Determination of Vitamin D Metabolites in Human Serum Using Automated ITSP Solid Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry

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Abstract
Due to their hydrophobic nature and strong binding to Vitamin D binding protein, accurate quantitation of the clinically relevant 25-hydroxy metabolites of Vitamins D3 and D2 is difficult. Acetonitrile, methanol or, alternatively, multi-step liquid-liquid extraction with hexane or heptane has been used to disrupt protein binding, but must be followed by further sample cleanup with offline or online solid phase extraction (SPE). LC-MS allows for quantitation of the two compounds simultaneously.

We have developed a quantitative method for the determination of 25-OH D3 and 25-OH D2 that utilizes calibrators and internal standard stabilized in a protein-based matrix, mimicking human serum. Salts, proteins and phospholipids are removed via solid phase extraction (SPE) using an advanced restricted access material with Instrument Top Sample Preparation (ITSP). Reverse phase liquid chromatography with multiple reaction monitoring (MRM) under atmospheric pressure chemical ionization (APCI) mass spectrometric condition is used for specific and sensitive detection.

Experimental

Sample Preparation

25-OH D3 and 25-OH D2 standard materials (Sigma Aldrich) and Internal Standard (IS; 25-hydroxy Vitamin D3-26,26,26,27,27,27-d6; Medical Isotopes) were dissolved in ethanol and stored in the dark at –80ºC. Concentrations were confirmed against 264 nm molar UV absorptivities of 18300 and 19400 (1/m-cm)[1], respectively. Bovine serum albumin (6%) in phosphate buffered saline (BSA/PBS) was supplemented with the analytes to provide 5, 10, 25, 50, 75 and 100 µg/L. Quality control was Tri-Level Vitamin D Plus Serum Toxicology Controls (UTAK Laboratories).

Sample aliquots (200 µL) were mixed with internal standard (20 µL; 1920 µg/L in BSA/PBS) and then protein binding was disrupted by addition of 400 µL of 1% formic acid in acetonitrile. Samples were well mixed, then incubated in the dark for 15 min, followed by centrifugation. Finally 250 µL of supernatant was mixed with 100 µL water in preparation for SPE using ITSP.

ITSP SPE Method

A CTC Analytics HTC PAL sample handler was configured with a 100 µL syringe and a cold stack with 3 tray holders, one to hold sample extracts, one for ITSP cartridges and one for final eluates. The SPE program, adapted from a standardized procedure[2] to maximize sample cleanliness[3] and recovery of the analytes, was as follows:

SPE Cartridges: ITSP Evolute ABN 10mg (Microliter Product 07-BABN10-20A)
Solvent A: Acetonitrile
Solvent B: Methanol
Solvent C: 1% Formic acid in water
Solvent D: 40% Acetonitrile in water
Solvent E: Water

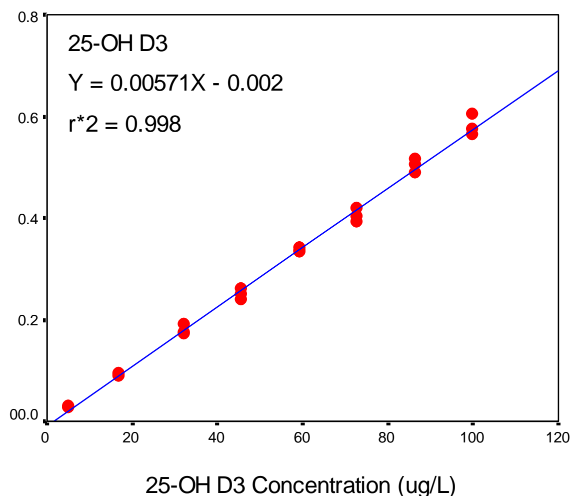
Step	Solvent	Volume (µL)	Flow Rate (µL/sec)
Clean SYR	A	100 x3	200
Condition	B	100	15
Equilibrate	C	100	15
Flush	Air	50	15
Load	Sample	100 x3	5
Flush	Air	50	15
Clean SYR	E	100 x3	200
Clean SYR	A	100 x3	200
Clean SYR	E	100 x3	200
Wash	C	100	15
Wash	D	100	15
Flush	Air	50	15
Elute	B	50 x2	5
Flush	Air	50	20

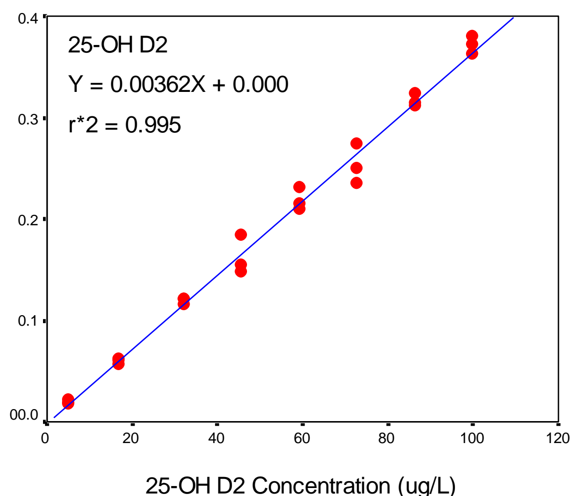
Analysis Method

Instrument: Agilent 6410 triple quadrupole with Agilent Model 1200 HPLC system
Solvent A: 0.1% Formic acid in methanol
Solvent B: 0.1% Formic acid in water
Column: Agilent Zorbax Eclipse Plus C18 Narrow Bore RR (2.1 x 50 mm, 3.5µ)
Mobile Phase: 87% A, 0.25 mL/min, 5.0 min end
Column Temp: 50ºC
Injection Vol: 10 µL
Ionization Mode: APCI+ (multimode source)
Mass Transition/ Collision Energy:
401.3>383.4; 2 (25-OH D3 quantifier)
401.4>159.1; 24 (25-OH D3 qualifier)
407.4>389.4; 2 (IS quantifier)
407.4>159.1; 24 (IS qualifier)
413.4>395.4; 2 (25-OH D2 quantifier)
413.4>159.1; 24 (25-OH D2 qualifier)

Results

Linearity across the concentration range was confirmed by analysis in triplicate of 8 samples prepared by proportional dilution of the lowest with the highest calibrator standard[4]. Deviations from expected were +0.1±4.7% (range –7.7 to +13.8%), 25-OH D3; 0.5±6.3% (range –10.9 to +15.3%), 25-OH D2. Correlation coefficients met or exceeded 0.995.





Functional sensitivity was assessed on 4 separate days using 4 injections of 5 samples prepared by diluting quality control materials with BSA/PBS to concentrations near to the anticipated LOQ[5]. Respectively, percentage coefficient of variation and deviation at the lowest levels assessed were 5.9% and –1.1% for 25-OH D3 at 4 µg/L; 12.8 and –5.5% for 25-OH D2 at 4.9 µg/L.

Recovery of the analytes from serum matrix was assessed by supplementing quality control materials with 10 and 25 µg/L of the analytes. Triplicate replicates were compared to control material. From the Low control, 9.82 (98.2%) and 22.9 (91.6%) µg/L of 25-OH D3 were recovered and 9.48 (94.8%) and 22.8 (91.3%) µg/L from the Level 1 control. For 25-OH D2, 10.6 (106.5%) and 23.3 (93.1%) µg/L were recovered from the Low control and 9.56 (95.6%) and 23.4 (93.5%) µg/L from the Level 1 control.

Precision experiments to determine within run and total imprecision were performed using two replicates of each of three levels of the quality control materials across six independent analytical runs (n=12)[6]. Each run was controlled using a 6-point calibration curve.

Analyte	Target Conc (µg/L)	Mean Conc (µg/L)	Imprecision (%)	
			Within	Total
25-OH D3	**11.1**	11.3	2.0	4.6
	27.6	24.4	3.0	3.4
	65.1	60.5	3.0	4.8
25-OH D2	**13.6**	13.4	2.4	7.5
	39.4	38.3	3.3	5.0
	97.2	92.8	3.9	5.5

No injection-to-injection carryover of 25-OH D3, 25-OH D2 or IS by the LC-MS system was observed on any occasion when a methanol blank was injected immediately following the highest BSA/PBS calibrator. Neither was any sample-to-sample carryover by the PAL observed on any of three occasions when a BSA/PBS blank was processed immediately following the highest BSA/PBS calibrator and injected into the LC-MS system prior to any analyte- or IS-containing sample.

Conclusions

ITSP SPE for the determination of vitamin D metabolites utilizes small sample volume and the benefits of using an advanced restricted access material. Unlike offline SPE, it is automated; this allows operator freedom for other activities and improves sample turnaround time. The procedure met or exceeded all expectations. Samples that may contain epi-25-hydroxy or 1-OH metabolites of Vitamins D3 or D2 will require an alternative chromatographic method.

References

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