pre-purification by paper chromatography. The activity of the 11ß-HSD2 can be inhibited by glycyrrhetinic acid (GA), a constituent of liquorice. Administration of GA results in increased ratios of cortisol to cortisone in saliva, plasma and urine (7). In patients where liquorice-induced hypertension is suspected, screening for increased ratios of cortisol to cortisone in saliva might become a non-invasive and fast alternative for measurement of urinary GA (8).

Conclusion

We have developed a rapid, robust UPLC-MS/MS assay for the combined measurement of salivary cortisol and cortisone. This method can be used as a non-invasive and highly-specific tool to assess the value of salivary cortisol as a surrogate for free serum cortisol and as a potential novel way to assess 11ß-HSD2 activity, e.g. in studies on liquorice-induced hypertension.

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Determination of serum 25-OH vitamin D_3 and 25-OH vitamin D_2 using LC-MS/MS with comparison to radioimmunoassay and automated immunoassay*

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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). The most reliable assessment of vitamin D status is measuring the concentration of serum 25-OH vitamin D (25(OH)D). The volume of 25(OH)D testing has markedly increased over the last few years. Serum 25(OH)D concentration can be measured by protein binding assay, radioimmunoassay, HPLC and more recently 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)D is technically demanding. We employed isotope-dilution LC-MS/MS for the measurement of both serum 25(OH)D_3 and 25(OH)D_2 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.

Materials and Methods

Sample preparation

After addition of 50 µl of internal standard (IS, 6.3 µmol/L hexadeuterated 25(OH)D_3, Synthetica AS,

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* The data from this communication have also been published as ref. 4.

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

Oslo, Norway) to 250 µl of serum, samples were vortex mixed and equilibrated at room temperature (RT) for 10 min. Fifty µl of a 4 mol/L sodium hydroxide solution was added to release protein-bound analyte during another 10 min incubation. One ml of AcN/MeOH solution (9: 1, v/v) was added for protein precipitation and the sample was incubated 15 min before centrifugation (10 min at 16,000 g) at 4 °C. Solid-phase extraction was by Strata C18-E columns, 55 µm particle size (Phenomenex, Utrecht), with elution by 250 µl MeOH in glass tubes containing 100 µl of water. The content of the glass tubes was transferred to LC-vials which were sealed. Calibration standards were prepared by diluting stock solutions (25(OH)D_3 and 25(OH) D_2 (Sigma Aldrich, Zwijndrecht, The Netherlands) in MeOH) with phosphate-buffered saline (PBS) containing 60 g/l albumin (2).

LC-MS/MS assay

20 µl of the reconstituted samples were injected onto an ACQUITY Ultra Performance LC (UPLC) BEH C18, 1.7 µm, 2.1 x 50 mm column (Waters Milford, MA, USA) and chromatographed at 45 °C at a flow rate of 0.35 mL/min on a ACQUITY UPLC system (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. The gradient eluted over the column consisted of: initial 60% B; 0 to 3.0 min: a gradient to 98 % B; 3.0 to 3.5 min: rinse 98 % B; 3.5 to 4.0 min: reversion of the mobile phase to 60% B; 4.0 to 5.0 min: 60 % B. We quantified the analytes by using selected reaction monitoring (SRM) on a Waters ACQUITY TQ tandem quadrupole mass spectrometer, interfaced with an Atmospheric Pressure Electrospray Ionisation (AP-ESI) source, monitoring mass-to-charge (m/z) transitions 401.5→159.2 (25(OH) D_3, 413.4→83.1 (25(OH)D_2) and 407.5→159.2 (IS). 25(OH) D_3 and 25(OH) D_2 were partially separated chromatographically eluting at 3.01 and 3.06 min, respectively with a 5 min total runtime.

Method validation

We used commercial calibrator (160 nmol/l) and control samples (72.7 and 238 nmol/l) of human serum origin (Chromsystems, Germany) 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 were tested by analysis of three self-prepared control serum samples with low, medium and high concentrations of 25(OH)D_3 (27, 117 and 209 nmol/l) and 25(OH) D_2 (36, 117 and 205 nmol/l) according to the NCLS-EP10A3 protocol [3]. The limit of detection (LOD) and of quantification (LOQ) was based on analyte signal/noise ratio of 3 and 10, respectively in serum samples containing 25(OH)D_3 and 25(OH) D_2 after serial dilution in PBS with 60 g/L albumin. Linearity was evaluated by measuring four replicates of four dilutions of 25(OH)D_3 and 25(OH) D_2 in PBS containing 60 g/l albumin in the range of 25-550 nmol/l (4). Analyte recovery was tested by adding two concentrations of 25(OH)D_3 (49.9 and 99.9 nmol/l) and 25(OH) D_2 (54.3 and 108.6 nmol/l) to three serum samples with 25(OH)D_3 concentrations ranging from 29.6-124.1 nmol/l, all with unmeasurable basal 25(OH)D_2 concentrations. 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/MS assay to other LC-MS participants (n=52). For comparison we analysed 125 routine serum samples, all with unmeasurable 25(OH)D_2 concentrations, with a manual 25-OH vitamin D ^{125}I radioimmunoassay (DiaSorin) and a recently modified automated electrochemiluminescent immunoassay (ECLIA) for 25(OH)D_3 (Roche Diagnostics).

Results

Intra-assay and total imprecision from NCLS-EP10 analysis were all below 8% for both 25(OH)D_3 and 25(OH) D_2. For CS calibrator and control material, the intra- and inter-assay imprecision were below 6.1%. LOD was 1.5 nmol/l for 25(OH)D_3 and 1.2 nmol/l for 25(OH)D_2. Respective quantification limits were 3.5 and 2.0 nmol/l. Both 25(OH)D_3 and 25(OH) D_2 were linear to at least 550 nmol/l, with regression curves $y = 0.970x + 1.35$ for 25(OH)D_3 ($r^2 = 0.9985$) and $y = 0.989x + 0.67$ for 25(OH)D_2 ($r^2 = 0.9985$). Observed errors (0.39 nmol/l (3.9%) for 25(OH)D_3 and 0.63 nmol/l (2.5%) for 25OHD_2) were within allowable systematic error (0.4 (4%) and 1 (4%) nmol/l, respectively). The mean recoveries were 99.5% (range 94.9-106.9%) for 25(OH)D_3 and 95.4% (range 82.7-100.3%) for 25(OH) D_2. Our LC-MS/MS results from DEQAS showed a mean bias of -7.2%. Least-squares regression analysis resulted in LC-MS external method mean = 1.01 x LC-MS/MS + 4.40 ($r^2 = 0.99$)(n=5). Analysis of the CS calibrator and control samples showed a bias of -11.5% for 25(OH)D_3 and -9.5% for 25(OH)D_2. The following correlations from Deming regression analysis were found: DiaSorin RIA = 0.975 (95% Confidence Interval (CI): 0.919-1.031) x LC-MS/MS +3.02 (95% CI: -0.42-6.46); Sy/x = 8.01; r^2=0.90, and Roche ECLIA = 0.948 (95% CI: 0.830-1.067) x LC-MS/MS + 13.01 (95% CI: 5.76-20.26)); Sy/x= 16.90; r^2=0.58 (figure 1A and B). 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. When applying the cut-off of 50 nmol/l for defining deficient versus normal results, as proposed by Holick (1), the LC-MS/MS and DiaSorin RIA classify 56.0% and 60.8%, respectively as normal (>50 nmol/l) whereas Roche ECLIA classifies more individuals (75.2%) with 25(OH)D concentrations above 50 nmol/l (table 1).

Discussion

Several LC-MS(/MS) methods have recently been described for the determination of 25(OH)D (2, 5), with an inter-laboratory imprecision similar to most immunoassays as based on results from laboratories participating in DEQAS (6). How the assays are calibrated is a major factor to the LC-MS inter-laboratory CV's. Recently, it was demonstrated that LC-MS inter-laboratory precision significantly improved after the use of a common calibrator (7). We decided to cali-

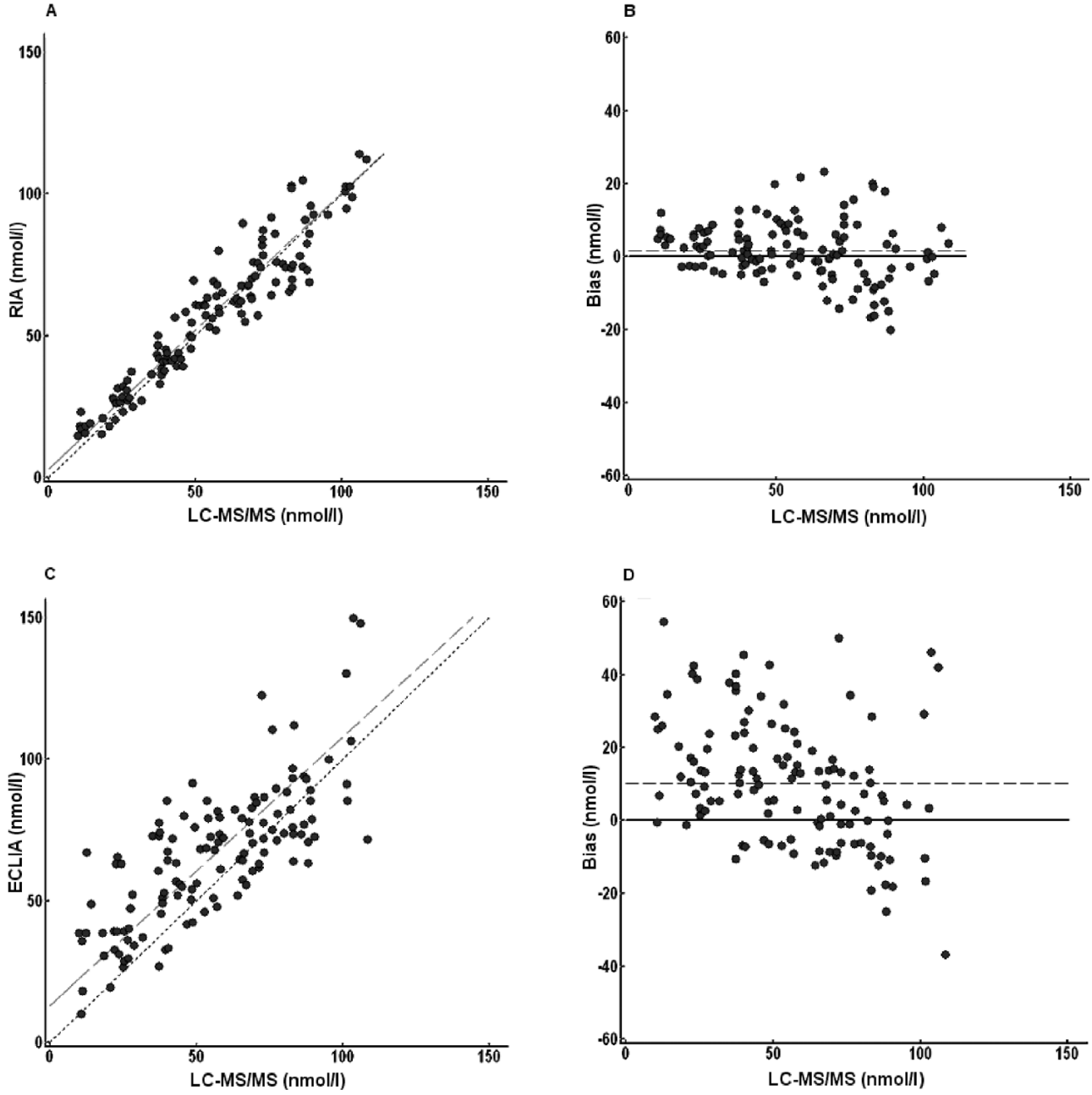

Figure 1. Comparison of LC-MS/MS with manual radioimmunoassay (A, B) and automated immunoassay (C,D) in 125 patient serum samples. A and C: scatter plots; B and D: bias plots.

brate our LC-MS/MS assay on dilutions of pure standards of 25(OH)D_3 and 25(OH) D_2 in PBS 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 an approximate -10% deviation from target value for both 25(OH)D_3 and 25(OH) D_2. In line with this are our results from DEQAS with a -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.

The LC-MS/MS agreed well with the results obtained by using the DiaSorin RIA. The scatter around the regression curve between both methods is 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

Table 1. Assay method dependent accuracy in % of classification according to Holick (1) on the basis of ranges of 25(OH) D_3 (n=125)

	Severe Deficiency < 25 nmol/l	Deficiency 25-50 nmol/l	Relative Insufficiency 51-75 nmol/l	Sufficiency >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

re-standardised their ECLIA assay to LC-MS/MS (8) giving approximately 10% lower values (Data from Roche Diagnostics). However, in comparison to our LC-MS/MS the ECLIA still overestimates 25(OH)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's results were seen differing up to ± 50 nmol/l of 25(OH)D_3. Matrix effects distorting effective displacement of 25(OH)D_3 from its binding protein may be responsible for the large inter-method variability in some individual patient sera. Another possibility is cross-reaction with other vitamin D metabolites in the ECLIA. In conclusion, the described LC-MS/MS method provides a rapid, accurate and sensitive alternative to other methods for determination of 25(OH)D, with a real advantage being the ability to report separate results for 25(OH)D_3 and 25(OH) 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.

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Geautomatiseerde morfologische analyse van perifeer bloed, liquor cerebrospinalis en andere lichaamsvloeistoffen met het digitale microscopiesysteem DM96*

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Introductie

Morfologische analyse van perifeer bloed en andere lichaamsvochten is een belangrijk diagnostisch hulpmiddel voor de clinicus. De huidige generatie bloedceltellers biedt een vrij complete analyse van perifeer bloed en veelal een 5-part-leukocytendifferentiatie. Voor pathologische bloedmonsters en andere lichaamsvochten zijn celtellers echter beperkt geschikt, aangezien ze niet in staat zijn om voorlopercellen en andere afwijkende cellen betrouwbaar te classificeren. Hierdoor blijft de manuele microscopische beoordeling de gouden standaard, ondanks alle technische en statistische beperkingen (1, 2).

Een aantal jaren geleden verscheen er een nieuwe generatie digitale microscopiesystemen op de markt. Het Zweedse bedrijf Cellavision bracht de Diffmaster Octavia en later de DM96 uit, waarvan werd gesteld dat ze volautomatische morfologische analyse van perifeer bloed mogelijk maakten. Tezamen met

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