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Development of 2D-LC/MS/MS Method for Quantitative Analysis of 1α,25-Dihydroxylvitamin D3 in Human Serum

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Introduction

Developments of LC/MS/MS methods for accurate quantitation of low pg/mL levels of 1α,25-dihydroxy vitamin D2/D3 in serum were reported in recent years, because their levels in serum were found to be important indications of several diseases associated with vitamin D metabolic disorder in clinical research and diagnosis [1]. However, it has been a challenge to achieve the required sensitivity directly, due to the intrinsic difficulty of ionization of the compounds and interference from matrix [2,3]. Sample extraction and clean-up with SPE and immunoaffinity methods were applied to remove the interferences [4] prior to LC/MS/MS analysis. However, the amount of serum required was normally rather high from 0.5mL to 2mL, which is not favourite in the clinical applications. Direct analysis methods with using smaller amount of serum are in demand. Research efforts have been reported in literatures to enhance ionization efficiency by using different interfaces such as ESI, APCI or APPI and ionization reagents to form purposely NH3 adduct or lithium adduct [4,5]. Here, we present a novel 2D-LC/MS/MS method with APCI interface for direct analysis of 1α,25-diOH-VD3 in serum. The method achieved a detection limit of 3.1 pg/mL in spiked serum samples with 100 uL injection.

Experimental

High purity 1α,25-dihydroxyl Vitamin D3 and deuterated 1α,25-dihydroxyl-d6 Vitamin D3 (as internal standard) were obtained from Toronto Research Chemicals. Charcoal-stripped pooled human serum obtained from Bioworld was used as blank and matrix to prepare spiked samples in this study. A 2D-LC/MS/MS system was set up on LCMS-8050 (Shimadzu Corporation) with a column switching valve installed in the column oven and controlled by LabSolutions workstation. The details of columns, mobile phases and gradient programs of 1st-D and 2nd-D LC separations and MS conditions are compiled into Table 1. The procedure of sample preparation of spiked serum samples is shown in Figure 1. It includes protein precipitation by adding ACN-MeOH solvent into the serum in 3 to 1 ratio followed by vortex and centrifuge at high speed. The supernatant collected was filtered before standards with IS were added (post-addition). The clear samples obtained were then injected into the 2-D LC/MS/MS system.

Table 1: 2D-LC/MS/MS analytical conditions

LC condition

Column	1st D: FC-ODS (2.0mmI.D. x 75mm L, 3μm) 2nd D: VP-ODS (2.0mmI.D. x 150mm L, 4.6μm)
Mobile Phase of 1st D	A: Water with 0.1% formic acid B: Acetontrile
Mobile Phase of 2nd D	C: Water with 0.1% formic acid D: MeOH with 0.1% formic acid
1st D gradient program & flow rate	B: 40% (0 to 0.1min) → 90% (5 to 7.5min) → 15% (11 to 12min) → 40% (14 to 25min); Total flow rate: 0.5mL/min
2nd D gradient program & flow rate	D: 15% (0min) → 80% (20 to 22.5min) → 15% (23 to 25min); Peak cutting: 3.15 to 3.40; Total flow rate: 0.5 mL/min
Oven Temp.	45°C
Injection Vol.	100 uL

MS Interface condition

Interface	APCI, 400°C
MS mode	Positive, MRM
Heat Block & DL Temp.	300°C & 200°C
CID Gas	Ar (270kPa)
Nebulizing Gas Flow	N2, 2.5 L/min
Drying Gas Flow	N2, 7.0 L/min

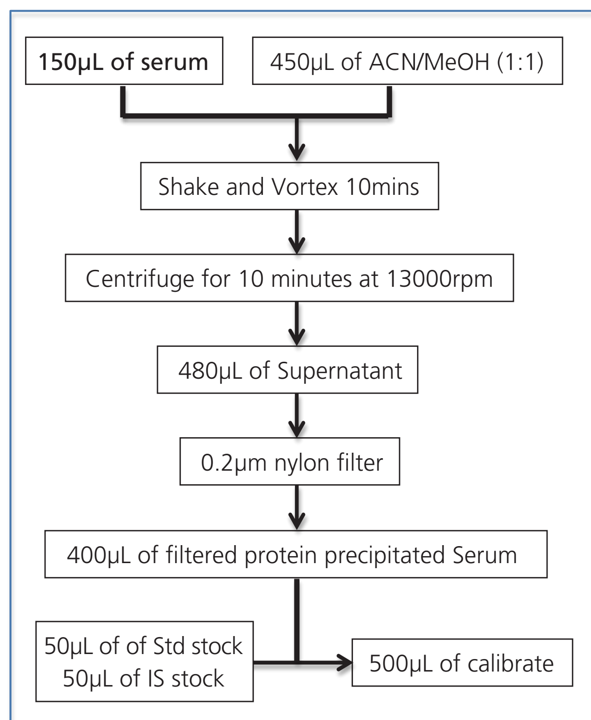

Figure 1: Flow chart of serum sample pre-treatment method

Results and Discussion

Development of 2D-LC/MS/MS method

An APCI interference was employed for effective ionization of 1α,25-diOH-VitD3 ($C_{27}H_{44}O_3$, MW 416.7). A MRM quantitation method for 1α,25-diOH-VitD3 with its deuterated form as internal standard (IS) was developed. MRM optimization was performed using an automated MRM optimization program with LabSolutions workstation. Two MRM transitions for each compound were selected (Table 2), the first one for quantitation and the second one for confirmation. The parent ion of 1α,25-diOH-VitD3 was the dehydrated ion, as it underwent neutral lost easily in ionization with ESI and APCI [2,3]. The MRM used for quantitation (399.3>381.3) was dehydration of the second OH group in the molecule.

Table 2: MRM transitions and CID parameters of 1α,25-diOH-VitD3 and deuterated IS

Name	RT[1] (min)	Transition (m/z)	CID Voltage (V)		
			Q1 Pre Bias	CE	Q3 Pre Bias
1α,25-dihydroxyl Vitamin D3	22.74	399.3 > 381.3	-20	-13	-14
		399.3 > 157.0	-20	-29	-17
1α,25-dihydroxyl-d6 Vitamin D3 (IS)	22.71	402.3 > 366.3	-20	-12	-18
		402.3 > 383.3	-20	-15	-27

1, Retention time by 2D-LC/MS/MS method

Development of 2D-LC/MS/MS Method for Quantitative Analysis of 1α,25-Dihydroxylvitamin D3 in Human Serum

The reason to develop a 2-D LC separation for a LC/MS/MS method was the high background and interferences occurred with 1D LC/MS/MS observed in this study and also reported in literatures. Figure 2 shows the MRM chromatograms of 1D-LC/MS/MS of spiked serum sample. It can be seen that the baseline of the quantitation MRM (399.3>381.3) rose to a rather high level and interference peaks also appeared at the same retention time.
The 2-D LC/MS/MS method developed in this study involves "cutting the targeted peak" in the 1st-D separation precisely (3.1~3.4 min) and the portion retained in a stainless steel sample loop (200 uL) was transferred into the 2nd-D column for further separation. The operation was accomplished by switching the 6-way valve in and out by a time program. Both 1st-D and 2nd-D separations were carried out in gradient elution mode. The organic mobile phase of 2nd-D (MeOH with 0.1% formic acid) was different from that of 1st-D (pure ACN). The interference peaks co-eluted with the analyte in 1st-D were separated from the analyte peak (22.6 min) as shown in Figure 3.

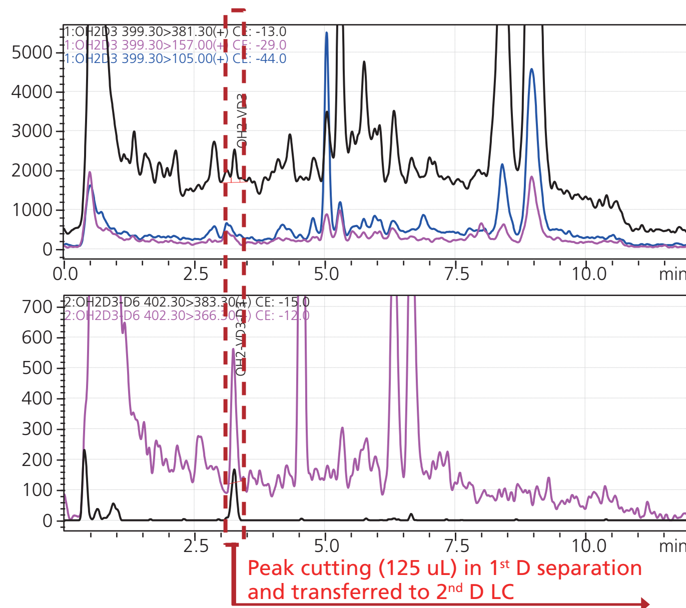

Figure 2: 1D-LC/MS/MS chromatograms of 22.7 pg/mL 1α,25-diOH-VitD3 (top) and 182 pg/mL internal standard (bottom) in serum (injection volume: 50uL)

Calibration curve (IS), linearity and accuracy

Two sets of standard samples were prepared in serum and in clear solution (diluent). Each set included seven levels of 1α,25-diOH-VitD3 from 3.13 pg/mL to 200 pg/mL, each added with 200 pg/mL of IS (See Table 3). The chromatograms of the seven spiked standard samples in serum are shown in Figure 3. A linear IS calibration curve (R2 > 0.996) was established from these 2D-LC/MS/MS analysis results, which is shown in Figure 4. It is worth to note that the calibration curve has a non-zero Y-intercept, indicating that the blank (serum) contains either residual 1 α,25-diOH-VitD3 or other interference which must be deducted in the quantitation method. The peak area ratios shown in Table 3 are the results after deduction of the background peaks. The accuracy of the method after this correction is between 92% and 117%.

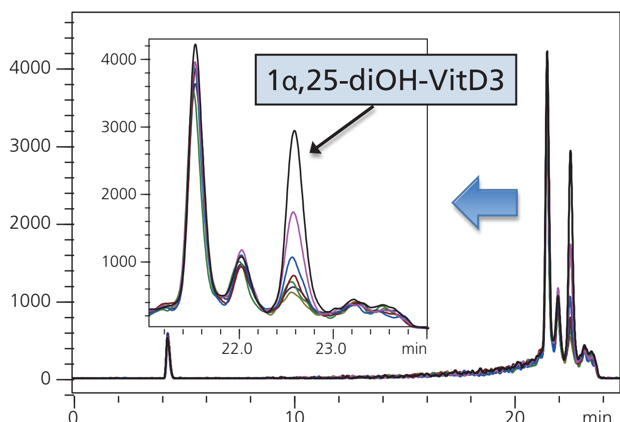

Figure 3: Overlay of 2nd-D chromatograms of 7 levels from 3.13 pg/mL to 200 pg/mL spiked in serum.

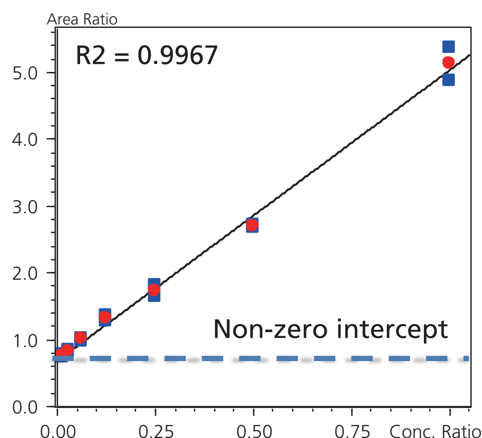

Figure 4: Calibration curves of 1α,25-diOH VD3 in serum by IS method.

Table 3: Seven levels of standard samples for calibration curve and performance evaluation

Conc. Level of Std.	1α,25-diOH VD3 (pg/mL)	Conc. Ratio[1] (Target/IS)	Area Ratio[2] (in serum)	Area Ratio[2] (in clear solu)	Accuracy[3] (%)	Matrix Effect (%)
L1	3.13	0.0156	0.243	0.414	103.8	58.7
L2	6.25	0.0313	0.321	0.481	100.0	66.8
L3	12.5	0.0625	0.456	0.603	117.3	75.6
L4	25.0	0.1250	0.757	0.914	115.9	82.9
L5	50.0	0.2500	1.188	1.354	95.5	87.7
L6	100.0	0.5000	2.168	2.580	92.15	84.0
L7	200.0	1.0000	4.531	4.740	102.0	95.6

1, Target = 1α,25-diOH VD3; 2, Area ratio = area of target / area of IS; 3, Based on the data of spiked serum samples

Matrix effect, repeatability, LOD/LOQ and specificity

Matrix effect of the 2D-LC/MS/MS method was determined by comparison of peak area ratios of standard samples in diluent and in serum at the seven levels. The results are compiled into Table 3. The matrix effect of the method are between 58% and 95%. It seems that the matrix effect is stronger at lower concentrations than at higher concentrations. Repeatability of peak area of the method was evaluated with L2 and L3 spiked serum samples for both target and IS. The Results of RSD (n=6) are displayed in Table 4.

The MRM peaks of 1α,25-diOH VD3 in clear solution and in serum are displayed in pairs (top and bottom) in Figure 5. It can be seen from the first pair (diluent and serum blank) that a peak appeared at the same retention of 1α,25-diOH VD3 in the blank serum. As pointed out above, this peak is from either the residue of 1α,25-diOH VD3 or other interference present in the serum. Due to this background peak, the actual S/N ratio could not be calculated. Therefore, it is difficult to determine the LOD and LOQ based on the S/N method. Tentatively, we propose a reference LOD and LOQ of the method for 1α,25-diOH VD3 to be 3.1 pg/mL and 10 pg/mL, respectively.

The specificity of the method relies on several criteria: two MRMs (399>381 and 399>157), their ratio and RT in 2nd-D chromatogram. The MRM chromatograms shown in Figure 5 demonstrate the specificity of the method from L1 (3.1 pg/mL) to L7 (200 pg/mL). It can be seen that the results of spiked serum samples (bottom) meet the criteria if compared with the results of samples in the diluent (top).

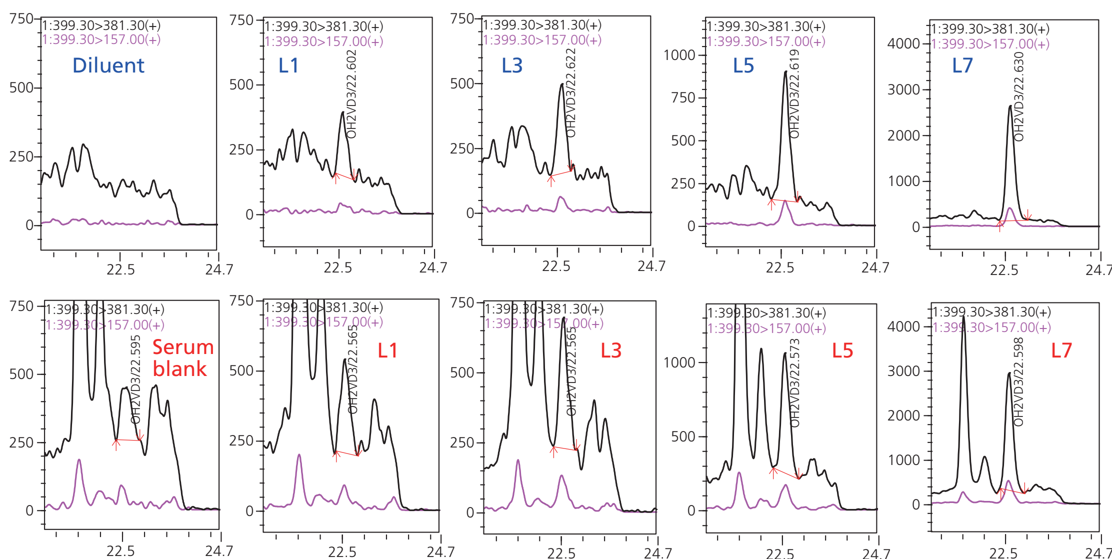

Figure 5: MRM peaks of 1α,25-diOH-VitD3 spiked in pure diluent (top) and in serum (bottom) of L1, L3, L5 and L7 (spiked conc. refer to Table 3)

Table 4: Repeatability Test Results (n=6)

Sample	Compound	Spiked Conc. (pg/mL)	%RSD
L2	1α,25-diOH VD3	6.25	10.10
	IS	200	7.66
L3	1α,25-diOH VD3	12.5	9.33
	IS	200	6.28

Conclusions

A 2D-LC/MS/MS method with APCI interface has been developed for quantitative analysis of 1α,25-dihydroxylvitamin D3 in human serum without offline extraction and cleanup. The detection and quantitation range of the method is from 3.1 pg/mL to 200 pg/mL, which meets the diagnosis requirements in clinical applications. The performance of the method was evaluated thoroughly, including linearity, accuracy, repeatability, matrix effect, LOD/LOQ and specificity. The results indicate that the 2D-LC/MS/MS method is sensitive and reliable in detection and quantitation of trace 1α,25-dihydroxylvitamin D3 in serum. Further studies to enable the method for simultaneous analysis of both 1α,25-dihydroxylvitamin D3 and 1α,25-dihydroxylvitamin D2 are needed.

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