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Multicenter comparison study of current methods to measure 25-hydroxyvitamin D in serum

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ABSTRACT

Objectives: Measurement of serum 25-hydroxyvitamin D [25(OH)D] is generally considered to be a reliable indicator of vitamin D status. The recent increase in diversity of 25(OH)D assays prompted us to evaluate the performance of chromatographic methods (two in-house ID–LC–MS/MS and HPLC (ClinRep, Recipe)), a protein binding method (Cobas-25(OH)D-total, Roche) and immunochemical methods (Liaison and RIA (Diasorin), iSYS (IDS), ADVIA Centaur (Siemens), and Architect i1000 and i2000 (Abbott)).
Methods: Blood was drawn from randomly selected outpatients ($N = 60$) at one site after informed consent. DEQAS and SRM 972 samples were obtained from the scheme organizer and NIST, respectively. Serum aliquots were prepared, frozen and transported to participating centers. Method comparison was performed according to CLSI-EP9 specifications.
Results: With these patient samples, and in comparison with ID–LC–MS/MS, Deming regression parameters slope, intercept and R were found to be within the ranges [0.57–1.07], [−1.7 to 6.9 nmol/L] and [0.88–0.98], respectively. 25(OH)D2 in DEQAS and SRM samples was fully recognized by chromatographic methods, but only partially by protein binding and immunochemical methods. Chromatographic methods, and to a lesser extent the protein binding assay, showed cross-reactivity with 3-epi-25(OH)D3. Agreement of 25(OH)D assays to ID–LC–MS/MS in sorting patients into distinct 25(OH)D categories varied between 53% and 88%.
Conclusions: Significant bias exists between ID–LC–MS/MS and many, but not all, other 25(OH)D assays. The variable response among different assays for 25(OH)D metabolites impedes the use of uniform cut-off values for defining vitamin D status. Our results indicate the need towards further standardizing assays for 25(OH)D measurement.

1. Introduction

The most reliable assessment of vitamin D status is measurement of 25-hydroxyvitamin D [25(OH)D] concentration in human serum and/or plasma. Two distinct forms of 25(OH)D exist: 25(OH)D3 from cutaneously-derived vitamin D3 (cholecalciferol), being the predominant natural source of vitamin D in humans, and 25(OH)D2 from plant-derived vitamin D2 (ergocalciferol). The ratio between both forms may vary depending on supplementation or fortification of food. It is the total 25(OH)D concentration that is of clinical importance and therefore assays should be used that can detect both the D2 and D3 forms. High-performance liquid chromatography (HPLC) and isotope-dilution liquid chromatography-tandem mass spectrometry (ID–LC–MS/MS) methods measure 25(OH)D2 and 25(OH)D3 separately, whereas competitive binding assays are capable of measuring only total 25(OH)D. Significant concentrations of C-3 epimers of 25(OH)D2 or 25(OH)D3 have been found in samples from children <1 yr of age and to a lesser extent in adults as well [1,2]. Epimers may contribute up to 60% of total

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25(OH)D in the infant population. In the majority of chromatographic methods, C3-epimers are not resolved which may lead to an overestimation of the true 25(OH)D concentration.

Determination of 25(OH)D is no easy task given the hydrophobic nature of 25(OH)D, its binding to the vitamin D-binding protein (DBP) with high affinity and with lower affinities to lipoproteins and albumin, and the impact of sample matrix on assay performance. High variability in 25(OH)D measurements due to utilized test and assay technologies, non-equimolar detection of 25(OH)D2 and 25(OH)D3, interferences from other hydroxylated vitamin D metabolites, and the lack of a definite reference method often confounds proper assessment of vitamin D status [3,4]. Recently, two reference measurement procedures for 25(OH)D3 and 25(OH)D2 have been described using ID–LC–MS/MS [5,6]. Availability of reference materials from native human origin will be of help getting 25(OH)D methods properly standardized.

The two main methodologies for measuring 25(OH)D are competitive binding methods (immunoassay, protein-binding assay) and those based on chromatographic separation followed by direct detection (HPLC, LC–MS/MS). Binding methods demonstrate a more or less considerable deviation of individual 25(OH)D values compared with ID–LC–MS/MS defined target concentrations [7–11]. The increased diversity of 25(OH)D assays over the last few years prompted us to evaluate the performance of new and established methods using patient samples, vitamin D External Quality Assessment Scheme (DEQAS) samples and Standard Reference Material (SRM) 972. The tested assays were commercially available radioimmunoassay (RIA), HPLC and six automated protein binding or immunoassays from five manufacturers as well as two different in-house ID–LC–MS/MS methods. In addition, we evaluated the clinical accuracy of each assay by examining the agreement of 25(OH)D assays to ID–LC–MS/MS in sorting patients into distinct 25(OH)D categories using the cutoffs for deficiency (<25 nmol/L), insufficiency (25–50 nmol/L), normal range (50–75 nmol/L) and optimal range (>75 nmol/L).

2. Experimental

Blood (serum–gel, S-Monovette, Sarstedt, Nümbrecht, Germany) was drawn at one site from random outpatients (N = 60, 46 women and 14 men) after informed consent. Mean age was 56 (range 15–84, SD 19). Laboratory requests originated from mainly general practitioners (N = 39) and internists (N = 14). DEQAS sample 390 was obtained from the scheme organizer and SRM 972 (N = 4, levels 1–4) was bought from the National Institute of Standards and Technology (NIST). Sample aliquots were prepared, frozen and transported to participating centers. Method comparison was performed according to Clinical and Laboratory Standards Institute (CLSI) Evaluation Protocol 9 (EP9) specifications. Evaluated assays were chromatographic methods (two different in-house ID–LC–MS/MS methods and a commercially available HPLC method (ClinRep HPLC, Recipe, München, Germany)), a protein binding method (COBAS 25(OH)D total, Roche, Penzberg, Germany) and immunochemical methods (Liaison (Diasorin, Stillwater, MN, USA), RIA (Diasorin), iSYS (IDS, Boldon, UK), ADVIA Centaur (Siemens, Deerfield, IL, USA) – adjusted, Architect i2000 and i1000 (Abbott, Abbott Park, IL, USA) – adjusted). Lower limits of detection are 2.0–4.0 nmol/L for chromatographic assays (D2 and D3 isoforms), 10 nmol/L for the majority of immunochemical methods and COBAS D total assay, and 20 nmol/L for the Architect assays (information leaflets).

The Architect assays as well as the ADVIA Centaur assay have been adjusted by the supplier relatively quickly after release on the market. The reagent kits used in this study were from after that period. All assays were compared to an established LC–MS/MS method (LC–MS/MS-a)[12]. In ID–LC–MS/MS-a, internal standard (IS) 25(OH)D3–d6 is added to calibrators (prepared in phosphate-buffered saline with 60 g/L albumin), controls and patient sera and 25(OH)D is released from vitamin D binding protein by adding sodium hydroxide prior to protein precipitation by acetonitrile/methanol (9:1, v/v), followed by off-line solid-phase extraction (SPE). A second ID–LC–MS/MS-b uses a reference measurement procedure (6) assigned calibrator and a proprietary protein disruption buffer to release 25(OH)D from its binding proteins. 25(OH)D3–d6 (IS) is added and samples are extracted by on-line SPE and analyzed by mass spectrometry (10). The 3-epimers of 25(OH)D are not resolved by both ID–LC–MS/MS methods. Therefore, the relative content of 3-epi-25(OH)D3 was measured by a modification of ID–LC–MS/MS-a using a fluorophenyl column [2].

Individual patient samples were measured in duplicate, with exception of HPLC, RIA and the Architect-i1000 where single measurements were performed. Concentrations of 25(OH)D in conventional units ng/mL may be obtained by dividing concentrations in the International System (SI) units nmol/L by a factor 2.5.

2.1. Statistical analysis

For method comparison studies we applied Deming regression and Bland–Altman bias plots using EP Evaluator Release 9 (Data Innovations LLC, South Burlington, VT, USA) or Analyse-it software program (Microsoft Corporation). Module CLSI EP9 Method Comparison was used for analysis of duplicate measurements, whereas module Alternate (Quantitative) Method Comparison was used for single measurements.

3. Results

All 25(OH)D values represent total 25(OH)D concentrations covering a range between 5.0 and 108.0 nmol/L with a mean value of 35.2 nmol/L based on the ID–LC–MS/MS-a measurements. All patient cases had undetectable 25(OH)D2 values, as well as non-significant levels of C3-epi-25(OH)D3 (mean (median) relative content 3.6 (3.1)%; range 2.0–10.6%). Details of the Deming regression analysis and coefficient of correlation (R) with ID–LC–MS/MS as the comparative method are listed in Table 1. The results of regression analysis with other assays (with duplicate measurements available) as the comparative method can be found in the Supplemental Tables. The slope of the Deming regression line for the evaluated 9 methods relative to ID–LC–MS/MS-a varied from 0.57 for the ADVIA Centaur to 1.07 for the ClinRep HPLC, and the intercept from −1.7 nmol/L for the COBAS D total and ID–LC–MS/MS-b to 6.9 nmol/L for the Architect i2000. Statistically significant bias

Table 1
Comparison of ID–LC–MS/MS-a with other methods using the CLSI EP9 Method Comparison protocol and Deming regression analysis (n = 60 specimens). Shaded fields indicate a statistically significant difference with ID–LC–MS/MS-a.

Method	R	Slope (95%CI)	Intercept (95% CI) nmol/L
ID–LC–MS/MS-b	0.98	1.05 (1.01–1.08)	−1.7 (−3.4 to (−0.1))
ClinRep HPLC (Recipe)[a]	0.96	1.07 (0.99–1.15)	1.2 (−2.3 to 4.6)
Liaison (Diasorin)	0.93	0.90 (0.84–0.96)	−0.6 (−3.0 to 1.9)
RIA (Diasorin) [a]	0.96	0.88 (0.81–0.95)	4.6 (1.8–7.5)
iSYS (IDS)	0.95	1.02 (0.97–1.08)	0.9 (−1.5 to 3.4)
ADVIA Centaur (Siemens)	0.92	0.57 (0.53–0.61)	4.6 (2.8–6.4)
Cobas D total (Roche)	0.88	1.01 (0.92–1.10)	−1.7 (−5.5 to 2.1)
Architect-i2000 (Abbott)	0.94	0.87 (0.82–0.93)	6.9 (4.6–9.1)
Architect-i1000 (Abbott)[a]	0.96	0.83 (0.77–0.90)	4.3 (1.5–7.0)

[a] No duplicate measurements.

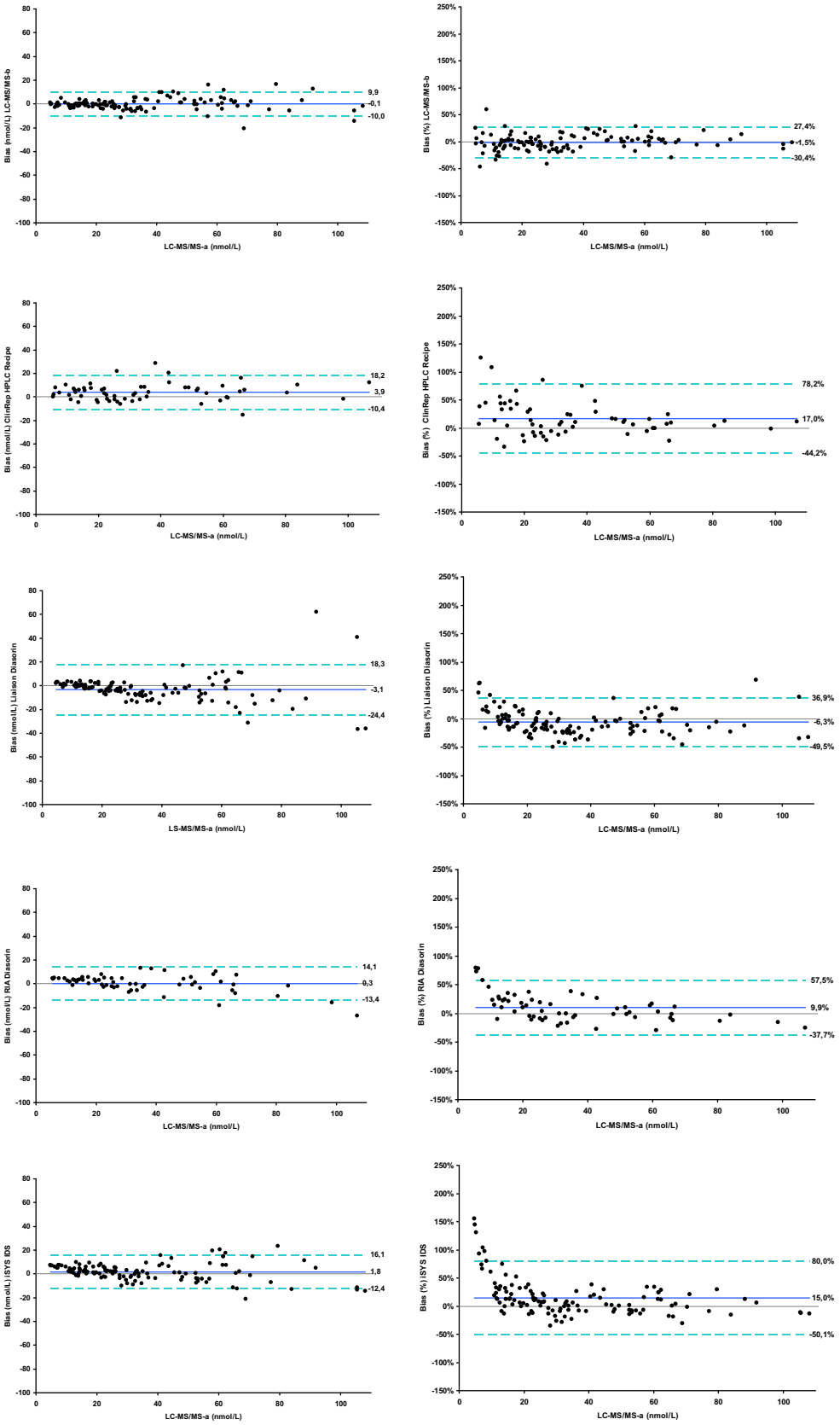

Fig. 1. Difference plots of nine assays to measure serum 25(OH)D against ID–LC–MS/MS-a showing bias in nmol/L (left panels) and percentage (right panels). Bold lines: mean bias. Dashed lines: 2SD limits of bias.

was detected in the majority of methods. The ClinRep HPLC, iSYS and COBAS D total assays showed no statistically significant bias, albeit the coefficient of correlation for the COBAS D total assay ($R = 0.88$) was suboptimal. Difference plots displaying absolute

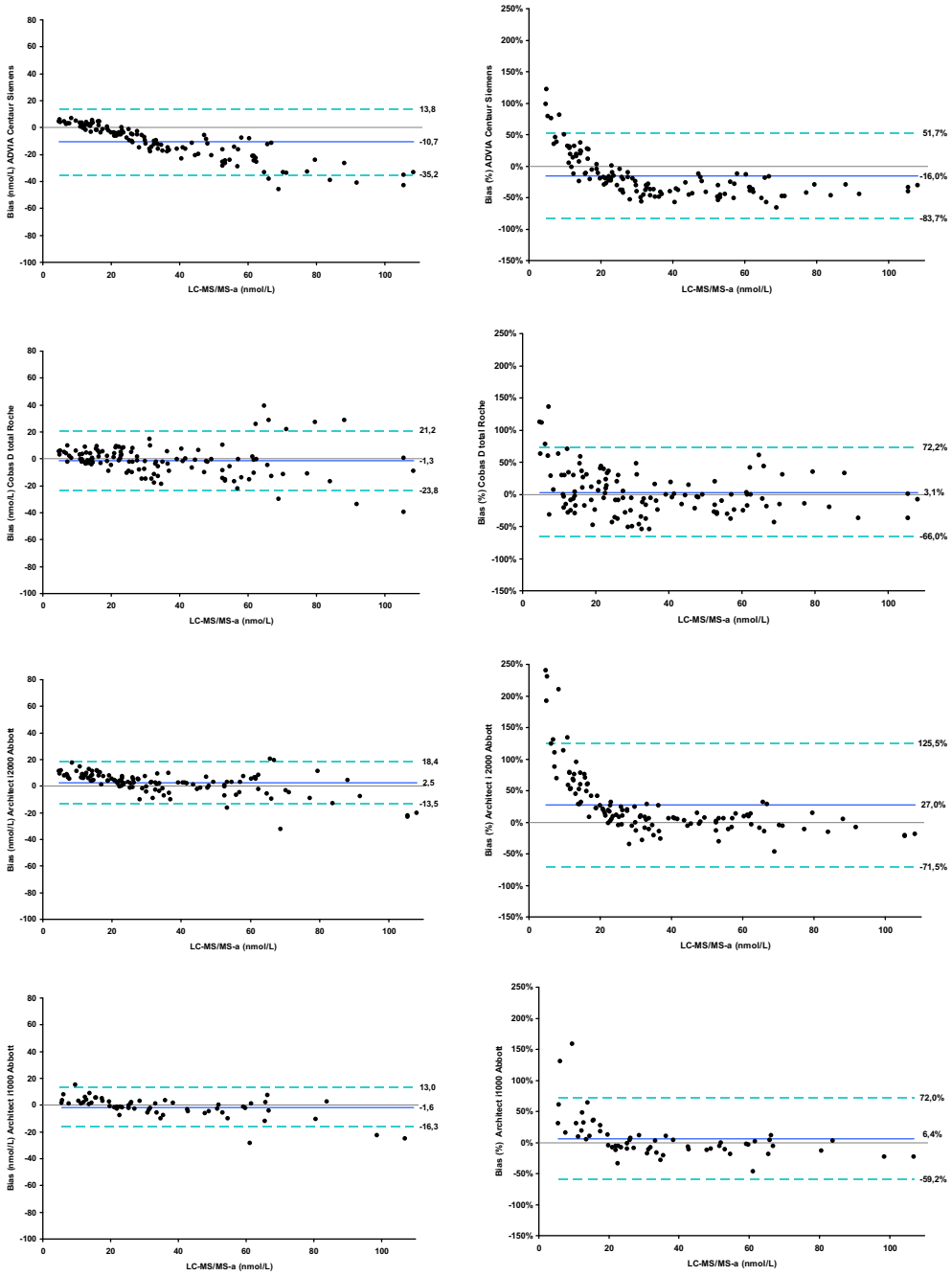

Fig. 1. (*continued*)

and relative difference against ID–LC–MS/MS-a values were applied to all evaluated samples (Fig. 1). A considerable proportional bias is demonstrable for the ADVIA Centaur assay. The Architect i2000 shows relatively high positive bias, up to 250%, at low concentration 25(OH)D. This is likely to be caused by the limited sensitivity (20 nmol/L) of the assay. The COBAS D-total assay shows increasing bias at increasing concentration of 25(OH)D. Mean absolute bias varied from −10.7 to 3.9 nmol/L, mean relative bias varied from −16% to 27% with ID–LC–MS/MS-b showing the smallest mean bias (−0.1 nmol/L; −1.5%).

There is no consensus on optimal concentrations of 25(OH)D making that recommendations differ between 50 nmol/L [13] and 75 nmol/L [14]. We have chosen to sort patient results into four categories being < 25, 25–50, 50–75 and >75 nmol/L for vitamin deficiency, insufficiency, normal or optimal levels, respectively. The proportion of patient samples falling into each category for each of the ten methods is displayed in Fig. 2a. Also shown is the percentage of patient samples for each assay that share the same category with ID–LC–MS/MS-a (overall concordance, Fig. 2b). Overall concordance varied between 53% and 88%. For the majority of methods the agreement to ID–LC–MS/MS-a deviated by no more than 1 category. Most striking is the near absence of patient samples with 25(OH)D levels >75 nmol/L in the ADVIA Centaur assay. The ADVIA Centaur assay showed the poorest overall agreement to ID–LC–MS/MS-a in sorting individual patient samples into the same category, with patient results even differing by more than 1 category in 3% of cases.

Results of the analysis of DEQAS sample 390 is listed in Table 2. Values shown are the mean of duplicate measurements. Virtually all measurements deviated at most ±1 SD from the overall method

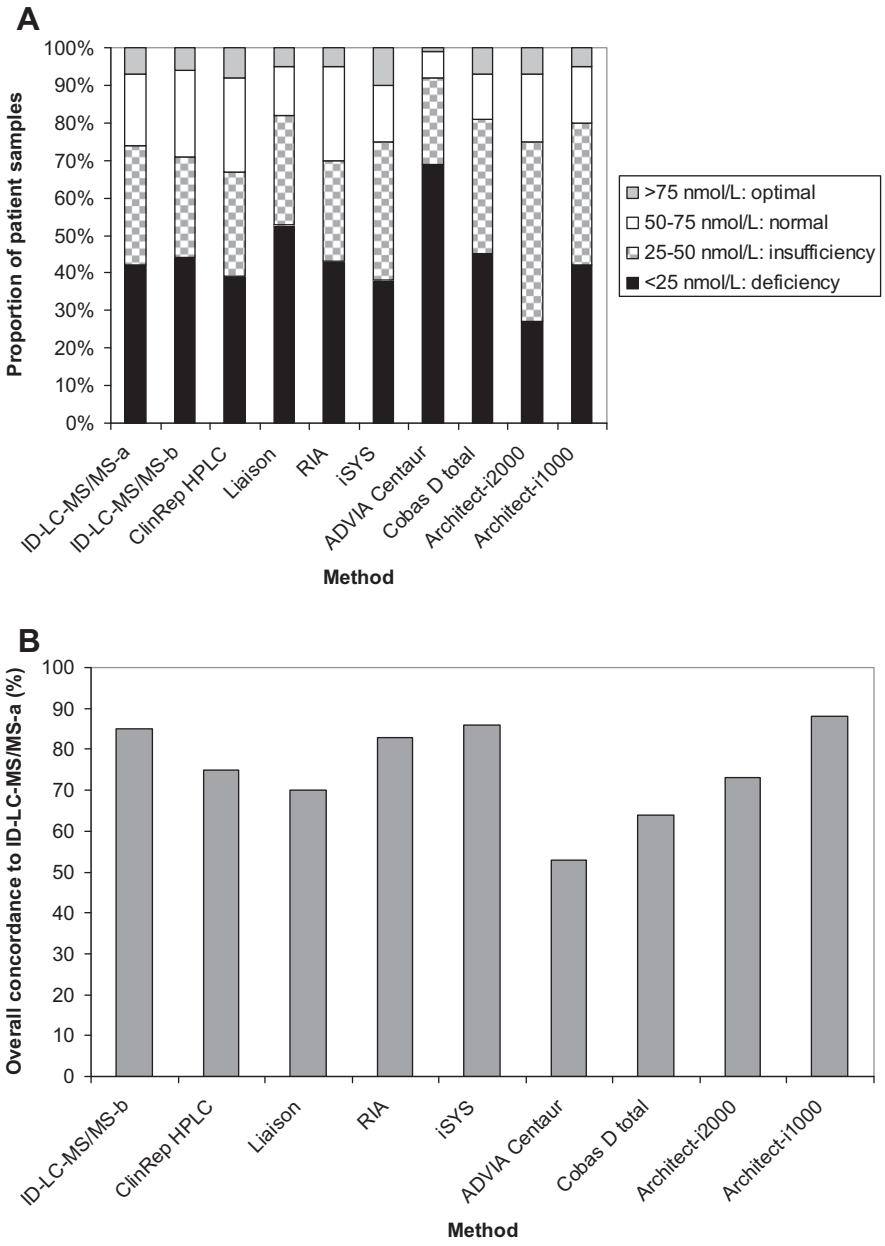

Fig. 2. (a) Proportion of patient samples (%) by 25(OH)D category for ten different assays to measure 25(OH)D. (b) Overall concordance to ID–LC–MS/MS-a category for nine assays to measure 25(OH)D.

Table 2
Measurement of 25-hydroxyvitamin D (nmol/L) in Vitamin D External Quality Assessment Scheme (DEQAS) sample 390.

Method	25(OH)D2		25(OH)D3		25(OH)D total	
	Result	DEQAS mean ± SD	Result	DEQAS mean ± SD	Result	DEQAS mean ± SD
ID–LC–MS/MS-a	44.0	40.3 ± 5.0	31.3	32.3 ± 4.0	75.3	72.6 ± 7.3
ID–LC–MS/MS-b	39.2		34.6		73.8	
ClinRep HPLC (Recipe)[a]	38.0	37.9 ± 4.5	38.0	32.0 ± 4.3	76.0	69.8 ± 8.5
Liaison (Diasorin)	–	–	–	–	45.0	50.7 ± 6.3
RIA (Diasorin) [a]	–	–	–	–	61.0	58.9 ± 12.8
iSYS (IDS)	–	–	–	–	48.3	47.4 ± 5.0
ADVIA Centaur (Siemens)	–	–	–	–	47.2	–
Cobas D total (Roche)	–	–	–	–	44.0	–
Architect-i2000 (Abbott)	–	–	–	–	45.8	–
Architect-i1000 (Abbott) [a]	–	–	–	–	42.5	–

[a] No duplicate measurements.

Table 3
Measurement of 25-hydroxyvitamin D (nmol/L) in National Insititute of Standards & Technology (NIST) Standard Reference Material (SRM) 972.

Method	Level 1		Level 2		Level 3		Level 4	
	Result	Certified value	Result	Certified value	Result	Certified value	Result	Certified value
ID–LC–MS/MS-a	<2.0 (D2) 62.6 (D3)	59.6 ± 2.1 (D3)	4.5 (D2) 28.9 (D3) 33.4 (total)	4.1 ± 0.2 (D2) 30.8 ± 1.5 (D3)	56.7 (D2) 49.3 (D3) 106.0 (total)	64.1 ± 4.8 (D2) 46.2 ± 2.8 (D3)	5.2 (D2) 207.0 (D3) 212.2 (total)	5.8 ± 0.5 (D2) 82.3 ± 2.0 (D3) 94.1 ± 2.9
ID–LC–MS/MS-b	<4.0 (D2) 65.4 (D3)		7.7 (D2) 31.3 (D3) 39.0 (total)		60.2 (D2) 47.8 (D3) 108.0 (total)		<4.0 (D2) 208.1 (D3)	(3-epi-D3)
ClinRep HPLC (Recipe) [a]	<2.0 (D2) 63.0 (D3)		<2.0 (D2) 31.0 (D3)		63.0 (D2) 50.0 (D3) 113.0 (total)		<2.0 (D2) 181.0 (D3)	
Liaison (Diasorin)	53.0		46.7		85.7		70.0	
RIA (Diasorin) [a]	60.0		43.0		92.0		75.0	
iSYS (IDS)	50.6		41.2		71.9		72.5	
ADVIA Centaur (Siemens)	n.d.		n.d.		n.d.		61.8	
Cobas D total (Roche)	57.5		24.5		67.0		124.0	
Architect-i2000 (Abbott)	50.5		36.5		60.7		64.6	
Architect-i1000 (Abbott) [a]	51.3		37.0		59.3		59.3	

n.d.: not determined.
[a] No duplicate measurements.

mean. Only one measurement was within the ±1 to 2 SD range. This means that samples were intact at time of analysis and that the quality of our assays in this study was comparable to the quality by the DEQAS participants in general. For those assays that are most recently introduced on the market (COBAS 25(OH)D total, ADVIA Centaur and Architect i2000 and i1000) there is no overall mean yet. DEQAS sample 390 contains endogenous 25(OH)D2, representing about 50% of the total 25(OH)D. All chromatographic methods fully detected 25(OH)D2 whereas all competitive binding assays report significantly lower values for total 25(OH)D, which indicates that these assays face difficulties in equimolar detection of 25(OH)D3 and 25(OH)D2.

Results from SRM 972 analysis are illustrated in Table 3. Values shown are the mean of duplicate measurements. Level 1 was prepared from human serum and has been unaltered. The chromatographic methods, RIA and COBAS D total assay reached the certified value within 10% deviation. Level 2 was prepared by diluting level 1 with horse serum to achieve a lower 25(OH)D concentration. Now, only the chromatographic methods agreed with the certified values. Level 3 contains human serum that has been fortified with 25(OH)D2. Chromatographic methods showed complete detection of 25(OH)D2 in this sample. However, the COBAS D total and immunochemical methods could only partially detect this compound. Level 4 contains human serum that has been fortified with 3-epi-25(OH)D3. This compound was completely detected by chromatographic methods and partially detected only by the COBAS D total assay.

4. Discussion

Since the principle of isotope dilution with stable-isotope-labeled internal standard compound, with its physicochemical behavior identical to that of the native analyte, and mass spectrometry detection yields the highest attainable analytical accuracy, this methodology has been selected as the comparative method. Both ID–LC–MS/MS methods agreed well in our study using randomly selected outpatient samples ($R = 0.98$). Except for ClinRep HPLC, iSYS [15] and COBAS D total all evaluated methods showed a variable systematic bias in measured 25(OH)D values when compared with ID–LC–MS/MS-a. This variable systematic bias limits the use of certain medical decision points for 25(OH)D as has been shown in Fig. 2. A large bias has been observed for the ADVIA Centaur assay, which may lead to unjustified treatment for vitamin D deficiency or insufficiency in a significant number of patients when using this assay. The performance of the ADVIA Centaur assay is unacceptable and this assay should be re-adjusted before clinical use.

We evaluated 25(OH)D2 cross-reactivity in competitive binding methods using SRM and DEQAS material, as no significant 25(OH)D2 concentrations were present in the patient cohort. Using the SRM 972 level 3, the RIA and Liaison assays showed the highest, but not complete, cross-reactivity with 25(OH)D2 followed by the iSYS and COBAS D-total assays. Exogenous 25(OH)D2, 25(OH)D3 or serum from other species added to human samples as in the SRM 972, however, has been suggested to be inappropriate in determining the analytical recovery of vitamin D compounds when using binding assays [16,17]. Therefore we also examined the cross-reactivity with 25(OH)D2 using the DEQAS 390 sample containing endogenous 25(OH)D2. Still, the levels of cross-reactivity using the DEQAS 390 sample are more or less comparable with those using the SRM 972 level 3 sample. Nevertheless, a significant limitation of this study is that no light could be shed onto how the various assays perform with real patient samples that contain both 25(OH)D2 and 25(OH)D3 as opposed to the reference standard material and DEQAS material.

It is worth noting that the COBAS D-total assay is the only assay employing human DBP as a binding agent, which in theory makes this protein binding assay more susceptible to matrix effects and potential cross-reactivity with other vitamin D metabolites such as 24,25-hydroxyvitamin D, 25(OH)D3 sulfate or 3-epi-25(OH)D3, than the antibody-based assays i.e. immunoassays. Uniquely different in comparison to all other evaluated methods is that the COBAS D-total assay shows increasing random bias at increasing concentration of 25(OH)D, which is most likely related to this design of competitive protein binding. As a consequence, the COBAS D-total assay has a relatively low coefficient of correlation. Moreover, the COBAS D-total assay appears the only binding assay that, partially, cross-reacts with 3-epi-25(OH)D.

A limitation of our study is that, due to the collection of samples in winter, relatively few patients displayed normal (50–75 nmol/L) to optimal (>75 nmol/L) 25(OH)D concentrations.

In conclusion, significant bias exists between ID–LC–MS/MS and many, but not all, other 25(OH)D assays tested in this study. Protein binding and immunochemical methods do not show equimolar detection of 25(OH)D2. 3-epi-25(OH)D3 is completely detected by chromatographic methods and partially recognized only by the COBAS D total assay. The variable response among different assays

for 25OHD impedes the use of uniform cut-off values for defining ones vitamin D status. Major effort is needed towards further standardizing assays for 25(OH)D measurement.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2012.07.013.

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