Journal of Chromatography B, 878 (2010) 1163–1168

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Measurement of 25-OH-vitamin D in human serum using liquid chromatography tandem-mass spectrometry with comparison to radioimmunoassay and automated immunoassay

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ARTICLE INFO

Article history:
Received 23 October 2009
Accepted 17 March 2010
Available online 25 March 2010

Keywords:
25-Hydroxyvitamin D
LC-MS/MS
Radioimmunoassay
Method comparison

ABSTRACT

The plasma 25-OH vitamin D concentration is a reliable biomarker for vitamin D status but assay's variability makes adequate monitoring of vitamin D status difficult. We employed isotope-dilution liquid chromatography (LC) tandem-mass spectrometry (MS/MS) for the measurement of both 25-OH vitamin D_3 and 25-OH vitamin D_2 in human serum. Hexadeuterium labelled 25-OH vitamin D_3 internal standard (IS) was added to calibrators (prepared in phosphate-buffered saline with 60 g/L albumin), controls or patient sera and 25-OH vitamin D metabolites were released from vitamin D binding protein by adding sodium hydroxide prior to protein precipitation by acetonitrile/methanol (9:1, v/v). Subsequent off-line solid-phase extraction was followed by chromatographic separation on a C-18 column using a water/methanol/ammonium acetate gradient. Detection was by Atmospheric Pressure Electrospray Ionisation (AP-EI) followed by selected reaction monitoring. We compared the LC-MS/MS assay to the DiaSorin radioimmunoassay (RIA) and a recently re-standardised version of an automated electrochemiluminescent immunoassay (ECLIA) from Roche Diagnostics. We also analysed external quality control samples from the International Vitamin D External Quality Assessment Scheme (DEQAS) for comparison with other participating laboratories using LC-MS. The method was linear from 5 to at least 550 nmol/L with intra- and interday CV's ≤6% for both 25-OH vitamin D_3 and 25-OH vitamin D_2. Recoveries ranged between 94.9 and 106.9% for 25-OH vitamin D_3 and 82.7 and 100.3% for 25-OH vitamin D_2. Our results for the DEQAS serum pools averaged −7.2% from the overall LC-MS method mean. The DiaSorin RIA agreed well with the LC-MS/MS method (r^2 = 0.90; average bias 1.61 nmol/L), the Roche ECLIA considerably disagreed (r^2 = 0.58; bias 10.13 nmol/L). This LC-MS/MS method is reliable and robust for the measurement of both 25-OH vitamin D_3 and 25-OH vitamin D_2 in human serum.

1. Introduction

In addition to the well known effect of vitamin D deficiency on bone metabolism, there is now growing evidence that vitamin D deficiency is involved in other diseases such as certain cancers [1]. Vitamin D exists in two forms; vitamin D_3 (cholecalciferol) produced in the skin on exposure to sunlight and vitamin D_2 (ergocalciferol), a plant derivative, found in many supplementation products. Vitamin D is metabolised in the liver to form 25-hydroxy (25-OH) vitamin D, which is further metabolised in the kidney to form the active metabolite 1,25-dihydroxy (1,25-OH) vitamin D. Measurement of 25-OH vitamin D is accepted as a reliable clinical indicator of vitamin status. Assessment of vitamin D status is important in the diagnosis of vitamin D deficiency and monitoring supplementation therapy. In the United States vitamin D_2 is the form available by prescription, unlike in The Netherlands and many other European countries, where vitamin D_3 is prescribed. There is no consensus on appropriate reference range and cut-offs for deficiency or insufficiency, as well as optimal and possible toxicity status. Recognition that vitamin D deficiency, as defined by most experts as a serum 25-OH vitamin D level of less than 50 nmol/L, may be more prevalent in most populations than earlier assumed has resulted in an marked increase in the volume of 25-OH vitamin D testing in clinical laboratories [1].

Serum 25-OH vitamin D concentration can be measured by competitive binding assay, radioimmunoassay, HPLC and more recently

Abbreviations: LC, liquid chromatography; UPLC, ultra performance liquid chromatography; MS/MS, tandem-mass spectrometry; AcN, acetonitrile (methylcyanide); MeOH, methanol; AP-EI, Atmospheric Pressure Electrospray Ionisation; IS, internal standard; SRM, selected reaction monitoring; DEQAS, vitamin D external quality control assessment scheme.

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doi:10.1016/j.jchromb.2010.03.035

liquid chromatography (LC) tandem-mass spectrometry (MS/MS) as well as automated immunoassay. Due to its hydrophobic character and strong protein binding, measurement of 25-OH vitamin D is technically demanding. We employed isotope-dilution LC-MS/MS for the measurement of both 25-OH vitamin D_3 and 25-OH vitamin D_2 in human serum and compared the assay to popular comparison methods, being radioimmunoassay (RIA) from DiaSorin and a recently re-standardised version of the automated chemiluminescence-based immunoassay (ECLIA) from Roche.

2. Material and methods

2.1. Materials and chemicals

25-OH vitamin D_3 and 25-OH vitamin D_2 were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). The internal standard (IS) 26,27-hexadeuterium labelled 25-OH vitamin D_3 was from Synthetica AS (Oslo, Norway). Water was prepared by USF-ELGA, Rossmark (Ede, The Netherlands) and LC/MS grade acetonitrile (AcN), methanol (MeOH), ammonium acetate and formic acid were supplied by Biosolve BV (Valkenswaard, The Netherlands).

2.2. Stock solutions, calibration standard solutions and control samples

Stock calibrator solutions of approximately 100 μmol/L 25-OH vitamin D_3 and 50 μmol/L 25-OH vitamin D_2 were prepared in MeOH. The absolute concentrations of the calibrators were checked by spectrophotometry with a molar absorption coefficient of 18,200 for both metabolites at 265 nm in MeOH [2]. Four calibration standards were prepared by diluting both stock calibrator solutions in phosphate-buffered saline with 60 g/L albumin [3] to give final concentrations 25–545 nmol/L for both 25-OH vitamin D_3 and 25-OH vitamin D_2. A 125 μmol/L IS stock solution was prepared in MeOH. A working solution of IS was prepared by diluting the stock with water to a final concentration of 6250 nmol/L. Stock solutions and working standard solutions were stored frozen at −80 °C in small aliquots. At this temperature these are stable for at least 6 months.

Additional commercial calibrator (order no. 38033) and control samples (order no. 0028), from human serum origin, were from Chromsystems (CS), Germany. In addition, a low serum control was prepared from pooled human sera containing low concentrations of 25-OH vitamin D_3 (27 nmol/L) and spiked 25-OH vitamin D_2 (36 nmol/L). The high serum control was obtained from spiking the low serum pool with 25-OH vitamin D_3 and 25-OH vitamin D_2 resulting in concentrations of 209 and 205 nmol/L, respectively. The medium sample was prepared from intermixing the high and low serum pool in a 1:1 ratio.

2.3. Sample preparation

After addition of 50 μl of IS solution to 250 μl of patient sera, controls or calibrator solutions samples were vortex mixed and equilibrated at room temperature for 10 min. We added 50 μl of a 4 mol/L sodium hydroxide solution to release protein-bound analyte during another 10 min incubation. Then, we added 1 ml of AcN/MeOH solution (9:1, v/v) for protein precipitation and incubated 15 min before centrifugation for 10 min at 16,000 × *g* at 4 °C. We transferred 850 μl of supernatant to a glass tube containing 1.5 ml of water. For solid-phase extraction Strata C18-E columns, 55 μm particle size (Phenomenex, Utrecht) were used. Columns were pre-equilibrated with 1 ml of MeOH, followed by 1 ml of water. The diluted supernatant was added to the column in two steps. The column was washed with 1 ml of water, followed by 1 ml of MeOH/water (60/40, v/v). The analytes were eluted by 250 μl of 100% MeOH in glass tubes containing 100 μl of water. The content of the glass tubes was transferred to LC-vials which were sealed.

Calibrators ($n = 4$) and controls ($n = 3$) were run with every batch (up to 56 patient sera) at the beginning as well as at the end of a working list.

2.4. LC-MS/MS system

Solvent delivery and sample introduction were performed using a Waters ACQUITY Ultra Performance LC (UPLC) system (Waters, Milford, MA, USA) equipped with a thermostat for both the sample and column compartments maintained at 4 and 45 °C, respectively. A Waters ACQUITY TQ tandem quadrupole mass spectrometer, interfaced with an Atmospheric Pressure Electrospray Ionisation (AP-ESI) source was used for the analysis. Separation was performed on a C18-column, 2.1 mm × 50 mm, packed with 1.7 μm particles (ACQUITY UPLC BEH C18, Waters). Mobile phases A and B consisted of ammonium acetate (2 mmol/L) containing 0.1% (v/v) formic acid, and MeOH (100%) with 0.3% (v/v) formic acid, respectively with a flow rate in all steps at 0.35 mL/min. The gradient program was as follows: initial: 60% B; 0–3.0 min: a gradient to 98% B; 3.0–3.5 min: rinse 98% B; 3.5–4.0 min: reversion of the mobile phase to 60% B; 4.0–5.0 min; 60% B. We used full loop injection to introduce 20 μL of sample into the system. 25-OH vitamin D_3 and IS under these conditions were eluted at about 3.01 min, 25-OH vitamin D_2 eluted at 3.06 min.

The AP-ESI was operated in the positive ion mode. Nitrogen was used as the nebulizing and desolvation gas at a flow rate of 15 and 900 L/h, respectively; argon at a pressure around 4.4×10^{-3} mbar was used as collision gas at a flow rate of 0.23 ml/min. The ion source and the desolvation temperature were maintained at 120 and 400 °C, respectively. Fragments of 25-OH vitamin D_3, 25-OH vitamin D_2 and IS were detected by selected reaction monitoring using the following mass-to-charge (m/z) transitions: 401.5 → 159.2 for 25-OH vitamin D_3, 413.4 → 83.1 for 25-OH vitamin D_2, and 407.5 → 159.2 for the labelled IS (Fig. 1), with a dwell time of 0.10 s; cone gas was set at 50 L/h and cone voltage at 20.0 V for 25-OH vitamin D_3 and IS and 25.0 V for 25-OH vitamin D_2. Collision energy was set at 27, 23 and 27 electron volt, respectively, for the above mentioned transitions at a delay time of 0.005 s. All aspects of system operation and data acquisition were controlled using Masslynx v4.1 software with automated data processing using the Quanlynx Application Manager (Waters).

2.5. Method validation

2.5.1. Ion suppression

Under continuous monitoring, we infused a constant flow of 250 nmol/L of either 25-OH vitamin D_3 or 25-OH vitamin D_2 solution directly into the MS, while we also started the auto-sampler to inject once 20 μL of a serum extract containing approximately 200 nmol/L of 25-OH vitamin D_3 and 100 nmol/L of 25-OH vitamin D_2.

2.5.2. Linearity

Linearity was evaluated by measuring four replicates of four dilutions of 25-OH vitamin D_3 and 25-OH vitamin D_2 in phosphate-buffered saline containing 60 g/L albumin in the range of 25–550 nmol/L. The responses were considered to be linear if the correlation coefficient (r^2) was greater than 0.99, calculated by least-squares linear regression using the EP Evaluator statistical module (D.G. Rhoads Associates Inc.).

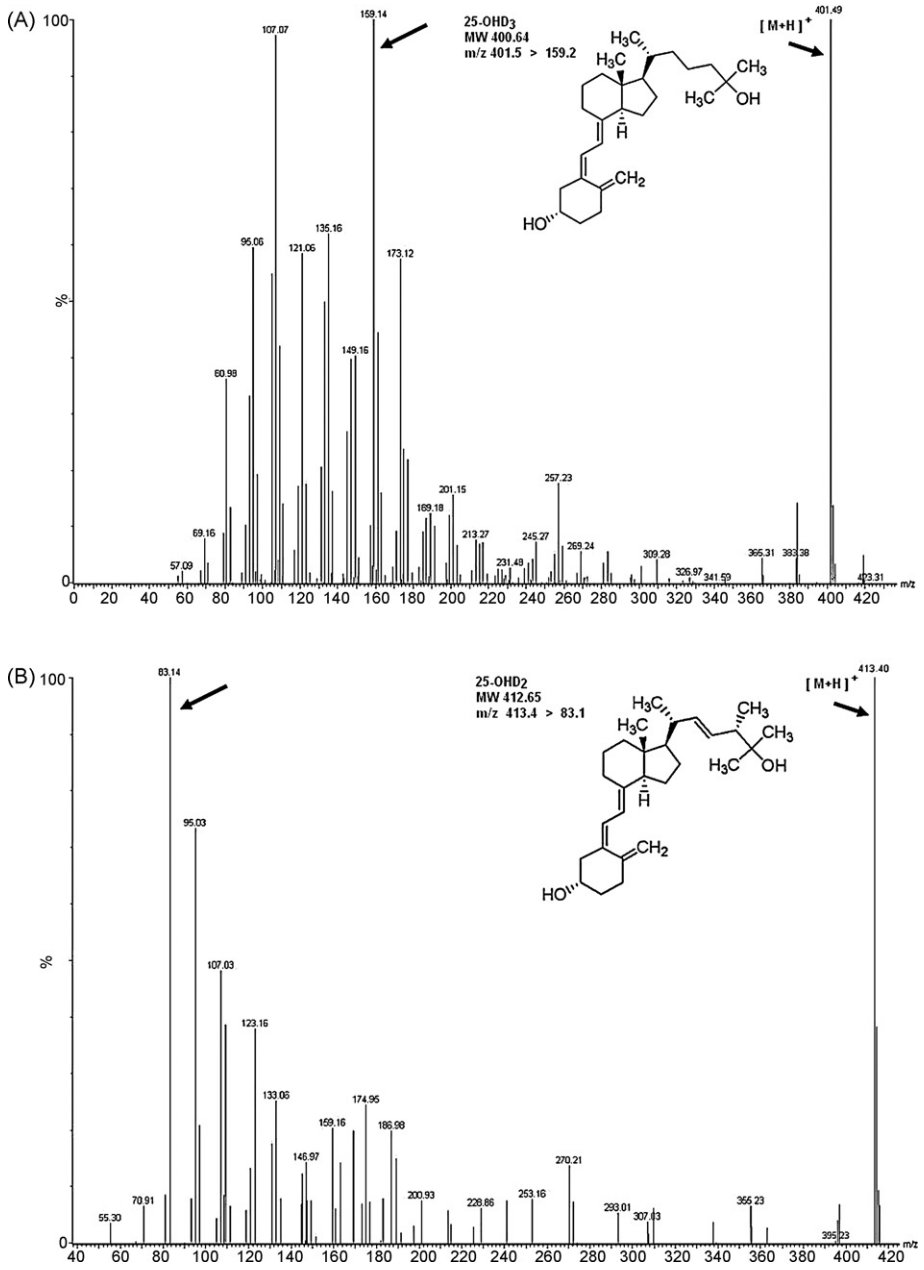

Fig. 1. Product ion scans for 25-OH vitamin D_3 (A) and 25-OH vitamin D_2 (B).

2.5.3. Detection limits

We determined limit of detection (LOD) and limit of quantification (LOQ) in serum samples containing 25-OH vitamin D_3 and 25-OH vitamin D_2 after serial dilution in phosphate-buffered saline with 60 g/L albumin. LOD and LOQ were defined as the injected amount that produced a signal-to-noise ratio of 3 and 10, respectively.

2.5.4. Imprecision

We used CS calibrator and quality control samples (level I and II) of human serum origin and obtained intra-assay variation from 14 replicate measurements in a single series and inter-assay variation from 14 assays over a 31 days period. In addition, intra-assay and total imprecision was tested by analysis of three self-prepared control serum samples with low, medium and high concentrations of 25-OH vitamin D_3 and 25-OH vitamin D_2 according to the NCLS-EP10A3 protocol [4]. In this protocol each level is assayed three times within a run over a total of 5 days.

2.5.5. Recovery

We estimated mean relative recoveries by adding two different concentrations of 25-OH vitamin D_3 and 25-OH vitamin D_2 to three plasma samples containing various basal concentrations of 25-OH vitamin D_3 and 25-OH vitamin D_2.

2.5.6. Accuracy

Five samples from the April 2009 distribution of DEQAS (an international vitamin D external quality assessment scheme) were analysed to determine the agreement of our LC-MS assay to other LC-MS participants ($n = 52$). These samples do not contain significant amount of 25-OH vitamin D_2. In addition, CS calibrator and control samples (levels I and II) were analysed as patient samples.

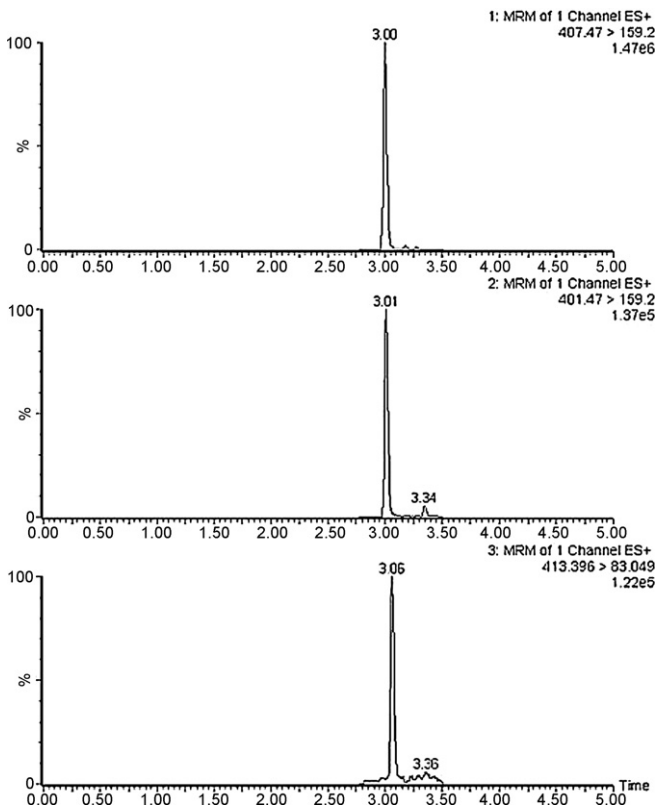

Fig. 2. Chromatogram of a serum control sample containing both 25-OH vitamin D_3 (212 nmol/L) and 25-OH vitamin D_2 (105 nmol/L). Registrations of peaks are shown with the m/z transitions: 407.5 → 159.2 for the IS (top), 401.5 → 159.2 for 25-OH vitamin D_3 (middle) and 413.4 → 83.1 for 25-OH vitamin D_2 (bottom).

2.6. Method comparison

Serum samples (n = 125) from routine measurement of 25-OH vitamin D were analysed after storage at −80 °C for 1 month. The LC-MS/MS method was compared to a manual 25-OH vitamin D ^{125}I radioimmunoassay (DiaSorin) as well as to a recently modified automated electrochemiluminescent immunoassay (ECLIA) for 25-OH vitamin D_3 (lot no. 00155233; Roche Diagnostics). We applied Deming regression and Bland-Altman analysis for method comparison using EP Evaluator (D.G. Rhoads Associates Inc.).

3. Results

3.1. Assay performance

25-OH vitamin D_3 and 25-OH vitamin D_2 were partially separated chromatographically (Fig. 2). The retention time for 25-OH vitamin D_3 and IS was 3.01 min and for 25-OH vitamin D_2 3.06 min. No attempt was made to resolve the analytes chromatographically, because the specificity of the mass selection and fragmentation (m/z transitions) gave the necessary compound specificity. When present, ion suppression (i.e. a decrease of 25% in the base line between retention time 0.40 and 0.80) was outside the typical retention times for both 25-OH vitamin D_3 and 25-OH vitamin D_2. Anyhow, the use of the co-eluting labelled IS compensates for the variation in matrix effects between individual patient samples.

3.2. Linearity

Both 25-OH vitamin D_3 and 25-OH vitamin D_2 were linear to at least 550 nmol/L. Regression lines were $y = 0.970x + 1.35$ for 25-OH vitamin D_3 ($r^2 = 0.9985$) and $y = 0.989x + 0.67$ for 25-OH vitamin D_2 ($r^2 = 0.9985$). Observed errors (0.39 nmol/L (3.9%) for 25-OH vitamin D_3 and 0.63 nmol/L (2.5%) for 25-OH vitamin D_2) were within allowable systematic error (0.4 (4%) and 1 nmol/L (4%), respectively).

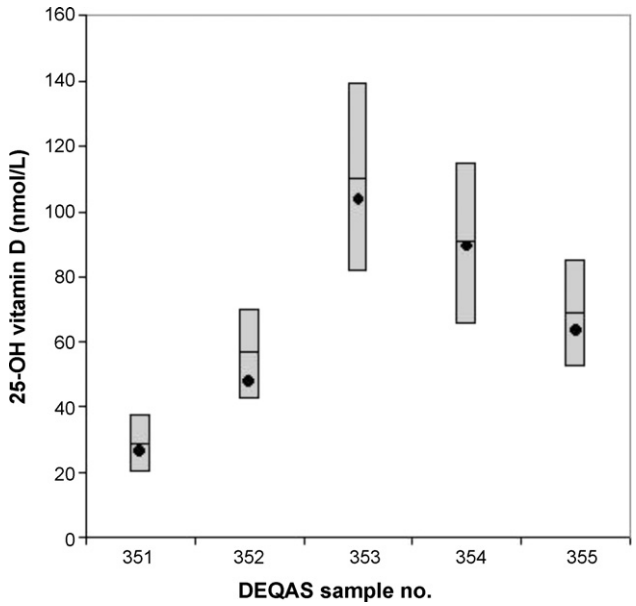

Fig. 3. Scores of LC-MS/MS results with those from the DEQAS survey. Shown are the mean (±2SD) concentrations of total 25-OH vitamin D of five samples from the April 2009 distribution from the LC-MS method group (n = 52) in grey. No significant 25-OH vitamin D_2 concentrations were present in these samples. Black dots represent the individual LC-MS/MS results.

3.3. Detection limits

LOD was 1.5 nmol/L for 25-OH vitamin D_3 and 1.2 nmol/L for 25-OH vitamin D_2. Respective quantification limits (at a signal-to-noise ratio of 10) were 3.5 and 2.0 nmol/L.

3.4. Imprecision

Table 1 shows the intra- and inter-assay imprecision CV's for the CS calibrator and control materials (levels I and II). CV's for both 25-OH vitamin D_3 and 25-OH vitamin D_2 were all ≤6%. Intra-assay and total imprecision CV's from NCLS-EP10 analysis of three, self-prepared, control serum samples with low, medium and high concentrations of 25-OH vitamin D_3 (27, 117 and 209 nmol/L) and 25-OH vitamin D_2 (36, 117 and 205 nmol/L) were all below 8%.

3.5. Recovery

Two concentrations of 25-OH vitamin D_3 (49.9 and 99.9 nmol/L) and 25-OH vitamin D_2 (54.3 and 108.6 nmol/L) were added to three serum samples with 25-OH vitamin D_3 concentrations ranging from 29.6 to 124.1 nmol/L, all with unmeasurable basal 25-OH vitamin D_2 concentrations. The mean recoveries were 99.5% (range 94.9–106.9%) for 25-OH vitamin D_3 and 95.4% (range 82.7–100.3%) for 25-OH vitamin D_2.

3.6. Accuracy

Fig. 3 shows the LC-MS/MS results of five samples from DEQAS in comparison to other LC-MS participants (n = 52). All samples were within the 2SD range from the LC-MS method mean, with four of five within 1SD, with a mean bias of −7.2%. Least-squares regression

Table 1
Intra- and inter-assay imprecision for CS calibrator and control (levels I and II) material.

	25-OH vitamin D_3				25-OH vitamin D_2			
	Target (nmol/L)	Mean (nmol/L)	Intra-assay[a] % CV	Inter-assay[b] % CV	Target (nmol/L)	Mean (nmol/L)	Intra-assay[a] % CV	Inter-assay[b] % CV
CS calibrator	160.0	142.1	1.3	5.0	71.1	64.0	3.0	5.8
CS level I	72.7	63.9	2.7	6.0	35.6	33.3	4.2	3.8
CS level II	238.0	211.5	1.4	5.0	119.0	104.7	3.1	6.0

Abbreviations: CV = coefficient of variance; CS = Chromsystems.
[a] Intra-assay imprecision was obtained from 14 replicates measured in a single series.
[b] Inter-assay imprecision from 14 assays over a 31 days period.

analysis resulted in LC-MS method mean = 1.01 × LC-MS/MS + 4.40 (r^2 = 0.99) (n = 5). Analysis of the CS calibrator and control samples (levels I and II) showed a bias of −11.5% for 25-OH vitamin D_3 and −9.5% for 25-OH vitamin D_2 (Table 1).

3.7. Method comparison

Both LC-MS/MS and DiaSorin RIA are methods that recognise both 25-OH vitamin D_3 and 25-OH vitamin D_2, whereas the ECLIA exclusively measures 25-OH vitamin D_3. In all 125 patient samples 25-OH vitamin D_2 levels were undetectable. So, all comparison data from LC-MS/MS, DiaSorin RIA and Roche ECLIA represent 25-OH vitamin D_3 solely. The following correlations from Deming regression analysis were found: DiaSorin RIA = 0.975 (95% Confidence Interval (CI): 0.919–1.031) × LC-MS/MS + 3.02 (95% CI: −0.42 to 6.46); Sy/x = 8.01; r^2 = 0.90, and Roche ECLIA = 0.948 (95% CI: 0.830–1.067) × LC-MS/MS + 13.01 (95% CI: 5.76–20.26)); Sy/x = 16.90; r^2 = 0.58 (Fig. 4A and C). The LC-MS/MS biased only 1.61 ± 8.11 nmol/L (bias ± SD) from the DiaSorin RIA, but 10.13 ± 17.31 nmol/L from ECLIA (Fig. 4B and D).

When applying the cut-off of 50 nmol/L for defining deficient versus normal results, as proposed by Holick [1], the LC-MS/MS

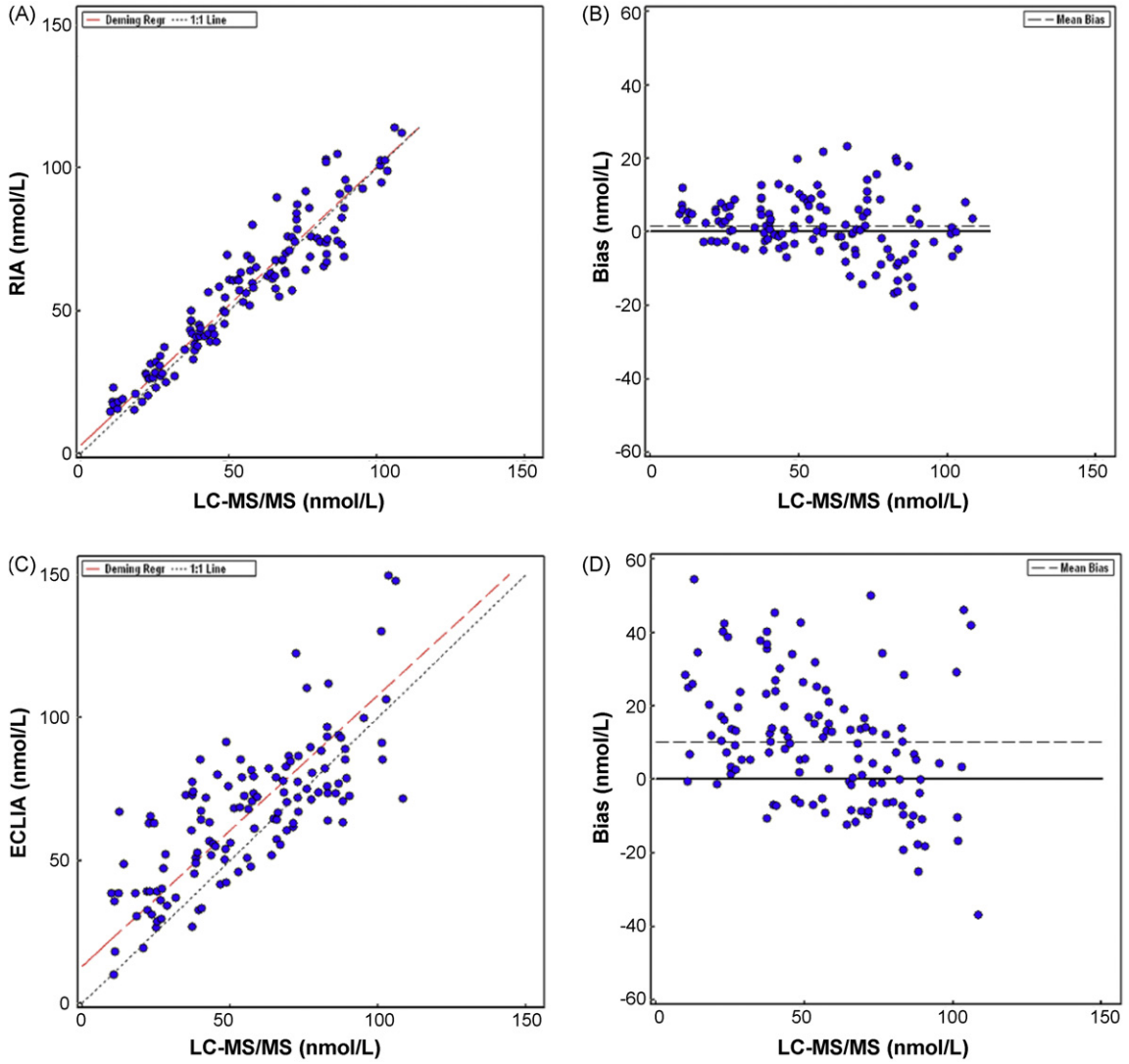

Fig. 4. Comparison of LC-MS/MS with manual radioimmunoassay (A and B) and automated immunoassay (C and D) in 125 patient serum samples. Scatter (A and C) and bias plots (B and D) are shown.

Table 2
Accuracy of classification for various concentration ranges of 25-OH vitamin D_3 ($n = 125$).

	Severe deficiency	Deficiency	Relative insufficiency	Sufficiency
	<25 nmol/L (%)	25–50 nmol/L (%)	51–75 nmol/L (%)	>75 nmol/L (%)
LC-MS/MS	13.6	30.4	30.4	25.6
RIA DiaSorin	10.4	28.8	36.8	24.0
ECLIA Roche	2.4	22.4	43.2	32.0

and DiaSorin RIA classifies 56.0% and 59.8%, respectively as normal (>50 nmol/L) whereas Roche ECLIA, with 75.2%, clearly overestimates the number of individuals having 25-OH vitamin D concentrations above 50 nmol/L (Table 2).

4. Discussion

Several LC-MS(/MS) methods have recently been described for the determination of 25-OH vitamin D [3,5–11], with an inter-laboratory imprecision similar to most immunoassays as based on results from laboratories participating in DEQAS [12]. How the assays are calibrated is a major factor to the LC-MS inter-laboratory CV's. It was recently demonstrated that LC-MS inter-laboratory precision significantly improved after the use of a common calibrator [2,13]. In this perspective, it will be interesting to see whether the recently released standard reference materials for 25-OH vitamin D (SRM 972 and SRM 2972) will reduce inter-laboratory variation. We decided to calibrate our LC-MS/MS assay on dilutions of pure standards of 25-OH vitamin D_3 and 25-OH vitamin D_2 in phosphate-buffered saline containing albumin. This was preferred above the CS calibrator, as no details are given on how the CS assigned value was determined. When we measured the CS calibrator, as it was a patient sample, we found a −10% deviation from target value for both 25-OH vitamin D_3 and 25-OH vitamin D_2. In line with this are our results from DEQAS where we found −7.2% bias of the LC-MS method mean. Depending on the number of laboratories participating in DEQAS using CS material for calibration, this might partly explain the negative bias of our LC-MS/MS to the LC-MS methods mean. Likely, calibrating on the CS material introduces a bias for which a correction is needed.

The strong protein binding of 25-OH vitamin D metabolites requires the use of suitable conditions to ensure release of bound analytes prior to protein precipitation. We started our method development using the sample preparation procedure as described by Vogeser et al. [5]. During the course of method development, we found out that the time for sample preparation prior to SPE could be reduced from 4 h [5] to less than an 1 h, without affecting recoveries of the IS.

The LC-MS/MS agreed well with the results by DiaSorin RIA, in agreement with other studies [6–8] and to a lesser degree also with Chen et al. who found a 12% bias [3]. The scatter around the regression line between our LC-MS/MS and RIA attributed to the relatively high imprecision (≥10% CV) of the RIA, as judged from repeated measurements of some of the patient sera using both methods. Roche has recently re-standardised their assay to LC-MS/MS [5] giving approximately 10% lower values (Data from Roche Diagnostics). However, in comparison to our LC-MS/MS the ECLIA still overestimates 25-OH vitamin D_3 concentrations up to 4-fold, particularly in the lower concentration range (<30 nmol/L). This might somehow be related to the limited sensitivity of the ECLIA having a LOD of 10 nmol/L. Also at higher concentrations (>75 nmol/L) large individual discrepant patient results were seen differing up to ±50 nmol/L of 25-OH vitamin D_3. Matrix effects distorting effective displacement of 25-OH vitamin D_3 from its binding protein may be responsible for the large inter-method variability in some individual patient sera. Another possibility is that in these samples significant concentrations of other vitamin D metabolites are present that may cross-react to some extent in the ECLIA (e.g. natural 24, 25-$(OH)_2$ vitamin D or 25, 26 $(OH)_2$ vitamin D, or the pharmaceutical compound 1-α-OH vitamin D_3 (alfacalcidol)). To our knowledge, there are only two reports describing the performance of an earlier version of Roche ECLIA for 25-OH vitamin D_3 in comparison to LC-MS/MS [9,10]. In one of these, the authors conclude good overall agreement between ECLIA and LC-MS/MS, although also in their study large between-method differences (up to 40 nmol/L) were seen in some individual samples [9].

The LC-MS/MS method creates 25% savings in labour and 80% savings in reagent costs when compared to our previously used routine assay for 25-OH vitamin D (DiaSorin RIA), although instrument cost is substantial and highly trained operators are needed. With processing up to 56 patient samples per batch, at present twice a week analysis meets our daily demand. In potential, up to 10,000 samples can be measured when analysis is performed on a daily basis. With even larger workloads, up to 20,000–30,000 samples per year, automation of the sample-workup using 96-wells plate formats and robotics might become a more cost-effective approach [11].

In conclusion, the LC-MS/MS method described here provides a rapid, accurate, sensitive and cost-effective alternative to other methods for determination of 25-OH vitamin D, with a real advantage being the ability to report separate results for 25-OH vitamin D_3 and 25-OH vitamin D_2. It compares well to the established DiaSorin radioimmunoassay but to a lesser extent to the recently re-standardised ECLIA vitamin D_3 assay from Roche.

Acknowledgements

We thank JPM Wielders (Meander Medical Center, Amersfoort, The Netherlands) for providing us with the DEQAS samples and Roche Diagnostics (Almere, The Netherlands) for the gift of kits free of charge for this study.

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