Clinica Chimica Acta 462 (2016) 49–54

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

# Various calibration procedures result in optimal standardization of routinely used 25(OH)D ID-LC-MS/MS methods

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ARTICLE INFO

*Article history:*
Received 8 July 2016
Received in revised form 24 August 2016
Accepted 24 August 2016
Available online 25 August 2016

*Keywords:*
Mass spectrometry
Method comparison
Standardization
Vitamin D

ABSTRACT

*Background:* The variety of LC-MS/MS methods measuring total 25(OH)D used today is vast and the comparability among these methods is still not well assessed.
*Methods:* Here, we performed a comparison in samples of healthy donors between the currently routinely used 25(OH)D LC-MS/MS methods in the Netherlands and the Ghent University reference measurement procedure to address this issue (n = 40). Additionally, an interlaboratory comparison in patient serum samples assessed agreement between the Dutch diagnostic methods (n = 37).
*Results:* The overall correlation of the routine methods for 25(OH)$D_3$ with the reference measurement procedures and with the mean of all diagnostic methods was excellent ($r > 0.993$ and $r > 0.989$, respectively). Three out of five methods aligned perfectly with both the reference measurement procedure and the median of all methods. One of the routine methods showed a small positive bias, while another showed a small negative bias consistently in both comparisons.
*Conclusion:* The biases most probably originated from differences in calibration procedure and may be obviated by reassessing calibration of stock standards and/or calibrator matrices. In conclusion, five diagnostic centers have performed a comparison with the 25(OH)D Ghent University reference measurement procedure in healthy donor serum samples and a comparison among themselves in patient serum samples. Both analyses showed a high correlation and specificity of the routine LC-MS/MS methods, yet did reveal some small standardization issues that could not be traced back to the technical details of the different methods. Hence, this study indicates various calibration procedures can result in perfect alignment.



## Aabbreviations

| | |
|---|---|
| LC-MS/MS | liquid chromatography-tandem mass spectrometry |
| 25(OH)D | 25-hydroxyvitamin D |
| NIH | National Institute of Health |
| CDC | Centers for Disease Control and Prevention |
| NIST | US National Institute of Standards |
| UGhent | Belgian Laboratory of Analytical Chemistry in Ghent |
| VDSP | Vitamin D Standardization Program |
| RMP | reference measurement procedure |
| SKML | Dutch Foundation for Quality Assessment in Medical Laboratories |

## 1. Introduction

Assessment of Vitamin D status in patients relies on accurate measurement of 25-hydroxyvitamin D (25(OH)D) concentration in serum or plasma, which can be achieved through appropriate standardization [2]. In a joined effort to implement standardized measurements for 25(OH)D, the National Institute of Health (NIH), the US Center for Disease Control and Prevention (CDC), the US National Institute of Standards and Technology (NIST) and the Belgian Laboratory for Analytical Chemistry in Ghent (UGhent), in collaboration with other researchers and organizations, established the Vitamin D Standardization Program (VDSP) [9]. The goal of this collaboration is to make 25(OH)D measurements traceable to the highest order reference, the NIST Standard Reference Material 2972a, by using recognized reference measurement procedures (RMP) operated at NIST, CDC, and UGhent and high quality

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http://dx.doi.org/10.1016/j.cca.2016.08.016
0009-8981/

serum based reference materials with values assigned by these RMPs [8,10,11]. Although several immunoassays and liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays have been standardized by CDC's Vitamin Standardization Certification Program [1], information about the accuracy of routinely used liquid chromatography-tandem mass spectrometry (LC-MS/MS) 25(OH)D methods is very limited. Since LC-MS/MS methods are known to generate more specific and accurate measurements than immunoassay-based methods, many laboratories have implemented this technique for patient assessment [4–6]. While in theory all LC-MS/MS based methods should deliver similar results, the actual procedures for preparing samples, standards or operating the instruments can be vastly different which leads to differences in measurement accuracy and performance. To address this matter, we performed a method comparison study between the currently used routine 25(OH)D LC-MS/MS methods in the Netherlands and the UGhent RPM using healthy donor serum samples and an inter-laboratory method comparison to assess agreement between the different laboratories using routine patient serum samples.

## 2. Materials and methods

### 2.1. Samples

Forty single healthy donor serum samples from the CDC Vitamin D Standardization Certification Program (VDSCP) (so called 'phase 1 samples'), which had been assigned a reference value by the UGhent RMP were used. These sera were obtained and processed according to CLSI protocol C37 [13] and covered a range of 23 to 198 nmol/L for 25(OH)$D_3$, <1 to 14 nmol/L for 25(OH)$D_2$, and 2 to 43 nmol/L for epi-25(OH)$D_3$. In addition, 37 single patient donor serum samples were obtained by drawing an extra tube of blood from patients who already underwent a venipuncture for diagnostic purposes in our outpatient clinic (VU University Medical Center, Amsterdam). These patient sera had concentrations ranging from <1 to 134 nmol/L for 25(OH)$D_3$, <2 to 27 nmol/L for 25(OH)$D_2$ and <1 to 7 nmol/L for epi25(OH)$D_3$ as determined by [12]. All samples were anonymized immediately after withdrawal and processed like regular patient samples. After centrifugation, serum was separated, aliquotted and frozen at −20 °C until analyses. Samples were distributed frozen on dry ice. Studies were approved by the local medical ethical committees.

### 2.2. Analytical methods

Five laboratories (the Radboud University Medical Center Nijmegen (Method A) (in duplicate), the University Medical Center Groningen (Method B) (in duplicate), the Canisius Wilhelmina Hospital in Nijmegen (Method C) (in singlicate), Medlon in Enschede (Method D) (in singlicate) and the VU University Medical Center in Amsterdam (Method E) (in duplicate) measured total 25(OH)$D_3$ concentrations with their respective routine LC-MS/MS methods. Duplicate or singlicate measurements were based on the way routine patient samples are measured in each laboratory. Methods B, C and E measured 25(OH)$D_3$ and 25(OH)$D_2$ while Method A and D only measured 25(OH)$D_3$. Technical details of the measurement and calibration procedures are given in Tables 1 and 2, respectively. The characteristics of the UGhent RMP have been described elsewhere [10,11].

Ideally, the comparisons would be based on total 25(OH)D, which is defined as the sum of 25(OH)D3 and 25(OH)D2. However, here we chose to compare the sum of 25(OH)D3 and epi-25(OH)D3 for the RMP and 25(OH)D3 for the routine LC-MS/MS 25(OH)D methods. We thus excluded 25(OH)D2 as it is rarely seen in patient samples in the Netherlands and two of the five routine 25(OH)D LC-MS/MS methods therefore do not include it in their routine measurements. Moreover, we included epi-25(OH)D3 for the RMP, because all Dutch routine LC-MS/MS 25(OH)D methods co-measure it with 25(OH)D3. By doing so

**Table 1**
Technical details of LC-MS/MS methods measuring total 25(OH)D.

| | Instrument | Analytical column | Sample preparation | Internal standard | | Measured quantifier transitions (*m/z*) | | | | Calibrator matrix | Calibrator source | Calibration range (nmol/L) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 25(OH)$D_3$ | 25(OH)$D_2$ | 25(OH)$D_3$ | SIL* 25(OH)$D_3$ | 25(OH)$D_2$ | SIL 25(OH)$D_2$ | | | 25(OH)$D_3$ | 25(OH)$D_2$ |
| Method A | Agilent 1290 Infinity VL UPLC and Agilent 6490 Triple Quad LC/MS | Waters ACQUITY UPLC® BEH C18 1.7 μm 2.1 × 50 mm | Protein dissociation and precipitation using NaOH and acetonitrile/methanol and subsequent SPE extraction (HLB 1 $cm^3$ 10 mg extraction cartridges, waters) | $^2H_3$–25(OH)$D_3$ | n.a. | 401.4 → 159.2 | 404.2 → 162.1 | n.a. | n.a. | Mobile phase | Gravimetric (25(OH)$D_3$ from Sigma) correction based on UV absorbance (at 18,200) | 3.91–250 | n.a. |
| Method B | Spark Holland Symbiosis system and Waters Quattro Premier XE MS/MS | Phenomenex, Synergi 4 μm Hydro-RP 80 Å, 2 × 100 mm | Protein disrupting buffer and online SPE using HySphere $C_8$ EC-SE, 10 μm, 2 × 10 mm cartridges | $^2H_6$–25(OH)$D_3$ | $^2H_6$–25(OH)$D_2$ | 401.2 → 257.25 | 407.2 → 263.25 | 413.2 → 159.15 | 419.2 → 159.15 | 25(OH)$D_3$ depleted plasma | Calibrated using Standard Reference Material 2972 25-Hydroxyvitamin $D_2$ and $D_3$ Calibration solutions. | 9.60–192 | 9.30–186 |
| Method C | Waters Acquity UPLC and Waters Quattro Premier XE MS/MS | Waters ACQUITY UPLC® BEH C18 1.7 μm 2.1 × 50 mm | Protein precipitation using acetonitril/methanol followed by SPE (Oasis HLB) | $^2H_6$–25(OH)$D_3$ | $^2H_3$–25(OH)$D_2$ | 401.1 → 159.0 | 407.3 → 159.0 | 413.3 → 159.0 | 416.3 → 82.8 | 6% BSA in PBS | Calibrated using Standard Reference Material 2972 25-Hydroxyvitamin $D_2$ and $D_3$ Calibration solutions. | 2.5–500 | 2.0–500 |
| Method D | Shimadzu LC 20 AD and QTRAP 3200 (AB SCIEX) | Agilent, Zorbax SB-C8, 3.5 μm, 3 × 100 mm | Protein precipitation using acetonitril followed by heated evaporation and reconstitution in 70% MeOH | $^2H_3$–25(OH)$D_3$ | n.a. | 383.4 → 257.4 | 389.4 → 263.4 | n.a. | n.a. | MeOH | Calibrated using H-083 CERILLIANT 25-Hydroxyvitamin D3 solution (Sigma) | 25–250 | n.a. |
| Method E | Waters Acquity UPLC and Waters Quattro Premier XE MS/MS | Phenomenex, Kinetex C18, 2.6 μm, 2.1 × 75 mm | Protein precipitation using acetonitril and liquid-liquid extraction using hexane | $^2C_5$–25(OH)$D_3$ | $^2H_6$–25(OH)$D_2$ | 401.1 → 383.3 | 406.1 → 388.3 | 413.2 → 395.2 | 419.2 → 401.2 | 6% BSA in PBS | Gravimetric (25(OH)$D_3$ and 25(OH)$D_2$ from Toronto Research Chemicals) | 4.27–346 | 1.31–106 |

* SIL, Stable-isotope-labelled.