We report a low sample volume LC/MS method for 25OH Vitamin D analysis. The method requires only 40 µl serum, is fully automatable, fast and sensitive. The method was successfully implemented for clinical analysis of infants for Vitamin D deficiency. The 6 min method has a sensitivity range from 2 to 100 ng/mL. A 3 min method was validated from 5 to 100 ng/mL range. Fast analysis, low sample volume and high sensitivity were achieved by combination of supported liquid extraction sample preparation, UHPLC chromatography and fused-core chromatographic column for separation, and highly sensitive mass spectrometry for detection. We emphasize the importance of sample preparation quality for rugged LC/MS analysis. Using SLE-based sample preparation we successfully used only 40 µl of serum while achieving a LLOQ of 2 ng/mL. We found from assayed samples, that 3-12 month old infants were not Vitamin D deficient, compared to adults. The average level of 25(OH)D3 in infants was 57.3 compared to 38.0 ng/mL for adults in a Bronx, NY patient population.

Keywords: Vitamin D; Mass spectrometry; Liquid Chromatography

Introduction

Vitamin D (cholecalciferol) and vitamin D2 (ergocalciferol) are the most abundant forms of vitamin D in the human body. Vitamin D is synthesized in the skin from 7-dehydrocholesterol in response to UV light (sunlight). 25OH Vitamin D2 and 25 OH vitamin D3 are derived from 25-hydroxylation of ergocalciferol and cholecalciferol, respectively, in the liver. They are precursors of active 1,25(OH)2D. Vitamin D3 is synthesized in the skin from 7-dehydrocholesterol in response to UV light (sunlight). Vitamin D2 and D3 are precursors of active 1,25(OH)2D. The latter 1,25 dihydroxy vitamin D metabolites have a short half life, exist at low abundance, and their levels must be interpreted in the context of precursor abundance and renal function (responsible for 1-hydroxylation). 25-OH vitamin D2 and D3 levels are felt to represent vitamin D stores in the human body and are used to diagnose vitamin D deficiency or toxicity and are tightly linked endocrinologically to inadequate bone mineralization and elevations in PTH with deficiency and hypercalcemia with toxicity. Vitamin D has long been recognized as essential for healthy bone and mineral metabolism, and its replacement in vitamin D deficiency has proven to cure rickets. Vitamin D is now recognized as a prohormone, which has multiple roles in maintaining optimum health [1-3]. Since Vitamin D deficiency is a widespread clinical problem and has been associated with many adverse health outcomes, analysis of Vitamin D2 (ergocalciferol) and D3 (cholecalciferol) and their major metabolites 25(OH)D2 and 25(OH)D3 has become a high priority topic in clinical analysis.

Traditional immunoassay-based analysis of 25-OH Vitamin D requires a large sample volume, typically 1-1.5 ml of serum. Such volume is easily available for analysis of adults. In contrast, for infants and newborns, sample volume is limited; as a result clinical analysis targets only critical analytes, while indicative, but non-essential analytes are routinely omitted, due to very limited sample availability. In addition, the Vitamin D immunoassays, such as RIA (DiaSorin) and automated chemiluminescent immunoassays, for example Abbott Diagnostics (Architect), DiaSorin (LIAISON), IDS (ISTS), Roche Diagnostics (E170), and Siemens (Centaur) are only able to measure total Vitamin D, and do not distinguish between the D2 and D3 forms as well as their inactive epimers [4]. Another disadvantage of immunoassays is higher assay variability and lack of specificity compared to LC/MS based assays. Typical LC/MS assays however consume 0.1-0.2 ml of serum, which is high for pediatric specimen analysis [5].

We successfully overcame this constraint by a combination of a highly sensitive triple quad mass spectrometer 6490 (Agilent Technologies) for detection and quantitation, use of a fused-core chromatographic column (Supelco) for a fast, 6 min separation, and supported liquid-liquid extraction (Biotage) as a simple and automatable sample preparation step. This combination allowed the use of only 40 microliter of serum per assay, while maintaining a LLOQ of 2 ng/mL. In a subsequent development step, the method was shortened to a 3 min run time with a validated LLOQ of 5 ng/mL [6].

Material and Methods

Chemicals and reagents

25-OH D (25-OH D2 Cat. # S176UNL-0.1 and 25-OH D3 Cat. # S163UNL-0.1) certified stock solutions were purchased from IsoSciences (PA, USA). Human serum Tri-Level Vitamin D Plus controls were purchased from Utak (CA, USA). 25-OH D2 (26,26,27,27-d6) (IS1) Cat. D5337 Lot 303 was obtained from Medical Isotopes Inc. and 25-OH D3 (26,26,27,27-d6) (IS2) Cat. 11-11576 DLM-7708-0 was obtained from Cambridge Isotopes Inc. Optima LC/MS Formic acid was obtained from Fisher Scientific; HPLC grade Methanol, 2-propanol and Heptane were obtained from Fisher Scientific; Millipore Q deionized water; Stripped human serum cat. # MSG 1000 was purchased from Western Gold Biologicals, Inc. (CA, USA). ISOLUTE SLE+200 mg Supported Liquid Extraction 96 Fixed Well Plates were obtained from Biotage. 0.5 mL 96 well plates were obtained from Agilent. The collection 2 mL well/Con B plates and

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piercible cover mats were obtained from Microliter Analytical supplies, now part of Wheaton (Millville, NJ).

Stock solutions, calibration standard, Internal standard and controls

An internal standard solution containing two standards (IS1 and IS2) was prepared at the concentration of 41.6 ng/mL 25-OH D2 (26,26,26,27,27,27-d6), 80 ng/mL 25-OH D3 (26,26,26,27,27,27-d6) in 50% 2-propanol.

Procedure

- 5.0 g Hydroxy Propyl β-cyclodextrin is dissolved in 121.5 g water to get 125 mL 4% HPB solution.
- 25-OH D2 (26,26,26,27,27,27-d6) 1.3 mg is added to 4% HPB water solution to get 10.4 µg/mL 4% HPB water solution, aliquoted 0.5 mL per 5 mL vials, then dried by N2 and stored at -40°C freezer.
- 25-OH D3 (26,26,26,27,27,27-d6) 5.0 mg is added to 4% HPB water solution to get 40 µg/mL 4% HPB water solution, aliquoted 0.5 mL per 5 mL vials, then dried by N2 and stored at -40°C freezer.
- The 25-OH D2 (26,26,26,27,27,27-d6) of 2 Vials and 25-OH D3 (26,26,26,27,27,27-d6) of 1 Vial are dissolved in 50% 2-propanol, transferred into 250 mL volumetric flask, then wash the vials 3 times by 50% 2-propanol, at last, add 50% 2-propanol to the line of the flask.
- The calibrators for calibration curves were prepared from the Isosciences 25-OH D2 (101.75 µg/mL) and 25-OH D3 (104.5 µg/mL) certified solutions by appropriate dilution in stripped serum (MSG 1000) at 7 different concentrations above the zero calibrator (2 ng–104 ng/mL (25-OH D3) and 2 ng–102 ng/mL (25-OH D2)), procedures as followed.
- Stock solution 101.75 ng/mL 25-OH D2 and 104.5 ng/mL preparation First, add MSG 2000 strip serum (less than 90 mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 40 ng/mL, 60 ng/mL) to each vial to be used.
- Use one of the 2 mL stock solution to get the calibrators (2 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 40 ng/mL, 60 ng/mL).
- 60 ng/mL- 600 ul (100 ng/mL) + 400 ul strip serum=1 mL into 1.5 mL glass vial
- 40 ng/mL- 400 ul (100 ng/mL) + 600 ul strip serum=1 mL into 1.5 mL glass vial
- 20 ng/mL 200 ul (100 ng/mL) +800 ul strip serum=1 mL into 1.5 mL glass vial
- 10 ng/mL 100 ul (100 ng/mL) + 900 ul strip serum=1 mL into 1.5 mL glass vial
- 5 ng/mL 50 ul (100 ng/mL) + 950 ul strip serum=1 mL into 1.5 mL glass vial
- 2 ng/mL 100 ul (20 ng/mL) + 900 ul strip serum=1 mL into 1.5 mL glass vial
- Store the stock solution and calibrators in freezer (-40°C)
- Tri-Level Vitamin D Plus controls (Low 13 ng/mL 25-OH D2, 14 ng/mL 25-OH D2, 25-OH D3, Lot # 7039, ref # 10060; Level 1 32 ng/mL 25-OH D2, 25-OH D3, Lot # 7039, ref # 10061; Level 2 73 ng/mL 25-OH D2, 75 ng/mL 25-OH D3 Lot # 7040, ref # 10062, Utah, CA, US) were reconstituted as per company's recommendations. Procedure as followed: Remove cap from each vial to be used.
- Reconstitute the dried control material by adding 5.0 mL of deionized water, using a 5 mL volumetric pipette. Replace cap and let sit 10-15 minutes.
- Swirl gently 3-4 minutes to ensure a homogeneous mixture.
- After each use, promptly return the reconstituted control material to -40°C freezer.

Patients sample (serum)

All human samples were collected through protocols approved by the Albert Einstein College of Medicine Committee on Clinical Investigations (IRB), Blood was collected and processed following routine clinical laboratory procedure. The collected serum was stored at -20°C until assayed.

Sample preparation

The SLE method was adapted. Briefly, 40 µL of patient sample calibrator or control was transferred in a 0.5 mL 96 well plate (Agilent). After dispensing of 40 µL of internal standards IS1/IS2, the mixture was shaken for 3 min manually. 80 µL 25% 2-propanol was added and the mixture was shaken for 3 min manually again. Then, the mixture was transferred into SLE+ 200mg fixed well plate (Biotage) with 96 wells collection plate (Microliter Analytical supplies, 2 mL well/Con B ) in collection position. After 5 minutes of waiting, 900 uL of heptane was added to the each well, and heptane by gravity flow was passed through the SLE sorbent. After 10 minutes, the collection plate was placed into a Speedvac concentrator to evaporate solvent at room temperature for 50 minutes followed by reconstitution in 80 µL 50% methanol. The collection plate was covered with a piercible cover and agitated at 200 RPM for 10 minutes prior to LC/MS analysis.

Chromatography

LC–MS/MS configurations

A Agilent LC system (Alpharetta, GA, USA) was used for HPLC chromatography. The system includes a 1290 infinity autosampler G226A, 1290 Flex Cube G4227, 1290 TCC G1316C, and 1290 Bin pump G226A, 1290 Flex Cube G4227, 1290 TCC G1316C, and 1290 Bin pump G4220A. For MS/MS analysis, an Agilent 6490 Triple Quadrupole LC/MS equipped with Jet Stream source. The instrument was operated in positive ion electrospray mode. The source parameters were: gas temperature 200°C, gas flow 11 L/min, nebulizer 30 psi, sheath gas temperature 250°C, sheath gas flow 11 L/min, capillary voltage 3000 V, Nozzle voltage 2000V. Agilent MassHunter software was used for data analysis and operation of the LC/MS system. Table 1 summarizes the LC–MS/MS configurations.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Q1 mass (Da) precursor</th>
<th>Q3 product</th>
<th>Dwell Time, ms.</th>
<th>Collision Energy, V</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-OH D b6D2</td>
<td>419.3</td>
<td>355.3</td>
<td>50</td>
<td>7</td>
</tr>
<tr>
<td>25-OH D2</td>
<td>413.3</td>
<td>355.3</td>
<td>50</td>
<td>7</td>
</tr>
<tr>
<td>25-OH D b6D3</td>
<td>407.3</td>
<td>371.3</td>
<td>50</td>
<td>9</td>
</tr>
<tr>
<td>25-OH D3</td>
<td>401.3</td>
<td>365.3</td>
<td>50</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 1: Quantification MRM precursor/product ion transitions selected for measurement of 25-OH D.
the selected MRM transitions and other optimized mass spectrometry parameters.

**Chromatographic conditions**

A Supelco C18 Ascentis Express column 50 × 2.1 mm (Cat# 53822-U, Sigma-Aldrich Inc., PA, USA), 2.7 µm was used. Mobile phase A contained methanol: 2-propanol: water (40:10:50) with 0.4% formic acid with 0.1% formic acid. Mobile phase B contained methanol: 2-propanol (90:10) with 0.1% formic acid. Chromatographic separation was achieved at 40°C, gradient flow rate was 0.5 mL/min; injection volume was 10 µL for both 3 and 6 min methods (Tables 2 and 3).

**Results**

Assay linearity was estimated using a curve with 7 non-zero calibrators in duplicates and a best fit curve was established using linear or quadratic weighting 1/x mode, respecting a least squares linear regression with an r > 0.98. Figure 1A and 1B LLOQ was determined using 2 ng/mL 25-OH D2, 25-OH D3 calibrator prepared in stripped...
Assay imprecision was evaluated using quality control samples (low, medium, high) with defined calibrators in a stripped serum matrix or defined calibrators in stripped serum matrix. Samples were measured in duplicate 15 times over 5 separate days (75 total measurements) to determine the repeatability and reproducibility of the assay (i.e. within assay and between assay variability), see Table 4. Analytes recovery during SLE was found around 70% including analyte decomposition.

serum and established. 2 ng/mL 25-OH D2, 25-OH D3 were accepted as the LLOQ because the following conditions are met: 1. The analyte response at the LLOQ is at least 5 times the response compared to blank response. 2. Analyte peak (response) is identifiable and reproducible with a precision of 20% and accuracy of 80%-120%. Accuracy was calculated from calibration curves and found that error is around 7% for ULOQ (Figure 1C).

<table>
<thead>
<tr>
<th>Serum</th>
<th>Repeatability QC1</th>
<th>QC2</th>
<th>QC3</th>
<th>QC1</th>
<th>QC2</th>
<th>QC3</th>
<th>QC1</th>
<th>QC2</th>
<th>QC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OH Vit. D3</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>Mean (ng/ml)</td>
<td>13.0</td>
<td>36.0</td>
<td>81.6</td>
<td>13.1</td>
<td>41.1</td>
<td>92.6</td>
<td>13.1</td>
<td>41.1</td>
<td>92.6</td>
</tr>
<tr>
<td>SD (ng/ml)</td>
<td>0.32</td>
<td>0.77</td>
<td>2.41</td>
<td>0.51</td>
<td>0.96</td>
<td>4.79</td>
<td>0.51</td>
<td>0.96</td>
<td>4.79</td>
</tr>
<tr>
<td>CV (%)</td>
<td>2.4%</td>
<td>2.1%</td>
<td>3.0%</td>
<td>3.9%</td>
<td>2.3%</td>
<td>5.2%</td>
<td>3.9%</td>
<td>2.3%</td>
<td>5.2%</td>
</tr>
</tbody>
</table>

Table 4: Assay imprecision for SLE-based LC/MS assay.

Figure 2: Pressure profile of 3 min LC/MS method.

Figure 3: Pressure profile of 6 min LC/MS method.
during drying.

Discussion

Typically, lowering of analysis cost is achieved by simplification of sample preparation. Paradoxically, we found that plasma protein precipitation in the long run is not cost efficient, since this approach reduces column lifetime and requires much more frequent instrument maintenance. We were able to lower sample volume to 40 ul and implement supported liquid-liquid extraction (Biotage) as a very simple sample preparation step. Unusually low sample volume was compensated by high sensitivity of mass spectrometry detection using a 6490 triple quad mass spectrometer from Agilent Technologies. Currently, it is one of the most sensitive instruments in the market. Fast gradients were accomplished by using ultra low dead volume 1290 UHPLC liquid chromatography and sharper chromatographic peaks were achieved by using fused core chromatographic column at higher flow rates. As a result of the combination of these method and instrumental features, we were able to maintain a LLOQ of 5 ng/ml for a 3 min run time and 2 ng/ml for a 5 min method.

We observed that reduction of the LC run time from 6 (Figure 2) to 3 min (Figure 3) leads to incomplete column equilibration. In general, incomplete column equilibration can cause instability of retention time for early eluting analytes. However, Vitamin D is a hydrophobic analyte and its retention time stability was not adversely impacted. In addition, we found that shortening the chromatographic separation did not impact assay linearity and selectivity, since the analyte to internal standard ratio was not affected (Figure 4).

As seen in Figure 4, the analyte (25OHD3) and its stable isotope labeled internal standard ratio is very similar for the 3 and 6 minute chromatographic methods, within the full calibration curve range. This overlap of calibration curves is evidence, that the quality of assay performance was maintained despite shortening the duration of the chromatographic separation by 50%.

Conclusions

Using SLE-based sample preparation we successfully used only 40 ul of serum while maintaining a robust and clinically relevant LLOQ of 5 ng/ml for 3 min method and 2 ng/ml for 5 min method. We found that reduction of LC separation leads to incomplete pressure equilibration and only partial mobile phase equilibration of the chromatographic column. Despite this fact however, assay performance was maintained.

We found that in this cohort, 3-12 month old infants were not Vitamin D deficient compared to adults. The infant’s average 25(OH)D3 was 57.3 ng/ml compared to the 38.0 ng/ml for adults living in the Bronx, NY.

References

6. Rogatsky E, Stein DT. Low Cost, Low Sample Volume, Rapid Vitamin D Assay. Poster 33b at MSACL conference. San Diego, CA 1-4 March 2014

\[ y = 0.0198x - 0.022 \quad R^2 = 0.9992 \]

\[ y = 0.0197x + 0.0318 \quad R^2 = 0.997 \]