamin D, it is important that food consumption frequency questionnaires be used, as well as an assessment of sun exposure, in order to estimate the person's overall vitamin D concentration, and thus avoid the excessive cost of these tests. This type of testing requires a more rational approach in its use, since this would avoid inappropriate expenditures both at the patient and the hospital level, and it would also lead laboratories to redirect their funds to other more necessary tests (19,22).

The controversy surrounding this issue is so great that there is no universal agreement among physicians, researchers and the public on the issues related to vitamin D and its analytical determination, nor on its influence on the etiopathogenesis of various diseases. Therefore, the need for quality research is paramount due to the aforementioned aspects, which cannot be avoided (23).

ANALYTICAL DETERMINATION OF VITAMIN D

The measurement of vitamin D is usually done in the serum by determining the amount of the metabolites [25(OH)D₂] and [25(OH)D₃], given their higher proportion in the blood. However, the need to evaluate the amount of other metabolites for a better idea about vitamin D status has been indicated. Table I shows the list of metabolites that have been determined simultaneously from a sample (24).

The main problem in the analytical determination of vitamin D is the wide range of methods used and the variations in the results due to problems with extraction and calibration. Therefore, today, there is a need to find an international method that calibrates measurement methods, as this will make the various studies in this field more comparable and reproducible, thus avoiding misinterpreting the large number of studies at the international level that are cited in the scientific literature. In line with this, the vitamin D standardization programs that are emerging (20) are worth mentioning. Through these programs, it is possible to improve clinical practice and public health worldwide (1).

Until international standardization is established, the determination of vitamin D levels should be reserved for special risk groups and types of disease (20). The methods for measuring the metabolites of vitamin D can be divided into two main groups: immunochemical methods (chemiluminescence immunoassays [CLIA], radioimmunoassay [RIA], enzymatic assays, and chemical binding assays) and physical detection methods (high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and LC-MS/MS) (1,8).

Immunoassay techniques present several problems such as cross-reactivity due to polyreactive antibodies, the ability to analyze only one analyte at a time with no discrimination between them, the inability to achieve structural validation of the analyte, and a highly fluctuating sensitivity. Immunological techniques are generally not able to distinguish between [25(OH)D₂]

Table I. Vitamins D₂ and D₃ and their metabolites determined simultaneously in human serum using LC-MS/MS

Name	Abbreviation
Vitamin D ₂	D ₂
Vitamin D ₃	D ₃
25-hydroxyvitamin D ₂	25(OH)D ₂
25-hydroxyvitamin D ₃	25(OH)D ₃
24,25-dihydroxyvitamin D ₂	24,25(OH) ₂ D ₂
24,25-dihydroxyvitamin D ₃	24,25(OH) ₂ D ₃
1,25-dihydroxyvitamin D ₂	1,25(OH) ₂ D ₂
1,25-dihydroxyvitamin D ₃	1,25(OH) ₂ D ₃
D ₂ Sulfate	D ₂ -S
D ₃ Sulfate	D ₃ -S
D ₂ Sulfate 25-hydroxyvitamin	25(OH)D ₂ -S
D ₃ Sulfate 25-hydroxyvitamin	25(OH)D ₃ -S

and [25(OH)D₃] due to the cross-reactivity of the antibodies, and it is not possible to obtain the same information provided using chromatographic determination (25). In addition, immunological techniques have limited sensitivity and dynamic range, difficulties in the displacement of DBP, non-equimolar detection of [25(OH)D₂] and [25(OH)D₃], interference of heterophile antibodies, gel and clot activator interference in blood collection tubes, and a lack of adequate standardization. Furthermore, within the cross-reactivity to other circulating metabolites of vitamin D mentioned above, [24,25(OH)₂D₃] is the most predominant (1).

In recent years, due to the growing interest in the role of vitamin D in the body, the number of determinations requested has been increasing, giving rise to the need to move from the antiquated manual radioimmunoassay to the automated immunoassay on random access analyzer platforms in the majority of clinical laboratories (20).

The reactive protein binding assay, although cheap and used in small samples, has the disadvantage of underestimation in low amounts and overestimation in high amounts. The RIA has the advantages that it is economical, fast and specific, determines small sample sizes, and is also specific (8).

As far as chromatographic techniques are concerned, they are less susceptible to the effects of the matrix than immunoassays (1). In the field of liquid chromatography, high-performance liquid chromatography (HPLC) is used, which is stable, reproducible, and discriminates between metabolites. However, it requires a larger sample size, requires a preparation step before chromatography, and sometimes the assay is subject to interference from other compounds measured in the ultraviolet spectrum, in addition to requiring a high level of technical expertise (8,25). Between liquid chromatography with ultraviolet detection (LC-UV), liquid chromatography with diode array detection (LC-DAD), and liquid chromatography-mass spectrometry (LC-MS), however, LC-MS provides better results than the two liquid chromatographic techniques listed above (25).

The gold standard method for the determination of vitamin D is LC-MS/MS. However, its high cost is an expense that many routine analysis laboratories cannot assume. It is also necessary to have a very specialized and qualified staff to carry out this determination. Therefore, the cheaper immunoassay is used, although it does not allow for the differentiation of vitamin D₂ and vitamin D₃ (20,25). In addition, LC-MS/MS requires expensive hardware and a laborious process that includes: pretreatment of the sample, calibration, chromatographic separation mode, and the selection of an internal standard (1).

LC-MS/MS provides multiple advantages including greater sensitivity, flexibility and specificity. This is fundamentally due to the use of internal standards that have a crucial role in the determination. In addition, it has the ability to accurately quantify multiple analytes that are of interest in a single assay, which speeds up and makes this determination very complete (25).

Unfortunately, no reference method or reference materials are available for the [1,25(OH)₂D] analysis to date (1). Vitamin D status is defined by the measurement of 25(OH)D, a term which refers to both the [25(OH)D₂] and [25(OH)D₃] circulating forms of the

vitamin. There are a number of reasons why the total $[1,25(\text{OH})_2\text{D}]$ concentration cannot be used as a vitamin D marker. Its short half-life of less than a day versus almost a month for $[25(\text{OH})\text{D}]$, the low concentrations of the final metabolite (picomole vs nanomole), and the fact that only a very small amount of $[25(\text{OH})\text{D}]$ is converted to $[1,25(\text{OH})_2\text{D}]$ give a false sense of vitamin sufficiency. It is likely that the quantitative applications of vitamin D metabolites in the HRMS will move from research to routine clinical laboratories in the near future, providing additional specificity in measurements. A multi-panel assay for the simultaneous measurement of vitamin D metabolites will improve future research on the optimal combination of vitamin D species for the assessment of vitamin D sufficiency, and will help us better understand the metabolism of vitamin D in both healthy and ill subjects (1).

HANDLING OF VITAMIN D SAMPLES

Methods for vitamin determination require a preconditioning of samples for analysis, including the separation of vitamins and their metabolites from complex matrices in biological fluids such as plasma and serum. In the literature, several procedures have been described for the preconditioning of samples, mainly for the separation of vitamins from the blood. To isolate these analytes from blood plasma, different techniques have been used, such as protein precipitation, liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Deproteinization and LLE result in endogenous matrix compounds passing into the supernatant, which can affect the separation and determination of the analytes. Therefore, an SPE technique is applied for the additional purification of the sample. When analyzing samples containing vitamin D, a well-established and standardized pretreatment phase is necessary, which distinguishes those elements that should be disregarded from those that interest us when proceeding with our analysis (26).

The handling of the samples is very complex, as it influences the association between vitamin D and DBP protein, albumin, and the analyte-antibody balance (20). The main problem with the DBP protein is that it has 3 polymorphic forms, which are derived in 6 allelic forms. These different alleles circulate in more or less variable concentrations and have a different degree of affinity for the different metabolites of vitamin D, which could compromise the treatment method, which binds this DBP protein to the vitamin D metabolite (27). Vitamin D is a hydrophobic compound and sensitive to the matrix. However, vitamin D analytes are stable for 2 weeks at 30 °C but for 1 year (or longer) at -20 °C, and are not affected by up to four freeze-thaw cycles of the serum samples. Ultraviolet rays also do not influence the calcidiol content of a serum sample (21). The hydrophobic nature of vitamin D and the strong binding to its transporter (vitamin D binding protein), the different forms that circulate in the blood, and the question of standardization are among the most important factors that influence the measurement of this metabolite. Since $[25(\text{OH})\text{D}]$ is a lipophilic substance closely linked to DBP, this generates some technical problems. In addition, endogenous lipids can affect binding and chromatographic separation, since plasma and serum are extracted together (27).

The problem with immunoassays in the preparation of samples is that an antibody that is so specific to such a small antigen must be determined very clearly, in addition to the hydrophobicity of the vitamin and its binding protein (DBP), which would make oscillations in the amount of DBP present in the sample affect the immunoassay. In addition, many laboratories rely on commercial immunoassay kits, which have their own pretreatment protocol that differs between laboratories. All of this causes sensitivity and specificity to fluctuate since many factors must be controlled (28,29).

The LC-MS/MS method, which is the one most widely accepted, begins with the pretreatment of the sample to separate $[25(\text{OH})\text{D}]$ from DBP and to eliminate phospholipids and other interferences from the matrix that cause alterations in the ionization. Sample preparation is usually performed using protein precipitation, LLE, SPE, supported liquid extraction (SLE) or extraction plates coated with an immobilized absorption phase (1).

The quantification of $[1,25(\text{OH})_2\text{D}]$ is difficult due to its extremely low concentrations in the serum, as well as the co-existence of many other abundant vitamin D metabolites that can interfere with its measurement. Additionally, accurate quantification of $[1,25(\text{OH})_2\text{D}_3]$ using LC-MS/MS is a challenge due to its low serum concentrations and the lack of ionizable polar groups resulting in low ionization efficiency in Electrospray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI). In addition, specific care is needed to avoid potential interference from other dihydroxylated vitamin D metabolites (1).

LC-MS/MS methods differ in sample preparation aspects, chromatography, ionization source, and fragmentation patterns detected. Therefore, despite the accuracy of the method, these factors cause the results obtained to vary between different LC-MS/MS methods. LC-MS/MS also has several variants in laboratories, since different methodologies are added to improve aspects of the method itself, thus also making the results less comparable. The choice of one method or another also depends on staff experience, the objective to which it is directed, as well as the volume of the center where the determination is done. Therefore, the immunoassays that provide guideline measurements, which are not completely accurate, will be used in smaller laboratories for clinical use. In addition to being inexpensive, the staff in these laboratories are often not very specialized, so they are not prepared for a more complex test like chromatography. In contrast, very large clinical laboratories and academic institutions use LC-MS/MS methods given that their sensitivity and specificity mean that they are used in those at-risk populations where the accuracy of the method may be very important, such as in the pediatric population, for example. Along the same line, the method can also be chosen depending on the metabolite that you aim to determine, using a more specific test that determines the last precursor of vitamin D in pathologies in which you want to precisely analyze this vitamin such as rickets (28,29).

Otherwise, even when a good sample treatment is carried out, as well as an appropriate analytical method of vitamin D is chosen, vitamin D levels can be increased or decreased in the same person who maintains similar eating habits and of exposure to the

sun during the year. This is due to the variation of the exposure to sunlight in the different stages of the year. In populations in which vitamin D was measured in different seasons of the year, greater deficiency was found in the hottest stages, so we must take into account the seasonality of the year, since in hotter seasons people take more sun in places like the beach and with less protection, besides that it is convenient that at the time of asking about the habit of exposure to the sun, the latitude and the month of the year are taken into account (30,31).

The tendencies that will arise in the future will go on to establish a methodology to automate extraction, without requiring any intervention from human beings. This is due to the fact that when people handle the samples, they increase the probability of operator error and biological risks (6). Future challenges of the assay include moving to SPE to allow better sample cleaning and minimize the extraction steps to those that can be automated and try to generate less waste (8).

The absence of certified reference material for the analytical determination of [25(OH)D] is the most important factor that determines the inaccuracy in identifying individuals with vitamin D levels below the optimal threshold (27).

CONCLUSION

The trend in vitamin D analysis is on the rise as there is a need for its determination due to its relationship with a wide range of pathologies. Therefore, it is necessary to look for a methodology, according to each situation, that is cheap, easy to perform and accurate. The most accurate determination technique for the situation mentioned above would be LC-MS/MS chromatography. However, this technique is not easy to perform because, as described, it requires considerable experience and is not cheap. To solve this problem, a standardization of this technique would be adequate. Currently, a wide range of entities including laboratories use this technique together with other processes independently, which makes the method more expensive as there is no single universal methodology that allows for mass production to lower costs. Additionally, it is not reproducible, which is a key factor when interpreting the scientific literature. If we use different measurement instruments, handle samples differently, and leave the human error intrinsic to the manual and non-technological handling of samples up to chance, we shall yield a result that will be suitable within a certain range when evaluating and extrapolating a single study, but which is hardly comparable at the global level.

No one technique is better than another, it is simply more interesting than others depending on the aim of the study. If we want, on the one hand, to avoid excessive economic expenditures, obtain reliable data but work with a broader or narrower range of concentrations, and the vitamin D determination is to obtain a guiding idea, but the exact value is not crucial for our study, we will opt for immunoassay techniques like in clinical settings. On the other hand, if what we want is precision in the data, even if that entails more effort, money and experience, our choice has to be chromatographic techniques, like in field studies.

The future of this field is to continue developing variants of LC-MS/MS and to replace the immunoassay as data accuracy is crucial, even if it leads to other associated problems.

Another line that could be opened would be the handling of samples according to temperature. Since vitamin D can withstand many freeze-thaw cycles and is sensitive to certain environmental variables, working conditions could be established, such as handling both the sample and internal standard directly in a cold chamber as characteristics are maintained longer in a cold room. By doing the pretreatment there, all the samples would be handled at a constant temperature and under the same conditions as in the chromatographic technique, since working with them at room temperature causes the temperature to fluctuate as well as an increase in exposure to external agents, which means that at the time of treating the sample using chromatography, some confounding factors will not have been suppressed.

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