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A Direct LC/MS/MS Method for Quantitative Determination of 25-Hydroxyvitamin D_2 and D_3 in Human Plasma

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Introduction

Vitamin-D is an essential nutrient for human health. Vitamin-D deficiency has been reported among populations, which is potentially a factor to develop chronic diseases. The level of vitamin-D is maintained by its metabolite 25OH-vitamin-D2/D3 in the bloodstream. Vitamin-D deficiency is normally defined as its serum level below 50~75nmol/mL. Therefore, accurate measurement of vitamin D status is required in clinical screening test. In recent year, LC/MS/MS has been used for quantitative analysis of vitamin-D metabolites. However, tedious sample preparation is often required such as extraction, derivatization and clean-up with SPE or immunoaffinity columns. This is to remove matrix interferences and increase sensitivity due to poor ionization of the compounds [1, 3]. Furthermore, the amount of serum/plasma required was rather high at 0.5~2 mL per analysis, which is not favourable in the clinical applications. Here, we present a direct high sensitivity LC/MS/MS method for quantitative determination of 25OH-vitamin-D2/D3 in plasma, achieving LLOQ of 3ng/mL with 10uL injection. The method exhibits good accuracy, reproducibility, linearity and specificity over the concentration range from 1-100 ng/mL.

Experimental

Stock solution of 25-hydroxyvitamin D2 (25-OH VD2) and 25-hydroxyvitamin D3 (25-OH VD3) were purchased from Sigma Aldrich (Cerilliant). The deuterated forms, 25-hydroxyvitamin D2-d6 (25-OH VD2-d6) and 25-hydroxyvitamin D3-d6 (25-OH VD3-d6) were used as internal standards, obtained from TRC (Toronto Research Chemicals). Pooled human plasma was purchased from i-DNA Biotechnology. A Shimadzu LCMS-8050 system was used in this work. The details of column, mobile phases and gradient program of LC separations and MS conditions are compiled into Table 1. The procedure of sample preparation for spiked human plasma samples (pre-spiked) is illustrated in Figure 1. It includes spiking of standards and IS into the plasma and protein precipitation by adding ACN-MeOH solvent into the human plasma followed by vortex and centrifugation at high speed. The supernatant collected was filtered and injected directly into the LC/MS/MS system.

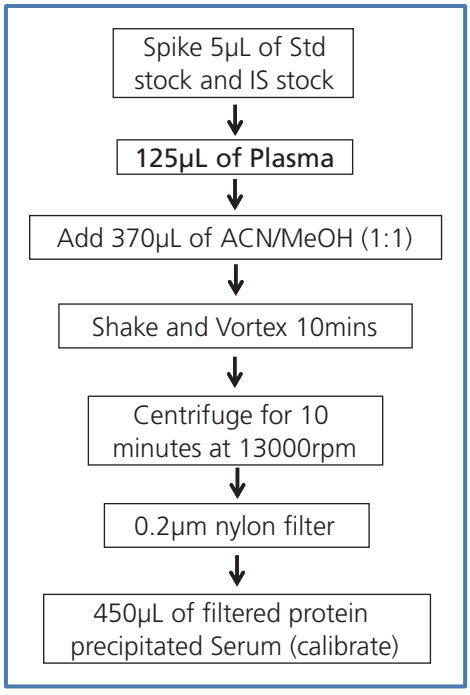

Figure 1: Flow chart of plasma sample pre-treatment by protein precipitation

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Table 1: Analytical conditions of direct analysis of 25-OH VD2/VD3 on LCMS-8050

LC condition	
Column	Kinetex 1.7u C18 100A (100x2.1mm) P/N:00D-4475-AN
Flow Rate	0.5 mL/min
Mobile Phase	A: 0.1% formic acid in Water B: 0.1% formic acid in Methanol
Elution Mode	Gradient elution, LC program 15 minute: 10% (0.0 to 0.5min) → 70% (1.5min) → 80% (6.5 to 9.0min) → 90% (9.1min to 11.0min) → 10% (12.0 to 15.0min)
Oven Temperature	45 °C
Injection Volume	10uL

MS Interface condition	
Interface	APCI
MS mode	Positive
Block Temperature	200 °C
Interface Temp.	400 °C
DL Temperature	200 °C
CID Gas	Ar (270kPa)
Nebulizing Gas Flow	N_2, 2.5L/min
Drying Gas Flow	N_2, 5.0L/min

Results and Discussion

Development of Direct LC/MS/MS method

A MRM quantitation method for 25-OH-VD2 and 25-OH-VD3 with their deuterated forms as internal standard (IS) was developed on LCMS-8050 using an APCI interface. The MRM optimization was performed in positive mode using an automated MRM optimization program with LabSolutions workstation. Three MRM transitions for each compound were selected (Table 2), and the transition with highest intensity for quantitation and the second & third ones for confirmation. The MRM used for quantitation was actually the dehydration of the OH group in the molecule. The plasma samples after protein precipitation and centrifugation were injected onto the LC/MS/MS directly without any further clean-up step.

Table 2: MRM transitions and CID parameters of 25-OH-VD2/VD3 and deuterated internal standards

Name	RT (min)	MRM Transition (m/z)		CID Voltage (V)		
		Precursor $[M+H]^+$	Product	Q1 Pre Bias	CE	Q3 Pre Bias
25-OH VD2	8.388	413.3	395.3	-20	-9	-28
			355.3	-20	-11	-26
			377.3	-20	-12	-24
25-OH VD2-d6	8.340	419.4	401.4	-20	-12	-28
			355.3	-21	-12	-25
			337.3	-21	-13	-24
25-OH VD3	7.927	401.4	383.2	-20	-11	-18
			365.2	-20	-13	-25
			257.2	-21	-15	-29
25-OH VD3-d6	7.884	407.4	389.4	-12	-11	-27
			371.3	-20	-14	-26
			263.2	-20	-17	-27

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Calibration curve (IS), linearity and accuracy

Three sets of standard samples were prepared by pre-spiking and post-spiking in plasma, as well as spiking in the diluent. Each set includes seven levels (triplicate) from 1 ng/mL to 100 ng/mL with IS of 25 ng/mL (Table 3). The processing of the calibration curves includes setting up the weighing method to 1/C. Linear IS calibration curves with R2 greater than 0.996 were established as shown in Figure 2. The MRM chromatograms of pre-spiked samples are shown in Figure 3.

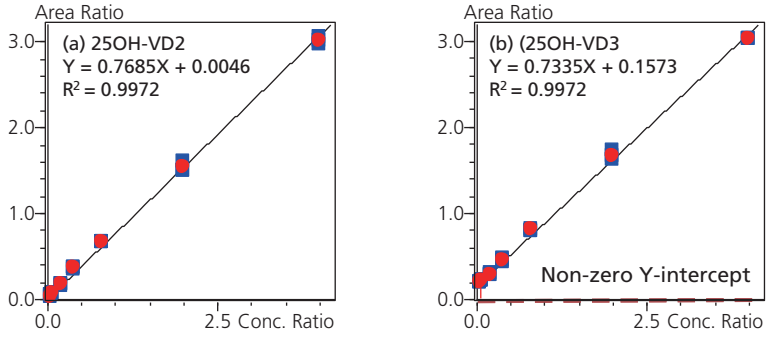

Figure 2: Internal standard calibration curves of 25-OH VD2 (a) and 25-OH VD3 (b) with pre-spiked plasma samples

It is worth to note that the calibration curve of 25-OH-VD3 has a non-zero Y-intercept, indicating that the blank (plasma) contains residual 25-OH-VD3. The level of the residue in the blank was determined to be at 5.14 ~ 5.36 ng/mL (the two values were obtained from post-spiked samples and pre-spiked samples, respectively). This corresponds to 15.42 ~ 16.08 ng/mL of residual 25-OH-VD3 present in the original plasma (the dilution factor of sample pre-treatment is 3.0). The peak area ratios shown in Table 3 are the results after deduction of the background peaks of 25-OH-VD3. The accuracy of the calibration curves are between 78.70% and 119.07%.

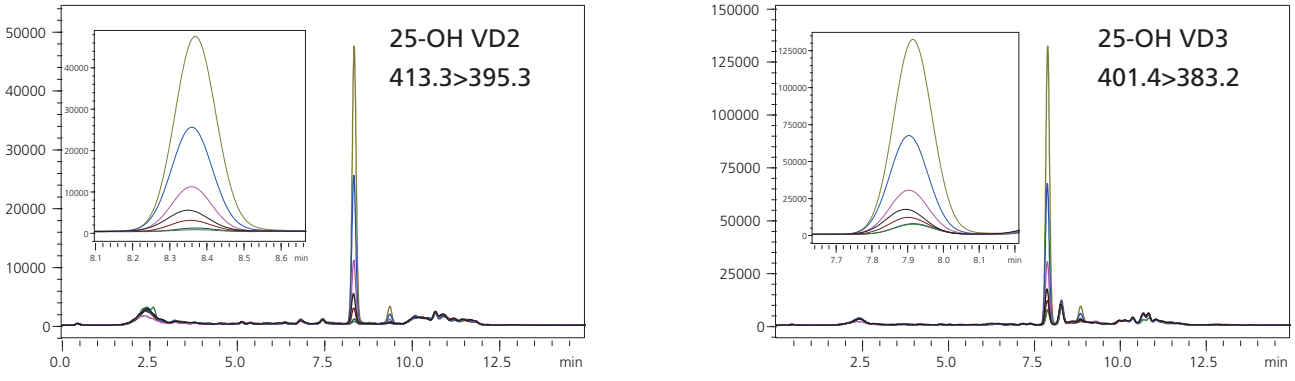

Figure 3: MRM chromatograms of 25-OH VD2 (left) & 25-OH VD3 (right) in pre-spiked in plasma L1 ~ L7

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Method Performance validation

Matrix effect of the MRM method was evaluated by comparison of the peak area ratios of post-spiked in plasma to the standard in diluent. Recovery efficiency of the simple pre-treatment method (protein precipitation) was evaluated by comparison of peak area ratios of pre-spiked to post-spiked in plasma. Process efficiency of the whole process is evaluated by comparison of peak area ratios of pre-spiked in plasma to standard spiked in diluent. The results are compiled into Table 3. The matrix effects, recovery efficiency and process efficiency of the method are within the ±15% except for a few data points out of the range. One data (the P.E of 25-OH-VD3 at 1 ng/mL) is greater than 20% (126.5%).
Repeatability of the method was evaluated with standard samples in solvent and in spiked plasma samples (pre-spiked and post-spiked of both target and IS). The results of %RSD (n=7) are below 7.5% for L3 and above. The %RSD of L1 (1 ng/mL) are 7% ~ 22%.

Table 3(a): Summary of method performance evaluation of 25-OH-VD2 spiked in plasma

Std Conc. Level	25-OH-VD2 (ng/mL)	Conc. Ratio (Target/IS)	Area ratio			Accuracy (%)			M.E (%)	R.E (%)	P.E (%)
			Neat	Pre-spiked	Post-spiked	Neat	Pre-spiked	Post-spiked			
L1	1	0.04	0.0329	0.0319	0.0307	78.7	88.7	99.1	93.3	103.9	96.9
L2	2	0.08	0.0677	0.0578	0.0574	99.3	86.5	91.5	84.7	100.7	85.3
L3	5	0.2	0.1602	0.1681	0.1583	103.3	106.4	100.0	98.8	106.2	104.9
L4	10	0.4	0.3374	0.3567	0.3355	112.5	114.5	105.7	99.4	106.3	105.7
L5	20	0.8	0.6612	0.6646	0.6789	111.9	107.4	106.8	102.7	97.9	100.5
L6	50	2.0	1.4035	1.5251	1.5525	95.8	98.9	97.6	110.6	98.2	108.7
L7	100	4.0	2.8774	3.0074	3.1653	98.6	97.7	99.5	110.0	95.0	104.5

Table 3(b): Summary of method performance evaluation for 25-OH-VD3 spiked in plasma

Std Conc. Level	25-OH-VD3 (ng/mL)	Conc. Ratio (Target/IS)	Area ratio			Accuracy (%)			M.E (%)	R.E (%)	P.E (%)
			Neat	Pre-spiked	Post-spiked	Neat	Pre-spiked	Post-spiked			
L1	1	0.04	0.0276	0.0349	0.0318	101.1	119.1	110.0	115.1	109.9	126.5
L2	2	0.08	0.0549	0.0509	0.0509	94.0	86.7	88.3	92.9	99.8	92.7
L3	5	0.2	0.1495	0.1264	0.1402	97.9	86.2	97.1	93.8	90.2	84.6
L4	10	0.4	0.3114	0.2877	0.2805	100.5	98.1	97.1	90.1	102.6	92.4
L5	20	0.8	0.6709	0.6434	0.6161	107.5	109.5	106.6	91.8	104.4	95.9
L6	50	2.0	1.5744	1.5041	1.4937	100.6	102.5	103.4	94.9	100.7	95.5
L7	100	4.0	3.0835	2.8683	2.8185	98.4	97.7	97.6	91.4	101.8	93.0

The MRM peaks of 25-OH VD3 in clear solution and plasma matrix are displayed in Figure 4. As mentioned above, due to the present of residual 25-OH VD3 in the plasma, the actual S/N ratio could not be obtained directly. As a result, LOD and LOQ of 25-OH VD3 could not be determined.

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Table 4: Summary of repeatability of IS method (n=7)

Cali. Level	Compound	Conc. (ng/mL)	RSD% (Conc.)		
			Clear Soln	Pre Spiked	Post Spiked
L1	25-OH VD2	1	21.7	20.1	15.6
	25-OH VD3	1	10.2	14.2	7.0
L3	25-OH VD2	5	5.2	3.7	6.4
	25-OH VD3	5	2.7	6.1	6.6
L4	25-OH VD2	10	4.9	4.2	3.9
	25-OH VD3	10	4.2	7.4	3.7
L5	25-OH VD2	20	4.1	3.6	3.6
	25-OH VD3	20	4.0	4.1	3.1

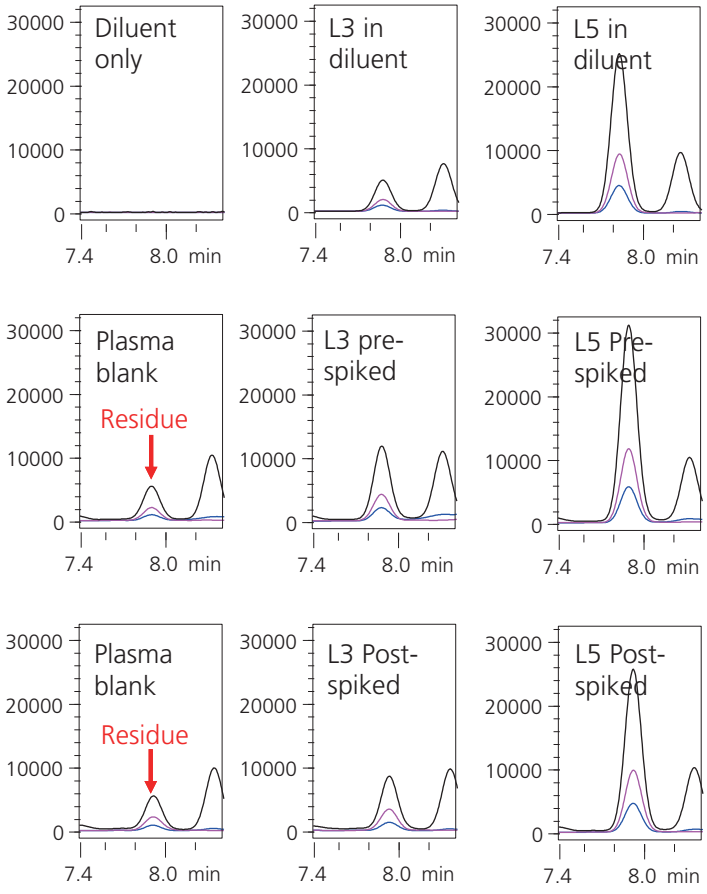

Figure 4: MRM peaks of 25-OH-VD3 in diluent (top row) and pre-spiked in plasma (middle row) & post-spiked in plasma (bottom row)

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They were estimated from extrapolation of S/N values after deduction of the residual level (5.1 ~ 5.4 ng/mL). Hence, we propose a reference LOD and LOQ of the method for 25-OH VD3 to be equal or better than 1 ng/mL and 3 ng/mL respectively. As for the LOD and LOQ of 25-OH VD2, it was determined directly from pre-spiked plasma samples to be 0.9 ng/mL and 2.7 ng/mL (average of three injections) respectively. The specificity of the method relies on several criteria: three MRM transitions (m/z), their intensity ratios and RT of the analytes. It can be seen in Figure 4 that the criteria (MRMx3, ratio and RT) could ensure a high level of reliability for the analysis in confirmation and quantitation of residual vitamin in blank plasma.

Conclusions

A direct LC/MS/MS method with APCI has been developed for quantitative determination of 25-hydroxyvitamin D2/D3 in human plasma. The performance of the method was evaluated thoroughly, including linearity, accuracy, repeatability, LOD/LOQ and specificity. The detection and quantitation range of the MRM-based method was 1 ng/mL to 100 ng/mL with 10ul of injection volume. The LOD and LOQ of the method was proposed to be 1ng/mL and 3ng/mL respectively. Furthermore, matrix effect, recovery efficiency and process efficiency were also obtained and the results indicated that the LC/MS/MS method with simple sample pre-treatment is highly sensitive and reliable in detection and quantitation of 25-hydroxyvitamin D2 and D3 in human plasma.

References

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Note: The method and data shown in the poster are for research use only, Not for Use in Diagnostic Procedures.

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