POSTER NOTE

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Quantification of 25-hydroxyvitamin D2 and D3 with Chromatographic Resolution of the C3-epimer in Human Plasma or Serum by Liquid Chromatography Tandem Mass Spectrometrys

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ABSTRACT

A clinical research analytical method for the quantification of 25-hydroxyvitamin D2 and D3 in human plasma or serum with chromatographic resolution of 3-epi-25-OH-vitamin D3 is reported. The method involves a simple protein precipitation step followed by online SPE using a Thermo Scientific™ Transcend™ II system; a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer with heated electrospray ionization is used for detection by single reaction monitoring (SRM) using 25-hydroxyvitamin D3-d6 as the internal standard. Method performance was evaluated using the MS7000 ClinMass® LC-MS/MS Complete Kit 25-OH-Vitamin D2/D3 in Plasma and Serum – On-Line Analysis from RECIPE to obtain limits of quantification, linearity ranges, accuracies and intra- and inter-assay precisions for both analytes.

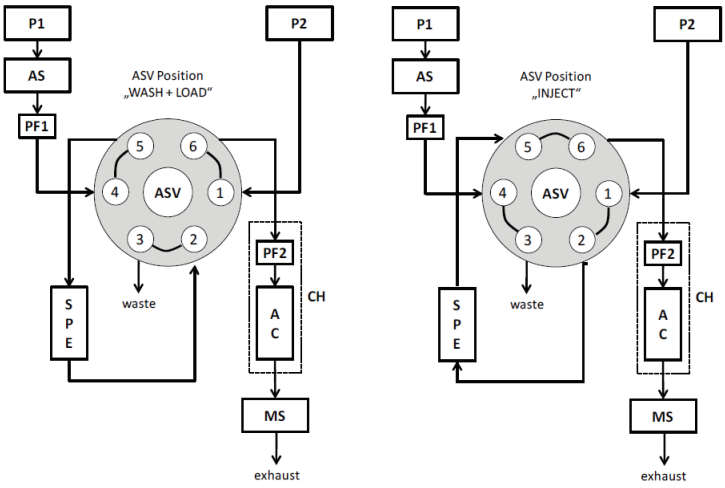
INTRODUCTION

An analytical method for clinical research for the quantification of 25-hydroxyvitamin D2 and D3 in human plasma or serum is reported. The method, based on the MS7000 ClinMass LC-MS/MS Complete Kit 25-OH-Vitamin D2/D3 in Plasma and Serum – On-Line Analysis from RECIPE, allows for the chromatographic separation of the C-3 epimeric form of 25-hydroxyvitamin D3 (3-epi-25-OH-vitamin D3). Sample extraction is performed by protein precipitation followed by online SPE using a Thermo Scientific Transcend II system; a Thermo Scientific TSQ Endura triple quadrupole mass spectrometer with atmospheric pressure chemical ionization is used for detection by single reaction monitoring (SRM) using 25-hydroxyvitamin D3-d6 as the internal standard. Implementation of the kit included the evaluation of the limits of quantification, linearity ranges, accuracy and intra- and inter-assay precision for each analyte.

MATERIALS AND METHODS

The reported analytical method for clinical research is based on the MS7000 ClinMass LC-MS/MS Complete Kit 25-OH-Vitamin D2/D3 in Plasma and Serum – On-Line Analysis from RECIPE and allows for the quantification of 25-hydroxyvitamin D2 and D3 in human plasma or serum. In this method, the C-3 epimeric form of 25-hydroxyvitamin D3 (3-epi-25-OH-vitamin D3), is separated from the analyte peak with a sufficient degree to avoid false high analytical values.

FIGURE 1. Schematic representation of the LC configuration used for on-line SPE



ABBREVIATIONS

P1 – SPE pump
P2 – HPLC pump
AS – autosampler
PF1 – inline-Filter
ASV – automatic switching valve
SPE – SPE column
PF2 – prefilter
AC – analytical column
CH – column heater
MS – tandem mass spectrometer

Sample Preparation

The kit includes calibrators and controls at four and two different levels, respectively, covering a concentration range between 8.2 and 68.5 µg/L for 25-hydroxyvitamin D2 and between 9.4 and 77.3 µg/L for 25-hydroxyvitamin D3. 25-hydroxyvitamin D3-d6 is used as the internal standard for the quantification of both analytes. Sample clean-up is performed by a preliminary protein precipitation with internal standard addition followed by on-line solid phase extraction (SPE) on a Transcend II system using mobile phases, SPE cartridge and analytical column provided with the kit. A schematic representation of the LC configuration is reported in Figure 1.

Liquid Chromatography

The LC method used for on-line SPE and chromatographic separation is reported in Table 1.

Mass Spectrometry

Detection was performed by SRM on a TSQ Endura triple quadrupole mass spectrometer with atmospheric pressure chemical ionization operated in positive mode. Two SRM transitions for each analyte were included in the acquisition method for quantification and confirmation, respectively. SRM transitions are reported in Table 2.

TABLE 1. LC method description

Time (min)	ASV Position	Pump P1 (SPE buffer)		Pump P2 (Mobile phase)	
		Flow (mL/min)	Event SPE column	Flow (mL/min)	Event Analytical column
0.00	Load	0.1	Loading	0.5	Equilibration
0.01		5.0			
0.75	Inject	5.0	Elution	0.5	Loading
0.85		0.1			Separation
2.15		0.1			
2.20	Load	2.0	Equilibration	0.5	Equilibration
2.85		2.0			
2.90		0.1			
3.00		0.1			

The following MS conditions were used:

Source type	Atmospheric Pressure Chemical Ionization (APCI) in positive mode
Vaporizer temp	350ºC
Ion Transfer Tube temp	275ºC
Discharge current	4.5 µA
Sheath gas	50 AU
Sweep gas	0 AU
Auxiliary gas	5 AU
Data acquisition mode	Selected reaction monitoring (SRM)
Chrom filter peak width	3.0 s
Collision gas pressure	1.5 mTorr
Cycle time	0.4 s
Q1 (FWMH)	0.7
Q3 (FWMH)	0.7

RESULTS

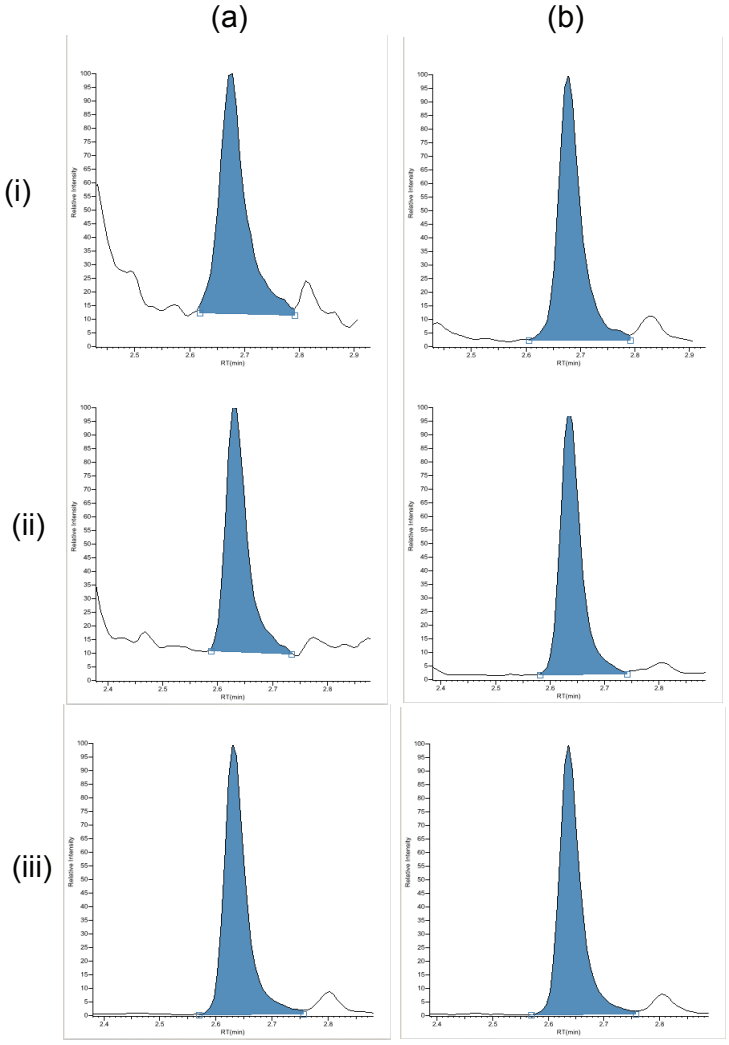
The method performance was evaluated by obtaining limits of quantification, linearity ranges, accuracy and intra- and inter-assay precision for each analyte; the method proved to be linear in the calibration range covered by the kit, with limits of quantification (LOQ) of 2.1 µg/L for both analytes, an upper limit (ULOQ) of 154.7 and 157.0 µg/L for 25-hydroxyvitamin D2 and D3, respectively and a correlation factor (R^2) always above 0.997.

TABLE 2. SRM transitions and parameters

Analyte	Precursor (m/z)	Product (m/z)	Collision Energy (V)
25-hydroxyvitamin D2	395.3	209.2	26
		269.2	18
25-hydroxyvitamin D3	383.3	257.3	15
		211.2	25
d6-25-hydroxyvitamin D3	389.4	263.4	17
		211.2	27

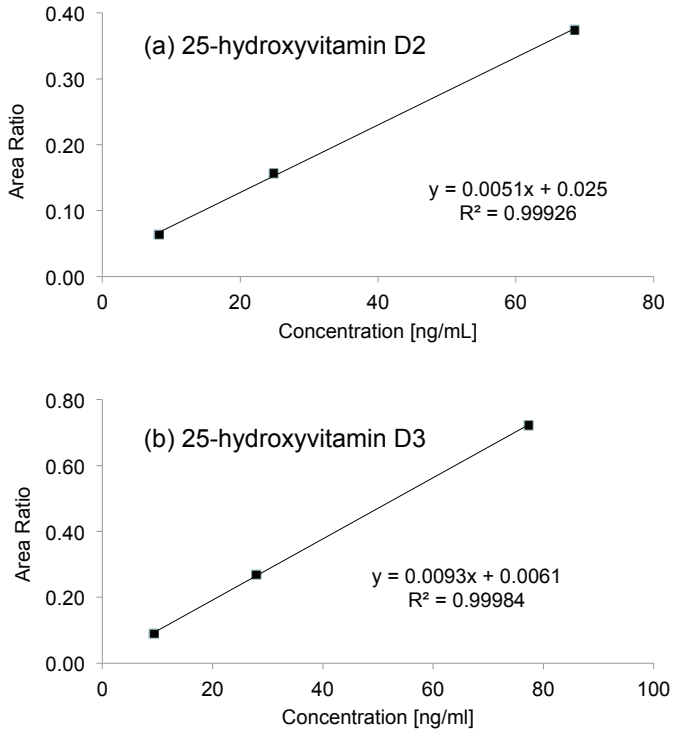
Representative chromatograms at the LOQ and ULOQ are reported in Figure 2 for the analytes and the internal standard.

FIGURE 2. Representative chromatograms at the (a) LOQ and (b) ULOQ for (i) 25-hydroxyvitamin D2 and (ii) D3 and (iii) their internal standard 25-hydroxyvitamin D3-d6



Representative calibration curves for both analytes are reported in Figure 3.

FIGURE 3. Representative calibration curves for (a) 25-hydroxyvitamin D2 and (b) 25-hydroxyvitamin D3



Analytical accuracy was evaluated in terms of trueness of measurement using the DEQAS Proficiency Test Sample #466 and #467 from DEQAS prepared and analyzed on 4 different days in single runs each day; the percentage bias between nominal and average back-calculated concentration for these control samples was 2.4 and 2.5% for 25-hydroxyvitamin D2 and D3, respectively. Results are reported in Table 3

TABLE 3. Analytical accuracy for (a) 25-hydroxyvitamin D2 and (b) 25-hydroxyvitamin D3

	Sample	n	Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	CV (%)	Bias (%)
(a)	DEQAS 466	5	25.8	26.4	4.3	2.4
(b)	DEQAS 467	5	17.8	18.2	6.5	2.5

Intra-assay precision was evaluated in terms of percentage coefficient of variation (%CV) using the kit controls at both levels in replicates of eight (n=8) prepared and analyzed in one batch; the %CV for intra-assay precision was 4.5% and 3.1% for the lower and upper control for 25-hydroxyvitamin D2, respectively and 2.2% and 2.8% for the lower and upper control for 25-hydroxyvitamin D3, respectively. Results are reported in Table 4.

TABLE 4. Intra-assay precision for (a) 25-hydroxyvitamin D2 and (b) 25-hydroxyvitamin D3 (n=8)

	Level I Measured Concentration (ng/mL)	CV (%)	Level II Measured Concentration (ng/mL)	CV (%)
(a)	15.4	4.5	37.1	3.1
(b)	19.9	2.2	45.8	2.8

Inter-assay precision was evaluated on the same controls in replicates of three (n=3) prepared and analyzed on five different days; the %CV for inter-assay precision was 10.0% and 8.3% for the lower and upper control for 25-hydroxyvitamin D2, respectively and 4.3% and 3.0% for the lower and upper control for 25-hydroxyvitamin D3, respectively. Results are reported in Table 5.

TABLE 5. Inter-assay precision for (a) 25-hydroxyvitamin D2 and (b) 25-hydroxyvitamin D3 (n=15)

	Level I Measured Concentration (ng/mL)	CV (%)	Level II Measured Concentration (ng/mL)	CV (%)
(a)	16.4	10.0	38.7	8.3
(b)	19.6	4.3	45.8	3.0

CONCLUSIONS

A liquid chromatography tandem mass spectrometry method for clinical research for the quantification of 25-hydroxyvitamin D2 and D3 in human plasma or serum using the MS7000 ClinMass® LC-MS/MS Complete Kit 25-OH-Vitamin D2/D3 in Plasma and Serum – On-Line Analysis from RECIPE was implemented and analytically validated on a Transcend II system connected to a TSQ Endura triple quadrupole mass spectrometer. This analytical method allows for the chromatographic resolution between 25-hydroxyvitamin D3 and its C-3 epimeric form, ensuring accurate quantitation of both Vitamin D epimers. The method meets research laboratory requirements for sensitivity and linearity of response covering analyte concentration ranges provided by the kit.

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