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Clinical

Quantification of five fat-soluble vitamins in human plasma or serum using the TSQ Altis MD Series system

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Keywords

Fat-soluble vitamins ADEK, Vanquish MD HPLC, TSQ Altis MD MS, plasma, serum

Highlights

- Simultaneous quantification of five fat-soluble vitamins in 30 µL of serum and plasma samples
- Simplified sample preparation using protein precipitation
- Method verified on a Thermo Scientific™ Vanquish™ MD HPLC coupled to a Thermo Scientific™ TSQ Altis™ MD mass spectrometer for clinical laboratories

Introduction

Vitamins are organic compounds that are essential for the normal growth and functioning of the body. They are largely divided into water-soluble vitamins and fat-soluble vitamins according to their solubilities. Fat-soluble vitamins include Vitamin A (VA), Vitamin D (VD), Vitamin E (VE), and Vitamin K (VK), whose presence in human tissues and body fluids is crucial for human health. VA (retinol, beta-carotene) is important for vision, immune function, and skin health. A deficiency in VA can cause degenerative changes in the eyes and skin, such as night blindness as an early symptom. Severe or long-term deficiency of VA can lead to dry eye syndrome, resulting in corneal ulcers and blindness. An overdose of VA can cause poisoning, with various symptoms including dry skin, glossitis, and hair loss. VE (tocopherol) is an antioxidant and free radical scavenger. It protects the integrity of unsaturated lipids in all cell membranes, thus maintaining the normal function of cell membranes, blood vessels, and the nervous system. Children lacking VE can develop reversible motor and sensory neuropathy. VD is an important nutrient that promotes the intestinal absorption of calcium and phosphate and promotes the deposition of these minerals

in newly formed bones. VD is metabolized in the liver mainly into 25-hydroxyvitamin D (25-OH-VD), which is the preferred biomarker to monitor VD nutritional status in the body. Since VD mainly exists in the forms of VD2 and VD3, it is necessary to simultaneously detect 25-OH-VD2 and 25-OH-VD3. Vitamin K1 (VK1, phylloquinone) plays a crucial role in controlling blood clotting and regulating bone metabolism.

This technical brief developed a method to simultaneously quantify five fat-soluble vitamins (VA, 25-OH-VD2, 25-OH-VD3, VE, and VK1) in human serum and plasma using the Thermo Scientific™ Vanquish™ MD high-performance liquid chromatography (HPLC) system coupled with the TSQ Altis MD triple quadrupole mass spectrometry (MS) system.[1] A simple protein precipitation step greatly improved the sample preparation efficiency while maintaining high analyte recovery. Analytes achieved good chromatographic separation from endogenous matrix components on the Thermo Scientific™ Acclaim™ C18 column. The established method demonstrated good linearity, robustness, high sensitivity and specificity; thus, it is suitable for routine vitamins biomonitoring in clinical laboratories.

Experimental

Instruments and reagents

HPLC:	Vanquish MD HPLC
MS:	TSQ Altis MD MS
Methanol:	UHPLC-MS grade, Thermo Scientific
Water:	Distilled water, Watsons
Formic acid:	LC-MS grade, Thermo Scientific

Analytes

Table 1. Formula, molecular weight, and the internal standards of the analytes

Analytes	Formula	Molecular weight (Da)	Internal standards
VA	$C_{20}H_{20}O$	286.2	d6
25OH-VD2	$C_{28}H_{44}O_2$	412.3	d3
25OH-VD3	$C_{27}H_{44}O_2$	400.3	d3
VE	$C_{29}H_{50}O_2$	430.3	d6
VK1	$C_{31}H_{46}O_2$	450.3	d7

Liquid chromatography conditions

Analytical column:	Acclaim RSLC 120 C18, 2.1 x 50 mm, 2.2 µm (P/N 068981)
Column temperature:	45 °C
Injection volume:	20 µL
LC solvent A:	0.1% formic acid in water
LC solvent B:	0.1% formic acid in methanol
LC gradient:	Table 2

Table 2. LC gradient

Time (min)	Flow rate (mL/min)	%B	Curve
0.00	0.5	80	Initial
0.20	0.5	80	5
1.00	0.5	95	5
2.20	0.5	95	5
2.21	0.5	100	5
4.80	0.5	100	5
4.81	0.5	80	5
6.00	0.5	80	5

Mass spectrometry parameters

Ionization source:	APCI
Scan mode:	SRM (Table 3)
Spray current:	4 µA
Ion transfer tube temperature:	350 °C
Sheath gas:	40 Arb
Aux gas:	10 Arb
Source temperature:	350 °C

Table 3. SRM transitions (* = quantifier ions)

Analyte	Precursor ions (*m/z*)	Product ions (*m/z*)	RF Lens (V)	CE (V)
VA	269.19	213.21*	13	55
	269.19	119.13	21	55
VA-d6	275.28	96.15	41	55
25OH-VD2	395.34	269.22*	20	67
	395.34	211.10	27	67
25OH-VD2-d3	398.30	269.22	20	67
25OH-VD3	383.34	257.22*	15	71
	383.34	229.20	19	71
25OH-VD3-d3	386.30	258.00	15	71
VE	431.35	165.16*	65	69
	431.35	137.00	65	68
VE-d6	437.30	171.10	23	69
VK1	451.40	187.20*	23	68
	451.40	199.00	24	68
VK1-d7	458.40	194.10	23	68

Table 4. Internal standard concentrations

Internal standards	VA-d6	VE-d6	25-OHVD2-d3	25-OHVD3-d3	VK1-d7
Concentration (ng/mL)	250	500	100	100	20

Sample preparation

A solution of 5 µL containing the five internal standards (IS, Table 4) was mixed with 30 µL of serum or plasma samples. Proteins were precipitated with the addition of 120 µL of acetonitrile followed by 3 min of vortexing. The mix was centrifuged at 12,000 rpm for 10 min, and 20 µL of the supernatant was injected for LC-MS/MS analysis.

Results and discussion

Representative chromatograms of analytes in pure solvent, serum, and plasma

The representative extraction ion chromatograms (EIC) of the five vitamins in the pure solvent, serum samples, and plasma samples are shown in Figures 1 to 3. After protein precipitation, all analytes achieved good chromatographic separation, ensuring the accuracy of the quantification of real biological samples.

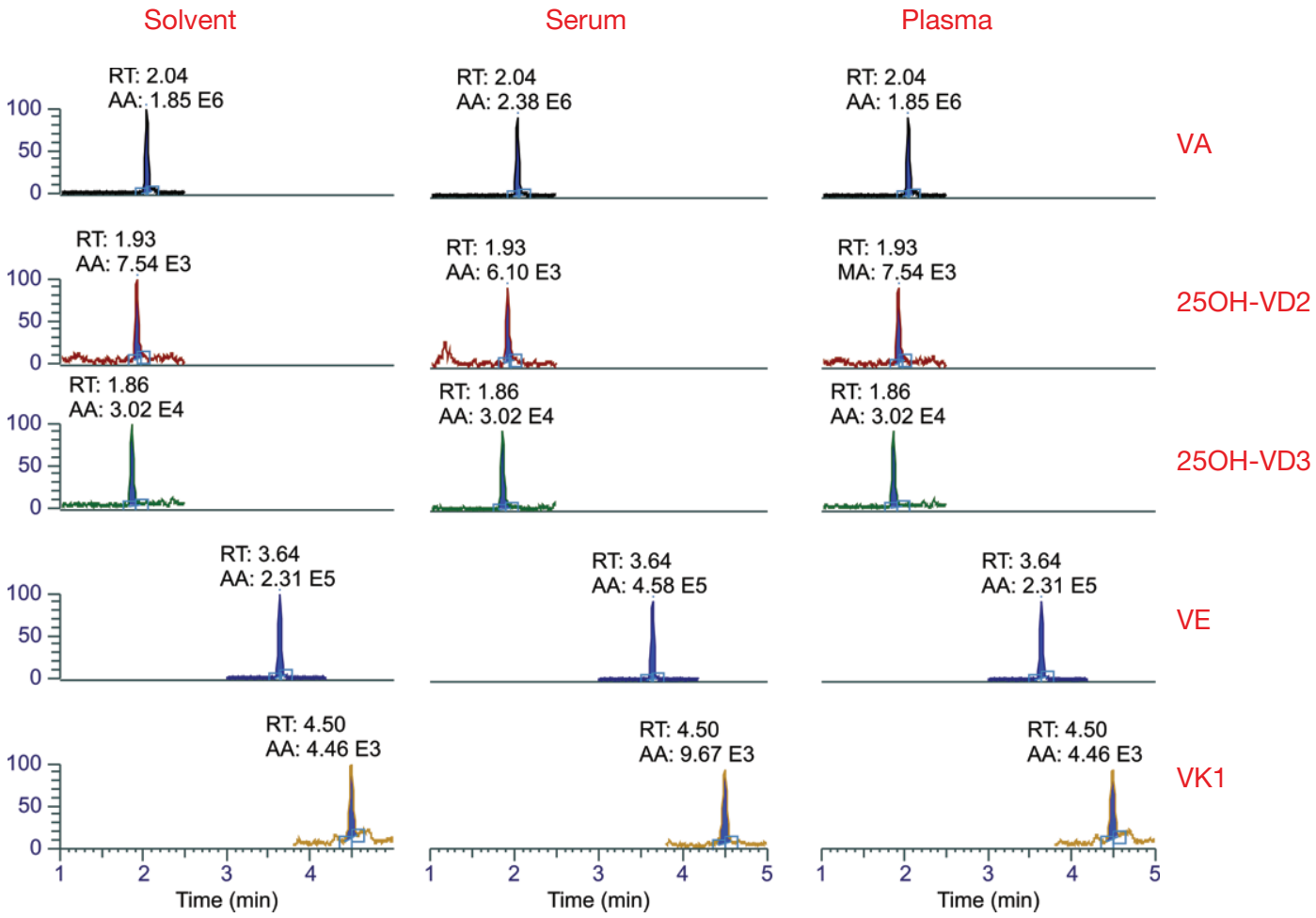

Figure 1. Representative EIC of analytes in solvent, serum, and plasma

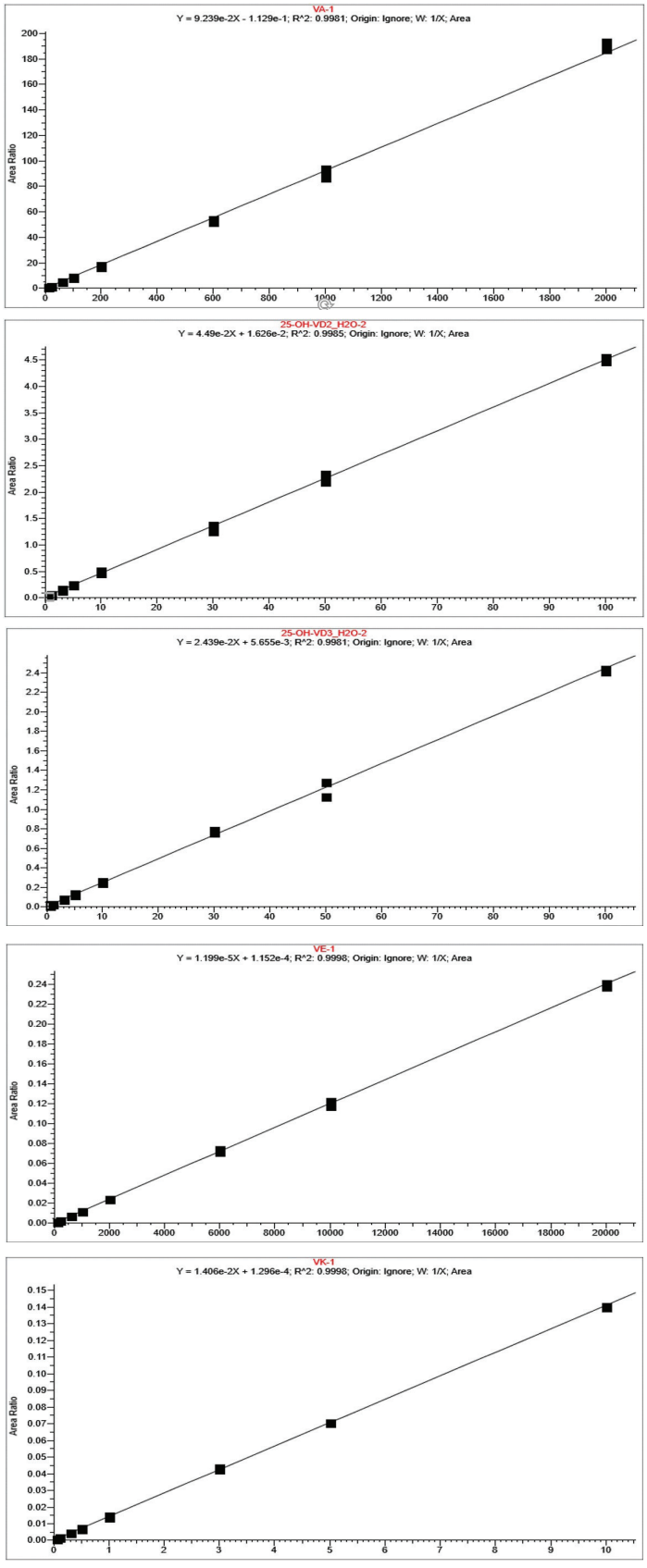

Figure 2. Linear curves of the analytes

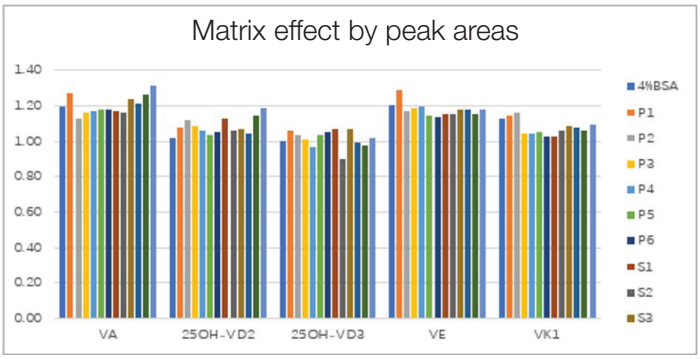

Figure 3A. Matrix effect in 4% BSA, six human serum, and six human plasma, calculated using analyte peak areas only, were between 0.90 to 1.31

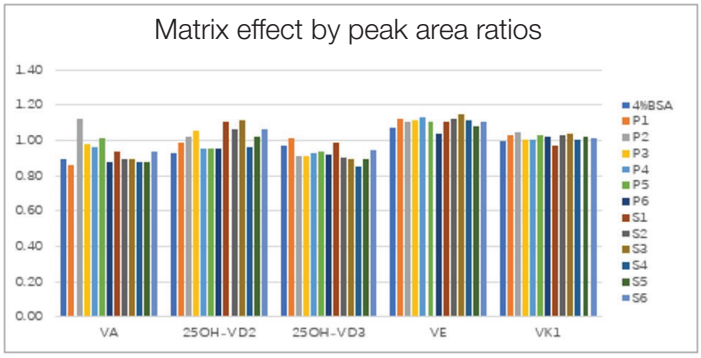

Figure 3B. Matrix effect in 4% BSA, six human serum, and six human plasma, calculated using peak area ratios between analytes and their IS, were between 0.86 to 1.12

Calibration curves

Fat-soluble vitamins are endogenous compounds. The laboratory did not have vitamins ADEK-free serum, plasma, or 4% BSA in PBS as the true blank matrix. Thus, water was used as the surrogate to generate the calibration curves. Good linearity ($R^2 > 0.99$) was achieved for all analyte in the eight-point calibration curves (Table 5).

Table 5. Linearity of the analytes

Analyte	Linear range	Linear equation	R^2
VA	10–2000	Y=9.239e-2X-1.129e-1	0.9981
25OH-VD2	1–100	Y=4.49e-2X+1.626e-2	0.9985
25OH-VD3	1–100	Y=2.439e-2X+5.655e-3	0.9981
VE	0.1–20 µg/mL	Y=1.199e-5X+1.152e-4	0.9998
VK1	0.05–10	Y=1.406e-2X+1.296e-4	0.9998

Analyte recovery

Pooled serum and plasma were spiked with the five analytes at low, medium, and high levels to prepare the quality control samples (LQC, MQC, and HQC, respectively, in Table 6). Analyte concentrations in the pooled serum and plasma prior to spiking were measured with the addition of pure solvent of the same volume. Analyte concentrations were calculated using the calibration curves from above. The spiked analyte recoveries were calculated with the equation shown below.

$$\text{Recovery (\%)} = \frac{\text{Measured concentration} - \text{concentration prior to spiking}}{\text{spiked concentration}} \times 100\%$$

The analyte recoveries in serum and plasma from each QC sample are listed in Tables 6 and 7. In summary, analyte recoveries in serum matrix were 88.33–113.33%, and those in plasma matrix were 86.58–112.40%. The results meet the requirements specified by the Clinical Mass Spectrometry Committee, Chinese Medical Doctor Association of Laboratory Medicine, for LC-MS method development for clinical use.[2]

Table 6. Analyte recoveries in serum

Analyte	Concentration prior to spiking (ng/mL)	Analyte spiking		Measured concentration (ng/mL)	Recovery (%)
		QC levels	Spiked concentration (ng/mL)		
VA	365.14	LQC	200.00	541.81	88.33
		MQC	600.00	908.13	90.50
		HQC	1000.00	1231.97	86.68
25-OH-VD2	1.00	LQC	1.00	1.90	89.65
		MQC	3.00	3.86	95.18
		HQC	5.00	6.44	108.82
25-OH-VD3	24.16	LQC	10.00	33.44	92.68
		MQC	30.00	51.14	89.89
		HQC	50.00	67.26	86.17
VE	6253.88	LQC	2000.00	8430.98	108.85
		MQC	6000.00	12251.24	99.96
		HQC	10000.00	15831.01	95.77
VK1	0.36	LQC	0.10	0.48	113.33
		MQC	0.30	0.68	107.11
		HQC	0.50	0.83	93.87

Table 7. Analyte recoveries in plasma

Analyte	Concentration prior to spiking (ng/mL)	Analyte spiking		Measured concentration (ng/mL)	Recovery (%)
		QC Levels	Spiked concentration (ng/mL)		
VA	279.65	LQC	200.00	480.55	100.45
		MQC	600.00	799.12	86.58
		HQC	1000.00	1136.35	85.67
25-OH-VD2	0.89	LQC	1.00	1.82	93.16
		MQC	3.00	4.14	108.23
		HQC	5.00	6.32	108.57
25-OH-VD3	9.63	LQC	10.00	14.59	99.20
		MQC	30.00	18.60	89.65
		HQC	50.00	35.88	87.51
VE	2540.18	LQC	2000.00	4734.93	109.74
		MQC	6000.00	8191.85	94.19
		HQC	10000.00	12880.19	103.40
VK1	0.08	LQC	0.10	0.19	112.40
		MQC	0.30	0.38	98.80
		HQC	0.50	0.53	90.35

Table 8. The inter- and intra-batch %CV of the five analytes in LQC, MQC, and HQC were all below 7.76%.

Analytes	LQC			MQC			HQC		
	Avg. concentration (ng/mL)	Intra-batch %CV	Inter-batch %CV	Avg. concentration (ng/mL)	Intra-batch %CV	Inter-batch %CV	Avg. concentration (ng/mL)	Intra-batch %CV	Inter-batch %CV
VA	114.74	2.23	1.76	238.05	3.02	3.33	606.09	2.44	1.53
25-OH-VD2	4.09	7.76	6.99	11.79	6.25	0.96	32.90	3.02	1.75
25-OH-VD3	3.71	7.51	2.02	9.94	4.85	5.16	27.78	2.30	1.81
VE	0.58	1.21	1.18	1.84	1.40	4.67	5.90	2.07	0.98
VK1	0.28	2.76	0.85	0.90	1.68	5.04	2.83	1.18	1.56

Inter- and intra-batch imprecision study

Due to the high concentrations of VA and VE in human serum and plasma, the imprecision experiments were performed using 4% BSA as the matrix to prepare the low-, medium-, and high-level QC samples. For the intra-batch imprecision, five samples from each level of low, medium, and high QCs were processed and injected in singlicate. Three batches were tested separately for the inter-batch precision. The results are summarized in Table 8. In all three QC samples, the inter- and intra-batch %CV were below 7.76%, indicating the reported method was highly reproducible.

Matrix effect

Analytes were spiked into 4% BSA, six human serum, and six human plasma samples after protein precipitation to assess the matrix effects in the individual matrices. The matrix effects were calculated in two ways: either by the analyte peak areas only, or by the peak area ratios between the analytes and their IS. When using the peak areas only, the matrix effects in all samples were between 0.90 and 1.31 (Figure 3A), while those calculated from the peak area ratios yielded values between 0.86 and 1.12 (Figure 3B).

Conclusion

The method described in this technical brief simultaneously quantified five fat-soluble vitamins in serum or plasma using the TSQ Altis MD MS. Simplified sample preparation using protein precipitation significantly enhanced efficiency and enabled high-throughput analytes detection required by the large clinical sample volumes. Recovery rates for the five analytes in plasma and serum ranged from 86.58% to 113.33%, with intra-batch and inter-batch precision both below 7.76%. The matrix effect, determined using the peak area ratio between analytes and their IS, was between 0.86 and 1.12. These results indicate that the reported method is highly accurate and reliable, making it suitable for routine vitamin biomonitoring in clinical laboratories.

References

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