Automated SPE-LC/MS/MS Assay for 25-OH Vitamin D Metabolites from Serum

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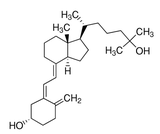
Introduction

25-OH Vitamin D and D2 metabolites have emerged in recent years as molecules of clinical diagnostic importance. Furthermore, LC/MS technology has been demonstrated as the most accurate tool for quantification of these metabolites in serum or plasma. The most pressing need is for a combined, automated sample preparation and analysis system. Herein we report on our development of an integrated and automated solid phase extraction (SPE) method and LC-MS/MS system for quantification of 25-OH Vitamins D3 and D2 metabolites in serum. The key advance is enhanced automation that is integrated into the same platform as the LC-MS/MS instrument system.

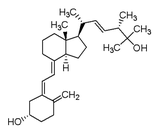
Background

Over the last few years there have been several reports of procedures for quantification of the 25-hydroxy metabolites of Vitamin D3 (synthesized in animals from 7-dehydrocholesterol) and D2 (derived from plant dietary sources and vitamin supplements) using liquid chromatography with mass spectrometric (LC-MS) detection. Assessment of Vitamin D status using these two metabolites is preferred because of their long half-life in serum. And LC-MS is becoming preferred over competitive binding immunoassays because of its ability to discriminate the Vitamin D3 and D2 forms in an equal manner and to quantify accurately relatively low biological levels. Moreover, the sample preparation techniques that can be used for LC-MS are better suited to release these compounds that are tightly bound to vitamin D binding protein in biological matrices. Potential problems for laboratories utilizing LC-MS technology for quantification of the 25-hydroxy metabolites of Vitamin D3 and D2 include instability of these compounds in the presence of light, preparation of calibration materials in a suitable matrix that is free of the compounds, the requirement for complex sample preparation in order to release these highly hydrophobic compounds from vitamin D binding protein and the propensity of the ionized forms of the compounds to lose water in the MS source.
Sample preparation techniques have involved either the use of hexane or heptane in liquid-liquid extractions to preferentially isolate these hydrophobic compounds; this then must be followed by a drying step and reconstitution in an LC compatible solvent mixture. Others have applied either offline or online solid phase extraction (SPE). Offline SPE has required large volumes of organic solvents (mLs per sample) and continual operator intervention. Online SPE followed directly by LC-MS compromises throughput because samples are processed one at a time and the dedicated SPE cartridge must be reconditioned and re-equilibrated between samples.

25-OH Vitamin D3



25-OH Vitamin D2



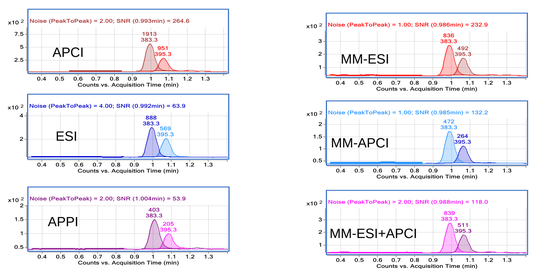
Agilent Optimized MS Ionization Conditions

MS: Agilent 6410A HS QQQ Triple Quadrupole

Ion Mode: Positive	APCI	ESI	APPI	MM-APCI	MM-ESI	MM-ESI+APCI
Source Conditions						
Drying Gas Temp (ºC)	325	250	325	250	250	250
Vaporizer Temp (ºC)	250	------	250	170	170	170
Drying Gas Flow (L/min)	5	11	5	5	5	5
Nebulizer Pressure (psi)	20	50	20	60	60	60
Capillary Voltage (V)	4000	5000	4000	4000	4000	4000
Corona Current (µA)	4	------	------	4	------	2
Charging Voltage (V)	------	------	------	------	2000	2000

MRM Acquisition Q1 peak width= 0.7 and Q2 peak width 0.7 m/z
Delta EMV (V) 200

Comparison of Ionization Methods at 5 µg/L



Chromatographic Conditions (Agilent)

Instrumentation

LC: Agilent Model 1200 SL with Rapid Resolution™
MS: Agilent Model 6410A with HotBox with MultiMode source

LC Conditions

Solvent A: 0.1% formic acid in water
Solvent B: 0.1% formic acid in methanol
Column: Agilent Zorbax Eclipse Plus C-18 (2.1 x 50 mm,1.8µ)
Column Temp: 50ºC; Injection Vol: 5 uL; Flowrate: 0.5 mL/min
Gradient:

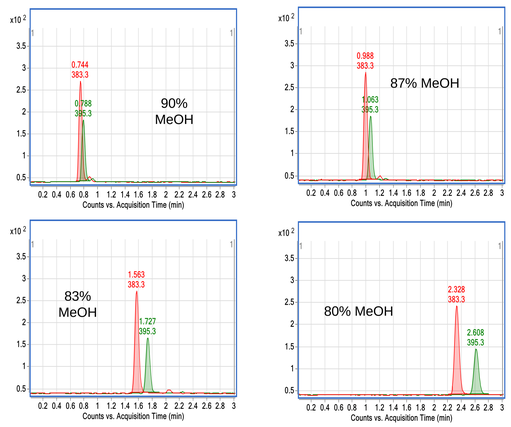
Time (min)	%B
0.0-3.0	80-90

MS/MS Conditions

Ionization: ESI+APCI (positive ion); Detection: MRM
Transitions:
25-OH Vitamin D3: 401.3>383.3
25-OH Vitamin D2: 413.2>395.3
25-OH Vitamin D3-d6 (IS): 407.3>389.3

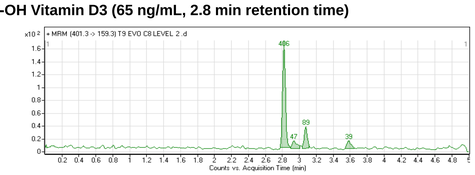
Chromatographic Optimization (Agilent)

The organic concentration was varied in the isocratic method.

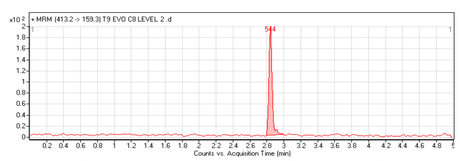


Example Chromatograms (OpAns)

25-OH Vitamin D3 (65 ng/mL, 2.8 min retention time)



25-OH Vitamin D2 (97.1 ng/mL, 2.8 min retention time)



Vitamin D3 (preliminary IS, ~100 ng/mL, 3.3 min retention time)

Analysis Conditions (OpAns, in development)

Instrumentation

LC: Agilent Model 1200 Rapid Resolution™
MS: Agilent Model 6410A with HotBox with MultiMode source

LC Conditions

Solvent A: 0.1% formic acid in water
Solvent B: 0.1% formic acid in methanol
Column: Agilent Zorbax Bonus RP (2.1 x 50 mm, 3.5µ
Column Temp: 30ºC, Injection Vol: 5 µL, Flowrate: 0.4 mL/min
Gradient::

Time (min)	%B
0.0-0.50	50
0.5-1.50	50-100 (linear)
1.5-3.25	100
3.25	50
3.25-5.00	50

MS/MS Conditions

Ionization: APCI (positive)
Transitions:

25-OH Vitamin D3	401.3>159.3
25-OH Vitamin D2	413.3>159.3
Vitamin D3 (preliminary IS)	385.3>159.3

Samples and Preparation (Agilent)

Vitamin D metabolite calibrators, quality controls and specimens (500 µL) were added to 16x125 mm glass screw cap culture tubes containing 100 uL of internal standard solution (400 µg/L vitamin D3-d6). The 1 mL of acetonitrile supplemented width 2% formic acid was added. The samples were mixed vigorously for 10 sec and the incubated for 15 min before centrifugation. SPE was performed using Biochemical Diagnostics GV 65Cc Gravity Flow columns.

Step	Solvent	Volume
Condition	Methanol	1 mL
Equilibrate	50% Methanol	1 mL
Load	Sample	1.5 mL
Wash 1	50% Methanol	1 mL
Wash 2	75% Methanol	1 mL
Apply Vacuum		0.5 min
Wash 3	n-Heptane	0.2 mL
Apply vacuum	Air	5-7 min
Elute	Methanol	0.3 mL
Adjust Polarity	Water	0.2 mL

Samples and Preparation (OpAns, in development)

Samples: Tri Level Vitamin D Serum Toxicology Controls were obtained from UTAK Laboratories. Nominal concentrations were 10, 30 and 73 ng/mL for 25-OH Vitamin D3 and 25-OH Vitamin D2. Dried control materials were reconstituted with water and are stable for 25 days at 2-8ºC. A stock solution of Vitamin D3 (Supeclo) was prepared in ethanol and stored at –80ºC; internal standard working solution (1 ng/uL) was prepared daily in 50% methanol/water and protected from light
Preparation: Twenty µL of internal standard solution was added to 1.5 mL polypropylene micro centrifuge tubes followed by 200 µL of serum. The samples were mixed fro 10 sec and then 400 µL of acetonitrile supplemented with 1% formic acid was added. The samples were mixed vigorously two times for 10 sec each and then allowed to incubate protected form light for 15 min. After centrifugation at high speed for 10 min, the clear supernatants were transferred into amber glass autosampler vials and capped in preparation for ITSP.

OpAns Instrument Top Sample Preparation (SPE)

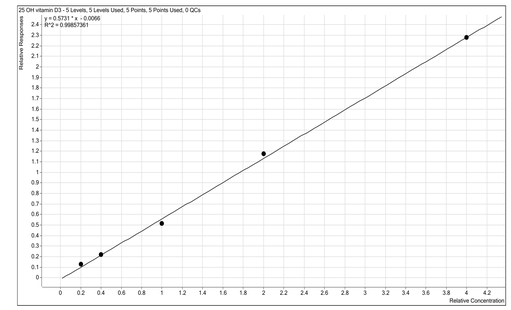
System: CTC Analytics HTC PAL with a 100 µL syringe and cold stack.
ITSP Cartridges: ITSP Evolute C8 (10 mg)
Microliter Product No. 07-BC10-20A
Solvent A: Acetonitrile, **Solvent B:** Methanol
Solvent C: 0.1% Formic Acid **Solvent D:** 50% Methanol in water

Step	Solvent	Volume	Flowrate
Clean SYR	A	100 x 2 uL	SYR Max uL/sec
Condition	B	100	10
Condition	C	100	10
Aspirate	Air	50	15
Load	Sample	100	5
Aspirate	Air	50	15
Clean SYR	A	100 x 4	SYR Max
Wash 1	C	100	10
Wash 2	D	100	10
Aspirate	Air	50	15
Elute	B	50 x 2	5
Aspirate	Air	50	15

Results (Agilent)

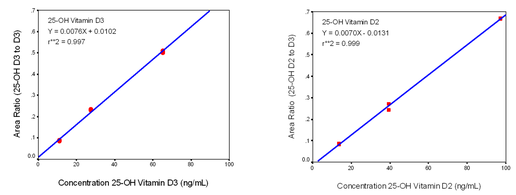
Agilent has fully validated their method per Clinical Laboratory Standards Institute (CLSI) guidelines. They have demonstrated and LOQ of 3 µg/L, linearity between 3 and 150 µg/L and inter-day imprecision of 9.5%

Example 25-OH Vitamin D calibration



Results (OpAns, preliminary)

The UTAK Vitamin D Plus controls were tested for absence/presence of Vitamin D3. In its absence, Vitamin D3 was chosen as the internal standard for preliminary investigations. An initial assessment of linearity was made using replicate samples of the Tri Level Vitamin D Serum Toxicology Controls. Regression of the ratios of the 25-OH Vitamin D metabolite peak areas to Vitamin D (preliminary IS) against the verified Vitamin D metabolite concentrations (11.1, 27.6 and 65.1 ng/mL for 25-OH Vitamin D3; 13.6, 39.4 and 97.2 ng/mL for 25-OH Vitamin D2) suggested a linear relationship for each of the two metabolites.



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