Establishment of a liquid chromatography tandem mass spectrometry method for vitamin D metabolites to detect 24-hydroxylase deficiency

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Introduction

Determination of 25-hydroxyvitamin D (25(OH)D) is recommended for the assessment of Vitamin D status. Concentrations in serum samples >100 nmol/L are considered desireable. However, in 24-hydroxylase deficient individuals higher than 100 nmol/L may be critical. The resulting endogenous Vitamin D excess can cause hypercalcemia and nephrocalcinosis with corresponding symptoms [1]. Simultaneous measurement of 25(OH)D_3, 25-hydroxyvitamin D_2 (25(OH)D_2), and 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) may help to identify affected individuals. Therefore, we established a liquid-chromatography-tandem-mass-spectrometry (LC-MS/MS system) method for determination of three Vitamin D metabolites.

Methods & patients

The method consists of protein precipitation followed by liquid-liquid-extraction and subsequent derivatization with 4-Phenyl-1,2,4-triazole-3,5-dione (PTAD) [2]. Samples were separated on an Agilent HPLC 1260 system using a Zorbax C18Eclipse column and a gradient elution. The eluate was introduced into a Sciex 4500 MS/MS instrument for the detection of the three metabolites within 17 min.
Analytical performance was assessed by determining linearity, limit of detection (LOD), limit of quantification (LOQ), imprecision and recovery. Accuracy was analyzed by method comparison with a commercial 25(OH)Vitamin D LC-MS/MS method (Recipe) at the Department of Clinical Pathology at Bolzano Hospital (Italy), with a validated in-house LC-MS/MS method from University of Liege (Belgium) and samples from the Vitamin D External Quality Assessment Scheme (DEQAS) which is traceable to the National Institute for Standards and Technology (NIST) [3,4]. In addition, the method was tested on four sera from patients that were referred to our laboratory, because of 25-hydroxyvitamin D concentration >100 nmol/L.

Table 2. Results of the Bland and Altman difference plot with the in-house method and the commercial 25-OH-Vitamin D2/D3 LC-MS/MS assay from RECIPE for 25(OH)D_3, and 25(OH)D_2 (Department of Clinical Pathology at Bolzano Hospital (Italy)) and with the in-house method and the validated in-house LC-MS/MS method at the Department of Clinical Chemistry, University of Liege (Belgium) for 24,25$(OH)_2D_3$

	25/OH)D_3	25(OH)D_2	24,25$(OH)_2D_3$
Mean difference	**-0.8798**	**-2.0260**	**-0.2335**
95 % CI	**-22.344 – 20.585**	**-25.1476 - 21.096**	**-1.7924 to 1.3254**
Intercept	**-1.842 – 9.303**	**-0.8333 – 10.200**	**-0.7975 – 1.098**
Slope	**0.79–1.03**	**0.76–1.00**	**0.72–1.14**

CI = confidents interval

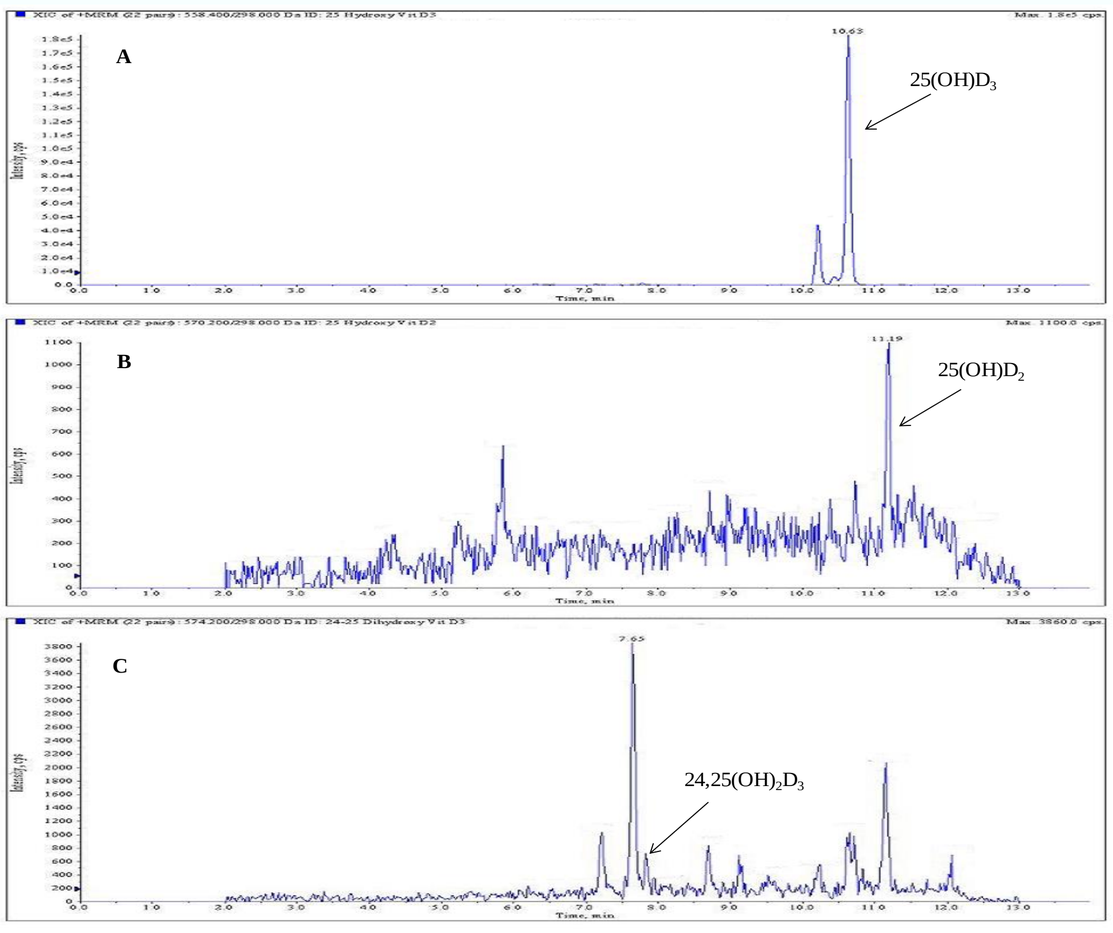

Figure 1. Chromatograms of Vitamin D metabolites in a patient with 24-hydroxylase deficiency.
A) 25(OH)D_3 concentration: 166 nmol/L (normal range: 50-100 nmol/L); B) 25(OH)D_2 concentration: 3.3 nmol/L (2-50nmol/L); C) 24,25$(OH)_2D_3$ concentration: 0.16 nmol/L (2-50 nmol/L)

Table 3. Concentrations of three Vitamin D metabolites in patients from four serum samples.

Patient	25(OH)D_3 [nmol/L]	25(OH)D_3 [nmol/L]	24,25$(OH)_2D_3$ [nmol/L]	Ca [mmol/L
1	166	3.3	0.16	3.7
2	445	0.3	6.5	2.5
3	182	1.04	11.1	2.4
4	134	1.77	10.9	2.4

LITERATURE
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(2) Laha TJ, Strathmann FG, Wang Z, de Boer IH, Thummel KE and Hoofnagele AN. Cross-reactivity for Immunoaffinity Purification of Analytes prior to Multiplexed Liquid Chromatography-Tandem Mass Spectrometry. Clinical Chemistry. 2012;58:(12)1711-16.
(3) Binkley N, Sempos CT; Vitamin D Standardization Program (VDSP). Standardizing vitamin D assays: the way forward. Journal of Bone and Mineral Research. 2014;29(8):1709-14.
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Results

The used transitions (m/z, mass to charge ratios) and analytical performances are summarized in Table 1. Results of the method comparison are shown in Table 2. Serum concentrations of all vitamin D metabolites obtained for all four patients are summarized in Table 3. Representative chromatograms for all vitamin D metabolites from the serum sample with 24-hydroxylase deficiency are shown in Figure 1 (A-C). Figure 2 shows the separation of the 24,25$(OH)_2D_3$ and 25,26$(OH)_2D_3$ metabolite and Figure 3 represents a possible physiolocial pathway in patient with a 24-hydroxylase defect.
In ten DEQAS samples the results for all three metabolites were within the allowable limits from the NIST target value.

Table 1. Performance characteristics of the Vitamin D in-house LC-MS/MS method

	25(OH)D_3	25(OH)D_2	24,25$(OH)_2D_3$
m/z ratio	558.4 / 298.1	570.2 / 298.1	574.2 / 298.1
Linear range (nmol/L)	7.8 - 250	1.5 - 48	1.5 - 48
Correlation factor (r^2)	0.999	0.997	0.998
Intraday assay (CV %)	1.8 – 10.4	3.5 – 7.3	5.3 – 8.3
Interday assay (CV %)	2.9 – 14.3	1.5 – 13.6	1.2 – 12.1
LoD (nmol/L)	1.5	0.3	0.3
LoQ (nmol/L)	3.1	1.0	1.0
Recovery (%)	72.2	73.1	69.3

m/z = mass to charge ratio; LoD = limit of detection; LoQ = limit of quantification; CI = confidents interval
Imprecision was assessed using two serum samples, commercial control material from RECIPE and in-house prepared calibrators.

Summary

- Valitation of this in-house method showed adequat analytical performance with good sensitivity, specificity, accuracy and precision for 25(OH)D_3, 25(OH)D_2 and 24,25$(OH)_2D_3$ Vitamin D metabolites.
- The performance in the DQAS programm confirmed acceptable accuracy for all Vitamin D metabolites tested.
- The clinical utility of this method has been confirmed in a patient with 24-hydroxylase deficiency.

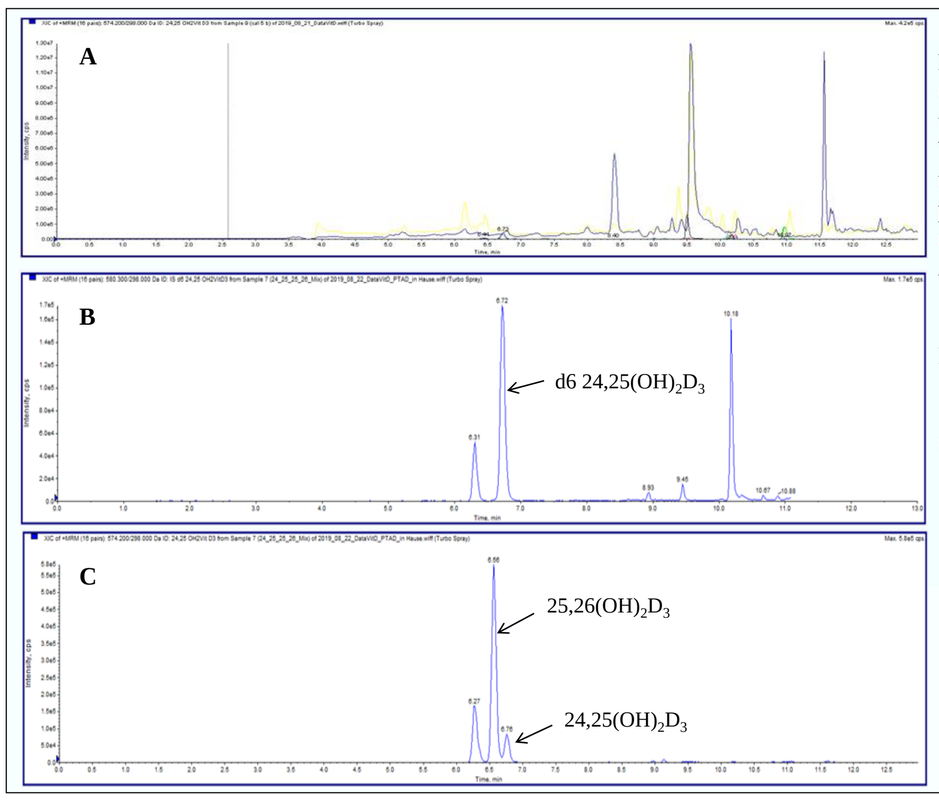

Figure 2. Chromatograms of the 25,26$(OH)_2D_3$ Vitamin D metabolite using 24 nmol/L (from pure substance (Biozol, Germany))
A) Total ion chromatogram (TIC);
B) Internal Standard: IS d6 24,25$(OH)_2D_3$;
C) Separation of the Vitamin D metabolites 24,25$(OH)_2D_3$ and 25,26$(OH)_2D_3$

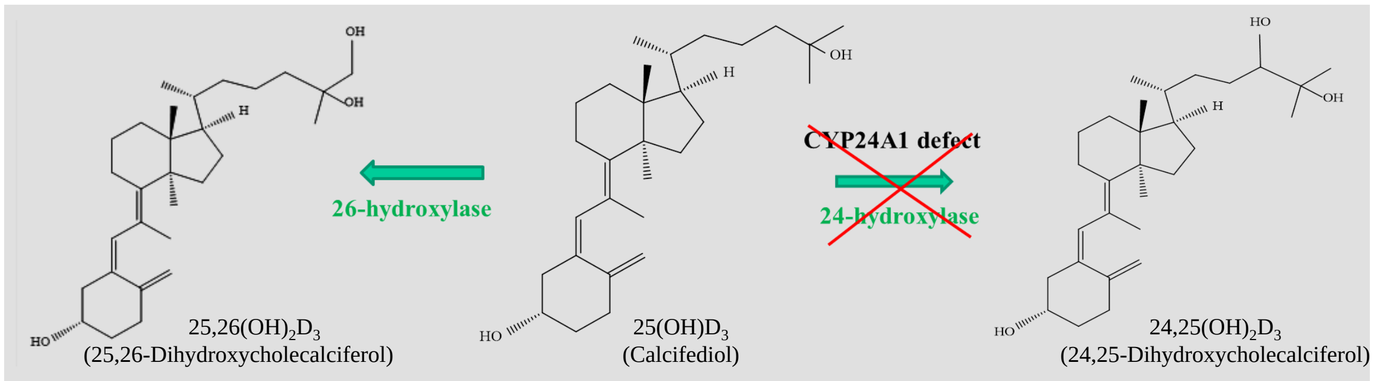

Figure 3. Possible physiological pathway in 24-hydroxylase deficient patients.