Recertification of 25-hydroxyvitamin D standards by Isotope Pattern Deconvolution (IPD)

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Introduction

Vitamin D (VTD) is an important prohormone widely known since its deficiency is directly related to development of rickets in children and osteoporosis in adults. Furthermore, recent studies have demonstrated that vitamin D has also an important role in non-skeletal conditions such as autoimmune diseases, cardiovascular diseases and cancer, among others[1]. This vitamin can be found in two main forms: vitamin D2 and vitamin D3. The metabolism of both forms of vitamin D are subjected to a first hydroxylation in the liver to form 25-hydroxyvitamin D (25(OH)D) and then to a second one in the kidney to form 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the active form of vitamin D. Nevertheless, the measurement of 25(OH)D in serum samples is preferred test for the assessment of vitamin D status over the $1,25(OH)_2D$. There are two main reasons for this choice: the longer lifetime (3 weeks versus 4 h) and its higher concentration levels (ng/mL versus pg/mL)[2].

Over the last years, a dramatic rise in vitamin D testing (as 25(OH)D) has been observed, due to two main reasons: the increased number of patients with VTD deficiency and the increase of the role of VTD as a biomarker related to several diseases.

In this work we propose a recertification approach for 25(OH)D2/D3 solvent standards based on Isotope Pattern Deconvolution (IPD) using NIST SRM 2972 as reference material. This approach could help to meet the requirements for external standardization criteria using in-house calibration curves.

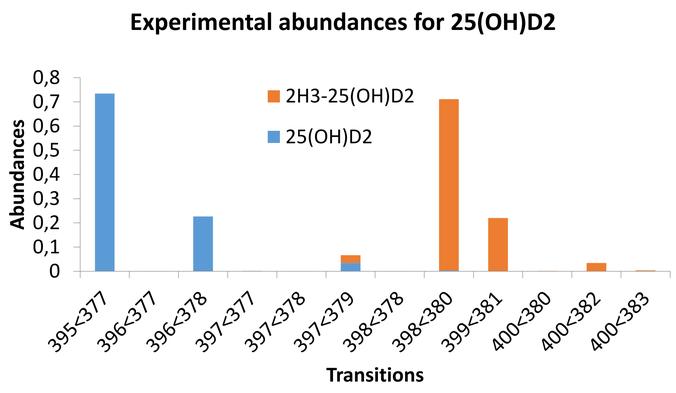
Results and Discussion

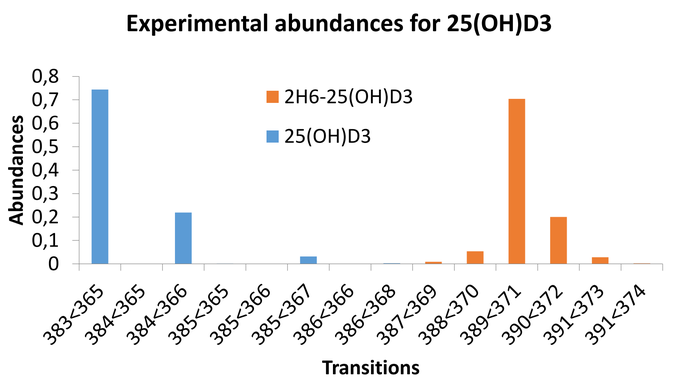
Characterization of natural and labelled solutions

+ 50 µL 1 µg/mL solution
+ 50 µL of water

vortex

UHPLC-MS/MS





Reverse Isotope Dilution (RID) of labelled compounds

+ 50 µL 0.5 µg/mL of labelled mix solution
+ 50 µL NIST SRM2972 (334,24 ng/mL of 25(OH)D3 + 231.32 ng/mL 25(OH)D2)
+100 µL of water

vortex

UHPLC-MS/MS

Concentration of labelled standards for spiking, determined by RID.

Compound	Mean[1] (µg/mL)	SD	%RSD
$^2H_3$-25(OH)D2	7.17	0.07	1.0%
$^2H_6$-25(OH)D3	5.12	0.13	2.5%

[1] *n* = 3, injected thrice each.
[2]SD=Standard Deviation, RSD=Relative Standard Deviation

Re-certification of the natural compound by IPD

+ 50 µL 0.5 µg/mL of labelled mix solution
+ 50 µL 0.5 µg/mL of natural mix solution
+100 µL of water

vortex

UHPLC-MS/MS

Re-certification of natural standards by IPD using their labelled analogues.

Compound	Theoretical concentration (µg/mL)	Mean[1] (µg/mL)	SD	%RSD	
25(OH)D2	976	1258	8	0.7%	+29%
25(OH)D3	765	732	9	1.2%	-4.7%

[1] *n* = 3, injected thrice each.
[2]SD=Standard Deviation, RSD=Relative Standard Deviation

Experimental

Instrumental conditions

UHPLC: Nexera X2 UPLC (Shimadzu)

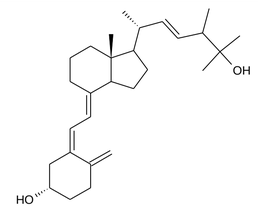
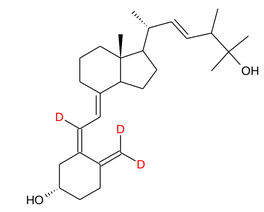
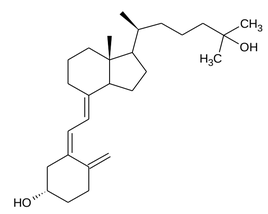
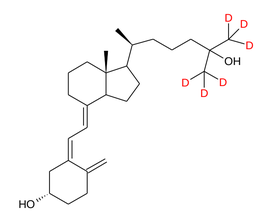
- **Column**: Kinetex®PFP 100Å Pentafluorophenyl (100 x 2,1mm, 2,6µm)
- **Flow: 0.4 mL/min**
- **Injection volume: 30 µL**
- **Mobile phases:**
  - **A: H_2O 0.1% HCOOH**
  - **B: MeOH HPLC 0.1% HCOOH**

Gradient conditions		
Time (min)	% A	% B
0	60	40
0.2	60	40
0.3	25	75
5.6	5	95
8	5	95
8.1	60	40
9.2	60	40

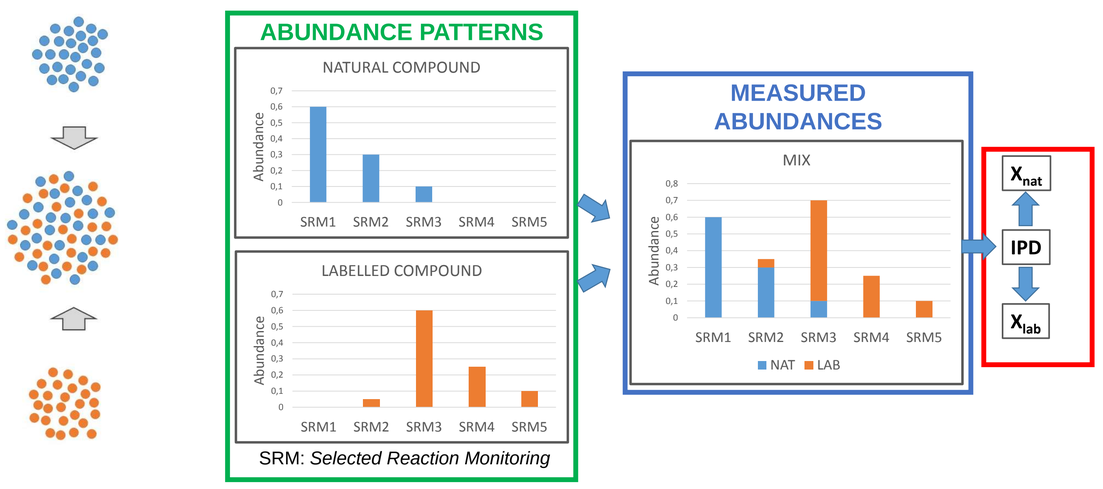
MS: Sciex QTRAP 6500

- Quadrupole-linear ion trap working triple quadrupole mode.
- Atmospheric Pressure Chemical Ionization (APCI+).
- Multiple Reaction Monitoring (MRM) .
- Further information of the method can be consulted in our previous publication[3].

Transitions monitored for natural and labelled 25-hydroxyvitamin D2 and D3

Analyte	Natural		Labelled	
25-hydroxyvitamin D2 (25(OH)D2)				
	395 > 377	396 > 378	398 >380	399 > 381
25-hydroxyvitamin D3 (25(OH)D3)				
	383>365	384>366	389>371	390>373

Isotope Pattern Deconvolution (IPD)



$$\begin{bmatrix} A_m^{SRM1} \\ A_m^{SRM2} \\ A_m^{SRM3} \\ A_m^{SRM4} \end{bmatrix} = \begin{bmatrix} A_{nat}^{SRM1} & A_{lab}^{SRM1} \\ A_{nat}^{SRM2} & A_{lab}^{SRM2} \\ A_{nat}^{SRM3} & A_{lab}^{SRM3} \\ A_{nat}^{SRM4} & A_{lab}^{SRM4} \end{bmatrix} \cdot \begin{bmatrix} x_{nat} \\ x_{lab} \end{bmatrix} + \begin{bmatrix} e^{SRM1} \\ e^{SRM2} \\ e^{SRM3} \\ e^{SRM4} \end{bmatrix}$$

Abundances in the mix | Abundances of the natural compound | Abundances of the labelled compound | Molar fractions | Error vector

MULTIPLE LINEAR REGRESSION (EXCEL)

$$\frac{C_{nat}}{C_{lab}} = \frac{X_{nat}}{X_{lab}}$$

NO CALIBRATION NEEDED!

Conclusions

- **We have demonstrated the power of Isotope Pattern Deconvolution for the easy and accurate re-certification of natural standards. Besides we have shown that the preparation of solvent standards from vials containing, in theory, 1 mg of solid can be a simple yet an important source of error.**
- **Re-certification by IPD using a reference standard in solvent is able to correct these problems in a fast, reliable and cost-saving way.**
- **IPD allowed to compensate for the observed biases of +4.7% and -29% in 25(OH)D3 and 25(OH)D2 standard concentrations.**

[1]R.M. Gathungu, et al, Mass Spectrom. Rev. 32 (2013) 72–86.
[2] M.L. Musteata et al., Bioanalysis. 3 (2011) 1987–2002.
[3] N. Fabregat-Cabello et al. Clin. Chim. Acta. 473 (2017) 116–123

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