Clin. Lab. 2013;59:XXX-XXX

ORIGINAL ARTICLE

Four Automated 25-OH Total Vitamin D Immunoassays and Commercial Liquid Chromatography Tandem-Mass Spectrometry in Finnish Population

MARJA-KAISA KOIVULA [1], NINA MATINLASSI [2], PÄIVI LAITINEN [3], JUHA RISTELI [4]

[1] *Institute of Diagnostics, Department of Clinical Chemistry University of Oulu and Laboratory, Oulu University Hospital, Oulu, Finland*
[2] *Laboratory, Oulu University Hospital, Oulu, Finland*
[3] *Department of Obstetrics and Gynecology, University of Oulu and Department of Clinical Chemistry, Helsinki University Central Hospital, Helsinki, and Central Ostrobothnia Hospital District, Kokkola, Finland*
[4] *Institute of Diagnostics, Department of Clinical Chemistry, University of Oulu, Finland*

SUMMARY

Background: **Comparing four fully automated 25-OH-D immunoassays to a commercially available liquid chromatography-tandem mass spectrometry (LC-MS/MS) method with human serum samples from Finnish population.**
Methods: **400 samples were analyzed with the Liaison® Total Vitamin D, the IDS-iSYS 25-Hydroxy Vitamin D, the ARCHITECT 25-OH Vitamin D, the ADVIA Centaur® Vitamin D Total, and a commercially available LC-MS/MS 25-OH D (PerkinElmer) method.**
Results: **The Liaison® method mean value (95% confidence intervals) was 65.6 nmol/L (62.6 - 68.6); the IDS-iSYS mean was 70.3 nmol/L (67.4 - 73.1); the ARCHITECT mean was 69.0 nmol/L (65.5 - 72.5); ADVIA Centaur® mean was 71.6 nmol/L (68.9 - 74.3), and the LC-MS/MS mean was 82.8 nmol/L (79.4 - 86.2). The regression coefficients (r) between the LC-MS/MS and immunoassays were 0.650 for Liaison®, 0.757 for IDS-iSYS, 0.721 for ARCHITECT and 0.684 for ADVIA Centaur®. With the Passing-Bablok analysis, none of the immunoassays gave results equivalent to LC-MS/MS. Two of the four automated 25-OH-vitamin D assays (IDS-iSYS, ADVIA Centaur®) were overall in good clinical agreement with LC-MS/MS, even though the results obtained with all compared methods were not equivalent.**
Conclusions: **In conclusion, in routine clinical laboratory both immunoassays and LC-MS/MS are useful for measuring 25-OH-vitamin D provided that these methods are correctly standardized and especially sample pretreatment is carefully performed.**
(Clin. Lab. 2013;59:xx-xx. DOI: 10.7754/Clin.Lab.2012.120527)

KEY WORDS

ADVIA Centaur, Architect, IDS-iSYS, immunoassays, LC-MS/ MS, Liaison, PerkinElmer, 25-OH-D, 25-OH-vitamin D

LIST OF ABBREVIATIONS

25-OH-D - 25-hydroxyvitamin D
25-OH-D_3 - 25-hydroxyvitamin D_3
25-OH-D_2 - 25-hydroxyvitamin D_2
HPLC - high pressure liquid chromatography
LC-MS/MS - liquid chromatography-tandem mass spectrometry
HPLC-UV - high pressure liquid chromatography with ultraviolet detector
VDBP - vitamin D binding protein
RLU - relative light unit
IS - internal standards
RT - room temperature
PTAD - 4-Phenyl-1,2,4-triazoline - 3,5-dione
MRM - multiple reaction monitoring
RMP - reference measurement procedures
CV - coefficient of variation

Manuscript accepted July 11, 2012

JCTLM - Joint Committee for Traceability in Laboratory Medicine
CI - confidence interval
SD - standard deviation
r - Pearson correlation coefficient
C - contingency coefficient
NIST - National Institute of Standard and Technology
SRM - standard reference material

INTRODUCTION

Circulating 25-hydroxyvitamin D (25-OH-D) is widely recognized as the best indicator of vitamin D status. In blood circulation the two major vitamin D metabolites are 25-hydroxyvitamin D_3 (25-OH-D_3) and 25-hydroxyvitamin D_2 (25-OH-D_2). 25-OH-D_3 is mainly derived from vitamin D_3 produced by sunlight in the skin, while 25-OH-D_2 is derived from plants in the diet. [1-4] There are currently two main types of methods used routinely for measuring 25-OH-D: methods based on chromatographic separation followed by non-immunological direct detection and competitive immunoassays. Most immunoassays depend on an antibody that can detect both 25-OH-D_2 and 25-OH-D_3, but the proportion of 25-OH-D_2 detection is variable [1-5]. Two non-immunological methods for direct detection are currently available: high pressure liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [1]. The HPLC is usually connected to an ultraviolet detector (HPLC-UV) or an electrochemical detector [2]. If HPLC is attached to mass detectors, the procedure is commonly termed LC-MS/MS or tandem mass spectrometry. Initial purification is required prior to chromatographic separation [1,3]. LC-MS/MS is considered a reference method [4,6] although for LC-MS/MS there is lack of consistency in the way these procedures are standardized [2,7]. The advantages of LC-MS/MS are the reliability, low batch-to-batch variation and low limit of detection (2.5 nmol/L) [3]. Additionally, LC-MS/MS methods are able to measure 25-OH-D_2 and 25-OH-D_3 independently with good recoveries [1-4]. The major disadvantage is the time-consuming and laborious extraction procedure that limits the number of samples that can be processed per day. However, several laboratories have now automated the sample pretreatment process, so the low throughput will not be a problem in the future [4]. Currently, LC-MS/MS methods are usually in-house assays. Recently, PerkinElmer launched the LC-MS/MS 25-OH-vitamin D kit.
Current commercial 25-OH-D immunoassays are delivered as kits. The first automated immunoassay was Nichols Advantage chemiluminescent 25-OH-vitamin D. At the end of 2005, Nichols withdrew the assay. Diasorin introduced a chemiluminescent immunoassay for the Liaison® automated platform in 2004; this assay was updated and replaced by Diasorin to improve sensitivity and precision, and renamed Liaison® Total in 2007. A commercial direct automated immunoassay specific for 25-OH-D_3 was introduced for use on Roche Diagnostics immunoassay analyzers in 2008 but the assay was withdrawn from the market due to its poor performance. IDS plc released a fully automated 25-OH-total-D assay on iSYS early in 2009. The assay received 510(k) clearances from the US Food and Drug Administration (FDA) in 2010. Abbott released a new diagnostic test, ARCHITECT 25-OH Vitamin D assay, to measure levels of 25-OH-vitamin D using Abbott's ARCHITECT automated instrument system in 2011. Additionally, in 2011 Siemens launched a new 25-OH-vitamin D Total assay for use with ADVIA Centaur® immunoanalyzer and in October 2011 the FDA cleared the Siemens Vitamin D Total assay. Currently automated immunoassays are the most popular methods, even though there is little information available on how commercial immunoassays are standardized [4]. However, there has been an increase in the use of LC-MS/MS [1].
The aim of this study is to compare four fully automated 25-OH-D immunoassays (Liaison® Total Vitamin D, IDS-iSYS 25-Hydroxy Vitamin D, ARCHITECT 25-OH Vitamin D, and ADVIA Centaur® Vitamin D Total) with a commercially available liquid chromatography-tandem mass spectrometry (LC-MS/MS; PerkinElmer) method in human serum samples from Finnish population.

MATERIALS AND METHODS

Serum samples

The leftover de-identified serum samples (n = 400, 263 females and 137 males) were randomly chosen from routine samples analyzed in the laboratory of Oulu University Hospital. The mean age of patients was 54.1 years (interval 0.8 - 100.8 years); that of females was 56.5 years (interval 0.8 - 100.8 years) and of males 49.4 years (interval 1.0 - 89.3 years). The number of samples from children (age < 16 years) was 21 : 14 girls (interval 0.8 - 15.9 years) and 7 boys (interval 1.0 - 15.7). All measurements were performed by single assays. Samples were separated within two hours after collection and analyzed first by Liaison® 25 OH Vitamin D TOTAL assay. After the analysis, the samples were divided into four aliquots and frozen (at -20°C) for further analysis because the analyzers used in this study were located in different hospitals. Before analysis by the ADVIA Centaur®, the samples were stored again at -20°C before re-analysis.

Liaison® 25OH Vitamin D TOTAL

The Liaison® 25OH Vitamin D TOTAL (DiaSorin, Stillwater, MN, USA) antibody-based 25-OH-vitamin D assay is a fully automated, random-access direct competitive two-step chemiluminescent system. During the first incubation, 25-OH-D is dissociated from its binding protein and binds to the specific antibody on the solid phase. After 10 minutes, the tracer (25-OH-vitamin D linked to an isoluminol derivate) is added. After a sec-

ond 10-minute incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added to initiate a chemiluminescent reaction.

IDS-iSYS 25-Hydroxy Vitamin D

The fully automated IDS-iSYS (IDS plc, Boldon, UK) 25-Hydroxy Vitamin D assay is intended for the quantitative determination of 25-OH-D and other hydroxylated metabolites. The assay is based on chemiluminescence technology. Samples are subjected to a pretreatment step to denature the 25-OH-vitamin D binding protein (VDBP). The treated samples are then neutralized in an assay buffer and a specific anti-25-OH-D antibody labeled with acridinium ester is injected. Following an incubation step, paramagnetic particles linked to 25-OH-D are added. After a washing step and addition of trigger reagents, the light emitted by the acridinium label is inversely proportional to the concentration of 25-OH-D in the original sample.

ARCHITECT 25-OH Vitamin D

The ARCHITECT 25-OH Vitamin D assay (Abbott Laboratories, Abbott Park, Illinois, US) is a delayed one-step immunoassay with six-point calibration. The method is used for the quantitative measurement of 25-OH-vitamin D in human serum and plasma samples. CMIA technology, referred to as Chemiflex with flexible assay protocol, is used in the measurement. In the first step the sample is pre-treated to dissociate the vitamin from its binding sites. An aliquot of the pre-treated sample then reacts with paramagnetic anti-25-OH-vitamin D coated microparticles. After incubation, a biotinylated 25-OH-vitamin D anti-Biotin acridinium-labeled conjugate complex is added. After washing, pre-trigger and trigger solutions are added to produce a chemiluminescent reaction and relative light units (RLUs) are measured. An indirect relationship exists between the amount of 25-OH-vitamin D in the sample and the RLUs detected.

ADVIA Centaur®

The ADVIA Centaur® Vitamin D Total assay (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) is a one-pass antibody competitive immunoassay. First, 25-OH-D is dissociated from its binding protein. Free 25-OH-D binds to the anti-VitD (mouse monoclonal) antibody labeled with acridinium ester. The anti-fluorescein (mouse monoclonal) coated paramagnetic particles (solid phase) and the 25-OH-vitamin D-analog conjugated to fluorescein are added and incubated. The unbound material is removed with a wash cycle. The starter reagents are added to initiate the chemiluminescent reaction.

Details of the automated immunoassays used in this study are presented in Table 1.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) 25-OH D

The CE marked LC-MS/MS method (MSMS VitD Kit from PerkinElmer/Wallac, Turku, Finland) was used for the measurement of 25-OH-D_2 and 25-OH-D_3. The kit contains deuterium-labeled internal standards (IS, $^2H_3$-25-OH-D_2 and $^2H_3$-25-OH-D_3). The calibrators consist of charcoal stripped human serum enriched with six levels (in the range of 4.7 - 150 ng/mL) of $^2H_6$-25-OH-D_2 and $^2H_6$-25-OH-D_3. The kit also includes three quality control levels of $^2H_6$-25-OH-D_2 and $^2H_6$-25-OH-D_3 in charcoal stripped human serum. The derivatization reagent used is PTAD (4-Phenyl-1,2,4-triazoline-3,5-dione).

The samples were prepared using a protein precipitation protocol. 100 µL of sample (serum, plasma, calibrator or control) was transferred into a 96-well plate (500 µL NUNC plate included in the kit) before the addition of 200 µL of a solution (acetonitrile with 0.1% formic acid) containing deuterium-labeled IS. The plate was covered and shaken at 750 rpm at room temperature (RT) for 10 minutes and the samples were centrifuged at 4210 RCF, at +4 to +30°C for 30 minutes. 150 µL of the supernatant obtained was transferred into another plate and dried under a gentle flow of nitrogen gas. 50 µL of PTAD reagent was added to the residue contained in each well, after which the plate was shaken at 750 rpm at RT for 10 minutes. Finally, 50 µL of quench solution was added to the plate, shaken again (at 750 rpm at RT for 10 minutes) and then loaded onto the auto-sampler (at +4 to 25°C) for the LC-MS/MS analysis.

The HPLC-MS/MS system consisted of a Waters 1525u binary HPLC system coupled to a Waters TQD triple quadrupole mass spectrometry (Waters Corp., Manchester, UK). The instrument was operated in a positive electrospray ionization mode. For HPLC analysis the Waters XBridge C8 3.5 µm 2.1 x 100 mm column was used. The mobile phase solvents were Mobile Phase A: 50% (v/v) solution of methanol in water containing 0.025% (v/v) of HPLC solvent additive and Mobile Phase B: methanol containing 0.025% (v/v) of HPLC solvent additive. Gradient composition started from 50% B and it was maintained for 1.0 minute. The mobile phase gradient profile involved two steps: increasing from initial conditions to 75% B within 5 minutes and then to 100% B within 0.1 minute holding for 2 minutes before returning to the initial state at 8.1 minutes, allowing 1 minute for column re-equilibration. The run time was 9 minutes. Flow rate was 300 µL/minute and 50 µL of sample was injected. The column temperature was set at 40°C. The MS-MS analysis was done in multiple reaction monitoring (MRM) mode following the ion transitions with appropriate instrumental parameters as described in table 2. The capillary voltage was 3.5 KV, source temperature was 100°C, desolvation gas flow 100 L/h at 450°C and collision gas argon kept at a pressure of 1.97 x 10^{-3}. Data were acquired with Mass Lynx 4.1 Software (Waters) and processed for calibra-

Table 1. Introducing the automated 25-OH-total-D immunoassays used in this study. All data of coefficient of variations (CVs) were generated by the authors.

Item	Liaison® 25OH Vitamin D Total	IDS-iSYS 25-Hydroxy Vitamin D	ARCHITECT 25-OH Vitamin D	Advia Centaur® Vitamin D Total
Sample type	Serum or plasma (EDTA, lithium-heparin)	Serum or plasma (EDTA, lithium-heparin, sodium-heparin, ammonium-heparin, sodium citrate)	Serum or plasma (EDTA, lithium-heparin, sodium-heparin)	Serum or plasma (EDTA, lithium-heparin, sodium-heparin)
Sample volume (µL)	25	10	10	20
Assay range (nmol/L)	10 - 375	12.5 - 350	20 - 400	9.3 - 375
LoB (nmol/L)	< 3	4.5	≤ 10	4.0
LoD (nmol/L)	NR	9.0	≤ 25	8.0
LoQ (nmol/L)	< 10	13.75	≤ 50	8.8
25-OH-D_2 cross-reactivity (%)	104	≥ 70	52	104.5
25-OH-D_3 cross-reactivity (%)	100	100	105	100.7
Time for the first result (minutes)	35	38	36	18
Throughput (tests/hour)	100	92	100	240
Between-day CVs for low control (%)	10.2 - 11.5 45 nmol/L	6.2 - 16.9 15 nmol/L	3.8 - 4.6 48 nmol/L	6.7 53 nmol/L
Between-day CVs for high control (%)	5.2 - 8.1 150 nmol/L	5.4 - 8.9 187.5 nmol/L	2.8 195 nmol/L	4.3 227.8 nmol/L

LoB = the limit of blank, LoD = the limit of detection, LoQ = the limit of quantification, CV = coefficient of variation, NR = not reported.

Table 2. MRM transitions for unlabelled and labelled analytes and their mass parameters in the LC-MS/MS method used in this study.

Analyte	Transition	Cone (V)	Collision Energy (eV)	Dwell Time (sec)
25(OH)D_2	619.4 → 298.1	25	20	0.100
25(OH)D_3	607.4 → 298.1	25	20	0.100
IS $^2H_3$-25(OH)D_2	622.4 → 301.1	25	20	0.100
IS $^2H_3$-25(OH)D_3	610.4 → 301.1	25	20	0.100
Calibrators and controls $^2H_6$-25-OH-D_2	625.4 → 298.1	25	20	0.100
Calibrators and controls $^2H_6$-25-OH-D_3	613.4 → 298.1	25	20	0.100

tion and for the quantitation of the analytes with Quan Lynx Software (Waters). The PerkinElmer MS/MS Vitamin D Derivatized Assay (used for this study) was successfully compared with the ID-LC-MS/MS Reference Measurement Procedures (RMP). This SI-traceable RMP based on ID-LC-MS/MS, for the quantification of 25-OH-D_2 and 25-OH-D_3 in serum, was recently developed by Stepman et al. from the University of Ghent, Belgium [8]. This method was nominated for listing (list I - Reference Measurement Procedures) in

Table 3. A) The mean and the 95% confidence intervals (CI) of assays compared this study in all samples. All the results are total vitamin D (the sum of 25-OH-D_3 and 25-OH-D_2). B) Pearson's linear correlation coefficients (r) and Passing-Bablok regressions of the 25-OH-total-D measured by immunoassays and LC-MS/MS in all samples.

A

	n	Mean (nmol/L)	95% CI (nmol/L)	SD (nmol/L)
Liaison®	400	65.6	62.6 to 68.6	30.9
IDS-iSYS	400	70.3	67.4 to 73.1	28.8
ARCHITECT	399	69.0	65.5 to 72.5	35.3
ADVIA Centaur®	400	71.6	68.9 to 74.3	27.6
LC-MS/MS-total-D	400	82.8	79.4 to 86.2	34.4

SD = standard deviation

B

			Passing-Bablok regressions			
	n	r	Slope	95% CI	Intercept	95% CI
Liaison®	400	0.650	0.88	0.80 to 0.95	-7.22	-13.27 to -2.66
IDS-iSYS	400	0.757	0.84	0.79 to 0.91	0.72	-3.15 to 4.55
ARCHITECT	399	0.721	1.00	0.93 to 1.08	-12.69	-17.64 to -7.76
ADVIA Centaur®	400	0.684	0.80	0.74 to 0.86	4.50	0.80 to 9.42

CI = confidence interval, All correlations were significant between methods ($p < 0.0001$).

Table 4. Clinically false negative and positive results. When the reference method (LC-MS/MS-total-D) gave clinically positive results (over the Finnish reference value ≥ 40 nmol/L) but the immunoassays gave clinically negative results (< 40 nmol/L), the results were encoded as false negatives. Results encoded as false positives were cases, when the reference method gave clinically negative results (< 40 nmol/L) but the immunoassays gave clinically positive results (≥ 40 nmol/L).

Immunoassay	False positive (n)	False negative (n)	C (0 ≤ C ≤1)
Liaison®	5	61	0.358
IDS-iSYS	3	25	0.522
ARCHITECT	5	59	0.364
ADVIA Centaur®	8	22	0.452

C= contingency coefficient.

listing (list I - Reference Measurement Procedures) in the database of the Joint Committee for Traceability in Laboratory Medicine (JCTLM).

Statistical analysis

Results of the assay methods were first presented by box plot (reported as mean 95% confidence interval (CI) and standard deviation (SD)) and compared by Pearson correlation coefficient (r). However, in the case of method-to-method comparison, unbiased Passing-Bablok regressions are suitable. Therefore Passing-Bablok fits were used to calculate slopes and intercepts between the analysis methods. If two methods are compared by Passing-Bablok fit and they give similar results, the 95% CI of slope should include value 1 and the 95% CI of intercept value 0 [9]. Box plots (with 95% CI and SD) and Passing-Bablok regressions were performed by Analyse-it for Microsoft Excel (Analyse-It Software Ltd, UK). The clinical values from the assays performed were compared by Pearson Chi-Squared test. Statistical significance was tested using the contingency coefficient (C, with $0 \leq C \leq 1$). For Pearson Chi-Squared test, the results of measurements were categorized as positive or negative according to Finnish (25-OH-total-D) reference ranges (positive ≥ 40 nmol/L and negative < 40 nmol/L). The Pearson Chi-Squared test was performed using IBM SPSS Statistics 19 (IBM, NY, USA).

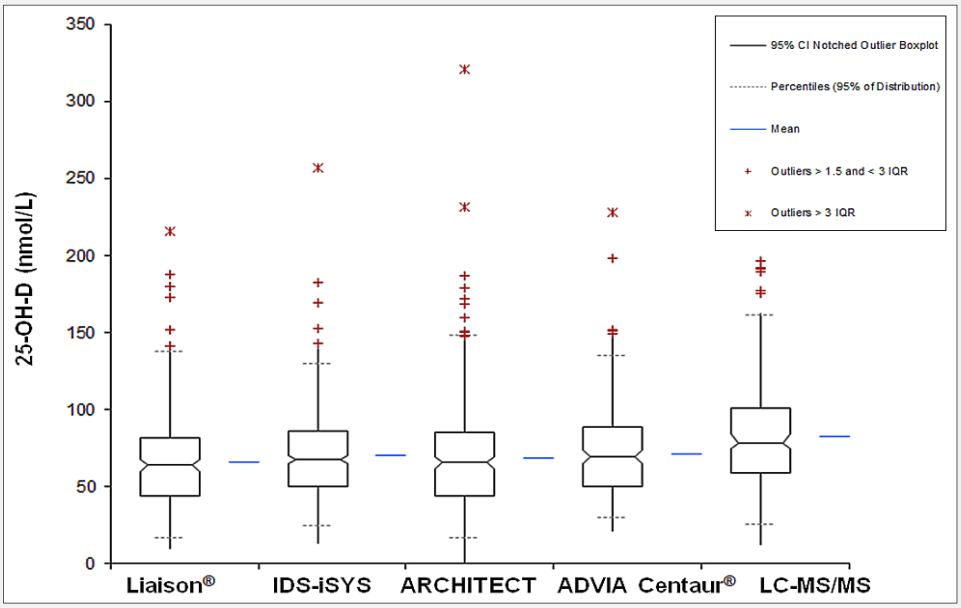
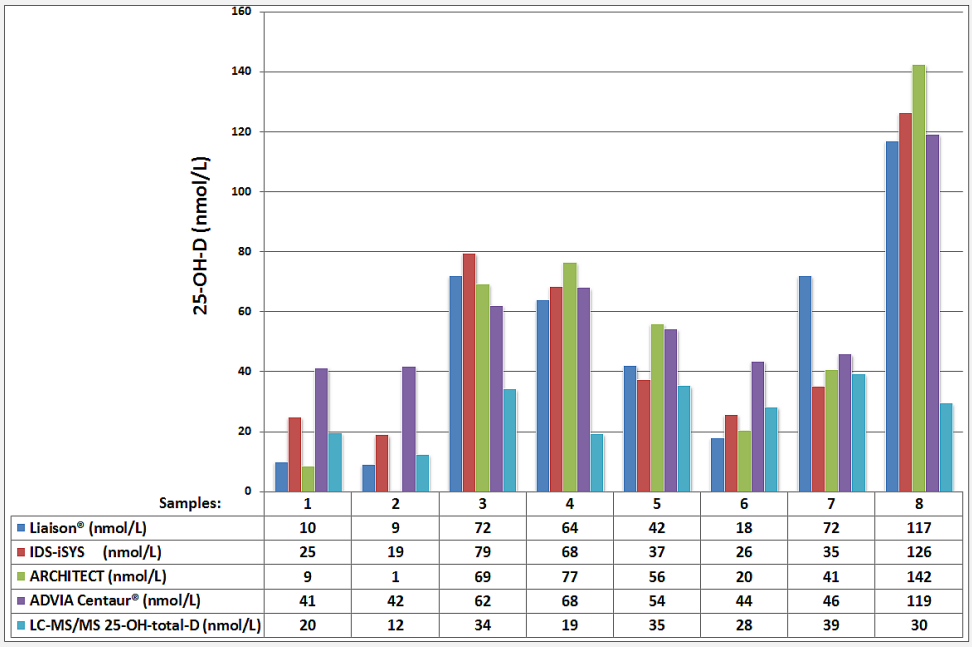


Figure 1. Boxplots of the mean and the 95% confidence intervals (CI) from serum total vitamin D (the sum of 25-OH-D_3 and 25-OH-D_2) results by the automated immunoassays and LC-MS/MS used in this study. The interquartile interval was abbreviated IQR.



Samples:	1	2	3	4	5	6	7	8
Liaison® (nmol/L)	10	9	72	64	42	18	72	117
IDS-iSYS (nmol/L)	25	19	79	68	37	26	35	126
ARCHITECT (nmol/L)	9	1	69	77	56	20	41	142
ADVIA Centaur® (nmol/L)	41	42	62	68	54	44	46	119
LC-MS/MS 25-OH-total-D (nmol/L)	20	12	34	19	35	28	39	30

Figure 2. False positive samples, when the results of the reference assay (LC-MS/MS-total-D) were less than 40 nmol/L but one or more of the immunoassays gave greater than or equal to 40 nmol/L.

RESULTS

The mean and 95% CI intervals of the assays compared in this study are presented in Figure 1 and in Table 3A. Furthermore, the LC-MS/MS mean of 25-OH-D_3 was 79.3 (75.9 to 82.7) nmol/L while the mean of LC-MS/MS 25-OH-D_2 was 3.5 (3.0 to 4.0) nmol/L.

Comparisons of four immunoassays to LC-MS/MS

Pearson's linear r between immunoassays and LC-MS/MS-total-D are presented in Table 3B. The low levels of 25-OH-D_2 in the samples could not contribute to the differences between the immunoassays and the LC-MS/MS method since Pearson's linear r values between the immunoassays and LC-MS/MS 25-OH-D_3 were similar (data not shown) as presented in Table 3B. All linear correlations were significant at the 0.0001 level but individual samples may have significant differences between the methods; linear correlation may thus not be the best statistical test.

In this study, regarding Passing-Bablok regression fits, none of the immunoassays gave equivalent results to LC-MS/MS (Table 3B). Under the Passing-Bablok regression analysis, these automated 25-OH-D assays did not yield similar results since the correlation slopes were not equaled to 1 and/or the intercepts were not value 0.

The results of measurements were categorized as positive or negative according to Finnish reference ranges (positive ≥ 40 nmol/L and negative < 40 nmol/L). When the reference method (LC-MS/MS) gave clinically positive results but immunoassays gave clinically negative results, the results were encoded as false negatives. Results encoded as false positives were cases where the reference method gave clinically negative results but immunoassays gave clinically positive results. False negative and positive results and the statistical significance of Pearson's Chi-Squared test are shown in Table 4. There were three patients (3, 4, and 8) who had a clinically negative result (25-OH-D < 40 nmol/L) measured by the commercial LC-MS/MS method but a positive result by all immunoassays (Figure 2). These samples were reanalyzed by another LC-MS/MS method [10]. The results of this in-house LC-MS/MS method were similar to all of the immunoassays (sample 3: 87 nmol/L, sample 4: 73 nmol/L, sample 8: 126 nmol/L). At the same time, there were patients who had a clinically positive result (25-OH-D ≥ 40 nmol/L) measured by the reference method but a negative result by all immunoassays (n = 10).

DISCUSSION

This study compared the performance of four fully automated 25-OH-D immunoassays and a commercially available LC-MS/MS assay for 25-OH-D concentration at four laboratories using 400 continuously collected samples from the Laboratory of Oulu University Hospital. It is recommended that any 25-OH-D assay in clinical laboratories should measure both 25-OH-D_2 and 25-OH-D_3 equally in order to report a total 25-OH-D value [11,12]. The evaluated immunoassays measure total 25-OH-D (both 25-OH-D_3 and 25-OH-D_2), but the cross-reaction with 25-OH-D_2 and 25-OH-D_3 differs from 52 to 100% in different methods (see Table 1). On the other hand, their major weakness is the inability to quantitate 25-OH-D_2 and 25-OH-D_3 separately when these commercial immunoassays measure total 25-OH-vitamin D concentrations.

It is shown here once again that 25-OH-D is a difficult analyte [13,14]. There are two main reasons for this. There are several different molecular forms of 25-OH-vitamin D in the serum [1]. These have a hydrophobic nature and will bind tightly to VDBP. Before analyzing 25-OH-vitamin D with any method, pretreatment of the sample is necessary. Pretreatment can include deproteinization or extraction, purification and finally quantification [1]. However, the details of the denaturing agents used in the immunoassays are not available, because they are regarded as commercial secrets. These matrix effects can lead to modest inter-laboratory variability in immunoassays [5]. However, the earlier 25-OH-vitamin D assays based on binding proteins were more susceptible to matrix effect than immunoassays that use antibodies for a sandwich assay [13]. In some individual patient sera, matrix effects distorting the displacement of 25-OH-D from its binding protein may cause great inter-method variability [15]. A second reason introducing variability is the standardization of the assay. Indeed, there is a need for a common international standard for the calibration of 25-OH-D assays. The National Institute of Standard and Technology (NIST) has developed standard reference material (SRM) for 25-OH-vitamin D analysis [1,2,4]. Some companies report using this SRM in the calibration of their assays. Regardless of the SRM calibration of the assays there is still discrepancy between the methods. When analyzing the samples in this study harmonization of the immunoassays had already been performed as suggested [16]. Recently, Holmes et al. [17] reported non-linear analytical recovery in the Liaison 25-OH-D immunoassay. The calibration of ADVIA Centaur® Vitamin D Total was also adjusted in low concentration (50 nmol/L) [18]. Abbott restandardized the calibrators and controls of the ARCHITECT 25-OH Vitamin D following our study [19].

LC-MS/MS is proposed as the reference method for 25-OH-vitamin D measurement. In this study, regarding Passing-Bablok regression fits, none of the immunoassays gave equivalent results to LC-MS/MS. Potential explanations may be sample treatment or a technical aspect of chromatography or mass spectrometry. We found that there were some problems with this commercial LC-MS/MS method (Figure 2). LC-MS/MS and HPLC release the 25-OH-D from its binding protein followed by protein precipitation before chromatography and final quantification [12]. Chromatographic

methods are less susceptible to sample matrix effects than immunoassays [7]. Chromatography has two areas of concern where analytical errors can occur. Firstly, the presence of interfering compounds is problematic when they elute at the same time as the analyte being measured. Secondly, the calibration issue noted above; the recent availability of NIST ethanol-based standards should resolve calibration problems and allow laboratories to trace their calibrators to the NIST material [5]. The measurement of 25-OH-D is technically demanding due to its strong binding to protein [15]. However, in LC-MS/MS ion suppression can reduce the performance of the mass detector. Ion suppression can be caused by non-volatile compounds [1,3].

In Finland, vitamin D deficiency is defined as a serum 25-OH-D level lower than the reference range (40 nmol/L). In this study, we found patients who had a clinically negative result measured by the LC-MS/MS method manufactured by PerkinElmer but positive results by all immunoassays. Three of these samples were reanalyzed using another LC-MS/MS method, and we found that the commercially available LC-MS/MS method gave too low concentrations at least in these three samples. Since this method was correctly standardized the problem should be the pretreatment of the samples before analysis. Additionally, in this study some patients had a clinically positive result measured by the reference method (LC/MS-MS) but negative results by all immunoassays.

There is continuous debate as to which is the best method of 25-OH-D measurement for routine clinical laboratory and research use. It is clear that both immunoassays and LC-MS/MS methods have advantages and limitations. The strengths of the measurement of 25-OH-D by immunoassays are convenience, speed, turnaround, and cost [12,20]. On the other hand, LC-MS/MS is a rapid, accurate, sensitive, and cost-effective alternative to other methods, its real advantage being the ability to report both 25-OH-D_3 and 25-OH-D_2 but reporting 25-OH-D_3 and 25-OH-D_2 separately may cause confusion among clinicians [4,15,21]. Two (the IDS-iSYS, the ADVIA Centaur®) out of four automated 25-OH-D assays were better than the others when compared with the commercial LC-MS/MS. In conclusion, in routine clinical laboratory analyses both immunoassays and LC-MS/MS are useful for measuring 25-OH-vitamin D, provided that these methods are correctly standardized and especially sample pretreatment is carefully done.

Acknowledgement:
The authors gratefully acknowledge the expert technical assistance with Liaison® and the collecting samples for this study at the Hormone Laboratory of Oulu University Hospital. They also thank Ms. Katja Koukkula (laboratory engineer) as well as the technical assistance by the staff of the laboratory of Central Ostrobothnia Central Hospital. This study was supported in part by Academy of Finland (MKK, JR).

Funding:
This research was funded in part by the Academy of Finland (MKK, JR), University of Oulu and Oulu University Hospital (MKK, NM, PL, JR), Helsinki University Central Hospital, and Central Ostrobothnia Hospital District, Kokkola (PL).

Ethical approval:
The Ethics Committee of Oulu University Hospital was consulted; approval for utilizing leftover de-identified clinical samples was not required. This study conforms to the Declaration of Helsinki.

Contributorship:
All authors of this research paper have directly participated in the conception and design of the study, acquisition of data, analysis and interpretation of data. Authors of this paper have drafted the article or revised it critically and approved the final version being submitted.

Declaration of Interest:
There is no potential conflict of interest.

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Correspondence:
Juha Risteli, MD, PhD
Professor of Clinical Chemistry
Institute of Diagnostics
P.O. Box 5000
90014 University of Oulu, Finland
Tel.: +358-40-5909512
Fax. +358-8-3154451
E-mail: juha.risteli@oulu.fi