ThP 036

Cost effective and rapid method for simultaneous determination of vitamin B12, 25-Hydroxyvitamin D2 and D3 from plasma using LC-MS/MS

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1. Overview

Lifestyle disorders are manifesting as deficiency of vitamin B12 and vitamin D. Clinicians have always corelated vitamin B12 and D deficiencies to heart diseases and many other health problems. This has led to the demand of simultaneous determination of vitamin B12, 25-hydroxyvitamin D2 and D3 in plasma.

To make the analysis cost effective and rapid, in this method the molecules of different polarity are been extracted and processed simultaneously.

2. Introduction

Vitamin B12 is ingested in its free form (nonprotein bound), which binds to a carrier protein known as R-binders or transcobalamin 1. This carrier protein is secreted by both the salivary glands in the oropharynx and the gastric mucosal cells within the stomach (Figure 1). Vitamin D is rapidly metabolized in the liver to form 25-hydroxyvitamin D and further it is hydrolyzed to form 1,25-Dihyroxyvitamin D (Figure 2)

First challenge in developing a cost effective and rapid method, is to simultaneously measure water soluble and fat soluble vitamin. Vitamin B12 (water-soluble) needs Electro Spray Ionization (ESI) for ionization whereas 25-hydroxyvitamin D2 and D3 (fat-soluble) needs Atmospheric Pressure Chemical Ionization (APCI) for ionization. LCMS-8045 (Shimadzu Corporation, Japan), equipped with heated ESI probe, helped to overcome the challenge. Second challenge of sample preparation is overcome by using simple preparation technique without any expensive chemicals or consumables.

Here, a cost-effective and rapid method is established for simultaneous analysis of these vitamins in plasma using LCMS-8045.

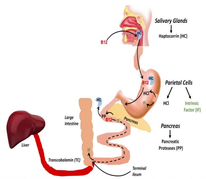

Figure 1. Vitamin B12 mechanism

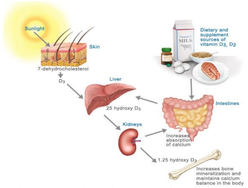

Figure 2. Vitamin D mechanism

3. Materials and methods

3-1.Sample preparation

• Preparation of matrix matched calibration standards and quality control (QC) samples

Aliquot of 100 uL of plasma was taken in vial to which 300 uL of 1% formic acid in acetonitrile is added to precipitate the protein in plasma and mixed well. Further the mixture was kept at 2-8 °C for 1 hour. After 1 hour the mixture was centrifuged at 7500 rpm for 10 mins and the supernatant was injected on the LCMS-8045 system.

25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and vitamin B12 (procured from Sigma-Aldrich) calibration standards at concentration levels of 1.56 ng/mL, 3.12 ng/mL, 12.5 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL were prepared in plasma. Quality control (QC) samples at concentration levels of LQC (1.5 ng/mL), MQC (12.5 ng/mL) and HQC (25 ng/mL) were also prepared in plasma. Samples were then injected in LCMS-8045 system.

3-2. LC-MS/MS analysis

Figure 3. Nexera with LCMS-8045

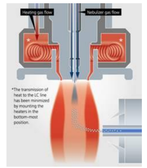

Figure 4. Heated ESI probe

LCMS-8045 triple quadrupole mass spectrometer by Shimadzu (Figure 3), sets a new benchmark in triple quadrupole technology with an unsurpassed sensitivity (UF sensitivity), ultra fast scanning speed of 30,000 u/sec (UF scanning) and polarity switching speed of 5 msec (UF switching). This system ensures highest quality of data, with very high degree of reliability.

In order to improve ionization efficiency, the newly developed heated ESI probe (Figure 4) combines high-temperature gas with the nebulizer spray, assisting in the desolvation of large droplets and enhancing ionization. This development allows high-sensitivity analysis of a wide range of target compounds with considerable reduction in background.

The instrument parameters are in Table 1.

Table 1. Instrument parameters

UHPLC condition (Nexera X2 system)	
Column	Shim-pack GIST C18 (50 mm L. × 3 mm I.D., 3 µm) (P/N: 227-30009-03)
Mobile phase	A: buffer; B: methanol
Flow rate	0.6 mL/min
Elution mode	Isocratic
Injection vol.	20 uL
Column temperature	40 °C
MS Parameters (LCMS-8045)	
MS interface	Electro Spray Ionization (ESI)
Nitrogen gas flow	Nebulizing gas- 2.5 L/min; Drying gas- 5 L/min
Zero air flow	Heating gas- 15 L/min
MS temperatures	Desolvation line- 250 °C; Heating block- 275 °C; Interface- 250 °C
MRM transition (positive)	25-hydroxyvitamin D2 413 > 395 413 > 355 25-hydroxyvitamin D3 401 > 383 401 > 159 Vitamin B12 678.5 > 359 678.5 > 147

4. Results

The details of calibration curve of 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and vitamin B12 which was plot by preparing calibrators using standards spiked in stripped plasma is presented in Table 2. The details of quality control for critical concentration of plasma which were prepared by spiking chemical standard in plasma sample is presented in Table 3. Linearity test was carried out using external standard calibration method with coefficient of regression of more than 0.99 for 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and vitamin B12 as shown in Figures 5, 6 & 7 respectively. The chromatographic representation is shown in Figure 8.

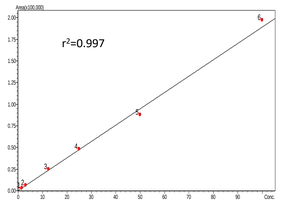

Figure 5. Calibration curve for 25-hydroxyvitamin D2

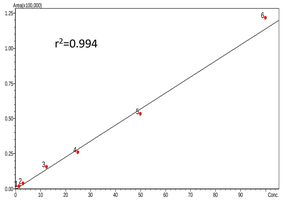

Figure 6. Calibration curve for 25-hydroxyvitamin D3

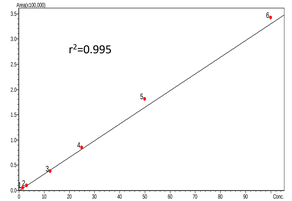

Figure 7. Calibration curve for Vitamin B12

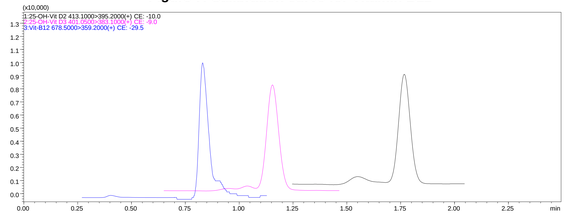

Figure 8. Chromatographic representation of analytes

Accuracy for standards was found to be between 90-110% and precision was found to be less than 10%, which are within acceptance criteria. Results are shown in Tables 2 and 3.

Table 2. Results of accuracy for 25-hydroxyvitamin D2, D3 and vitamin B12 spiked calibration standards

Name of compound	Standard concentration (ng/mL)	Calculated concentration from calibration graph (ng/mL)	% accuracy
25-hydroxyvitamin D2	1.56	1.53	98.5
	3.12	3.19	102.3
	12.50	12.94	103.6
	25	25.19	100.8
	50	46.24	92.5
	100	104.33	104.3

Name of compound	Standard concentration (ng/mL)	Calculated concentration from calibration graph (ng/mL)	% accuracy
25-hydroxyvitamin D3	1.56	1.51	96.9
	3.12	3.41	109.1
	12.50	13.76	110.1
	25	22.74	91.0
	50	46.73	93.5
	100	106.67	106.7

Name of compound	Standard concentration (ng/mL)	Calculated concentration from calibration graph (ng/mL)	% accuracy
Vitamin B12	1.56	1.63	104.8
	3.12	2.89	92.5
	12.50	11.54	92.4
	25	25.58	102.2
	50	54.88	109.8
	100	103.58	103.6

Table 3. Results of accuracy and repeatability for 25-hydroxyvitamin D2, D3 and vitamin B12 QC samples

Name of compound	Standard concentration (ng/mL)	Calculated average concentration from calibration graph (ng/mL)	Average % accuracy	Average % RSD for area counts (n=3)
25-hydroxyvitamin D2	1.56 (LQC)	1.46	97.0	2.31
	12.50 (MQC)	12.50	100.0	2.37
	25 (HQC)	24.16	96.6	1.24
25-hydroxyvitamin D3	1.56 (LQC)	1.48	98.9	2.01
	12.50 (MQC)	13.43	107.4	2.72
	25 (HQC)	23.08	92.3	3.42
Vitamin B12	1.56 (LQC)	1.64	109.2	3.06
	12.50 (MQC)	11.62	92.9	1.18
	25 (HQC)	24.69	98.7	3.31

5. Conclusion

A cost effective and rapid LC-MS/MS method is developed for simultaneous analysis of vitamin B12, 25-hydroxyvitamin D2 and D3 with LOQ of 1.56 ng/mL in spiked stripped plasma on Shimadzu LCMS-8045 system.

6. References

[1] Yuan C, Kosewick J, He X, Kozak M, Wan S. Communications in Mass Spectrometry. 2011; 25:1241–1249.
[2] Higashi T, Simada K, Toyo'oka T. Journal of Chromatography B. 2009; 878:1654–1661.