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Analysis of multiple vitamin D metabolites by ultra performance supercritical fluid chromatography-tandem mass spectrometry (UPSFC-MS/MS)

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Key words: Vitamin D; UPSFC-MS/MS; supercritical fluid chromatography-tandem mass spectrometry

Abstract

In recent years, increased interest in the human health benefits of vitamin D has led to demand for improved analysis of patient vitamin D 'status'. Studies to date have focused primarily on a single vitamin D metabolite, 25-hydroxyvitamin D, despite the existence of a broad range of vitamin D metabolites, referred to as the vitamin D metabolome. This study reports on the development of a rapid UPSFC-MS/MS method for the analysis of nine vitamin D metabolites in human serum. Optimum separation was obtained with a Lux-Cellulose chiral column. We observed an orthogonal elution order when compared with ultra high performance liquid chromatography (UHPLC). The order of elution was reversed based on hydroxyl- group number, however elution order did not differ between isomeric changes in hydroxyl- group position or epimers. Although UPSFC yielded superior resolution and selectivity over previously developed UHPLC-MS/MS methods, improvements in sensitivity could not be achieved owing to the lower injection volume required for UPSFC relative to UHPLC. Method validation was performed on the developed UPSFC-MS/MS method and found to be within acceptable limits. Applying the method to the analysis of human serum samples showed a significant correlation with serum concentrations of metabolites measured by UHPLC-MS/MS (25OHD3 r=0.997, P=<0.001, and 3-epi-25OHD3 r =0.996, P=<0.001). These data indicate that UPSFC provides an efficient analytical platform for rapid analysis of multiple vitamin D metabolites from serum.

1. Introduction

Vitamin D plays a vital role in calcium homeostasis and bone health, but it has also been reported to have extra-skeletal actions, including roles in muscle function, immunity, pregnancy and cardiovascular disease [1-4]. Studies of these new facets of vitamin D in human health have dramatically increased the demand for analysis of vitamin D 'status' for research and clinical laboratories. This, in turn, has prompted new strategies for improved analysis of vitamin D in biological tissues [5-7]. In particular, there is now increasing interest in the measurement of multiple vitamin D metabolites to better define optimal vitamin D levels for individual patients.

In humans, vitamin D is derived primarily from photolytic conversion of 7-dehydrocholesterol to vitamin D3 in the skin [8]. Another form of vitamin D, vitamin D2, is derived from dietary sources [9]. Endocrine metabolism of vitamin D involves an initial hydroxylation step in the liver to form 25-hydroxyvitamin D (25OHD), which is then further hydroxylated to the active form of vitamin D; 1α,25-dihydroxyvitamin D ($1\alpha,25(OH)_2D$). This step occurs primarily in the kidneys but can also occur at extra-renal sites [10]. The precursors of $1\alpha,25(OH)_2D$ are thought to be inactive, storage and transported forms of vitamin D, whilst $1\alpha,25(OH)_2D$ generates a biological response through nuclear receptor binding in target tissues [11-14]. Other metabolites produced from 25OHD include $24R,25(OH)_2D3$ which is thought to be inactive. Epimerisation of 25OHD2 and 25OHD3 occurs to form C3-epimers, 3-epi-25OHD [5, 15]. The physiological role of the C3-epimer metabolites is not well established, however it does not have the biological activity of 25OHD converted to $1\alpha,25(OH)_2D$ [5, 16, 17].

All vitamin D metabolites share a cyclopentanoperhydrophenanthrene ring structure [11]. The physiological function of 1α,25$(OH)_2$D is determined by the number and position of hydroxyl groups. Owing to the structural similarities between metabolites, one of the key challenges in the quantitation of multiple vitamin D analytes by mass spectrometry is the ability to separate and gain good resolution by chromatographic methods. Liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) methods have achieved the necessary resolution for accurate analysis of multiple vitamin D analytes, including separating 25OHD from 3-epi-25OHD and 1α,25$(OH)_2$D3 from 24,25$(OH)_2$D3 [6, 18, 19]. Whilst UHPLC-MS/MS can provide the necessary resolution and sensitivity for analytical methods, this platform does not achieve the resolution power of gas chromatography mass spectrometry (GC-MS), however the sensitivity achieved by GC-MS is generally not as high as UHPLC-MS/MS to provide lower levels of measurements. Supercritical fluid chromatography (SFC) is an alternative separation technique to liquid and gas chromatography which applies the use of supercritical fluid in the mobile phase, which can improve the resolution of analytical methods as supercritical fluids have diffusivity properties of a gas whilst maintaining a low viscosity [20, 21]. The application of SFC with a tandem mass spectrometer therefore has the potential to combine the resolving power of gas chromatography whilst maintaining low detection limits. The Waters Ultra-Performance Convergence Chromatography™ (UPC^2) system is an application of SFC which uses supercritical CO_2 with a co-solvent as mobile phases [20]. We have previously shown that UPSFC-MS/MS yields improved resolution of multiple steroid metabolites when compared to UHPLC-MS/MS [20]. Although, the separation of vitamin D metabolites using SFC has previously been described using a SFC-TOF/MS system, to the best of our knowledge no UPSFC-MS/MS methods for the analysis of multiple vitamin D metabolites been developed and applied to date. It is anticipated that detection limits could be enhanced using SFC-

MS/MS compared with SFC-TOF/MS [9]. The aim of this study was therefore to develop a UPSFC-MS/MS method to measure multiple vitamin D metabolites and to compare the resolving power, run time and sensitivity of the resulting method to that of a previously established UHPLC-MS/MS method [6].

2. Materials and methods

2.1 Materials

Vitamin D analytical reference standards (vitamin D3, vitamin D2, 25OHD3, 25OHD2, 3-epi-25OHD3, 1α,25$(OH)_2$D3, 24R,25$(OH)_2$D3 and 23R,25$(OH)_2$D3), deuterated internal standards (25OHD3-d_3, 3-epi-25OHD3-d_3 and 1α,25$(OH)_2$D3-d_3) and 4-phenyl-1,2,4-triazoline-3,5-dione were purchased from Sigma Aldrich (Pool, UK). Lux cellulose-3 chiral columns (100 mm, 2 mm, 3 µm) and (150 mm, 3 mm, 3 µm) were purchased from Phenomenex (Macclesfield, UK). Waters UPC^2 BEH column (100 mm, 3 mm, 1.7 µm), UPC^2 fluoro phenyl column (150 mm, 2.1 mm, 1.7 µm) and a 2 µm in line filter were purchased from Waters Corporation (Manchester, UK). Food grade CO_2 was purchased from BOC, UK and LC-MS grade methanol and formic acid was purchased from Greyhound Chromatography (Merseyside, UK).

Sample preparation

Vitamin D standards were purchased as stock solutions in ethanol. Working solutions were prepared in methanol at µg/mL concentration ranges (1-5 µg/mL) for preparation of standard curve concentrations and quality controls. Stock solutions were stored at -80 ^oC and working standards at -20 ^oC in amber salinized vials.

Serum samples were extracted as previously described using supportive liquid-liquid extraction (SLE) [6], with slight modification of using 125 µL methanol as reconstitution solvent. A derivatization reaction was performed using PTAD and was based on reactions previously described [22-25]. Following the SLE elution, 200 µL 0.5 mg/mL PTAD in acetonitrile was added to each sample and the reaction occurred at ambient temperature for 2 hours. The reaction was quenched by the addition of 20 µL water and vortexed before drying down with nitrogen and reconstituting with 125 µL methanol for analysis.

2.2 UPSFC-MS/MS

A Waters ACQUITY UPC^2 coupled to a Waters Xevo TQ-MS mass spectrometer (Waters Corporation, Milford, USA) was used for analysis. A Lux cellulose-3 chiral column (150 mm, 3 mm, 3 µm) with an ACQUITY UPLC HSS PFP VanGuard Pre-column (1.8 µm, 2.1 mm x 5 mm) and UPC^2 BEH column (100 mm, 3 mm, 1.7 µm) were used to separate underivatized and derivatized vitamin D metabolites respectively. The mobile phase was CO_2 and 0.1% FA in methanol with a make-up solvent of 0.1% FA, the gradient is described in **Table 1.** The back pressure was set by the automated back pressure regulator (ABPR) set at 1750 PSI. The column temperature and flow rate were set at 20 ^oC and 1.6 mL/min, respectively, for both methods.

Multiple reaction monitoring (MRM) mode was used for quantifying vitamin D metabolites using positive electrospray ionisation mode (ESI +). Capillary voltage was set to 3.8 kV and desolvation gas flow to 1000 L h^{-1}. The source temperature and desolvation temperature were set at 120 ^oC and 400 ^oC respectively when measuring underivatized analytes, and 150 ^oC and 500 ^oC when measuring derivatized 1α,25(OH)$_2$D3. The MRM transitions, cone voltage and collision energies for each analyte are displayed in **Table 2**. An inlet flow rate of 0.1 mL/min

was applied to a make-up pump for the flow of 0.1% formic acid prior to the mass spectrometer. Data analysis was performed using MassLynx V4.1.

2.3 Method validation

The optimised UPSFC-MS/MS method was subjected to method validation which was performed based on US Food and Drug Administration (FDA) guidelines [26]. The method parameters assessed where accuracy, precision, lower limit of quantitation (LLOQ), lower limit of detection (LLOD), linearity and selectivity. Vitamin D depleted charcoal stripped serum (Golden West Biologicals Inc.), certified for vitamin D UHPLC-MS/MS applications was used as a negative control matrix for validation experiments. Reference concentrations of vitamin D standards and internal standards were added to the negative control matrix to prepare calibration series and quality control (QC) standards.

The analysis of 41 routine serum samples was performed using the optimised UPSFC-MS/MS method and using a UHPLC-MS/MS method which has previously been developed for the analysis of vitamin D metabolites [6] to inform of the standardisation of measurements between platforms. Serum samples for this analysis were collected as described previously [6].

2.4 Data analysis

Data analysis was performed on Waters MassLynx software. SPSS statistics software was used for regression analysis and Bland-Altman plots for comparing measured concentrations in serum between methods.

3. Results

3.1 Optimisation of UPSFC conditions

Full description of optimisation of the UPSFC-MS/MS method is described in the **supplemental material**. The optimised method achieved separation and quantified 9 vitamin D metabolites and three deuterated internal standards. Optimal separation was achieved using a Lux Cellulose-2 chiral column (150 x 3mm, 3µm) at a flow rate of 1.6 mL/min, heated at 20 ^{o}C, which enabled an injection volume of 7 µL. Other optimised method parameters were make-up rate of 0.1 mL/min and ABPR set at 1750 psi. The run time of the method was 6 minutes.

An overall observation of this method compared with UHPLC-MS/MS was that the elution order of the UPSFC-MS/MS methods was reversed based on hydroxyl group number. UHPLC has previously separated metabolites based primarily on polarity [6]. The elution order observed by UHPSFC-MS/MS could be explained by hydrogen bonding and dipole-dipole interactions [17]. Under UPSFC conditions the stationary phase can act as acceptor of hydrogen bonds with the hydroxyl groups from vitamin D analytes. Hence, vitamin D analytes with greater number of hydroxyl are retained on column for longer. However, the order of elution between sub-groups of metabolites 25OHD3 and 3-epi-25OHD3, along with 24OHD2 and 25OHD2 was the same on both UPSFC and UHPLC platforms. Comparison of chiral column elution order between UPSFC and UHPLC is illustrated on **Figure 1**. Chromatograms of UHPLC and UPSFC-MS/MS methods are shown in **Figure 2**.

An optimised derivatization method for more sensitive quantitation of 1α,25$(OH)_2$D3 was achieved using a UPC^2 BEH column (100 mm, 3 mm, 1.7 µm) with a flow rate of 1.6

mL/min, heated at 20 ^{o}C. The make-up flow rate and ABPR were optimised at 0.1 mL/min and 1750 psi, respectively.

3.2 Optimisation of Mass spectrometry conditions

The UPSFC mobile phase was combined with make-up solvent from the make-up pump prior to ionisation. Optimal ionisation was achieved using make-up solvent of methanol with 0.1% FA at a flow rate of 0.1 mL/min. MRM transitions were determined by combined infusion into the MS using UHPLC-MS/MS. MRM transitions for PTAD derivatized analytes were determined by running full and daughter scans using UPSFC-MS/MS, followed by determining optimised cone voltage and collision energies by individual sample injections. The abundant ions using 400 ^{o}C desolvation temperature and 120 ^{o}C source temperature were $[M+H]^+$, apart from 24OHD2 and 1α,25$(OH)_2$D3 which were $[M-H_2O+H]^+$. PTAD derivatized abundant ions were $[M-H_2O+H]^+$ when the desolvation temperatures was increased to 500 ^{o}C.

3.3 Validation

Accuracy, precision and matrix effects are displayed in **Table 3**. The accuracy and precision values were within the 15% acceptable range of variability for medium and high concentration ranges and were within 20% variation at the low concentration. Analysis of matrix effects revealed little ion suppression or enhancement by UPSFC-MS/MS analysis. Ion suppression ranged from 0.9-5.8%, while ion enhancement was only observed in a low and high concentration sample of 3-epi-25OHD3, which was 2.5 and 9.8% respectively.

3.4 UPSFC Separation

The novel UPSFC-MS/MS method achieved the required separation to accurately quantify the vitamin D metabolites. Compounds of equal mass were resolved at baseline. The resolution factors obtained for pairs of analytes with equal mass are displayed in **Supplemental Table 2**. UPSFC achieved superior resolution factors for analytes of equal mass, apart from separation between 25OHD3 and 3-epi-25OHD3 which had a greater resolution on UHPLC. Nonetheless, resolution on both platforms between these analytes was above the recommended value of 2. It had not previously been possible to achieve baseline separation (resolution factor 0.95) between 25OHD2 and 24OHD2 when developing UHPLC-MS/MS methods manually or using automated method development software [6, 27] (**figure 2b**). However, baseline separation of 25OHD2 and 24OHD2 (resolution factor 2.5) was achieved using the UPSFC with a Lux-Cellulose 2 chiral column (**Figure 2a)**.

Comparison of elution order of vitamin D analytes on a Lux Cellulose chiral column between UPSFC and UHPLC revealed a reversal in elution. This was based on hydrogen bonding of the hydroxyl groups with the stationary phase using UPSFC, whereas elution order was based on polarity with UHPLC (**Figure 1**). The increase methanol mobile phase in UHPLC eluted dihydroxy metabolites followed by single hydroxyl metabolites and vitamin D analogues. However, this was reversed on UPSFC, the vitamin D analogues initially eluted, followed by single hydroxyl metabolites and dihydroxy metabolites with increased methanol mobile phase. The elution order between isomers, 25OHD3 and 3-epi-25OHD3; 24OHD2 and 25OHD2, was the same on both platforms, although there was a difference in resolving power between isomers.

3.5 Performance of UPSFC-MS/MS and comparison with UHPLC-MS/MS

Analysis of 41 routine human serum samples was performed on both UPSFC-MS/MS and UHPLC-MS/MS to assess the standardisation between methods and determine potential method bias with either separation method. Regression analysis and deviation between methods for 25OHD3 and 3-epi-25OHD3 and 25OHD2 measurements is displayed in **Figure 3**. Comparison of methods for 25OHD3 (r =0.997, P=<0.001) and 3-epi-25OHD3 (r =0.996, P=<0.001) showed a significant correlation between measurements. Measured values of 25OHD3 between methods gave a mean difference of 1.41% (95% CI 3.576 - -6.410) bias towards the measurements by UPSFC-MS/MS, however this was not significant (t =1.907, P =0.064). There was a mean bias of 0.61% (95% CI 6.087 - -4.876) towards the UHPLC-MS/MS measurements of 3-epi-25OHD3 which was found to not be significant (t =1.065, P=0.293). Previous reported concentrations of 1α,25$(OH)_2$D3 range between 20-120 pg/mL [5]. The LLOQ of 1α,25$(OH)_2$D3 for this method when derivatized with PTAD was 80 pg/mL, hence the routine serums run as part of this method could not be quantified. It is anticipated that with future work applying this method on a later generation mass spectrometer would achieve the required detection limits to routinely quantify in serum.

4. Discussion and conclusions

Development of a novel UPSFC-MS/MS method described above has enabled the comparison of established UHPLC-MS/MS method for quantifying multiple vitamin D metabolites. The optimised UPSFC method achieved the required separation over a 6 minute run time, separating chiral metabolites 23,25(OH)2D3, 24,25$(OH)_2$D3 and 1α,25$(OH)_2$D3 along with the C3-epimer 3-epi-25OHD3 from 25OHD2 and 24OHD2 from 25OHD2. Separation achieved by UPSFC was overall superior compared to UHPLC, whilst reducing the run time to 6 minutes compared with 8 minutes required for UHPLC separation. This

method demonstrated the ability of UPSFC-MS/MS to resolve and quantify structurally similar metabolites, specifically where the position or bond direction of a hydroxyl group is the only structural difference, utilizing hydrogen bonding of the analytes with the stationary phase. This improved resolution has also been shown previously for other endocrine androgen metabolites [20].

Previous methods for vitamin D using UHPLC have enabled injection volumes of 20 µL whilst maintaining symmetrical peak shape. A limitation whilst developing this method was maximum injection volume which was limited dependant on column length and width. A maximum injection volume of 7 µL could be achieved with high injection volumes above 7 µL resulting in peak saturation **Supplemental Figure 4**. As less analyte could be injected onto the column on the UPSFC-MS/MS, the achieved limits of quantitation were less than observed for the UHPLC-MS/MS when compared on the same Waters Xevo TQ-MS mass spectrometer. Owing to the low endogenous levels of some metabolites in the vitamin D pathway it was not possible to quantify the metabolites 1α,25$(OH)_2$D3, 23,25$(OH)_2$D3 or 24OHD2 under current conditions. To achieve the required sensitivity necessary for routine serum analysis of these analytes by UPSFC, analysis in tandem with a later generation mass spectrometer is required. However, the reduced injection volume enables additional repeat injections from the same samples, to allow monitoring of sample reproducibility.

The UPSFC-MS/MS method described was developed as an alternative approach to UHPLC-MS/MS methods for measuring multiple metabolites of vitamin D in clinical applications to determine changes in vitamin D metabolism. The use of UPSFC has clear advantages over LC for routine analysis including higher throughput and improved separation for accurate quantitation. Matrix effects analysis ensured no signal interferences altering measured values.

The use of supercritical CO_2 is also advantageous owing to reduced costs and environmental effects compared with organic solvents used for reversed phase LC [20, 28]. This approach to analysis would be beneficial in a number of method development facilities which involve high throughput analysis separating structurally similar metabolites. However, measuring analytes at low concentration levels (pg/mL) will likely require latest generation mass spectrometry platforms or structural modifications to improve ionisation such as derivatization owing reduced sample injection volumes for UPSFC application columns.

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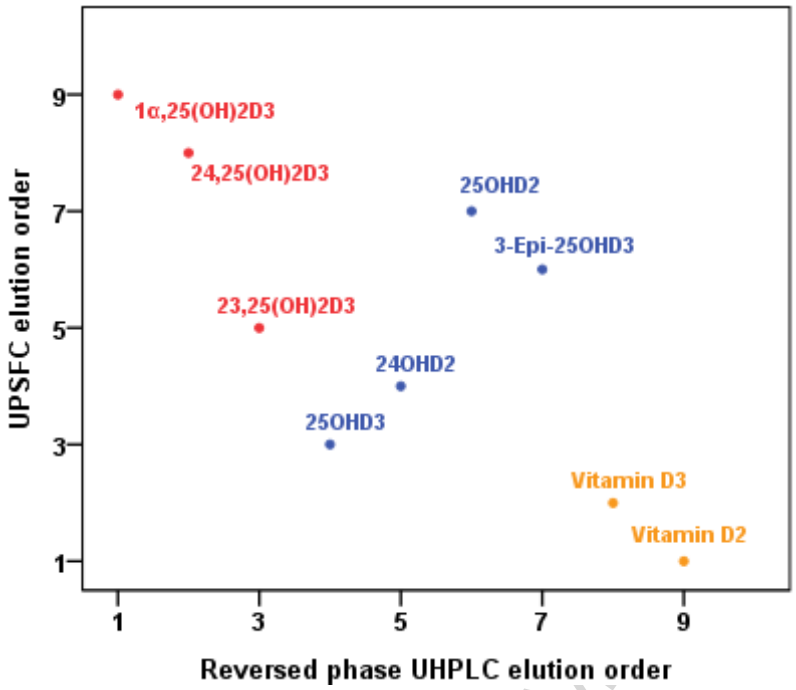

Figure 1: Elution order depicted between UPSFC and UHPLC.

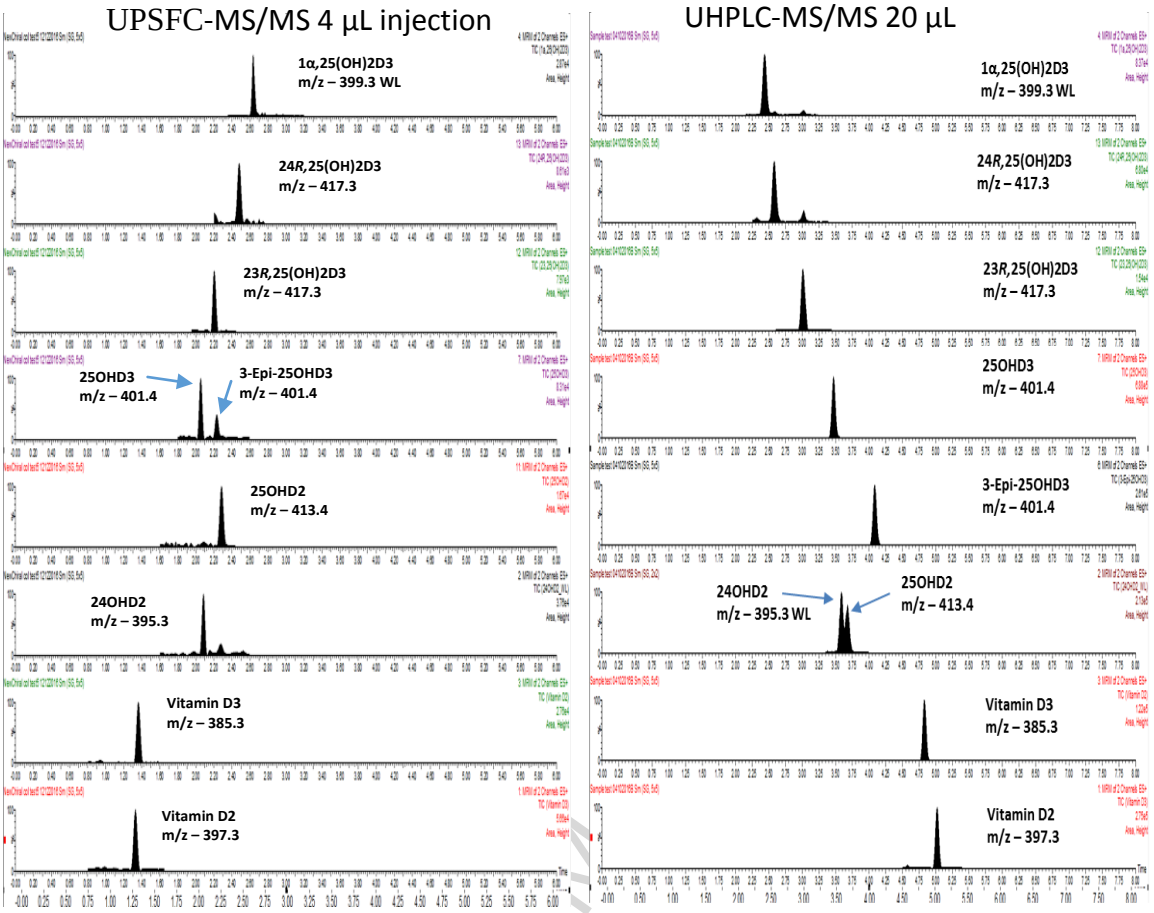

Figure 2: Chromatographic separation of vitamin D metabolites by UPSFC-MS/MS and UHPLC-MS/MS.

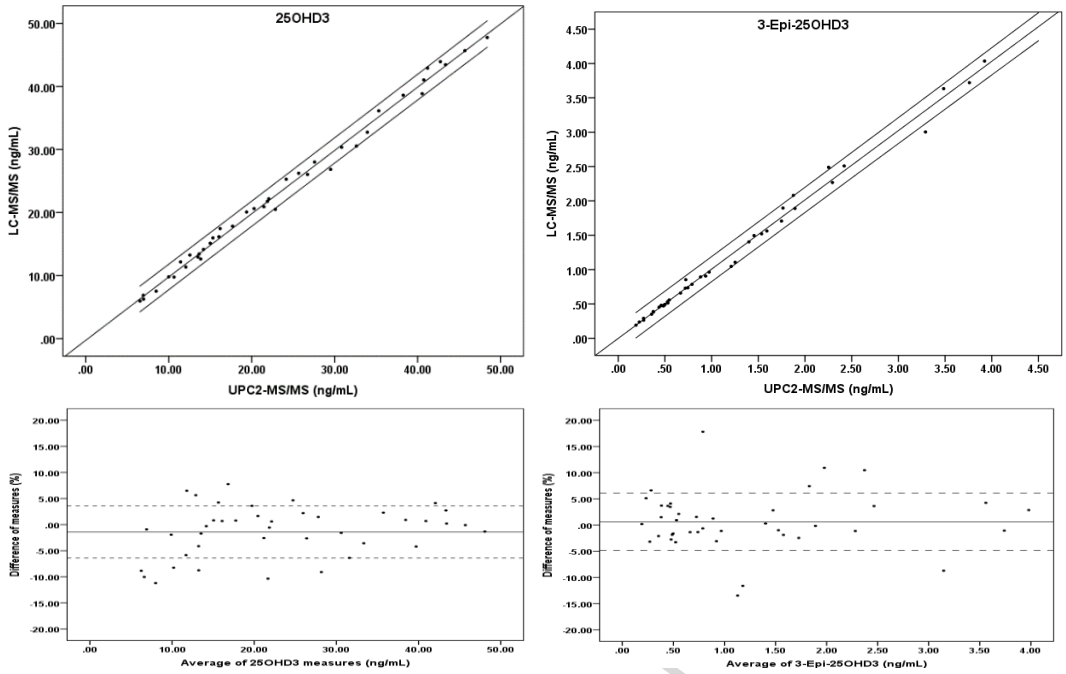

Figure 3: Regression analysis of the UPSFC-MS/MS and UHPLC-MS/MS methods for 25OHD3 and 3-epi-25OHD3 concentrations analysed with a cohort of serum samples (n=41). The regression line and 95% confidence intervals are represented. Bland Altman plots for the comparison of UHPLC-MS/MS and UPSFC-MS/MS for analysis of 25OHD3 and 3-epi-25OHD3, showing the mean bias (solid line) and ±SD (dotted lines).

Table 1 – Mobile phase gradients for two analytical methods.

Underivatized vitamin D	Mobile phase A	Mobile phase B
Run time (minutes)	CO_2 (%)	Methanol (%)
0	95.0	5.0
2.8 - 3.0	73.0	27.0
4.5 - 6.5	95.0	5.0
Derivatized $1\alpha,25(OH)_2D3$		
Run time (minutes)	CO_2 (%)	Methanol (%)
0	95.0	5.0
2.0 - 2.2	60.0	40.0
2.7 - 3.5	95.0	5.0

Table 2 - MRM transitions of vitamin D metabolites, mass spectrometry parameters and retention time.

Abbrev.	Name	Mass transitions	Cone voltage	Collision energy	Retention Time (min) UPSFC-MS/MS	Retention Time (min) UHPLC-MS/MS
25OHD3	25-Hydroxyvitamin D3	**401.4 > 135.1** 401.4 > 151.1	18 18	22 12	2.06	3.47
25OHD2	25-Hydroxyvitamin D2	**413.4 > 105.0** 413.4 > 353.3	16 16	40 8	2.38	3.68
24OHD2	24-Hydroxyvitamin D2	**395.3 > 119.1** 395.3 > 269.1	26 26	26 22	2.18	3.57
3-Epi-25OHD3	3-Epi-25-hydroxyvitamin D3	**401.4 > 159.1** 401.4 > 365.3	18 18	26 10	2.24	4.10
1α,25(OH)$_2$D3	1α,25-Dihydroxyvitamin D3	**399.3 > 135.1** 399.3 > 151.1	24 24	18 24	2.64	2.43
24R,25(OH)$_2$D3	24,25-Dihydroxyvitamin D3	**417.4 > 121.1** 417.4 > 381.3	16 16	18 10	2.48	2.57
23R,25(OH)$_2$D3	23,25-Dihydroxyvitamin D3	**417.3 > 325.2** 417.3 > 121.1	16 16	10 24	2.21	3.01
Vitamin D2	Vitamin D2	**397.3 > 69.0**	20	22	1.33	5.02

		397.3 > 104.5	20	32		
Vitamin D3	Vitamin D3	**385.3 > 105.0** 385.3 > 259.2	20 20	40 14	5.02	4.84
25OHD3-d3	25-Hydroxyvitamin D3-d3	**404.4 > 135.1** 404.4 > 151.1	16 16	12 16	2.06	3.47
3-Epi-25OHD3-d3	3-Epi-25-hydroxyvitamin D3-d3	**404.4 > 159.1** 404.4 > 365.3	16 16	22 10	2.24	4.10
1α,25$(OH)_2$-d3	1α,25-Dihydroxyvitamin D3-d3	**402.3 > 135.1** 402.3 > 151.1	20 20	18 20	2.64	2.43
Vitamin D2-d3	Vitamin D2-d3	**400.3 > 69.0** 400.3 > 104.5	20 20	32 16	1.33	5.02
1α,25$(OH)_2$D3-PTAD	1α,25-Dihydroxyvitamin D3-4- Phenyl-1,2,4-triazoline-3,5-dione	**574.3 > 314.1** 574.3 > 298.2	22 22	16 16	1.87	3.95
24R,25$(OH)_2$D3-PTAD	24,25-Dihydroxyvitamin D3- Phenyl-1,2,4-triazoline-3,5-dione	**574.3> 298.2** 574.3> 279.9	22 20	18 28	2.00	3.76
1α,25$(OH)_2$D3-d3-PTAD	1α,25-Dihydroxyvitamin D3-4- Phenyl-1,2,4-triazoline-3,5-dione	**577.3> 317.1** 577.3> 301.2	16 20	15 16	1.87	3.95

Table 3 - Method validation data.

Compound	Conc. (ng/mL)	Level	Precision (%) RSD		Accuracy (%)	Matrix effect %	
			Intra-day N=6	Inter-day N=18	N=6	Conc (ng/mL)	N=3
25OHD3	5.0	Low	8.78	15.9	99.5	5.0	-1.0
	20.0	Medium	5.28	7.5	87.2	25.0	-2.8
	100.0	High	4.05	4.2	99.0	100.0	-2.3
3-Epi-25OHD3	1.00	Low	11.8	9.8	99.1	5.0	2.5
	5.00	Medium	8.3	6.8	98.4	25.0	-5.8
	25.00	High	6.2	4.0	91.6	100.0	9.8
$1\alpha,25(OH)_2D3$-PTAD	0.70	Low	6.0	7.6	93.5	0.50	-0.5
	1.40	Medium	2.5	4.6	97.8	2.50	-3.0
	3.00	High	2.7	4.7	100.3	8.0	-1.1
$24R,25(OH)_2D3$	0.70	Low	3.9	9.4	92.5	2.0	-1.0
	1.40	Medium	6.8	13.0	95.8	3.0	-0.9
	3.00	High	5.5	10.6	100.9	7.0	-1.0
25OHD2	1.00	Low	8.7	10.3	123.2		
	5.00	Medium	10.0	13.2	104.3	10.0	-1.5
	25.0	High	9.1	11.5	97.4	100.0	-3.8
24OHD2	0.80	Low	7.7	6.9	102.1	1.0	-2.1
	4.00	Medium	8.0	9.2	101.9	5.0	-2.4
	20.00	High	8.2	8.4	103.9	50.0	-3.2

Highlights

- High throughput UPSFC-MS/MS to measure multiple vitamin D metabolites.
- Order of elution was reversed compared with LC-MS/MS based on hydroxyl group number.
- Chiral column separation enabled separation of analytes on UPSFC.
- Analysis of serum samples shows correlation between UPSFC-MS/MS and LC-MS/MS for routine serum measurements for 25OHD3 measurements.