High-Throughput Simultaneous Measurement of Vitamins A, D and E in Human Plasma by LDTD-MS/MS

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1. Introduction

Lipohilic vitamins have important roles in physiological functions to maintain good health. Deficiencies in vitamin A, D and E circulating levels are associated with increased risks of degenerative diseases. Such deficiencies, and especially for Vitamin D, have been found to be wide spread within world population. Therefore, as awareness and the need for large epidemiologic studies is increasing, the measurement of vitamins A, D and E status has greatly increased too. Then laboratories need to face this demand with high-throughput, accurate and multiplexed methods.

Here we present a method to simultaneously quantify these vitamins by Laser Diode Thermal Desoprtion-tandem mass spectrometry (LDTD^{TM}-MS/MS) in human plasma.

2. Materials and Method

2-1. Reagents

All-trans retinol (Vitamin A), a-tocopherol (Vitamin E), 25-OH vitamin D2 (ergocalciferol) and 25-OH vitamin D3 (cholecalciferol) were selected as target compounds. 2H6-tocopherol and 2H6-cholecalciferol were used as internal standards. Certified standard solutions of each compound were purchased from Sigma-Aldrich.

As double-charcoal-stripped plasma was not free of the target components, the calibration standards and quality controls were prepared in a plasma surrogate (Bovine serum albumin at 50 mg/mL in aqueous NaCl at 0.9% (w/v). The calibration range was from 1 to 250 ng/mL for 25-OH vitamins D2/D3, 10 to 4500 ng/mL for Vitamin A and 0.1 to 40 µg/mL for Vitamin E. Seven calibration levels, regularly dispatched and four quality controls levels were independently prepared (LLOQ, 3x LLOQ, 50% of the range and 90% of the range) . Internal standard solution contained 2H6-tocopherol and 2H6-cholecalciferol at a concentration of 20 and 2.5 µg/mL in ethanol, respectively.

BHT (butylhydroxytoluene) was added at a concentration of 1% (w/v) in all solvents used to prevent Vitamin A degradation.

Solvents used were of LC-MS or Pesticide analysis grade from Wako chemicals.

2-2. Sample Preparation

Calibration standards, QC or samples were assayed the same way. One-hundred microliters of sample were mixed with 10 µL of ISTD solution and 100 µL of water/isopropanol (1/1 v/v). After vortexing, the samples were incubated at room temperature for 15 minutes. Then 200 µL were loaded on SLE sorbent (SLE+ 200, Biotage, Sweden) by gravity. After 5 minutes, compounds were eluted with 2x300 µL of hexane. Extracts were then directly deposited in LazWell 96 plate (see fFigure 1).

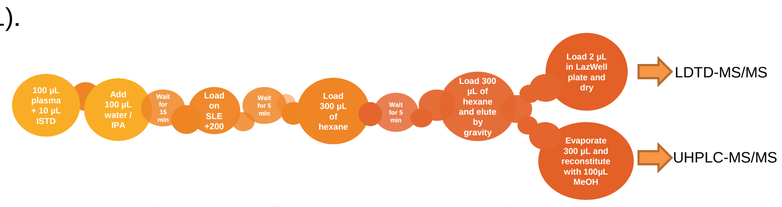

Figure 1: Sample Preparation Process

2-3. Analytical Conditions

Analysis was performed using a LDTD-SH-960 system (Phytronix, Québec, Canada) coupled with LCMS-8060 triple quad mass spectrometer (Shimadzu Corp. Kyoto, Japan) (Figure 2). Parameters are described in Table 1 and 2.

Three 96-well plates could be assayed in 1 hour.

Figure 2: Overview of the Analytical System

Table 1: LDTD conditions

Parameter	Value
System	: SH-960
Laser pattern	: 45 % in 3 seconds
Carrier gas flow	: 3 L/min (Air)
Injection Volume	: 2 µL
Total Run Time	: 12 seconds

Table 2: MS/MS Parameters

Parameter	Value		
System	: LCMS-8060		
Ionization	: APCI (+5 kV)		
MRM	: Compound	Quan	Qual
	25-OH D2	395.35 > 269.20	295.35 > 251.20
	25-OH D3	383.35 > 257.25	401.35 > 257.25
	$^2H_6$-25-OH D3	389.35 > 263.25	
	Vitamin A	269.20 > 93.20	269.20 > 81.05
	Vitamin E	431.40 > 137.10	431.40 > 55.15
	$^2H_6$-Vitamin E	437.40 > 143.10	
Dwell time / Pause time	: 24 ms / 1 ms		
Temperatures	: DL 150°C	Heater Block 200°C	

3. Results

3-1. Calibration

Calibration curves were calculated by internal standardization using a linear regression model with 1/x weighting. Acceptance criteria was an accuracy comprised between 85 to 115%.

Some typical calibration curves are presented in Figure 3 and mass chromatograms at the LLOQ in Figure 4.

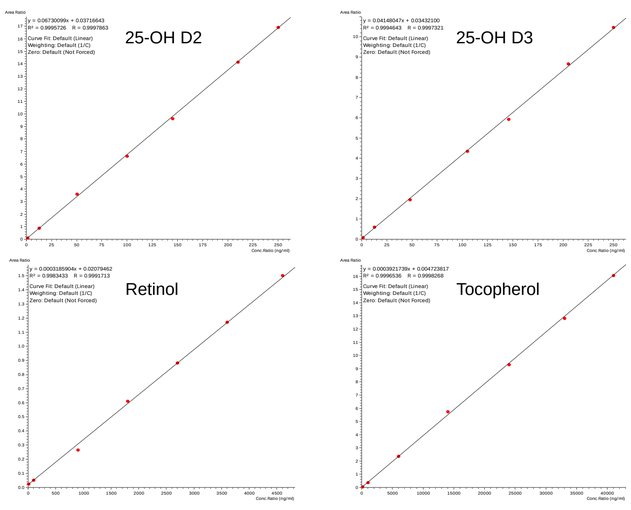

Figure 3: Typical Calibration Curves

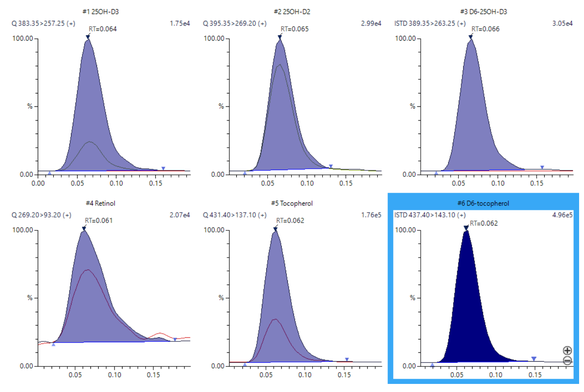

Figure 4: Chromatograms at the LLOQ

3-2. Recovery and Matrix Effect

As no plasma was found to be free of the targeted vitamins, recovery and matrix effect were evaluated on ISTD peaks. Six individual plasma samples (3 males, 3 females, age 25-67) were tested. Extraction recovery was ranging from 90 to 97% for $^2H_6$-Vit D3 and from 87 to 93% for $^2H_6$-Vit E. Matrix effect was within 0.95 to 1.10, and within 0.97 to 1.05 for $^2H_6$-Vit D3 and $^2H_6$-Vit E, respectively.

3-3. Precision and Accuracy

Precision and accuracy were evaluated by measuring the concentration of QC samples (5replicates) at 4 levels across 4 independent runs. Results are presented in Table 3.

Table 3: Precision and Accuracy

	25-OH D2	25-OH D3	Retinol	Tocopherol
Intra-run precision (%)	9.3 / 5.5 / 5.1 / 4.8	7.6 / 6.4 / 3.2 / 4.1	8.2 / 2.3 / 3.1 / 2.5	5.1 / 4.4 / 4.6 / 6.5
Inter-run precision (%)	10.2 / 6.3 / 5.2 / 5.6	8.0 / 6.4 / 5.0 / 4.8	8.5 / 4.2 / 3.0 / 4.8	7.2 / 4.5 / 6. 5 / 7.1
Average accuracy	92.5%	101%	96.2%	105%

3-5. Real Sample Analysis – LDTD versus UHPLC-MS/MS

Because there is no chromatographic separation, LDTD-MS:MS could be subjected to quantitative bias due to isobaric interferences. To confirm this point, commercially available individual plasma samples were assayed by LDTD-MS/MS and UHPLC-MS/MS following the sample preparation process described in Figure 1. Prior to analysis, the UHPLC-MS/MS method was validated using the same standards and QC than LDTD-MS/MS. Performances of the method wee not significantly different (linearity, recovery, matrix effect, precision and accuracy). UHPLC-MS/MS method conditions are available upon request.

Figure 5 shows a typical chromatogram with interferences separation and Figure 6 shows the result comparison. 25-OH Vitamin D2 was not detected in these samples with both methods.

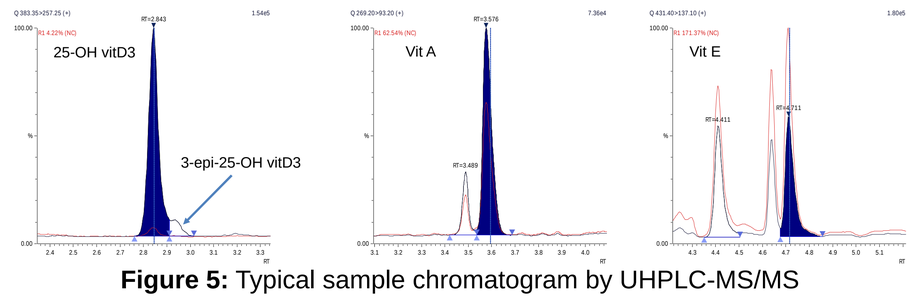

Figure 5: Typical sample chromatogram by UHPLC-MS/MS

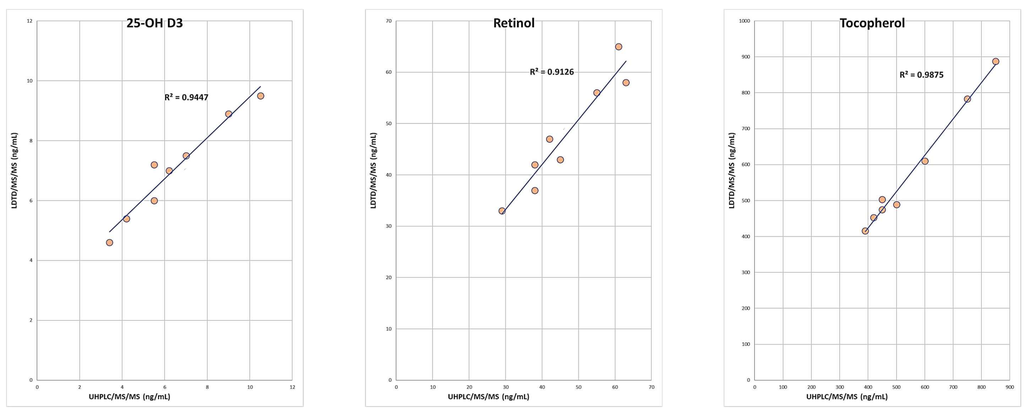

Figure 6: Comparison of LDTD/MS/MS method to UHPLC/MS/MS

4. Conclusions

- ✓ A sensitive and very high-throughput LDTD-MS/MS method was developed,
- ✓ All targeted vitamins are covered despite large differences in reference ranges,
- ✓ Method performances were in agreement with current bioanalytical guidelines,
- ✓ Despite the lack of separation the method is comparable to UHPLC-MS/MS but 20x faster. Correlation of retinol was slightly less good. This is probably due to the absence of analogue ISTD.
- ✓ The two methods are complementary. The proposed strategy is to use LDTD for very high-throughput sample screening and the UHPLC method for confirmation when needed. The two methods can be switched on the same system without replacing the ion source.