Sensitive LC-MS/MS Method for Determination of 25-Hydroxyvitamin D3 and its C3-Epimer after Diels-Alder Derivatization

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Overview

- Purpose: Develop a highly sensitive LC-MS/MS method for quantitative determination of 25-hydroxyvitamin-D3 (25-OH-D3), its C3-epimer (3-epi-25-OH-D3) and 24,25-dihydroxyvitamin-D3 (24,25-diOH-D3) in small volumes of human plasma from infants under one year of age.
- Methods: A sample preparation procedure involving derivatization with a substituted triazolinedione and a highly selective HPLC setup were developed. An API 4000 or TSQ Vantage triple quandrupole mass spectrometer equipped with electrospray source was used. Derivatization products of metabolites of vitamin D2 and D3 were monitored in MRM (SRM) mode.
- Results: The analytical method demonstrated high sensitivity, accuracy, precision, specificity and linearity. Complete separation of minor derivatized products of 25-OH-D3 and 3-epi-25-OH-D3 was achieved in less than 6 minutes and allowed determination of vitamin D levels in infants using only 20 mcL of plasma.

Introduction

The status of vitamin D in infants is receiving considerable attention from clinical researchers. It is generally accepted that the 3α epimers of both 25-hydroxyvitamins D3 and D2 (25-OH-D) account for a significant part of the total circulating 25-OH-D in infants under the age of 1 year. Until recently, most LC-MS/MS methods developed for the quantification of 25-OH-D were not sufficiently specific and the sum of concentrations of epimers of each analyte was reported. Notwithstanding that some researchers have developed LC-MS/MS assays that separate the 3α epimers, a sensitive high-throughput method remains a challenging task, especially in light of the fact that volume of sample available from very young patients often does not exceed 50 mcL.

Derivatization of vitamin D and it metabolites with substituted triazolinediones in a Diels-Alder cycloaddition reaction has been used often as a means to increase the MS response of these analytes.[1,2] On the other hand, published results describing the HPLC separation of epimers of 25-OH-D3 and D2 were obtained only with underivatized analytes, but their MS responses were many times lower than those achieved after derivatization.[3,4,5]

We therefore developed a method whereby chromatographic separation of epimers after derivatization was achieved thus ensuring sufficient sensitivity suitable for analysis of samples of small volume.

The conjugated double bonds near the A-ring of 25-OH-D may be attacked from either side during Diels-Alder cycloaddition. Since the C-3 hydroxyl is normally in β position, the major product (6S isomer)[6] is formed via attack from less hindered side of the ring. In the case of the 3α epimer, the attack occurs preferentially from the other side of the A-ring, yielding the 6R isomer as the major product (**Fig. 1**).

These two major derivatization products could not be resolved chromatographically within a reasonable run time, while the minor reaction products, 3β-6R and 3α-6S derivatives, were well separated using C18 reverse phase.

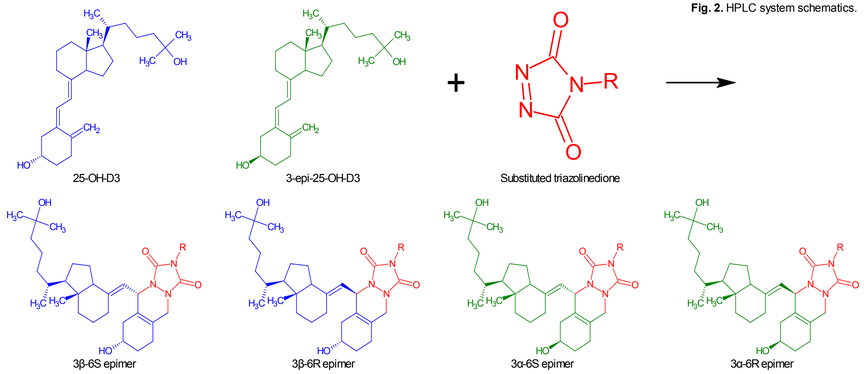

Fig. 1. Derivatization of a mixture of 25-hydroxyvitamin D3 and its C3-epimer by a substituted triazolinedione

Methods

The standard curve was prepared in organic solvent by dilution from stock solutions. For 25-OH-D3 and 25-OH-D2, NIST SRM-2972 calibration solutions were used as stocks. For 3-epi-25-OH-D3, the concentration of stock solution was determined from UV absorbance at 264 nm. Pooled serum samples from DEQAS were used as QC samples for 25-OH-D3. Deuterium labeled 25-OH-D3 (d6) was used as internal standard for 25-OH-D3, 3-epi-25-OH-D3 and 25-OH-D2. Deuterium labeled calcitriol (d6) was used as internal standard for 24,25-diOH-D3. The analytical ranges were 5-100 ng/mL for 25-OH-D3, 3-epi-25-OH-D3 and 25-OH-D2 and 0.5-10 ng/mL for 24,25-diOH-D3.

Sample preparation from 20 mcL of plasma or serum included the following steps:

- Dilution with water
- protein precipitation with isopropanol followed by sonication
- liquid-liquid extraction of the resulting mixture with hexanes followed by evaporation
- derivatization at room temperature with a solution of appropriate substituted triazolinedione
- evaporation and reconstitution in 50 mcL of acetonitrile followed by 150 mcL of water.

Typically, 20 mcL of extract were injected in the HPLC system with a SecurityGuard C8 2x4 mm (Phenomenex) precolumn installed on a switching valve (**Fig. 2**) with a mobile phase composed of acetonitrile/water/acetic acid 25/75/0.01 by volume pumped at 0.6 mL/min. At 0.7 min the valve was switched in order to elute the analytes from the precolumn to the analytical column, composed of one Kinetex 2.6 µ C18 50x2.1 mm and one Kinetex 2.6 µ XB-C18 50x2.1 mm (Phenomenex) in line. Both columns were thermostated at 70°C. The analytical mobile phase was composed of acetonitrile/water/acetic acid 44/56/0.01 by volume and it was pumped at 0.5 mL/min. At 1.0 min the valve was switched back and the precolumn was washed with dimethylformamide.

The analytes were detected with an API 4000 triple-quadrupole mass-spectrometer (AB-Sciex) equipped with a Turbo V TIS assisted electrospray source, or with a TSQ Vantage triple-quadrupole mass-spectrometer (Thermo Scientific) equipped with a HESI-II source. All analytes were monitored using appropriate mass transitions in positive MRM (SRM) mode, using two acquisition periods, the first one for 24,25-diOH-D3 and its IS, the second – for 25-OH-D metabolites.

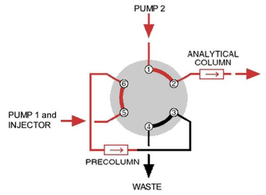

Fig. 2. HPLC system schematics.

Results

The minor Diels-Alder derivatives of 25-OH-D3 and its C3 epimer could be efficiently separated using the HPLC setup described (**Fig. 3**). Most likely, similar derivatives of 25-OH-D2 and its C3 epimer were chromatographically resolved as well, but we were unable to confirm this without a standard of 3-epi-25-OH-D2.

The chromatographic setup included a precolumn installed to a switching valve. Washing the precolumn after each injection permitted to complete removal of late eluting interference peaks. It also ensured signal stability by preventing the accumulation of any signal-supressing substances in the analytical columns and in the ionization source.

The recoveries of all four analytes using the extraction procedure described were higher than 95%, and no matrix effect was observed, therefore it was possible to use pure solutions of analytes for calibration purposes.

Despite the use of minor derivatization products for quantification, the sensitivity was very good. Our results for patient's samples suggest that the LLOQ for 3-epi-25-OH-D3 may be reduced to as low as 1 ng/mL without any modification to the method. The response for all analytes was a linear function of concentration on both instruments.

The intra-batch precision of the method was evaluated using four replicate aliquots of plasma samples from seven different healthy infants under 1 year of age. The results are summarized in **Table 1**.

To evaluate the suitability of this method for the assay of 25-OH-D3, recent DEQAS samples were extracted and injected together with patient's samples. The results are summarized in **Table 2**.

The use of NIST SRM-972 serum samples for quality control is planned in future.

Overall, in all the samples we measured from healthy infants under 1 year of age, concentrations as high as 37 ng/mL for 3-epi-25-OH-D3 and as high as 12 ng/mL for 24,25-diOH-D3 were observed. The concentrations of 25-OH-D2 were below the level of quantification in all human samples, except DEQAS 376.

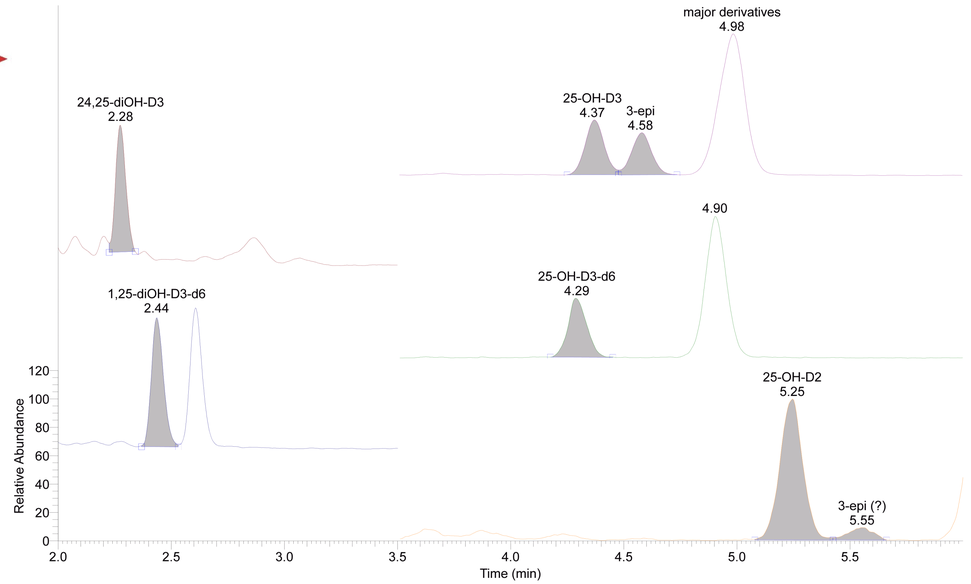

Fig. 3. Chromatograms of derivatization products. Plots for 25-OH-D3 and 24,25-diOH-D3 are for sample 94-3, the plot for 25-OH-D2 is for DEQAS sample 376.

Table 1. Intra-batch repeatability on four aliquots of patient's samples.

Sample	25-OH-D3		3-epi-25-OH-D3		24,25-diOH-D3	
	Average conc. (ng/mL)	%CV	Average conc. (ng/mL)	%CV	Average conc. (ng/mL)	%CV
36-2	32.8	1.8	6.54	4.3	3.27	4.0
94-3	64.4	4.0	37.4	2.0	8.48	3.7
65-6	51.5	0.8	11.5	2.7	7.52	4.9
59-8	50.4	2.0	8.02	4.4	3.54	2.0
75-9	25.4	2.2	N/d	N/a	0.833	7.1
48-12	24.9	3.2	1.76	3.9	2.13	1.9
133-8	47.3	8.6	7.58	9.6	3.38	6.9

Table 2. Results for DEQAS samples in comparison with ALTM (All methods mean)

DEQAS sample	Measured concentration of 25-OH-D3 only (except sample 376)		DEQAS ALTM nmol/L	Bias from ALTM, %	Batch date and instrument
	ng/mL	nmol/L			
376 (D2)	9.7	23.5			
376 (D3)	11.2	28.0	46.7	+10.2	
377	6.7	16.7	19.2	-12.9	Nov 2010 API-4000
378	31.6	78.9	68.2	+15.6	
379	17.1	42.7	45.0	-5.2	
380	24.4	60.9	60.3	+1.0	
381	31.4	78.4	85.1	-7.9	
382	13.1	32.7	37.7	-13.3	Dec 2010 TSQ Vantage
383	27.6	68.9	73.1	-5.8	
384	9.9	24.7	28.7	-13.9	
385	20.4	50.9	56.4	-9.7	

Conclusions

These results clearly show that Diels-Alder derivatization of metabolites of vitamin D is suitable not only to enhance the sensitivity in mass spectrometric detection of these substances, but also can improve the chromatographic separation of minor C3-epimers from main analytes. This offers new potential for developing sensitive LC-MS/MS methods with enhanced throughput for determination of these metabolites in infants under 1 year of age.

References

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