



Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: [www.elsevier.com/locate/clinbiochem](http://www.elsevier.com/locate/clinbiochem)

## Performance evaluation of four 25-hydroxyvitamin D assays to measure 25-hydroxyvitamin D<sub>2</sub>

James Freeman <sup>\*</sup>, Kimberly Wilson, Ryan Spears, Victoria Shalhoub, Paul Sibley

Siemens Healthcare Diagnostics Inc., 511 Benedict Ave, Tarrytown, NY 10591, USA

### ARTICLE INFO

#### Article history:

Received 17 March 2015

Received in revised form 27 May 2015

Accepted 28 May 2015

Available online xxx

#### Keywords:

Vitamin D total assay

ID-LC-MS/MS

VDSCP

25 Hydroxyvitamin D

Vitamin D

25(OH)D

25(OH)D<sub>3</sub>

25(OH)D<sub>2</sub>

### ABSTRACT

**Objectives:** The ability of current immunoassays to accurately measure equimolar amounts of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> has been recently questioned. This study determined serum 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub> and total serum 25(OH)D concentrations in healthy vitamin D<sub>2</sub>-supplemented subjects by isotope dilution liquid chromatography mass spectrometry (ID-LC-MS/MS); and, evaluated the ability of the Siemens, DiaSorin, Roche, and Abbott Vitamin D Total assays to monitor total serum 25(OH)D concentrations compared to an ID-LC-MS/MS method traceable to the National Institute of Standards and Technology (NIST), and that has achieved certification from the Centers for Disease Control and Prevention (CDC) Vitamin D Standardization Certification Program (VDSCP).

**Design and methods:** Twenty (20) healthy adults, with no history of prior vitamin D supplementation were administered oral vitamin D<sub>2</sub> (2400 IU/day for 6 months). Serum samples (140) from baseline and monthly blood draws were tested.

**Results:** After one month, the mean serum 25(OH)D<sub>2</sub> concentrations rose from 0.8 to 43.6 nmol/L, whereas 25(OH)D<sub>3</sub> concentrations declined from 84.0 to 63.4 nmol/L; total serum 25(OH)D concentrations rose from 86.6 to 107.0 nmol/L. The overall mean bias to ID-LC-MS/MS was −7.1% for the Siemens ADVIA Centaur assay, −15.3% for the DiaSorin LIAISON assay; −8.4% for the Roche ELECSYS assay and −16.3% for the Abbott ARCHITECT assay. Correlation coefficients (r) were 0.94, 0.79, 0.74, and 0.73; the mean bias for baseline [25(OH)D<sub>3</sub>-containing] versus six-month [25(OH)D<sub>2</sub>- and 25(OH)D<sub>3</sub>-containing] samples was −13.4% and −5.7%; −3.5% and 20.3%, 9.6% and −12.1%, and 0.2% and −17.8%, respectively.

**Conclusions:** The bias results obtained for the Siemens ADVIA Centaur assay and Roche ELECSYS assay were slightly lower than those for the DiaSorin LIAISON assay and the Abbott ARCHITECT assay, but all 25(OH)D assays demonstrated acceptable performance.

© 2015 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.

### Introduction

Vitamin D deficiency, as determined by serum concentrations of 25(OH)D [i.e., the sum of 25(OH)D<sub>3</sub> and 25(OH)D<sub>2</sub>] is known to compromise musculoskeletal health [1,2]. This knowledge has spurred an increase in demand for 25(OH)D testing which has led many laboratories to replace radioactive methods with fully automated immunoassays. The term “Vitamin D” represents a family of related secosteroids whose parent compound exists in two forms—one made by skin exposed to UVB sun rays (cholecalciferol, vitamin D<sub>3</sub>) and the other made by plants, fungi, and fish (ergocalciferol, vitamin D<sub>2</sub>) [1,3]. Both forms of vitamin D undergo identical hydroxylations to yield 25-hydroxyvitamin D [25(OH)D] in the liver and 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D] in the kidney [1]. Although 1,25(OH)<sub>2</sub>D is the biologically active metabolite of vitamin D, 25(OH)D generally represents the best marker of

vitamin D nutritional status that better reflects calcium absorption [4] and disease states that respond to supplementation [1,2,5–7]. Nutritional supplements may contain either vitamin D<sub>3</sub> or vitamin D<sub>2</sub> forms [2]. Depending on the dosage regimen and study population, supplementation with either form may augment total serum 25(OH)D concentrations [8–16], and correlate with efficacy [17–19]. Several reports have demonstrated that vitamin D<sub>2</sub> is less potent than vitamin D<sub>3</sub> in raising serum 25(OH)D concentrations; [8,10,20–26] whereas, other reports demonstrate equipotency [9,13].

How vitamin D<sub>2</sub> supplementation affects endogenous 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub> and total 25(OH)D has been the subject of several studies in recent years. Some reports demonstrate a compensatory decline in 25(OH)D<sub>3</sub> serum concentrations concomitant with an increase in 25(OH)D<sub>2</sub> serum concentrations compared to baseline [8,10,23,27]. Peak concentrations of serum 25(OH)D after dosing were found to be variable—greater [8], lower [23], or not changed [13,27]. The variable results likely reflect differences in study design that include dosing regimen, study population, season, and latitude. Endogenous 25(OH)D<sub>2</sub> concentrations are generally very low (less than five percent of total serum 25(OH)D concentrations); however, the 25(OH)D<sub>2</sub> to 25(OH)D<sub>3</sub>

<sup>\*</sup> Corresponding author.

E-mail addresses: [james.freeman@siemens.com](mailto:james.freeman@siemens.com) (J. Freeman), [kimberly.s.wilson@siemens.com](mailto:kimberly.s.wilson@siemens.com) (K. Wilson), [ryan.spears@siemens.com](mailto:ryan.spears@siemens.com) (R. Spears), [victoria.shalhoub@siemens.com](mailto:victoria.shalhoub@siemens.com) (V. Shalhoub), [paul.sibley@siemens.com](mailto:paul.sibley@siemens.com) (P. Sibley).

ratio may change substantially after vitamin D<sub>2</sub> supplementation [8,10,11,13,23,27]. Thus, current guidelines for the measurement of total serum 25(OH)D concentrations stipulate that assays must recognize serum 25(OH)D<sub>3</sub> and 25(OH)D<sub>2</sub> in equimolar amounts in order to avoid underrepresentation and misdiagnosis of total serum 25(OH)D concentrations [28].

In the past, variability was reported in the ability of automated immunoassays to accurately measure serum 25(OH)D<sub>2</sub> concentrations [29–32]. The affinity for 25(OH)D<sub>2</sub> and other vitamin D metabolites was found to differ between assays, which—depending on the assay—reportedly overestimated [29,30,32], or underestimated 25(OH)D<sub>2</sub> concentrations [29,30,32,33]. Assays reported to overestimate 25(OH)D<sub>2</sub> concentrations included Siemens ADVIA Centaur® Vitamin D Total (Siemens, Tarrytown, NY, USA) [29,30,32]. Assays reported to underestimate 25(OH)D<sub>2</sub> concentrations were Abbott ARCHITECT 25-OH Vitamin D (Abbott, Deerfield, IL, USA) [29,32], and Roche ELECSYS Vitamin D Total (Roche Diagnostics, Mannheim, Germany) assays [29]. The DiaSorin LIAISON 25 OH Vitamin D TOTAL assay (DiaSorin, Stillwater, MN, USA) has been reported to over-recover [32] and under-recover [29,30,33] 25(OH)D<sub>2</sub> concentrations, depending on the study.

The goal of this study was to evaluate the ability of four current Vitamin D Total immunoassays (from Siemens, DiaSorin, Abbott, and Roche) by comparison with a VDSCP-certified ID-LC-MS/MS method traceable to NIST to accurately measure and monitor serum total 25(OH)D concentrations in serum samples from subjects receiving vitamin D<sub>2</sub> supplementation.

## Materials and methods

### Sample population and study design

One hundred and forty seven (147) archived and non-identifiable clinical serum samples from 23 apparently healthy adult donors were purchased from a commercial vendor (Research Sample Bank, Inc., Delay Beach, FL, USA). Written informed consent was obtained from all participants. One subject withdrew from the study before, and two others withdrew from the study after the first monthly blood draw. Thus, results are presented for those 20 subjects that completed the study (total of 140 samples). The Roche ELECSYS Vitamin D Total assay and Abbott ARCHITECT 25-OH Vitamin D assay, each measured 25(OH)D concentrations in 139 total samples due to loss of one sample after the first month. ID-LC-MS/MS results at baseline confirmed virtually undetectable serum concentrations of 25(OH)D<sub>2</sub>. The average age for the 20 remaining subjects at the end of the study was 41 years (range 22–72 years). Seven subjects were females and 13 subjects were males. All subjects initiated the study between April 16, 2013 and April 17, 2013 and ended the study between October 15, 2013 and November 27, 2013. Blood was drawn from 23 healthy donors—not previously vitamin D supplemented—at baseline and at approximately 1, 2, 3, 4, 5, and 6 months after supplementation with ergocalciferol, vitamin D<sub>2</sub>–2400 IU daily by oral route (Deva Vegan Vitamins Vegan vitamin D<sub>2</sub>, DEVA Nutrition LLC, [info@devanutrition.com](mailto:info@devanutrition.com)). After collection, blood was placed at 4 °C, centrifuged, and serum aliquots were prepared. Serum samples were stored at –20 °C for less than nine months until they were sent to Siemens Healthcare Diagnostics Inc. (Tarrytown, NY, USA). At Siemens Healthcare Diagnostics Inc. samples were sent to LabCorp (Cranford, NJ, USA) where total serum 25(OH)D concentrations were measured using the DiaSorin LIAISON 25 OH Vitamin D TOTAL assay; and, 25(OH)D-total, 25(OH)D<sub>3</sub>, and 25(OH)D<sub>2</sub> were separated and measured by the Esoterix ID-LC-MS/MS method (test number 500116). Total serum 25(OH)D concentrations were measured using the Siemens ADVIA Centaur Vitamin D Total assay, Roche ELECSYS Vitamin D Total assay, and Abbott ARCHITECT 25-OH Vitamin D assay at Siemens Healthcare Diagnostics Inc. ADVIA Centaur is a registered trademark of Siemens Healthcare Diagnostics Inc. All other trademarks and brands are the property of their respective owners. The ID-LC-MS/

MS 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub> and total 25(OH)D values are expressed as nmol/L. Concentrations in nmol/L for 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub> and total 25(OH)D for each assay were calculated using the ID-LC-MS/MS 25(OH)D<sub>2</sub>/25(OH)D<sub>3</sub> ratio.

### Method comparison and traceability

Three of the assays evaluated in this study are competitive chemiluminescent immunoassays—Siemens ADVIA Centaur assay [34], DiaSorin LIAISON assay [35], and Abbott ARCHITECT assay [36]. The Roche ELECSYS Vitamin D Total assay is an electrochemiluminescence protein assay that involves capture of 25(OH)D by vitamin D binding protein [37]. Traceability of the assays can be found in the manufacturer's Instructions for Use as to the performance of the assays.

### ADVIA Centaur assay

[34] The assay is traceable to the Ghent University 25(OH)vitamin D Reference Measurement Procedure (RMP) and has also achieved certification from the CDC-VDSCP [28,38–40]. When this study was performed the ADVIA Centaur Vitamin D Total assay was the only assay of the ones tested that was CDC-VDSCP certified. The standardized assay is reported to demonstrate equimolar cross-reactivity with 25(OH)D<sub>2</sub> (104.5%) and 25(OH)D<sub>3</sub> (100.7%), minimal cross-reactivity with 3-epimer of 25(OH)D<sub>3</sub> (3-epi-25(OH)D<sub>3</sub>) (1.1%), and a broad assay range of 10.5–375 nmol/L (4.2–150.0 ng/mL). The Limit of Quantitation (LoQ) of the assay is 10.5 nmol/L (4.2 ng/mL). Precision analysis involved assaying six samples twice a day in replicates of 2, over 20 days (n = 80 replicates per sample) according to the Clinical and Laboratory Standards Institute (CLSI) protocol EP5-A2; [41] the run-to-run CVs were in the range of 4.2% and 11.9%. All samples were run in singlicate on both the ID-LC-MS/MS and a single ADVIA Centaur system.

### Abbott ARCHITECT assay

[36] The assay is reported to demonstrate 105% cross-reactivity with 25(OH)D<sub>3</sub>, 82% cross-reactivity with 25(OH)D<sub>2</sub>, 12.6% cross-reactivity with 1,25(OH)<sub>2</sub>D<sub>3</sub>, 112% cross-reactivity with 24,25(OH)<sub>2</sub>D<sub>3</sub>, minimal cross-reactivity with 3-epi-25(OH)D<sub>3</sub> (2.7%), and a measuring interval of 32.5–240.0 nmol/L (13.1–96.2 ng/mL). The LoQ of the assay is 20 nmol/L (8 ng/mL). Precision studies, as reported in the assay Instructions for Use, used six samples—3 Vitamin D Controls and 3 serum based samples, using two lots of reagents, in replicates of two, twice per day for 20 days (n = 80 replicates per sample), according to the CLSI protocol EP05-A2 [41]. The reported total between run CVs for the vitamin D assay control samples ranged from 2.7 to 4.6% (190.8–48.8 nmol/L, 76.3–19.5 ng/mL); and for the serum samples from 2.6 to 4.0% (178.3–57.5 nmol/L, 71.3–23.0 ng/mL).

### Roche ELECSYS assay

[37] The assay reportedly demonstrates 100% cross-reactivity with 25(OH)D<sub>3</sub>, improved 92% cross-reactivity with 25(OH)D<sub>2</sub>, 91% cross-reactivity with 3-epi-25(OH)D<sub>3</sub>, 149% cross reactivity with 24,25(OH)D<sub>3</sub>; and a measuring range of 12.5–150.0 nmol/L (5.0–60 ng/mL). The LoQ of the assay is 12.5 nmol/L (5.0 ng/mL). Precision, as reported in the assay Instructions for Use (IFU), was determined by 2 runs per day in duplicate and each for 21 days (n = 84 replicates per sample) according to a CLSI modified protocol EP05-A2; [41] the run-to-run CVs ranged from 1.6 to 7.2% (132.0–15.5 nmol/L, 52.6–6.2 ng/mL).

### DiaSorin LIAISON assay

[35] The assay is reported to demonstrate equimolar cross-reactivity with 25(OH)D<sub>2</sub> (104%) and 25(OH)D<sub>3</sub> (100%), cross-reactivity with 3-epi-25(OH)D<sub>3</sub> of <1.0%, and a measuring range of 10.0–375.0 nmol/L (4.0–150.0 ng/mL). The LoQ of the assay is 10.0 nmol/L (4.0 ng/mL). Precision, as reported in the assay Instructions for Use, was determined by assaying six serum samples and two levels of LIAISON 25 OH Vitamin D TOTAL controls over 20 days according to the CLSI protocol EP05-A2

[41] the total CVs were in the range of 12.6–10.8% (19.8–280.0 nmol/L, 7.9–112.1 ng/mL), for serum and 9.7–9.5% (45.0–154.5 nmol/L, 18.0–61.8 ng/mL) for kit controls.

#### Isotope dilution liquid chromatography mass spectrometry (ID-LC-MS/MS)

The serum 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> concentrations were determined by the Esoterix ID-LC-MS/MS method, which has been certified by the CDC through the VDSCP program. The lower LoQ for the assay was 2.5 nmol/L (1.0 ng/mL) for each 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>. The analytical measuring range was 2.5–625.0 nmol/L (1.0–250 ng/mL) for each 25(OH)D<sub>3</sub> and 25(OH)D<sub>2</sub>. The ID-LC-MS/MS method used in this study measures total and fractionated vitamin D, but does not separate the 3-epimer form of 25(OH)D [3-epi-25(OH)D].

#### Statistics

Correlation plots, difference plots, Bland–Altman plots, and bias ± standard deviation (SD) values were obtained using GraphPad Prism and Microsoft Excel (2010); Analyze-It add-in program in Excel was used to compare the different sets of data in order to obtain the 95% confidence interval (CI), 95% limits of agreement, and Deming fit.

#### Results

The ID-LC-MS/MS method was used to measure endogenous concentrations of serum 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub> and total 25(OH)D. At baseline, only three subjects had detectable 25(OH)D<sub>2</sub> [2.8, 3.2, 8.5 nmol/L (1.1, 1.3 and 3.5 ng/mL)]. After taking vitamin D<sub>2</sub> supplements for two months, all subjects exhibited greater than baseline concentrations of serum 25(OH)D<sub>2</sub>.

Consistent with previous reports, 25(OH)D<sub>2</sub> supplementation was associated with a compensatory decrease in serum 25(OH)D<sub>3</sub> concentrations (Table 1 and Fig. 1). At two months, serum 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> concentrations exhibited a 1:1 ratio and remained at their two-month concentrations until the end of the six-month study. The total serum 25(OH)D concentrations rose above those at baseline, reaching equilibrium by one month (Table 1 and Fig. 1).

The total 25(OH)D concentrations measured by the ADVIA Centaur assay and LIAISON assay, but not the ARCHITECT assay and ELECSYS assay, were greater than baseline in subjects taking supplements for one month. The ARCHITECT assay and ELECSYS assay results remained at baseline concentrations throughout the study (Table 1).

The ability of the four Vitamin D Total assays to accurately measure and monitor total serum 25(OH)D concentrations over a six-month period compared to the ID-LC-MS/MS method was determined. The samples containing both 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> numbered 133 out of 140 (>2.5 nmol/L, 1 ng/mL). Regression plots and summary of the results for total serum 25(OH)D concentrations from all subjects measured by each of the assays compared to total serum 25(OH)D concentrations by ID-LC-MS/MS are presented in Fig. 2. The Pearson correlation coefficients (r) were: ADVIA Centaur assay, 0.94 [95% confidence interval (CI) 0.91–0.95], LIAISON assay, 0.79 (CI 0.72–0.85), ELECSYS assay, 0.74 (CI 0.65–0.80), and ARCHITECT assay, 0.73 (CI 0.64–0.80) (Fig. 2).

The mean percent bias comparing each of the immunoassays to the ID LC-MS/MS assay was similar: ADVIA Centaur assay,  $-7.1 \pm 9.3\%$ , ELECSYS assay,  $-8.4 \pm 18.0\%$ ; LIAISON assay,  $-15.3 \pm 14.0\%$ ; and ARCHITECT assay,  $-16.3 \pm 14.2\%$  (data not shown). The results for overall average bias, overall average percent bias, along with 95% limits of agreement plotted as a function of total serum 25(OH)D<sub>2</sub> concentrations are shown in Fig. 3.

In order to compare the bias in samples containing relatively low 25(OH)D<sub>2</sub> concentrations (mainly those samples at baseline) with bias in samples containing greater concentrations of 25(OH)D<sub>2</sub>, results were analyzed by month (Table 1). The percent bias to ID-LC-MS/MS at each of the time-points demonstrated a negative trend for

the LIAISON, ELECSYS, and ARCHITECT assays, and a slight positive trend for the ADVIA Centaur assay (Table 1, Fig. 3).

Not unexpected, the results of assay comparisons to Bland Altman were similar to those of ID-LC-MS/MS: ADVIA Centaur assay,  $-7.8 \pm 10\%$  versus  $-7.1 \pm 9.3\%$ , ELECSYS assay,  $-10.5 \pm 18.6\%$  versus  $-8.4 \pm 18.0\%$ ; LIAISON assay,  $-17.8 \pm 16.0\%$  versus  $-15.3 \pm 14.0\%$ ; and ARCHITECT assay,  $-19.1 \pm 15.9\%$  versus  $-16.3 \pm 14.2\%$  (data not shown). Minimum performance requirements for the 25(OH)D assay have been set at a mean bias of  $\leq 15.8\%$  [42]. According to this criterion, the ADVIA Centaur assay, ELECSYS assay, and LIAISON assay results met the minimum performance goal for comparisons to ID-LC-MS/MS results.

Three participants did not show the typical increases observed in serum 25(OH)D<sub>2</sub> concentrations compared to baseline (ie., they did not achieve 25(OH)D<sub>2</sub> values above 48.4 nmol/L (20 ng/mL)). The serum 25(OH)D<sub>2</sub> concentration ranges for these three participants were as follows: Subject one, 3.2–13.3 nmol/L (1.3–5.5 ng/mL); Subject two, 0.0–33.9 nmol/L (0.0–14 ng/mL); and Subject three, 0.0–31.5 nmol/L (0.0–13.0 ng/mL); combined range for the three subjects: 0.0–33.9 nmol/L (0.0–14.0 ng/mL). The low serum 25(OH)D<sub>2</sub> concentrations could have been due to a problem with absorption or compliance. Two other subjects demonstrated gradual declines in serum 25(OH)D<sub>2</sub> concentrations after two months [range from three to six months: 31.5–7.3 nmol/L (13.0–3.0 ng/mL) and 50.8–13.6 nmol/L (21.0–5.6 ng/mL)] compared to their earlier highs of 53.2 nmol/L (22.0 ng/mL) and 67.8 nmol/L (28.0 ng/mL), respectively. Nevertheless, all five subjects demonstrated greater than 7.3 nmol/L (3.0 ng/mL) 25(OH)D<sub>2</sub> concentrations at each time-point after the baseline, so all samples from these subjects were included in the study [as mentioned in Methods, ID-LC-MS/MS LoQ was 2.5 nmol/L (1 ng/mL) for 25(OH)D<sub>2</sub>].

#### Discussion

In this study we determined serum concentrations for 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub>, and total 25(OH)D in healthy subjects receiving 2400 IU daily vitamin D<sub>2</sub> supplements; and, evaluated the ability of four commercially available Vitamin D Total assays compared with the ID-LC-MS/MS method to accurately measure and monitor total serum 25(OH)D concentrations. Optimal concentrations of total serum 25(OH)D are reported to be 75.0–110.0 nmol/L (30.0–44.0 ng/mL) based on requirements to maintain physiological concentrations of serum calcium, parathyroid hormone (PTH), and healthy bones [18, 43–45]. At baseline, eighty percent of subjects had total serum 25(OH)D concentrations in the sufficiency range [75.0 nmol/L (30.0 ng/mL) or greater]. The concentrations in the remaining twenty percent (four) subjects were between 62.5 and 65.0 nmol/L (26.0–27.0 ng/mL). Although the mean total serum 25(OH)D concentrations were 86.6 nmol/L (35.1 ng/mL), they rose on average over six months to 106.4 nmol/L (43.0 ng/mL)—a concentration reported to be safe (non-toxic). (The safe range has been reported to be <220.0 nmol/L (<88.0 ng/mL) [15,18].) The 20.0 nmol/L (8.0 ng/mL) mean increment is similar to the 15.0–17.5 nmol/L (6.0–7.0 ng/mL) increase in circulating 25(OH)D generally found for 1000 IU daily vitamin D<sub>3</sub> dosing [12, 16,46]. Heaney et al. [12] showed that healthy subjects [with a basal total serum 25(OH)D concentrations of 72 nmol/L (28.8 ng/mL) and 65.6 nmol/L (26.24 ng/mL) who were receiving 25.0 and 250.0 µg (1000 to 10,000 IU) daily vitamin D<sub>3</sub>] could increase their total 25(OH)D concentrations by 12.0 and 158 nmol/L (4.8 and 63.4 ng/mL) after five months, respectively. Thus, it is possible that higher than a 2400 IU daily dose of vitamin D<sub>2</sub> could have led to greater total serum 25(OH)D concentrations in these healthy individuals. The continuous daily dosing time-to-equilibrium for total serum 25(OH)D concentrations was about one month—faster than the five months reported for a 1000 IU daily dose of 25(OH)D<sub>3</sub> [12].

We do not know if 2,400 IU daily of vitamin D<sub>2</sub> would increase 25(OH)D concentrations in vitamin D deficient adults (<50.0 nmol/L, <20.0 ng/mL), into the optimal range; this was not a goal of the study.



**Table 1**  
Statistics for serum concentrations of total 25(OH)D, 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> as determined by ID-LC-MS/MS, ADVIA Centaur (Centaur), LIAISON, ELECSYS, and ARCHITECT assays.

	Analyte,	Mean ( $\pm$ SD)	Median nmol/L	Interquartile (IQ) range nmol/L	Minimum nmol/L	Maximum nmol/L	Percent bias to ID-LC-MS/MS ( $\pm$ SD)
Baseline, 0 h	ID-LC-MS/MS	86.61 (15.09)	86.35	76.70–99.38	62.50	112.50	
	D Total	0.83 (2.06)	0	00.00–00.00	00.00	8.47	
	D2	84.03 (16.46)	85.00	66.88–95.63	55.00	112.50	
	D3	74.64 (12.97)	73.95	64.78–85.43	51.70	99.40	–13.4 (8.2)
	Centaur	83.23 (17.22)	82.15	72.18–90.50	52.70	116.30	–3.5 (14.6)
	LIAISON	95.95 (31.03)	88.20	76.00–105.20	62.80	189.00	9.6 (21.2)
1 month	ELECSYS ARCHITECT	86.47 (21.08)	88.00	68.30–93.60	57.40	154.50	–0.2 (15.3)
	ID-LC-MS/MS						
	D Total	107.00 (18.32)	108.80	91.27–122.50	65.60	140.60	
	D2	43.55 (15.09)	44.77	36.30–53.24	5.57	67.76	
	D3	63.44 (16.27)	62.50	52.50–74.38	23.75	87.50	
	Centaur	101.40 (19.36)	98.60	88.78–117.00	63.80	145.50	–5.1 (7.5)
2 months	LIAISON	97.19 (23.55)	102.6	80.98–107.80	59.40	160.10	–9.6 (12.1)
	ELECSYS	97.17 (28.13)	91.70	81.70–108.50	62.30	176.10	–9.0 (17)
	ARCHITECT	87.58 (19.56)	88.20	69.30–102.30	58.60	138.00	–16.7 (11.6)
	ID-LC-MS/MS						
	D Total	106.60 (20.39)	105.70	88.88–124.90	73.30	150.00	
	D2	54.81 (18.10)	55.66	51.43–64.74	13.31	84.70	
3 months	D3	51.75 (15.86)	55.00	37.50–64.38	22.50	75.00	
	Centaur	98.69 (24.37)	90.45	78.83–121.00	55.90	138.80	–8.2 (8.7)
	LIAISON	87.18 (23.74)	88.45	65.33–102.30	58.70	133.10	–18.7 (12.6)
	ELECSYS	94.02 (29.84)	88.75	73.15–103.50	48.20	180.60	–12.6 (15.8)
	ARCHITECT	85.81 (19.83)	83.15	70.43–99.05	57.80	138.40	–19.1 (12.9)
	ID-LC-MS/MS						
4 months	D Total	111.70 (25.11)	106.9	96.27–128.50	78.30	167.30	
	D2	53.47 (18.60)	58.08	47.19–67.76	9.44	82.28	
	D3	58.25 (19.79)	60.00	40.00–71.88	27.50	97.50	
	Centaur	105.00 (25.56)	97.60	90.03–120.90	72.20	157.20	–6.1 (6.9)
	LIAISON	87.91 (25.12)	82.20	68.33–111.30	54.10	152.20	–21.5 (12.5)
	ELECSYS	96.96 (30.42)	95.20	72.45–116.20	57.80	185.40	–13.6 (15.1)
5 months	ARCHITECT	86.16 (20.30)	81.70	73.23–99.05	52.10	133.80	–22.5 (10.6)
	ID-LC-MS/MS						
	D Total	110.90 (24.09)	108.30	90.95–129.10	69.10	165.20	
	D2	51.62 (19.19)	58.08	31.46–67.76	17.42	79.86	
	D3	59.25 (15.07)	58.75	48.13–69.38	30.00	95.00	
	Centaur	105.30 (27.98)	104.20	84.08–129.80	58.10	155.90	–5.8 (9.3)
6 months	LIAISON	88.94 (24.15)	81.40	71.85–107.10	58.30	146.30	–20.1 (10.3)
	ELECSYS	101.90 (31.11)	92.70	84.23–120.10	64.20	206.50	–8.3 (15.1)
	ARCHITECT	90.29 (20.11)	92.00	72.48–105.50	58.30	133.00	–18.1 (9.4)
	ID-LC-MS/MS						
	D Total	109.3 (27.01)	106.7	89.00–124.4	61.20	159.90	
	D2	54.55 (24.89)	59.29	35.70–74.41	11.86	91.96	
7 months	D3	54.78 (14.38)	55.00	45.00–65.00	23.00	80.00	
	Centaur	104.00 (29.90)	93.90	86.18–118.40	55.20	170.40	–5.3 (9.2)
	LIAISON	93.69 (26.19)	86.65	75.80–106.40	62.00	165.20	–13.6 (14.0)
	ELECSYS	94.84 (30.40)	87.20	74.55–114.7	49.60	187.00	–13.1 (16.2)
	ARCHITECT	86.62 (23.45)	84.10	68.38–92.43	58.00	142.80	–19.8 (14.2)
	ID-LC-MS/MS						
8 months	D Total	106.40 (24.09)	100.3	91.25–121.80	66.10	159.70	
	D2	51.63 (26.73)	56.87	26.80–73.21	7.26	96.80	
	D3	54.75 (15.6)	50.00	42.50–70.00	25.00	85.00	
	Centaur	102.00 (32.74)	92.70	77.10–125.8	52.70	167.30	–5.7 (12.7)
	LIAISON	85.95 (28.97)	79.90	63.45–105.8	44.10	157.50	–20.3 (13.3)
	ELECSYS	93.93 (30.42)	86.70	74.28–105.30	56.30	189.20	–12.1 (15.1)
9 months	ARCHITECT	86.70 (21.19)	85.75	67.65–97.23	58.00	126.7	–17.8 (14.0)
	ID-LC-MS/MS						

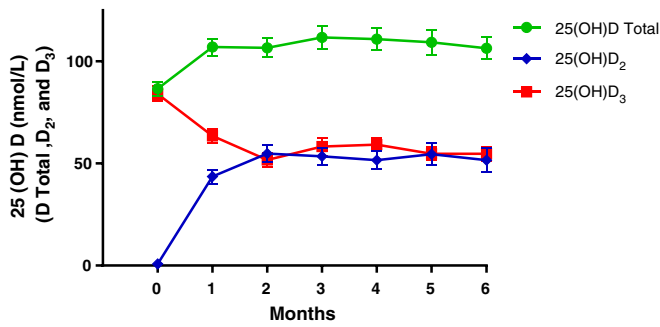
The number of subjects at each monthly measurement was 20, except at one month when the number of subjects for ELECSYS and ARCHITECT was 19 each. Total nmole/L for each assay was calculated using the ID-LC-MS/MS 25(OH)D<sub>2</sub>/25(OH)D<sub>3</sub> ratio as described in Methods. To convert 25(OH)D concentrations to nanograms per milliliter (ng/mL), divide by 2.5 for 25(OH)D<sub>3</sub> and 2.42 for 25(OH)D<sub>2</sub>. Interquartile (IQ) Range: Values ranging from the 25th to the 75th percentile.

However, we surmise that 2,400 IU daily may not be sufficient based on the results of several studies—albeit those studies used different regimens of vitamin D<sub>2</sub> or vitamin D<sub>3</sub> [13,46]—and the Endocrine Society guidelines that recommend initial treatment of vitamin D deficient adults with vitamin D<sub>2</sub> or vitamin D<sub>3</sub> doses of 50,000 IU once a week or 6000 IU per day to achieve a blood concentration of 25(OH)D above 30.0 ng/mL [2].

In the present study, 2400 IU daily of vitamin D<sub>2</sub> led to higher serum 25(OH)D<sub>2</sub> concentrations and lower serum 25(OH)D<sub>3</sub> concentrations than baseline; total serum 25(OH)D concentrations were higher than baseline. These compensatory findings are consistent with the results of others; [8,10,23,27] whereas, total serum 25(OH)D concentrations were reported to be higher [8], lower [23], or unchanged in those

studies [13,27]—likely a reflection of the study design, dosing regimen and baseline serum 25(OH)D concentrations, as mentioned earlier. A similar reciprocal compensatory response was reported after vitamin D<sub>3</sub> supplementation, (i.e., rise in serum 25(OH)D<sub>3</sub> and decline in serum 25(OH)D<sub>2</sub> concentrations). Both vitamin D<sub>2</sub>- and vitamin D<sub>3</sub>-induced compensatory responses likely involve similar feedback mechanisms or competition for common metabolic pathways [8,47].

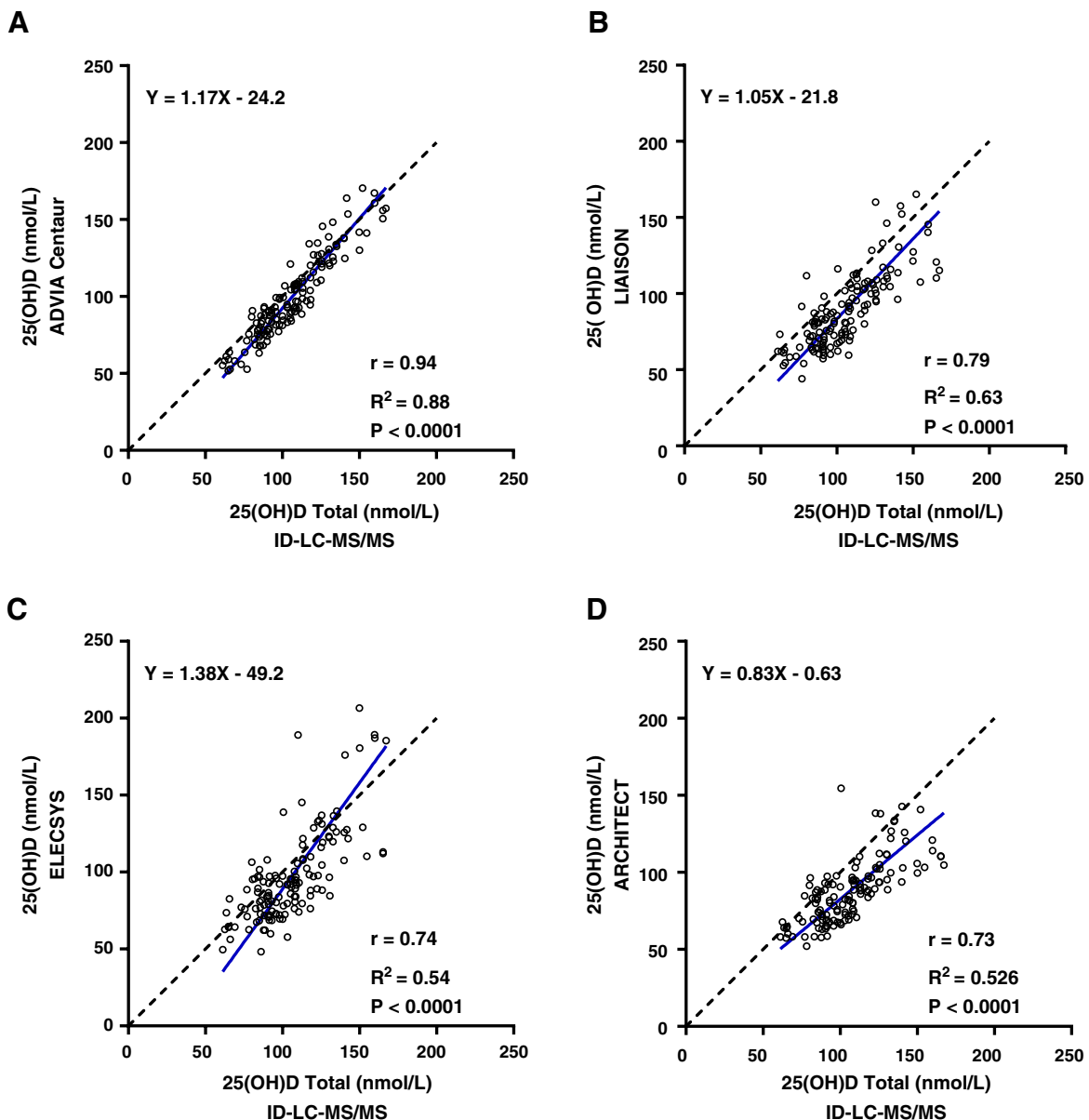
Vitamin D<sub>2</sub> metabolites appear to be less effective at increasing serum 25(OH)D concentrations, especially when given as a bolus dose compared with daily dosing [8,10,20–26,48,49]. The present study did not directly compare the efficacy of vitamin D<sub>2</sub> and vitamin D<sub>3</sub>, so we do not know whether a similar daily dose of 25(OH)D<sub>3</sub> would have



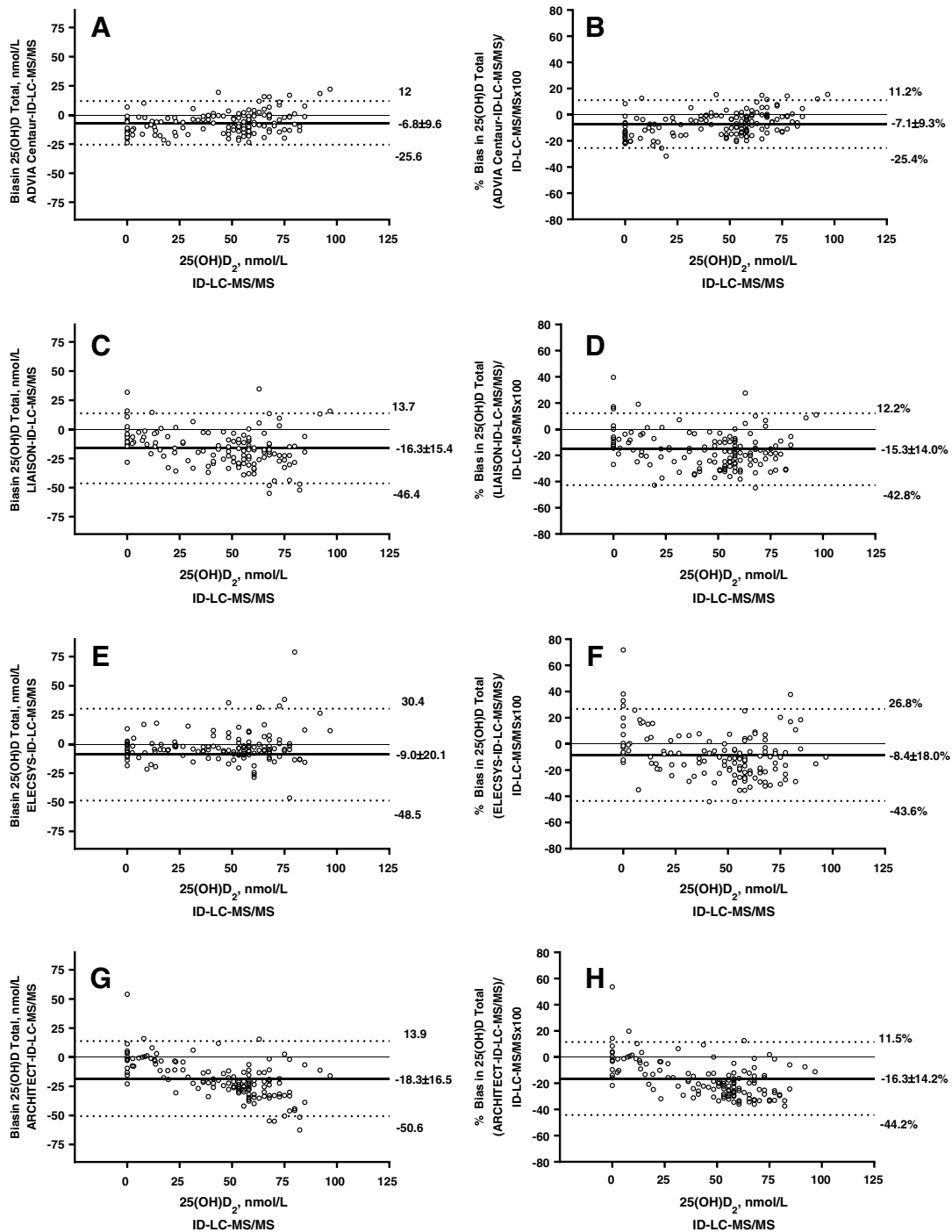
**Fig. 1.** Time-course of total serum 25(OH)D, 25(OH)D<sub>2</sub>, and 25(OH)D<sub>3</sub> concentrations in subjects receiving 2400 IU daily of vitamin D<sub>2</sub> (means ± standard error of the mean, SEM). Serum concentrations of 25(OH)D<sub>2</sub>, 25(OH)D<sub>3</sub> and total 25(OH)D were determined using the ID-LC-MS/MS method. To convert 25(OH)D concentrations to ng/mL divide by 2.5 for 25(OH)D<sub>3</sub> and 2.42 for 25(OH)D<sub>2</sub>.

raised total serum 25(OH)D concentrations to the same degree as 25(OH)D<sub>2</sub>. This study demonstrated that 2400 IU daily led to a mean increment in 25(OH)D of 20.0 nmol/L (8.0 ng/mL); this is more than two fold the amount of vitamin D<sub>3</sub> reported to raise 25(OH)D by the same amount (ie., 1000 IU daily of vitamin D<sub>3</sub> raised total serum 25(OH)D concentrations by about 15.0–17.5 nmol/L (6.0–7.0 ng/mL)) [12,16,46]. Although indirect, these assumptions support reports that 25(OH)D<sub>3</sub> supplementation is at least 30–50% more effective than 25(OH)D<sub>2</sub> in maintaining total serum 25(OH)D concentrations [8,10, 20–26,48,49].

Variability for automated and other methods in the measurement of 25(OH)D has been attributed to a variety of sources including differential recognition of 25(OH)D<sub>3</sub> and 25(OH)D<sub>2</sub> by the capture antibody [29–32], and 25(OH)D is a difficult analyte to measure due to its hydrophobic properties and tight binding to an abundance of serum binding proteins. Additionally, due to its lower affinity for binding proteins, 25(OH)D<sub>2</sub> in serum may be preferentially released and more



**Fig. 2.** Regression analysis of 25(OH)D measurement comparison. A. ADVIA Centaur and ID-LC-MS/MS; B. LIAISON and ID-LC-MS/MS; C. ELECSYS and ID-LC-MS/MS; D. ARCHITECT and ID-LC-MS/MS. To obtain approximate 25(OH)D concentrations in ng/mL divide by 2.5.



**Fig. 3.** Difference plots demonstrating bias and percent bias to ID-LC-MS/MS versus serum 25(OH)<sub>2</sub> concentrations. A. and B.: ADVIA Centaur; C. and D.: LIAISON; E. and F.: ELECSYS; G. and H.: ARCHITECT. The bold line represents bias; dotted lines represent bias  $\pm$  1.96 SD (the 95% limits of agreement). To obtain 25(OH)<sub>2</sub> concentrations in ng/mL divide by 2.42.

readily available for binding to capture antibody [50]. This may have been the case for the ADVIA Centaur assay which showed a slight positive trend for bias compared to ID-LC-MS/MS serum 25(OH)<sub>2</sub> concentrations increased. This is consistent with the product labeling [104.5% cross-reactivity with 25(OH)<sub>2</sub> and 100.7% cross-reactivity with 25(OH)<sub>3</sub>]. Compared with the ADVIA Centaur assay that was used in previous studies, the assay in the present study was calibrated with new standard values and is traceable to the Ghent University 25(OH)vitamin D RMP. A recent study demonstrated acceptable bias for individuals with different concentrations of vitamin D binding protein and 25(OH)-D<sub>2</sub> concentrations using this assay [51]. The Esoterix ID-LC-MS/MS method used in the present study detects 3-epi-25(OH)<sub>2</sub> or 3-epi-25(OH)<sub>3</sub> as part of

25(OH)<sub>2</sub> and 25(OH)<sub>3</sub>, so the ID-LC-MS/MS method may have overestimated 25(OH)<sub>2</sub> values.

Limitations of the study include the following: First, none of the subjects had total serum 25(OH)D concentrations lower than 48 nmol/L (20.0 ng/mL), so bias and clinical concordance at the medical decision point could not be assessed; however, the ADVIA Centaur assay and ELECSYS assay results for bias to ID-LC-MS/MS for subjects with 25(OH)<sub>2</sub> ranging from 0.0 to 48.0 nmol/L were acceptable when compared to the bias for subjects with total 25(OH)D or 25(OH)<sub>2</sub> concentrations over 48.0 nmol/L (Fig. 3 and Table 1). Second, no placebo group was included in the study to control for changes in serum concentrations of 25(OH)<sub>2</sub> and 25(OH)<sub>3</sub> over time. It is unlikely that the

subjects' diets would have changed such that serum 25(OH)<sub>2</sub>D and total serum 25(OH)D concentrations would rise as quickly as they did within the first few weeks concomitant with a decline in 25(OH)D<sub>3</sub> serum concentrations. In addition, samples were collected in spring/early summer making it unlikely that vitamin D<sub>3</sub> concentrations would decline due to lack of exposure to sunlight. Third, serum PTH and calcium concentrations were not measured. Serum 25(OH)D concentrations regulate physiological concentrations of serum PTH [12,45]. The highest total serum 25(OH)D concentration measured in this study by ID-LC-MS/MS was 167.3 nmol/L (68 ng/mL) which was reported to be within the safe range for maintaining serum PTH and calcium concentrations (<220.0 nmol/L, <88.0 ng/mL) [16,18]. Future studies will include serum PTH and calcium measurements. The strengths of the study include adequate number of samples representative of apparently healthy subjects receiving vitamin D<sub>2</sub> (D<sub>2</sub>:D<sub>3</sub> ratio of 1:1); relatively long study (6 months); good participant compliance with protocol; use of a comparison VDSCP-certified ID-LC-MS/MS method traceable to NIST; use of at least one assay, ADVIA Centaur Vitamin D Total assay, traceable to the Ghent University 25(OH)vitamin D RMP that has also achieved certification by the CDC VDSCP.

## Conclusions

The 25(OH)D bias results for the ADVIA Centaur assay and ELECSYS assay were slightly lower than those for the LIAISON assay and the ARCHITECT assay; however, this study demonstrated that all 25(OH)D assays achieved acceptable performance.

## Disclosure

All authors are employees of Siemens Healthcare Diagnostics Inc.

## Conflict of interests

All authors are employees of Siemens Healthcare Diagnostics Inc.

The authors declare that there is no conflict of interests regarding the publication of this article.

## Acknowledgments

This study was supported by Siemens Healthcare Diagnostics Inc.

## References

- Holick MF. Vitamin D, deficiency. *N Engl J Med* 2007;357:266–81.
- 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;96:1911–30.
- Holick MF, Chen TC, Lu Z, Sauter E. Vitamin D and skin physiology: a D-lightful story. *J Bone Miner Res* 2007;22(Suppl. 2):V28–33.
- Heaney RP, Barger-Lux MJ, Dowell MS, Chen TC, Holick MF. Calcium absorptive effects of vitamin D and its major metabolites. *J Clin Endocrinol Metab* 1997;82:4111–6.
- Bischoff-Ferrari HA, Dietrich T, Orav EJ, et al. Higher 25-hydroxyvitamin D concentrations are associated with better lower-extremity function in both active and inactive persons aged > or =60 y. *Am J Clin Nutr* 2004;80:752–8.
- DeLuca HF. Overview of general physiologic features and functions of vitamin D. *Am J Clin Nutr* 2004;80:1689s–96s.
- Trivedi DP, Doll R, Khaw KT. Effect of four monthly oral vitamin D<sub>3</sub> (cholecalciferol) supplementation on fractures and mortality in men and women living in the community: randomised double blind controlled trial. *BMJ* 2003;326:469.
- Armas LA, Hollis BW, Heaney RP. Vitamin D<sub>2</sub> is much less effective than vitamin D<sub>3</sub> in humans. *J Clin Endocrinol Metab* 2004;89:5387–91.
- Biancuzzo RM, Young A, Bibuld D, et al. Fortification of orange juice with vitamin D(2) or vitamin D(3) is as effective as an oral supplement in maintaining vitamin D status in adults. *Am J Clin Nutr* 2010;91:1621–6.
- Binkley N, Gemar D, Engelke J, et al. Evaluation of ergocalciferol or cholecalciferol dosing, 1,600 IU daily or 50,000 IU monthly in older adults. *J Clin Endocrinol Metab* 2011;96:981–8.
- Clemens TL, Zhou XY, Myles M, Endres D, Lindsay R. Serum vitamin D<sub>2</sub> and vitamin D<sub>3</sub> metabolite concentrations and absorption of vitamin D<sub>2</sub> in elderly subjects. *J Clin Endocrinol Metab* 1986;63:656–60.
- Heaney RP, Davies KM, Chen TC, Holick MF, Barger-Lux MJ. Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. *Am J Clin Nutr* 2003;77:204–10.
- Holick MF, Biancuzzo RM, Chen TC, et al. Vitamin D<sub>2</sub> is as effective as vitamin D<sub>3</sub> in maintaining circulating concentrations of 25-hydroxyvitamin D. *J Clin Endocrinol Metab* 2008;93:677–81.
- Malabanan A, Veronikis IE, Holick MF. Redefining vitamin D insufficiency. *Lancet* 1998;351:805–6.
- Vieth R. Vitamin D, supplementation, 25-hydroxyvitamin D concentrations, and safety. *Am J Clin Nutr* 1999;69:842–56.
- Vieth R, Chan PC, MacFarlane GD. Efficacy and safety of vitamin D<sub>3</sub> intake exceeding the lowest observed adverse effect level. *Am J Clin Nutr* 2001;73:288–94.
- Adams JS, Kantorovich V, Wu C, Javanbakht M, Hollis BW. Resolution of vitamin D insufficiency in osteopenic patients results in rapid recovery of bone mineral density. *J Clin Endocrinol Metab* 1999;84:2729–30.
- Bischoff-Ferrari HA, Shao A, Dawson-Hughes B, Hathcock J, Giovannucci E, Willett WC. Benefit-risk assessment of vitamin D supplementation. *Osteoporos Int* 2010;21:1121–32.
- Bischoff-Ferrari HA, Willett WC, Wong JB, et al. Prevention of nonvertebral fractures with oral vitamin D and dose dependency: a meta-analysis of randomized controlled trials. *Arch Intern Med* 2009;169:551–61.
- Heaney RP, Recker RR, Grote J, Horst RL, Armas LA. Vitamin D(3) is more potent than vitamin D(2) in humans. *J Clin Endocrinol Metab* 2011;96:E447–52.
- Houghton LA, Vieth R. The case against ergocalciferol (vitamin D<sub>2</sub>) as a vitamin supplement. *Am J Clin Nutr* 2006;84:694–7.
- Leventis P, Kiely PD. The tolerability and biochemical effects of high-dose bolus vitamin D<sub>2</sub> and D<sub>3</sub> supplementation in patients with vitamin D insufficiency. *Scand J Rheumatol* 2009;38:149–53.
- Logan VF, Gray AR, Peddie MC, Harper MJ, Houghton LA. Long-term vitamin D<sub>3</sub> supplementation is more effective than vitamin D<sub>2</sub> in maintaining serum 25-hydroxyvitamin D status over the winter months. *Br J Nutr* 2013;109:1082–8.
- Romagnoli E, Mascia ML, Cipriani C, et al. Short and long-term variations in serum calcitropic hormones after a single very large dose of ergocalciferol (vitamin D<sub>2</sub>) or cholecalciferol (vitamin D<sub>3</sub>) in the elderly. *J Clin Endocrinol Metab* 2008;93:3015–20.
- Trang HM, Cole DE, Rubin LA, Pierratos A, Siu S, Vieth R. Evidence that vitamin D<sub>3</sub> increases serum 25-hydroxyvitamin D more efficiently than does vitamin D<sub>2</sub>. *Am J Clin Nutr* 1998;68:854–8.
- Tripkovic L, Lambert H, Hart K, et al. Comparison of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> supplementation in raising serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. *Am J Clin Nutr* 2012;95:1357–64.
- Stephensen CB, Zerofsky M, Burnett DJ, et al. Ergocalciferol from mushrooms or supplements consumed with a standard meal increases 25-hydroxyergocalciferol but decreases 25-hydroxycholecalciferol in the serum of healthy adults. *J Nutr* 2012;142:1246–52.
- Thienpont LM, Stepman HC, Vesper HW. Standardization of measurements of 25-hydroxyvitamin D<sub>3</sub> and D<sub>2</sub>. *Scand J Clin Lab Invest Suppl* 2012;243:41–9.
- Farrell C, Soldo J, Williams P, Herrmann M. 25-Hydroxyvitamin D testing: challenging the performance of current automated immunoassays. *Clin Chem Lab Med* 2012;50:1953–63.
- Holmes EW, Garbincius J, McKenna KM. Analytical variability among methods for the measurement of 25-hydroxyvitamin D: still adding to the noise. *Am J Clin Pathol* 2013;140:550–60.
- Hsu SA, Soldo J, Gupta M. Evaluation of two automated immunoassays for 25-OH vitamin D: comparison against LC-MS/MS. *J Steroid Biochem Mol Biol* 2013;136:139–45.
- Le Goff C, Peeters S, Crine Y, Lukas P, Souberbielle JC, Cavalier E. Evaluation of the cross-reactivity of 25-hydroxyvitamin D<sub>2</sub> on seven commercial immunoassays on native samples. *Clin Chem Lab Med* 2012;50:2031–2.
- Moon HW, Cho JH, Hur M, et al. Comparison of four current 25-hydroxyvitamin D assays. *Clin Biochem* 2012;45:326–30.
- Siemens ADVIA Centaur®, ADVIA Centaur® XP Vitamin D Total (VitD) Assay [directional insert 10699279\_EN Rev. A, 2013-07]. Tarrytown, NY, USA: Siemens Healthcare Diagnostics Inc.; 2013.
- DiaSorin LIAISON® 25 OH Vitamin D TOTAL Assay [directional insert] DiaSorin, Stillwater, MN, USA; 2012.
- Abbott ARCHITECT® 25-OH Vitamin D Assay [directional insert 3L52, G2-3231/R03]. Abbott Park, IL, USA: Abbott Laboratories; 2011.
- Roche ELECSYS®. Vitamin D total assay [directional insert 06506780 160]. 3.0 Sandhofer Strasse 116, D-68305 Mannheim, Germany: Roche Diagnostics GmbH; 2013-07.
- Miller WG, Myers GL, Gantzer ML, et al. Roadmap for harmonization of clinical laboratory measurement procedures. *Clin Chem* 2011;57:1108–17.
- Sempow CT, Vesper HW, Phinney KW, Thienpont LM, Coates PM. Vitamin D status as an international issue: national surveys and the problem of standardization. *Scand J Clin Lab Invest Suppl* 2012;243:32–40.
- Stepman HC, Vanderroost A, Van Yttinghe K, Thienpont LM. Candidate reference measurement procedures for serum 25-hydroxyvitamin D<sub>3</sub> and 25-hydroxyvitamin D<sub>2</sub> by using isotope-dilution liquid chromatography-tandem mass spectrometry. *Clin Chem* 2011;57:441–8.
- Clinical and Laboratory Standards Institute (formerly NCCLS). Evaluation of precision performance of quantitative measurement methods: approved guideline. Wayne, PA: Clinical and Laboratory Standards Institute; 2004[NCCLS Document EP5-A2].
- Farrell CJ, Martin S, McWhinney B, Straub I, Williams P, Herrmann M. State-of-the-art vitamin D assays: a comparison of automated immunoassays with liquid chromatography-tandem mass spectrometry methods. *Clin Chem* 2012;58:531–42.

- [43] Bischoff-Ferrari HA, Giovannucci E, Willett WC, Dietrich T, Dawson-Hughes B. Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes. *Am J Clin Nutr* 2006;84:18–28.
- [44] Dawson-Hughes B, Heaney RP, Holick MF, Lips P, Meunier PJ, Vieth R. Estimates of optimal vitamin D status. *Osteoporos Int* 2005;16:713–6.
- [45] Pepe J, Romagnoli E, Nofroni I, et al. Vitamin D status as the major factor determining the circulating levels of parathyroid hormone: a study in normal subjects. *Osteoporos Int* 2005;16:805–12.
- [46] Aloia JF, Patel M, Dimaano R, et al. Vitamin D intake to attain a desired serum 25-hydroxyvitamin D concentration. *Am J Clin Nutr* 2008;87:1952–8.
- [47] MacDonald HM, Wood AD, Tang JC, Fraser WD. Comparison of vitamin D(2) and vitamin D(3) supplementation in increasing serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. *Am J Clin Nutr* 2012;96:1152–3 [author reply 3–4].
- [48] Glendenning P, Chew GT, Seymour HM, et al. Serum 25-hydroxyvitamin D levels in vitamin D-insufficient hip fracture patients after supplementation with ergocalciferol and cholecalciferol. *Bone* 2009;45:870–5.
- [49] Tjellesen L, Hummer L, Christiansen C, Rodbro P. Serum concentration of vitamin D metabolites during treatment with vitamin D2 and D3 in normal premenopausal women. *Bone Miner* 1986;1:407–13.
- [50] Hollis BW. Comparison of equilibrium and disequilibrium assay conditions for ergocalciferol, cholecalciferol and their major metabolites. *J Steroid Biochem* 1984; 21:81–6.
- [51] Freeman J, Wilson K, Spears R, Shalhoub V, Sibley P. Influence of vitamin D binding protein on accuracy of 25-hydroxyvitamin D measurement using the ADVIA centaur vitamin D total assay. *Int J Endocrinol* 2014;2014. <http://dx.doi.org/10.1155/2014/691679> Article ID 691679.