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Review article
Pregledni članak

ANALYTICAL METHODS AND PERFORMANCE OF THE IMMUNOASSAY METHODS FOR DETERMINATION OF VITAMIN D IN COMPARISON TO MASS SPECTROMETRY

ANALITIČKE METODE I IZVOĐENJE IMUNOMETRIJSKIH ODREĐIVANJA VITAMINA D U POREĐENJU SA MASENOM SPEKTROMETRIJOM

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Summary: Demand for vitamin D testing has been on a constant rise worldwide, partially due to mounting evidence linking vitamin D status to overall health and well-being. Currently available assays measure 25-hydroxy vitamin D (25-OHD), a major circulating form of vitamin D. Available methodologies include immunoassays and mass spectrometry based methods (LC-MS/MS). Until recently, the only immunoassays available for diagnostic use in the US have been DiaSorin radioimmunoassay (RIA) and an automated immunoassay on a LIAISON® platform. Within the last year, Siemens and Abbott successfully launched immunoassays for determination of total vitamin D on their respective automated platforms, Centaur® and *ARCHITECT*®. Development of robust and precise Vitamin D immunoassays has historically been plagued with difficulty. One of the major challenges is development of specific antibodies against such a small antigen. Vitamin D is also highly hydrophobic molecule predominantly bound to vitamin D binding protein (DBP). It is likely, therefore, that immunoassays might be affected to varying extent by the DBP concentration. Adoption of LC-MS/MS into clinical laboratories has enabled development of accurate and almost fully automated methods that could handle increasing volume demands, especially in large volume reference laboratories. Smaller to mid-size hospital laboratories as well as physician offices have neither funds nor technical expertise to implement LC-MS/MS based testing. Our laboratory at the University of Chicago Medical Center has also seen increase in vitamin D volume and currently performs close to 20,000 25-OHD assays per year. We have recently developed an LC-MS/MS method for quantitation of 25-OHD_2 (obtained from plant sources) and 25-OHD_3 (endogenous and animal sources). Prior to acquisition of LC-MS/MS

Kratak sadr`aj: Broj zahteva za određivanjem vitamina D je u konstantnom porastu širom sveta, delom zbog sve više dokaza koji povezuju status vitamina D sa opštim zdravljem. Trenutno raspoloživim testovima određuje se 25-hidroksi vitamin D (25-OHD), glavni oblik vitamina D u cirkulaciji. Postojeće metodologije uključuju imunometrijska određivanja i tehnike zasnovane na masenoj spektrometriji (LC-MS/MS). Do nedavno, jedine raspoložive imunometrijske metode korišćene za dijagnostiku u SAD su bile DiaSorin radioimunoodređivanje (RIA) i automatizovano imunoodređivanje na LIAISON® platformi. U toku prošle godine Siemens i Abbott su uspešno lansirali imunometrijske testove za određivanje ukupnog vitamina D na svojim odgovarajućim automatizovanim platformama, Centaur® i ARCHITECT®. Razvoj robusnih i preciznih imunometrijskih testova za određivanje vitamina D su, istorijski gledano, pratili problemi. Jedan od najvećih izazova je razvoj specifičnih antitela protiv malog antigena. Vitamin D je takođe jako hidrofoban molekul predominantno vezan za vitamin D vezujući protein (DBP). Stoga postoji verovatnoća da na imunoodređivanja u različitom stepenu može da utiče koncentracija DBP. Uvođenje LC-MS/MS u kliničke laboratorije je omogućilo razvoj tačnih i skoro potpuno automatizovanih metoda koje bi mogle da obrade rastući broj zahteva za analizu, naročito u referentnim laboratorijama sa velikim obimom posla. Kliničke laboratorije manjeg do srednjeg obima, kao i lekarske ordinacije, ne raspolažu finansijskim sredstvima niti tehničkim znanjem za implementaciju određivanja zasnovanog na LC-MS/MS. U našoj laboratoriji u Medicinskom Centru Univerziteta u Čikagu je takođe primećen porast broja zahteva za određivanje vitamina D i trenutno se izvrši blizu 20 000 određivanja 25-OHD godišnje. Nedavno smo razvili LC-MS/MS metodu za kvantifikaciju

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instrument, we performed 25-OHD analysis by RIA. During the transition period, we encountered several challenges, including the necessity to streamline sample preparation as well as the bias introduced by calibration differences. We chose to match our LC-MS/MS method to the RIA method in order to make this transition transparent to the clinician. Most immunoassays available today are acceptable for clinical use and might be method of choice for smaller laboratories. Larger clinical laboratories and academic institutions that possess technical expertise, particularly the ones with large pediatric population where assay sensitivity and specificity may be important, might find LC-MS/MS methodology a more suitable choice.

Keywords: vitamin D, 25-hydroxyvitamin D, LC-MS/MS, RIA, immunoassay

25-OHD_2 (dobijen iz biljnih izvora) i 25-OHD_3 (iz endogenih i životinjskih izvora). Pre nabavke LC-MS/MS aparata analiza 25-OHD je rađena RIA metodom. U prelaznom periodu, naišli smo na nekoliko izazova, uključujući neophodnost jednostavnije pripreme uzorka, kao i odstupanje nastalo razlikama u kalibraciji. Odlučili smo da uporedimo našu LC-MS/MS metodu sa RIA metodom da bi ovaj prelaz postao transparentan za kliničare. Većina imunoodređivanja koja su danas na raspolaganju su prihvatljiva za kliničku upotrebu i mogu biti metode izbora za manje laboratorije. Za veće kliničke laboratorije i akademske institucije koje poseduju tenhničku obučenost, naročito one sa velikom pedijatrijskom populacijom gde osetljivost i specifičnost mogu biti važne, LC-MS/MS metodologija može biti adekvatniji izbor.

Ključne reči: vitamin D, 25-hidroksivitamin D, LC-MS/MS, RIA, imunoodređivanje

Introduction

The essential role of vitamin D in bone metabolism and calcium homeostasis is well established (1, 2). In addition, a number of research studies demonstrated the role of vitamin D in blood pressure regulation, autoimmunity, regulation of cell growth and metabolic diseases and malignancy (2–9). This has all led to increase in vitamin D testing requests, with many laboratories in the United States reporting annual increase rates of 50% or more (10). In the University of Chicago Medical Center, for instance, we have observed an increase of approximately 10 fold in vitamin D testing volumes since 2005.

Although the treatment for vitamin D deficiency or insufficiency is generally affordable and straightforward, the correct diagnosis is dependent not only on reliable and reproducible methods of analysis but also on the choice of the appropriate test. Confusion still exists among clinicians regarding the most suitable test to assess vitamin D status. To correctly determine vitamin D insufficiency, 25-hydroxyvitamin D (25-OHD) should be ordered rather than the active metabolite 1, 25-dihydroxyvitamin D (1,25-$(OH)_2D$). Measurement of 1,25-$(OH)_2D$ should only be reserved for the cases of severe renal disease, and rare conditions such as vitamin D resistant rickets and granulomatous diseases (11–13).

Accuracy of 25-OHD Measurement

Accurate and precise measurement of vitamin D has been challenging. The methodology used to measure vitamin D includes immunoassays and liquid chromatography-tandem mass spectrometry (LC-MS/MS) and, is discussed in the next section. Recently, the US Centers for Disease and Control (CDC) have convened a roundtable to discuss the scientific challenges involved in the measurement of serum 25-OHD and the assessment of vitamin D status across several decades of US National Health and Nutrition Examination Survey (NHNES) (14). The panel of experts concluded that variability of serum 25-OHD measurements were likely the artifact caused by fluctuations in the assay performance over time rather than by true vitamin D status changes. This instance highlighted the need for robust methodology and accuracy-based standard. In 2009, the National Institute of Standards and Technology (NIST) developed Standard Reference Material (SRM) to assist accurate determination of 25-OHD in the serum or plasma (15, 16). This standard, SRM 972, consists of four pools of serum, each with different levels of vitamin D metabolites. Today, a number of clinical laboratories, mostly the LC-MS/MS users, participate in this standardization program. Unfortunately, due to matrix effects, only one SRM level could be used in immunoassay standardization. The other three levels are either spiked with exogenous vitamin D or diluted with horse serum and thus unsuitable for many immunoassays (17, 18). None of the commercial immunoassays are, therefore, aligned to SRM 972. Several candidate reference methods for accurate and sensitive 25-OHD measurement have also been published in the recent years (19–21).

Vitamin D Assays

Historically, gold standard methodology for Vitamin D measurement has been radioimmunoassay (RIA). With the increased adoption of the LC-MS/MS into the clinical laboratories, more laboratories are developing their own customized LC-MS/MS methods, using their own calibration preparations and value assignments. This, of course, initially introduced even more variability in vitamin D testing, a problem partially alleviated with the introduction of NIST SRM. Most of the clinical laboratories still lack either funds, expertise or both for mass spectrometry-based testing and are still relying on commercial immunoassays.

One source of variability for immunoassays are different, vendor specific, sample pr etreatment protocols used to release vitamin D from vitamin D binding protein (DBP). Effects of variable r ecoveries and DBP concentration changes on differ ent patient populations can have significant impact on assay accuracy and precision (22–25). Manufactur ers have r ecognized increasing demands for vitamin D testing and are working on impr oving the existing kits to pr ovide reliable and reproducible results. In the last year, two new total 25-OHD kits became available on the market, Abbott Architect and Siemens Centaur assays.

Radioimunoassays (RIA)

Two RIA assays are currently available for measurement of total vitamin D: DiaSorin (DiaSorin, Stillwater, MN) based on the assay originally developed by Hollis et al. (26) and Immunodiagnostic Systems (IDS) assay (Immunodiagnostic Sys tems, Inc., Scottsdale, AZ). Only DiaSorin is appr oved for diagnostic use in the US. Both RIAs involve extraction of 25-OHD with acetonitrile followed by equilibrium RIA using 25-OHD specific antibody and ^{125}I-labelled 25-OHD. As per their r espective package inserts, DiaSorin assay claims 100% cross-reactivity with both 25-OHD_2 and 25-OHD_3, while IDS claims 100% cross-reactivity with 25- OHD_3 and only 75% cr oss-reactivity with 25-OHD_2.

Chemiluminescence Immunoassays

Both RIA manufactur ers offer automated versions of their assays. The cur rent version of DiaSorin assay was introduced in 2007 and is available on the Liaison automated platform. IDS introduced their version of automated immunoassay in 2009 to be used on iSYS™ automated analyzer (not available for sale in the US).

Two most r ecent immunoassays, Abbot Ar chitect and Siemens Centaur, utilize 8-anilino-1-naphtalenesulfonic acid (ANSA), compound known to effectively displace hor mones from binding pr oteins (27, 28). While both assays claim 100% cr oss-reactivity with 25-OHD_3, Centaur package insert states 100% cross-reactivity with 25- OHD_2 and Architect states only 82% cr oss-reactivity with vitamin D $_2$. Only the Centaur immunoassay is traceable to LC -MS/MS, although it is not clear fr om documentation provided by manufacturer which LC-MS/MS methodology was used in calibrator value assignment.

Vitamin D assay is also available from Roche but this assay is only marketed for deter mination of 25-OHD_3 and thus cannot be used in the US.

LC-MS/MS Assays

Mass spectrometry is a methodology of choice for the majority of lar ge reference laboratories and academic centers in the US. LC-MS/MS methods are laboratory specific and could differ in aspects of sample preparation, chromatography, ionization sour ce and fragmentation patterns detected. It appears that, compared to electrospray, APCI ionization sour ce results in less variability in vitamin D measur ements (29). Unlike immunoassays that measur e total vitamin D, LC-MS/MS methods can separate 25- OHD_3 and 25-OHD_2 although most of the laboratories still report total 25-OHD to avoid confusion. Virtually all LC-MS/MS assays in the US ar e developed and validated by the individual testing laboratories. T o date, there is only one kit for vitamin D analysis on the LC-MS/MS system available fr om ChromSystems (Munich, Germany). This kit is CE-marked and the company will likely seek FDA approval to market this kit in the US for diagnostic use (30).

Our laboratory has r ecently developed LC-MS/MS method for quantitation of 25OH-D $_2$ and 25OH-D_3. During the transition period, we encountered several challenges, including the necessity to streamline sample pr eparation as well as the bias introduced by calibration differ ences. We chose to match the new LC-MS/MS method to our clinical RIA method, in or der to make this transition transpar ent to the clinician.

Immunoassays versus LC-MS/MS: Head to head Comparison

Several investigators per formed extensive side by side evaluation of commer cial vitamin D assays (23, 31, 32). Ong et al. (31) evaluated accuracy and precision of thr ee new automated immunoassays (Roche, Abbott and Siemens) and compared them to the existing RIA (DiaSorin) and in-house developed LC-MS/MS methods. These investigators found that all five assays had acceptable impr ecision at vitamin D levels >40 nmol/L. At lower vitamin D values, only RIA and LC-MS/MS performed well. To assess agreement between these methods, a set of 200 patient samples were used. While the three automated assays and RIA correlated well with LC-MS/MS assay, Abbott and Roche assay demonstrated significant biases of 25% and 31%, respectively.

In March 2012 edition of Clinical Chemistr y, two consecutive publications evaluated per formance of essentially all 5 available automated immunoassays (*ARCHITECT*, Centaur, iSYS, LIAISON and Elecsys), and one RIA (DiaSorin) in comparison to the LC -MS/MS methods (23, 32). F arrell et al. (32) compared immunoassay per formance against two independent, non-commercial LC-MS/MS assays aligned to the NIST SRM 972. A total of 170 ser um samples from routine vitamin D assay requests were used. The

only immunoassay that matched the performance of mass spectrometry assays was RIA, most likely due to complete extraction of vitamin D from DBP. Among the automated immunoassays, only LIAISON and IDS demonstrated acceptable performance. *ARCHITECT* and Centaur showed excessive bias (>25%). In addition, *ARCHITECT* assay demonstrated unacceptable concordance with LC-MS/MS. Roche Elecsys assay had low bias but did not correlate well with LC-MS/MS assays. Farrell et al. (32) also observed an increased imprecision of the automated platforms at low end (vitamin D <20 nmol/L), which is in agreement with the observations reported by Ong et al. (31).

Heijboer assessed the accuracy of automated immunoassays by stratifying the patient populations based on their DBP levels. The authors found major differences in 25-OHD concentrations between different assays tested. This is the first study to demonstrate an inverse relationship between DBP concentrations and deviations of measured 25-OHD from LC-MS/MS method (aligned to Thienpont candidate reference method (21)). Significant biases observed were likely due to fact that, in automated assays, 25-OHD is not completely extracted from DBP in sera that have relatively high DBP concentration. For example, in critically ill patients who have lower DBP concentrations compared to healthy individuals, Liaison significantly overestimated 25-OHD levels compared to LC-MS/MS. On the other hand, in pregnant women, who had higher DBP levels, Centaur and iSYS tended to underestimate vitamin D levels. Therefore, Hejinboer's data suggest that not all assays are suitable for 25-OHD assessment in all patient groups.

Choice of Method

Selection of the appropriate method is laboratory specific and depends on population tested, sample throughput and staff expertise (33).

In the US, for example, laboratories are required to measure both 25-OHD_2 and 25-OHD_3 as patients are still frequently supplemented with 25-OHD_2, unlike the laboratories in Europe, where there is no requirement to measure 25-OHD_2. Nonetheless, unless the laboratorians recognize limitations of their assay, significant confusion can arise. This was nicely illustrated in the case report from Belgium where physicians treated vitamin D deficient patient with vitamin D_2, while her serum vitamin D levels were measured using the vitamin D_3 assay (34). It is also important to recognize that none of the studies published on comparison of automated immunoassays with LC-MS/MS methodology recruited more than a few patients supplemented with vitamin D_2. This is important because, as mentioned earlier, not all immunoassays report 100% cross-reactivity with vitamin D_2. Further studies evaluating performance of these analyzers in 25-OHD_2 measurement are thus required. Finally, laboratories performing the significant volume of pediatric testing must evaluate potential cross-reactivity of their assay with vitamin D epimer (3-epi-25-OHD_3) present in significant amounts in neonates. This interference is only problematic for LC-MS/MS methodology, since the mass spectrometers cannot distinguish stereoisomers (35), and can potentially result in overestimation of 25-OHD_3. None of the main immunoassays in use today are susceptible to 3-epi-25-OHD_3 interference (14). Although the amounts of vitamin D epimer in adult serum are generally small, high concentrations have been reported in some individuals (36).

The use of mass spectrometry in the clinical laboratories has increased over the years due to its superior analytical characteristics and lack of interference from structurally related compounds. In addition,, low LC-MS/MS reagent costs result in significant cost-savings compared with the immunoassays, provided the testing volumes are high enough to justify initial capital investment. However, different laboratories are encountering different challenges brought upon by continuous increases in vitamin D testing volumes. Smaller and mid-size hospital laboratories and academic centers typically employ classically trained laboratory technologists and are, therefore, lacking technical expertise required to sustain this high complexity testing. On the opposite end of the spectrum are large reference laboratories that receive hundreds to thousands of vitamin D requests daily. With such high volumes, the throughput of LC-MS/MS systems becomes the limiting factor. Until recently, the only strategy available to LC-MS/MS users to improve throughput has been multiplex LC systems using the technology such as Thermo Fisher TLX systems. This strategy is utilized in author's own laboratory wherein up to 4 separate LC systems operate simultaneously in a staggered fashion. In 2011, a group at Mayo Clinic developed and implemented an elegant multiplexing method where up to 5 patient samples are multiplexed within one single LC-MS/MS injection, using the specifically designed mass tags. The throughput that can be achieved with this methodology is up to 300 specimens per hour or 7200 specimens per instrument per day, matching the throughput of automated immunoassays (37).

Conclusion

Considering superior precision and accuracy of the LC-MS/MS instrumentation, it is clear that, given the appropriate resources and technical expertise, it is the method of choice for vitamin D analysis. However, the reality is that many laboratories still posses neither financial resources nor technical know-how to adopt this technology and are still in the market for reliable automated immunoassay, a fact well recognized by

immunoassay manufacturers. Recent studies have found that automated immunoassay have suboptimal performance at measuring vitamin D levels below 20 nmol/L (31, 32). This might be acceptable to most laboratories considering that these levels ar e clearly deficient and it thus might be of little clinical significance. Finally, the laboratorians should be cognizant of the fact that accuracy of some immunoassays depends on patient population, especially if the pa - tient condition might cause significant changes in DBP levels.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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