[TECHNOLOGY BRIEF]

Simultaneous Quantification of 25-Hydroxyvitamin D_3, 25-Hydroxyvitamin D_2, and 24,25-Dihydroxyvitamin D_3 in Clinical Research Studies By UPLC-MS/MS

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GOAL

To accurately quantify vitamin D metabolites in low volume serum samples to facilitate human, rodent, and *in vitro* research studies.

Measure multiple vitamin D metabolites in a single analysis using only 100 µL of sample.

BACKGROUND

Total serum 25-hydroxyvitamin D (25(OH)D, the sum of the concentrations of 25(OH)D_2 and 25(OH)D_3), is the biomarker of vitamin D nutritional status.[1] The vitamin D catabolite, 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) is formed from 25(OH)D_3 by the action of cytochrome P450 CYP24A1, which is now accepted as a determinant of circulating 25(OH)D levels.[2] In current vitamin D research studies, there is renewed interest in quantifying 24,25$(OH)_2D_3$, in addition to 25(OH)D as a measure of the rate of clearance of 25(OH)D_3. However, the analytical tools to quantify multiple vitamin D metabolites in a single method are not widely available.

Figure 1. ACQUITY UPLC System with Xevo TQ-S Mass Spectrometer.

THE SOLUTION

To facilitate clinical research studies of vitamin D metabolism, we have developed a new method based on liquid-liquid extraction, chemical derivatization, and UPLC-MS/MS, using an ACQUITY UPLC® System with a Xevo® TQ-S (Figure 1). The method provides analytically sensitive and selective measurement of 24,25$(OH)_2D_3$ and 25(OH)D in the same run, requiring only 100 µL of serum. The recent availability of deuterated internal standard for 24,25$(OH)_2D_3$ from various sources enables a truly quantitative approach. The chromatographic separation of 3-epi-25(OH)D_3 from 25(OH)D_3 enabled by this method ensures that 25(OH)D_3 measurements are not confounded by the presence of this naturally-occurring stereoisomer. The method described here can be easily adapted for the analysis of rodent serum,[3] and for the study of vitamin D metabolism in *in vitro* cultured-cell models.

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EXPERIMENTAL

Materials

25(OH)D_3 and 25(OH)D_2 calibrators (6PLUS1) were purchased from Chromsystems. A 6-level calibrator set for 24,25$(OH)_2D_3$ was generated in-house using a matrix of 20% human serum in 0.1% bovine serum albumin dissolved in phosphate-buffered saline; supplemented with synthetic 24,25$(OH)_2D_3$. Internal standards d_3-25(OH)D_3 and d_3-25(OH)D_2 were purchased from Isosciences. d_6-24,25$(OH)_2D_3$ was supplied by Drs. Antonio Mourino and Miguel Maestro, University of Santiago de Compostela. All LC-MS/MS solvents, additives, and extraction solvents were Optima LC-MS grade and purchased from Fisher Scientific, with the exception of MTBE, which was purchased from Sigma Aldrich. DMEQ-TAD was purchased from Key Synthesis.

Sample preparation

In microcentrifuge tubes, 100-µL aliquots of test serum or calibrator were diluted with 200 µL of water and supplemented with the following internal standards: 80 ng/mL d_3-25(OH)D_3, 65 ng/mL d_3-25(OH)D_2, and 6 ng/mL d_6-24,25$(OH)_2D_3$. A 100 µL volume of 0.1M HCl was added, and protein precipitation was carried out by adding 150 µL 0.2 M zinc sulfate and 450 µL of methanol with vortex mixing after addition of each component. The mixture was centrifuged at 12,000 x g for 10 minutes and the supernatant was transferred to borosilicate glass tubes. Organic extraction was carried out by adding 700 µL of hexane and 700 µL of MTBE, with vortex mixing after addition of each component. The upper organic phase was transferred into maximum recovery vials (p/n 186000327c) and dried under a stream of prepurified N_2 at 37 °C. Samples were derivatized by redissolving the dry residue in 25 µL of 0.1 mg/mL DMEQ-TAD in ethyl acetate and incubating for 30 minutes at room temperature in the dark. A second aliquot of DMEQ-TAD was added and allowed to incubate for an additional 60 minutes.[4] A 40 µL volume of ethanol was added and the derivatized extract was dried, and redissolved in 60 µL of 60:40 (v/v) methanol/water UPLC® mobile phase.

Chromatography conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY UPLC BEH-Phenyl, Å300, 1.7 µm, 2.1 x 50mm (p/n 186002884)
Column temp.:	40 °C
Sample temp.:	4 °C
Injection vol.:	10 µL, full loop mode
Strong wash:	Methanol
Weak wash:	60:40 (v/v) methanol/water
Flow rate:	400 µL/min
Mobile phase	A: 2 mM ammonium acetate plus 0.1% formic acid in water
Mobile phase	B: 2 mM ammonium acetate plus 0.1% formic acid in methanol
Gradient:	Initial conditions were 35:65 (v/v) mobile phase A:mobile phase B at a flow rate of 400 µL/min. Mobile phase B was increased to 90% over 5 minutes using an exponential gradient (curve 8), before returning to starting conditions for 1 minute for a total run time of 6 minutes.

Mass spectrometry conditions

Mass spectrometer:	Xevo TQ-S
Ionization mode:	ESI positive
Capillary voltage:	1.0 kV
Desolvation temp.:	650 °C
Desolvation gas:	1000 L/h
Cone gas:	150 L/h
Acquisition mode:	multiple reaction monitoring (MRM) as shown in Table 1.

Data management

MassLynx® Software v4.1 with TargetLynx™ Application Manager

Compound	Time (min)	MRM *(m/z)*	Cone voltage (V)	Collision energy (eV)
25(OH)D_3	3.8	746.6 > 468.3	80	22
d_3-25(OH)D_3 (IS)	3.8	749.6 > 471.3	80	22
3-epi-25(OH)D_3	3.6	746.6 > 468.3	80	22
25(OH)D_2	4.0	758.6 > 468.3	80	18
d_3-25(OH)D_2 (IS)	4.0	761.6 > 471.3	80	18
24,25$(OH)_2D_3$	2.3	762.6 > 468.3	80	22
d_6-24,25$(OH)_2D_3$(IS)	2.3	768.6 > 468.3	80	22

Table 1. MRM conditions for the analysis of DMEQ-TAD derivatized vitamin D metabolites.

RESULTS AND DISCUSSION

For this clinical research method, derivatization of vitamin D metabolites with DMEQ-TAD offers the advantage of improving ionization efficiency relative to native metabolites. This derivatisation also produces an increase in molecular mass of 336 Da which results in reduced background interference to precursor and product ions.[4] The major characteristic ions for derivatized $25(OH)D_3$, $25(OH)D_2$, and $24,25(OH)_2D_3$, were the protonated species $[M+H]^+$ at *m/z* 746.6, 758.6, and 762.6, respectively (Table 1), and when subjected to collision-induced dissociation under optimized conditions yield A-ring/DMEQ-TAD fragment (*m/z* 468), and DMEQ-TAD fragment (*m/z* 247) moieties as the major products, as shown for the $25(OH)D_3$ adduct in Figure 2. The *m/z* 468 fragment ion was chosen for MRM analysis because of greater selectivity and lower background as compared with *m/z* 247. DMEQ-TAD adducts of the target analytes consisted of 6*R* and 6*S* isomers,[4] of which the more abundant 6*S* isomer was used for quantification.

In a representative serum sample, the 6*S* isomers of the DMEQ-TAD adducts of $25(OH)D_3$, $25(OH)D_2$, and $24,25(OH)_2D_3$ eluted at 3.80, 4.02, and 2.30 minutes respectively as shown in Figure 3. The peak eluting at 3.58 co-migrated with synthetic 3-epi-$25(OH)D_3$, characterized by a single broad peak suggesting co-elution of the 6*R* and 6*S* isomers for this analyte. 3-epi-$25(OH)D_3$ is a known isobar of $25(OH)D_3$. Since these two analytes were chromatographically resolved, the presence of 3-epi-$25(OH)D_3$ did not confound $25(OH)D_3$ measurement using the current system.

Over the calibration range of $25(OH)D_3$ (3.8-148 ng/mL), $25(OH)D_2$ (4.9-120 ng/mL), and $24,25(OH)_2D_3$ (0.4-11.6 ng/mL) the method was shown to be linear for each analyte giving representative r^2 values of at least 0.997 (Figure 4). Lower limits of quantification as defined by signal-to-noise ratios of ≥10, were estimated to be within the 0.1-0.2 ng/mL range and lower limits of detection

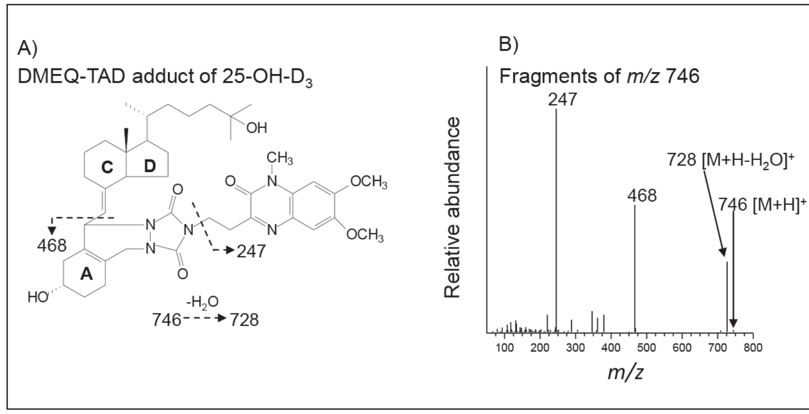

Figure 2. Proposed fragmentation pattern of $25(OH)D_3$ derivatized with DMEQ-TAD.

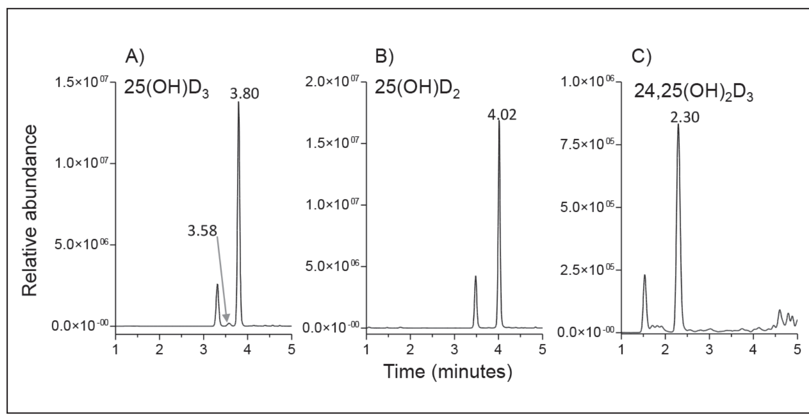

Figure 3. MRM analysis of vitamin D metabolites in a human serum sample.

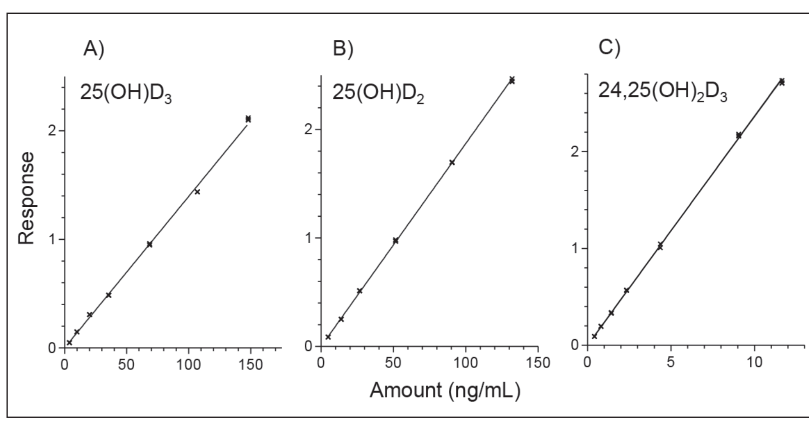

Figure 4. Representative in-serum calibration curves for the target vitamin D metabolites.

(signal-to-noise ≥3) were estimated to be as low as 0.04 ng/mL. Within-run and between-run imprecision was determined by analyzing five replicates of a serum sample on each of 14 assay days. Mean within-run CVs for the target analytes ranged from 3-4% and between-run CVs ranged from 4-7% (Table 2). For $25(OH)D_3$ and $25(OH)D_2$, method accuracy was assessed by analyzing serum samples distributed by the Vitamin D External Quality

Assessment Scheme (DEQAS) on each assay day. Mean discrepancies from the all-laboratory trimmed mean, and LC-MS/MS method mean were - 2% and -5% respectively based on 77 DEQAS samples analyzed over 14 days of analysis.

	$25(OH)D_3$ 22 ng/mL	$25(OH)D_2$ 34 ng/mL	Total 25(OH)D 56 ng/mL	$24,25(OH)_2D_3$ 2.5 ng/mL
Within-run CV (%)	3.7	2.9	2.6	4.0
Between-run CV (%)	4.2	6.9	5.1	5.6

Table 2. Within run (N=5 replicates) and between run (N=14 days) imprecision for the target analytes.

SUMMARY

This method demonstrates that $24,25(OH)_2D_3$ can be reliably measured using the Waters ACQUITY UPLC System and Xevo TQ-S Mass Spectrometer. A robust and analytically sensitive method has been developed, using small sample volumes, that is well suited to clinical research studies.

References

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