Chromatography Technical Note No AS122

Fully automated sample preparation and analysis of 25-OH-Vitamin D3/D2 and 3-Epi-25-OH Vitamin D3/D2 in serum using LC-MS/MS.

Paul H. Roberts & Dan Carrier. Anatune Ltd. Girton, Cambridgeshire.

Introduction

Vitamin D, along with calcium, promotes proper bone growth in children and aids in the prevention of osteoporosis in older adults. Vitamin D is present in two forms, Vitamin D3 and Vitamin D2. Both D2 and D3 vitamins are metabolised in the liver to form 25-Hydroxyvitamin D2 (25OH-D2) and 25-Hydroxyvitamin D3 (25OH-D3), respectively. In addition, biologically inactive 3-epi analogues of 25OH-D2 and 25OH-D3 have been reported, especially in young children [1]. The levels of the 25-Hydroxy metabolites are routinely measured for diagnostic assessment of vitamin D related diseases; however, recent studies have indicated that separation from the inactive 3-epi analogues may provide more accurate information for treatment and prevention. Analytical methods that can accurately quantitate both of the 25-Hydroxyvitamin D analytes in the presence of 3-epi analogues may become essential for diagnosis and monitoring of patients with vitamin D disorders. HPLC analysis of 25OH-D2 and 25OH-D3 is classically performed using C18 stationary phases. On this phase, the 3-epi analogues are not resolved and thus are included in the overall reported value. During recent application development studies, it was observed that a pentafluorophenyl (PFP, Ascentis Express F5) stationary phase provided increased selectivity towards 25OH-D3 and the corresponding 3-epi analogue relative to reported methods.

Presented in this application note is a fully automated push button end to end solution incorporating the protein crash step and extract injection. The method fully separates 25OH-D3/D2 from the inactive form Epi-25OH-D3/D2 which enables successful quantification of these analytes.

Instrumentation

GERSTEL MPS 3XL *xt*, fitted with a 250 µl syringe and LC injection valve
Anatune CF-100 centrifuge
ITSP Hardware Kit
Maestro Version 1.4.18.30
Agilent 6410 Triple Quadrupole Mass Spectrometer - multimode source.
Agilent 1200 Series HPLC
- G1312B Binary Pump SL
- G1316B Thermostatted Column Compartment SL
- G1379B Degasser

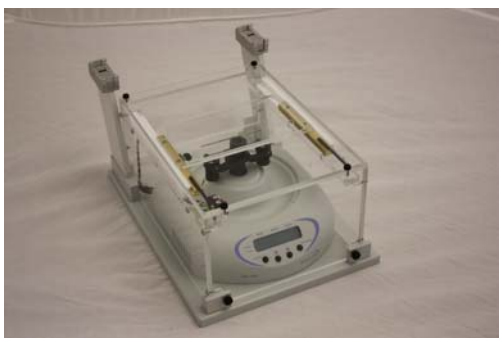


Figure 1 - Anatune CF-100 centrifuge

Figure 2 – Vitamin D Workstation

Methodology

Commercially available lyophilized four point calibration standards and QC materials were purchased (Chromsystems, Munchen, Germany) and reconstituted according to the manufacturer's instructions. For sample preparation 200 µl of serum sample is placed in a standard 2 ml glass screw top autosampler vial and the vial capped using a magnetically transportable PolyMag™ cap (GERSTEL, Germany). The sample is then placed on the vial tray of the multi purpose sampler (MPS).
The following aspects of sample preparation are fully automated, conducted via the MPS.

40 µl of internal standard solution (25OH-D3-d6 50 ng/ml) is added to the sample, followed by 200 µl of a 0.2 M zinc sulphate solution to enhance the sensitivity of the assay. Following this, 500 µl of methanol is added to the vial to precipitate the proteins.
The vial is then moved using magnetic transport to the CF-100 centrifuge whereby the contents are thoroughly vortexed for 1 minute to assist in the protein precipitation. The vial is then centrifuged at 3000 rpm for 1 minute to separate the proteins from the supernatant.
A 10 mg C18 Vitamin D ITSP SPE cartridge is solvated with 100 µl of methanol and then equilibrated with 100 µl of HPLC grade water. 500 µl of the supernatant is then loaded onto the SPE cartridge, before the cartridge is washed with 100 µl of 70 % methanol in water. The cartridge is then dried with 250 µl of air. Analytes are eluted with one 100 µl aliquot of methanol into a 300 µl high recovery vial. The polarity of the final solution is then adjusted by the addition of 40 µl of HPLC grade water, to improve the peak shape of the analytes.

Sample analysis is fully automated by means of an external injection valve and loop fitted onto the MPS. 20 µl of extracted sample is injected. Separation is achieved by means of a Supelco Ascentis Express F5 2.1 x 100 mm; 2.7 µm particle size. The chromatographic mobile phases consisted of 0.1 % acetic acid (v/v) in water (eluent A) and 0.1 % acetic acid (v/v) in methanol (eluent B). A gradient elution was performed from 40 % B to 95 % B over 6 minutes. The column flow rate was 0.3 ml/min throughout the chromatographic run and the column temperature was maintained at 40 °C.

Anatune Ltd, Unit 4, Wellbrook Court, Girton, Cambridge, CB3 0NA, UK
Tel: +44 (0) 1223 279210 Fax: +44 (0) 1223 279253 Email: info@anatune.co.uk Internet: www.anatune.co.uk

An Agilent 6410B tandem mass spectrometer with multimode source was used in positive APCI mode. Instrument analysis time was 8.5 minutes per sample using the conditions listed in table 1.

Parameter	25OH-D3	Epi-25OH-D3	25OH-D2	Epi-25OH-D2	25OH-D3-d6
Precursor ion	401.3	401.3	413.3	413.3	407.3
Product ion (Q)	383.3	383.3	395.3	395.3	389.3
Product ion (q)	159.1	159.1	159.1	159.1	159.1
Dwell	50	50	50	50	50
Fragmentor (V)	120	120	120	120	120
Collision Energy Q (V)	5	5	5	5	5
Collision Energy q (V)	25	25	25	25	25
Cell Acc. (V)	7	7	7	7	7

Gas Temp (°C):200
Gas Flow (l/min):5
Capillary (v):2500
Corona Current (µA):6
Vaporizer Temp (°C):170
Nebulizer (psi):40
Charging Voltage (V):2000

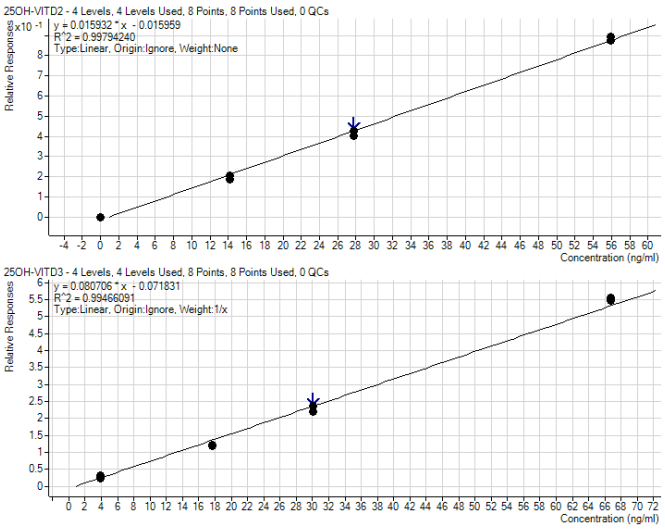
Table 1:- Selected MS conditions for analysis

Results

Calibration curves were run in duplicate, at the start and end of the analysis and constructed for 25OH-D2, 25OH-D3, Epi-25OH-D2 and Epi-25OH-D3. Linear calibrations were achieved from the Chromsystems four point serum calibration standards. Correlation coefficients of 0.997, 0.994, 0.996 and 0.997 were obtained for 25OH-D2, 25OH-D3, Epi-25OH-D2 and Epi-25OH-D3 respectively. See Table 2 and Figure 3.

Cal Level µg/L	25OH-D3	Epi-25OH-D3	25OH-D2	Epi-25OH-D2
Level 01	3.9	0	0	0
Level 02	17.7	9.4	14.2	8.7
Level 03	30.1	18.2	27.7	17.3
Level 04	66.7	28.3	55.9	26.3

Table 2:- Calibration levels for all compounds



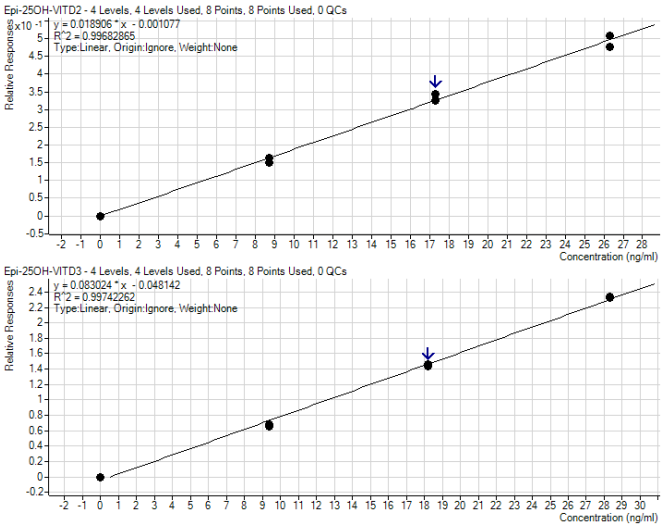


Figure 3 - Calibration curves for 25OH-D2, 25OH-D3, Epi-25OH-D2 and Epi-25OH-D3.

The method was validated by running two different QC materials from (Chromsystems, Munchen, Germany). Dried control materials were reconstituted with water to provide solutions with target concentrations of 16.2 and 35.7 µg/L for 25OH-D2 (13.0-19.4, 28.6-42.8), 15.6 and 36.7 µg/L for 25OH-D3 (12.5-18.7, 29.4-44.0), 13.5 and 22.4 µg/L for Epi-25OH-D2 (10.8.0-16.2, 17.9-26.9), 14.2 and 23.6 µg/L for Epi-25OH-D3 (11.4-17.0, 18.9-28.3). See Tables 3 & 4.

Compound µg/L	25OH-D3	Epi-25OH-D3	25OH-D2	Epi-25OH-D2
Target	15.6	14.2	16.2	13.5
Range	12.5 - 18.7	11.4 - 17.0	13.0 - 19.4	10.8 - 16.2
Mean	14.1	13.6	15.7	13.3
SD	0.4	0.5	0.7	0.7
% RSD	2.8	3.3	4.7	5.3
% Accuracy	90.3	96.0	96.7	98.5

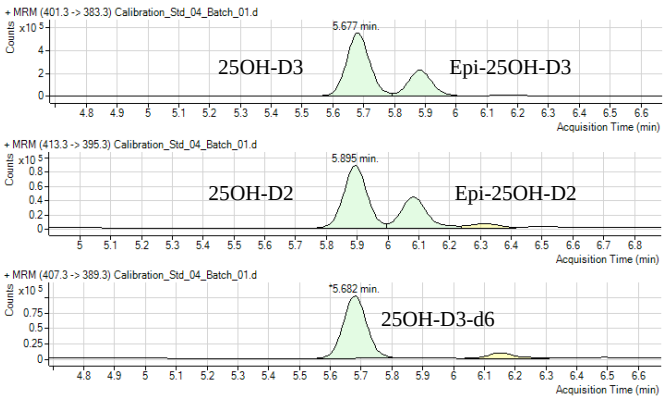
Table 3:- Showing results of the QC Level 1 serum control samples.

Compound µg/L	25OH-D3	Epi-25OH-D3	25OH-D2	Epi-25OH-D2
Target	36.7	23.6	35.7	22.4
Range	29.4 - 44.0	18.9 - 28.3	28.6 - 42.8	17.9 - 26.9
Mean	34.4	23.9	35.8	21.2
SD	1.8	1.6	2.3	0.9
% RSD	5.2	6.6	6.5	4.3
% Accuracy	93.8	101.2	100.2	94.6

Table 4:- Showing results of the QC Level 2 serum control samples.

Anatune Ltd, Unit 4, Wellbrook Court, Girton, Cambridge, CB3 0NA, UK
Tel: +44 (0) 1223 279210 Fax: +44 (0) 1223 279253 Email: info@anatune.co.uk Internet: www.anatune.co.uk

The solid phase extraction provided extracts free from matrix interferences resulting in clean chromatograms in which the 25OH-D2, 25OH-D3, Epi-25OH-D2 and Epi-25OH-D3 are the only major components.



Utilising the comprehensive prep ahead facility in Maestro the system is capable of processing 96 samples in just under seventeen hours. If a dual head MPS was utilised for this assay configured for parallel preparation, in which one head will perform all sample preparation and the second head is used solely for injection the system is capable of processing 96 samples in fourteen hours.

References

[1] Higashi, T., Shimada, K., Toyo'oka, T. Journal of Chromatography B 2010, 878, 1654–1661.

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