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Current 25-hydroxyvitamin D assays: Do they pass the test?

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ABSTRACT

Background: Vitamin D testing is becoming increasingly important with recent research demonstrating a correlation between vitamin D insufficiency and metabolic diseases, immunodeficiencies and other diseases. However, existing 25-hydroxyvitamin D (25OHD) assays lack comparability to the candidate reference method, causing difficulties in diagnosis and monitoring of vitamin D deficiency.
Methods: We looked at the accuracy of 3 automated assays (Roche Diagnostics Elecsys® Total 25OHD assay, Abbott Architect® Total vitamin D assay, Advia Centaur® vitamin D Total assay) and Diasorin® Radioimmunoassay (RIA) compared to a routine laboratory Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS).
Results: The correlation based on Passing Bablok regression was good with the slopes between 0.95 and 1.31 and the intercepts between −3.24 and 3.68. However, a significant positive bias was observed using the Abbott Architect and the Diasorin RIA. Using published analytical goals of coefficient of variation (CV) <10% and bias <5%, most methods did not meet these criteria. Using measurement of uncertainty of 9%, most methods were able to meet criteria using quality control materials but not patient samples.
Conclusion: Inadequacies of these assay performances are contributed by differences in method of extraction of vitamin D from vitamin D binding protein, cross-reactivities to $25OHD_2$, $25OHD_3$ and other vitamin D metabolites, matrix interferences and a lack of standardization.

1. Introduction

The importance of vitamin D in both bone and non-bone related diseases is increasingly being recognized, with more awareness about vitamin D deficiency worldwide. Research on vitamin D has multiplied in the last 10 years, showing its relationship with blood pressure regulation, immunomodulation, regulation of cell growth and metabolic diseases [1]. With better understanding of hazards of vitamin D deficiency, there is increased vitamin D testing worldwide and increasing evidence of deficiency in various populations [2–4]. Many laboratories in United States have reported an increase in vitamin D testing at a rate of 50% or more per year [5,6]. The treatment for vitamin D deficiency/insufficiency is generally affordable and easily available, however the diagnosis should be dependent on reliable and reproducible methods of analysis.

25OHD analysis is used to diagnose hypovitaminosis D, as it is the precursor to active 1,25-dihydroxyvitamin D (1,25OHD) and has a longer half life of 3 weeks compared to 24 hours. The conversion from 25OHD to 1,25OHD is unregulated and indicates availability of vitamin D substrate. 25OHD consists of 25-hydroxyvitamin D_3 ($25OHD_3$) and 25-hydroxyvitamin D_2 ($25OHD_2$), of which $25OHD_3$ is more potent and normally present in higher concentrations in the body compared to $25OHD_2$. Vitamin D supplementations are available in D_2 and D_3 formulations over the counter or as prescription medications. Vitamin D_2 formulations are available in higher doses and more common in some populations or countries for vitamin D replacement [7,8].

Although it is important to have an optimal 25OHD status, there is a lack of consensus with regards to the 25OHD concentrations used for diagnoses of deficiency and insufficiency. According to the Endocrine Society 2011 Clinical Practice Guideline on evaluation, treatment and prevention of vitamin D deficiency, vitamin D insufficiency is defined as having a 25OHD concentration of ≤72.5 nmol/l (29 ng/ml) and deficiency is defined as having a 25OHD concentration<50 nmol/l (20 ng/ml) [9]. This is incongruent with the 2010 Institute of Medicine report [10] where a 25OHD level<30 nmol/l

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(12 ng/ml) is considered deficient and a 25OHD concentration between 30 and 49 nmol/l (12–29 ng/ml) is considered inadequate.

The candidate reference method [11–15] for 25OHD assay, Liquid Chromatography-Tandem Mass spectrometry (LC-MS/MS), requires technical expertise, specialized equipment, and expensive deuterated internal standards. Commercial assays such as immunoassays and chemical binding assays are used more frequently in clinical laboratories, as they have automated pre-treatment steps and produce results within a shorter duration. However they vary in accuracy and precision [16–20]. With increasing interest in 25OHD testing, manufacturers are working to improve existing commercial kits to provide reliable and reproducible results. There are 2 new total 25OHD kits recently available from Abbott® Diagnostics and Siemens® Healthcare Diagnostics, as well as one assay from Roche® Diagnostics that was available from the manufacturer before regulatory approval. Of note, the Roche kit is purported to be an improvement of its previous $25OHD_3$ kit by measuring both $25OHD_2$ and $25OHD_3$.

This study aimed to evaluate the accuracy and precision performance of 4 different 25OHD assays (3 new automated assays and the Diasorin® Radioimmunoassay) compared to LC-MS/MS. In addition, we compared these assays using published recommendations on analytical requirements for 25OHD measurements [21–23] with the aim of identifying commercial kits that are accurate and precise and allow laboratorians to choose a suitable 25OHD assay to facilitate testing with certainty.

2. Materials and methods

2.1. Samples

Two hundred surplus single patient serum samples were collected from our laboratory archives for analysis, protected from light and stored at −80 °C until analysis. All samples were bar-coded before storage to allow anonymity. The only selection criterion was that the surplus serum volume was 2.8 ml or more. The serum 25-hydroxyvitamin D measurements were done using 2 principles on 5 platforms, namely Roche Diagnostics Elecsys® vitamin D total assay, Abbott Architect® 25-OH vitamin D assay, Advia Centaur® vitamin D Total assay, Diasorin® 25-hydroxyvitamin D I^{125} RIA Kit and compared to a routine laboratory LC–MS/MS assay. Institutional review board approval was obtained prior to start of the study.

2.2. Methodologies

2.2.1. Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

Serum samples were measured using LC–MS/MS method at the Biomedical Mass Spectrometry Unit, Department of Chemical Pathology, Prince of Wales Hospital, Shatin, Hong Kong.

2.2.1.1. Materials. Commercial multi-levels of $25OHD_2$ and $25OHD_3$ were from Chromsystems (Chromsystems Instruments and Chemicals GmbH, München, Germany). Quality control samples were from UTAK (UTAK Laboratories Inc, Valencia, CA). Calibrators and quality control samples were prepared according to the manufacturer's instructions. Deuterium-labeled $25OHD_2$ (d_6-$25OHD_2$) and $25OHD_3$ (d_6-$25OHD_3$), internal standards for quantitation, were from Iso-Sciences (King of Prussia, PA) and Medical Isotopes (Medical Isotopes Inc, Pelham, NH), respectively. An internal standard solution containing 1250 nmol/l of both d_6-$25OHD_2$ and d_6-$25OHD_3$ was prepared in 80% methanol. LCMS grade methanol and water were from Fisher Scientific (Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK). Ammonium acetate and zinc sulfate were from BDH VWR International BVDA (Leuven, Belgium); and n-hexane was obtained from Merck KGaA (Darmstadt, Germany). These chemicals were of analytical grade.

2.2.1.2. Sample preparation. To equilibrate endogenous $25OHD_2$ and $25OHD_3$ with the internal standards, 150 µl serum sample/quality control/calibrator and 30 µl internal standard solution were mixed in a polypropylene microcentrifuge tube and incubated in a 37 °C water bath for 1 hour. To deproteinize the samples, the incubated samples were vortex-mixed with 150 µl 0.4 mol/l zinc sulfate and then with 300 µl methanol. To extract the vitamin D metabolites from the samples, a liquid–liquid extraction with 1 ml of hexane was performed and followed by centrifugation at 13000 ×*g* for 2 min. This extraction was repeated for a second time. For each sample, a total of 1.8 ml of hexane was collected into a new microcentrifuge tube. Hexane was evaporated to dryness by a vacuum concentrator (Savant SpeedVac Concentrator, Thermo Electron Co., Waltham, MA) at 45 °C. The dried residue was reconstituted with 50 µl of 70% methanol and transferred to a sample vial ready for LC–MS/MS analysis.

2.2.1.3. Methodology of LC–MS/MS. LC was performed on a Waters Acquity® UPLC system (Waters Corporation, Milford, MA). Autosampler injected 10 µl of the extract into an Acquity® UPLC BEH C8 column (2.1 × 100 mm, 1.7 µm) maintained at 45 °C in column oven. LC separation of the 25OHD and matrix interference was performed using a gradient profile of mobile phase A and B solutions, consisting of 2 mmol/l ammonium acetate and 0.1% formic acid in water and methanol, respectively. The flow rate was at 400 µl/min and the injection to injection time was 5 min.

MS/MS was used to detect $25OHD_2$ and $25OHD_3$ and their corresponding deuterium-labeled internal standards on a Waters Acquity® TQ-Detector. At unit mass resolution, the mass analyzer had the following settings: capillary voltage at 1.0 kV; cone voltage at 24 V; collision energy at 20 V; source and desolvation temperatures at 120 °C and 400 °C, respectively; and desolvation gas flow at 900 l/h. The analysis was performed in multiple reaction monitoring (MRM) mode using the following transactions: mass to charge ratio (*m/z*) 413.2 → 83.0 (quantitation) and *m/z* 413.2 → 395.3 (confirmation) for $25OHD_2$; *m/z* 419.2 → 83.0 for d_6-$25OHD_2$; *m/z* 401.2 → 383.3 (quantitation) and *m/z* 401.2 → 159.1 (confirmation) for $25OHD_3$; and *m/z* 407.3 → 159.1 for d_6-$25OHD_3$. The dwell time for each MRM was 50 ms. Quantitation was performed by the TargetLynx Manager in the Waters MassLynx 4.1 software by linear regression of peak area ratios of $25OHD_2/d_6$-$25OHD_2$ and $25OHD_3/d_6$-$25OHD_3$ against the calibrator concentrations with 1/x weighting. The difference between the results of the confirmation MRMs and the quantitation MRMs must be <20%. The LC–MS/MS is unable to differentiate 3-epi-$25OHD_3$ from $25OHD_3$, which is present in neonates and possibly in adults at a lower concentration [24].

2.2.2. Radioimmunoassay (RIA)

The radioimmunoassay was the method of measurement of serum 25OHD at our Department of Laboratory Medicine. The Diasorin® 25OHD assay (Diasorin® Inc, Stillwater, MN, USA) consists of a two-step procedure involving rapid extraction of 25OHD and other hydroxylated metabolites using acetonitrile, followed by an equilibrium RIA procedure using an antibody with specificity to 25OHD. The standards, controls and samples were all analyzed in duplicates and samples were reanalyzed if the coefficients of variation (CV) of the duplicates were >10%.

2.2.3. Chemiluminescence immunoassays

2.2.3.1. Roche Elecsys® Total 25OHD assay. The Roche Elecsys ® Total 25OHD assay (Roche Diagnostics GmbH, Germany) consists of pretreatment using dithiothreitol and sodium hydroxide. This is followed by incubation with ruthenium labeled vitamin D binding protein, streptavidin-coated microparticles and biotin-labeled 25OHD. Serum 25OHD will bind to ruthenium labeled vitamin D