Low sample consumption for Vitamin D analysis of infants

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Overview

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.

Introduction

Traditional immunoassay-based analysis of 25-OH Vitamin D requires a large sample volume, typically 1 -1.5 ml of serum. Such volume is always easily available for analysis of adults. In contrast, for infants and babies, sample volume is always a bottleneck. Clinical analysis is always performed for critical target analytes. Indicative, but non-essential analytes can sometimes be skipped due to very limited sample availability.

We successfully overcame this constrain by 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. That combination allowed to use only 40 microliter of serum per assay and maintains LLOQ of 2 ng/ml. The original sample protocol was modified due to our finding of a differential binding of 25(OH)D3 and it's stable labeled Internal Standard to albumin.

Methods

Positive mode ESI LC/MS/MS analysis was performed on 6490 Agilent Technologies triple quadrupole mass spectrometer. MRM transitions *m/z* 413.2→355.2 for 25(OH)D2, and transitions *m/z* 401.2→365.2 were used. As an internal standard, hexadeuterated analogues of analytes of interest were used. LC separation was carried on 1290 Infinity UHPLC system, composed of Autosampler G226A, Binary UPLC pump G4220A and Thermostatted column Compartment G1316C using Ascentis Express 2.1x50 mm C18 2.7 um fused-core column. Water-methanol mobile phases containing 0.1% formic acid was used. Sample preparation based on supported liquid extraction was performed.

Results

An original, suggested by manufacturer sample preparation procedure was: mix of 50 µL HPLC grade water and 50 µL isopropanol (propan-2-ol), add 100 µL of sample (plasma) and mix for 10 seconds. Load pre-treated plasma (200 µL total volume) onto the ISOLUTE SLE+ plate, leave the samples to absorb for 5 minutes under gravity and elute analyte of interest by 1 ml of heptane. Evaporate eluate to dryness, reconstitute and analyze by LC/MS.

During initial development we noticed that analyte to internal standard ratio for 25(OH)D3 was aprox. 10% higher in neat calibrators, compared to samples prepared in stripped serum. For Vitamin D2 this effect was less expressed (data not shown).

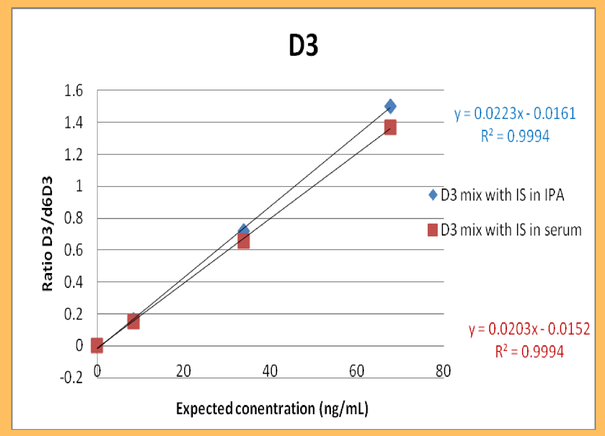

Fig1. 40uL of pure calibrators + 40uL IS+ 80uL 25% IPA
40uL calibrators+ 40uL IS+ 60uL serum + 20uL 100% IPA

We reproduced this adverse side effect by addition of stripped serum to pure, neat solution of the vitamin D calibrators for understanding of the hidden pitfalls in sample preparation. Vitamin D in serum is mostly bound to carrier proteins. In contrast, the internal standard in our assay was prepared as a neat, matrix-free solution in 50% Isopropanol. We chose matrix-free IS solution due to its long-turn stability at 4C. Supported liquid-liquid extraction process is a less denaturative, compared to a plasma crash approach. As result, at certain conditions, some part of the analyte could be remain bound. It shouldn't be assumed, that IS spiked in the sample will have the same degree of the binding by definition! Observed ratio must be confirmed with the ratio in neat sample. As we can see in Fig 2, the order of the matrix addition into the sample has a distinct impact on the analyte/IS ratio for 25(OH)D3.

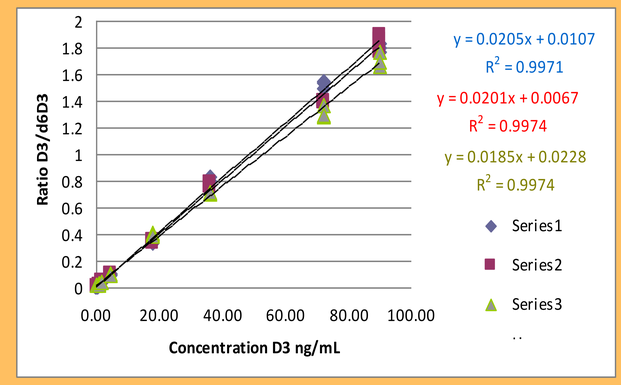

Fig 2. Blue: Calibrators diluted in 50% IPA without SLE
Conditions: 40 uL Calibrator +40 uL IS+ 20uL 100% IPA +60H_2O
Red- Calibrators diluted in strip serum with SLE
Conditions: 40 uL Calibrator +40 uL IS+ 20uL 100% IPA +60uL H_2O
Green: Calibrators diluted in 50% IPA with SLE
Conditions: 40 uL Calibrator +40 uL IS+ 20uL 100% IPA +60uL serum

Based on obtained results, the SLE preparation protocol was modified. First, to 40ul serum was added 40 ul of IS solution in 50% IPA followed by 2 min incubation. Next, 80 ul of 20% IPA was added. High aqueous content is essential to gain selectivity during extraction.

Conclusions

Using SLE-based sample preparation we successfully used only 40 ul of serum while appropriate LLOQ of 2 ng/ml was achieved. We found that in this cohort, babies (3-12 month) were not Vitamin D deficient, compare to an adults. The infant's average 25(OH)D3 was 57.3 ng/ml compared to the 38.0 ng/ml for the adults in Bronx, NY.