Analysis of 1α,25-dihydroxyvitamin D3 (calcitriol) in a biological fluid using SLE-SPE-LC-MS/MS

Joanna Denbigh, Tony Edge, Joanne Gartland, Tim Liddicoat, Kimberly Phipps

Thermo Fisher Scientific, Runcorn, Cheshire, UK, WA7 1TA

Overview

1α,25-dihydroxyvitamin D3 (calcitriol) is the biological form of vitamin D3 and is used as an indicator for a number of disease states such as chronic renal failure and hypoparathyroidism. With the increased interest to monitor calcitriol in the clinical environment, the design of a robust assay is essential to ensure that appropriate quantitation is obtained.

Here, a simple and sensitive method has been developed which uses a comprehensive sample preparation technique of supported assisted liquid liquid extraction (SLE) and solid phase extraction (SPE), coupled with LC-MS/MS analysis to give an accurate and precise measurement of calcitriol in plasma.

The assay developed has been shown to be precise, accurate and linear over the range 1 ng/mL to 100 ng/mL in accordance with the 2001 FDA guidleines [1].

Introduction

Vitamin D plays important roles in bone health and a variety of other pathophysiological conditions such as diabetes and cardiovascular disease. There are two common forms of vitamin D, cholecalciferol (D3) and ergocalciferol (D2). Vitamin D3 is produced in the skin of vertebrates after exposure to ultraviolet B light from the sun or artificial sources, and can also be obtained from diet, as it occurs naturally in a small range of foods. In some countries staples such as milk, flour and margarine are artificially fortified with vitamin D, and it is also available as a supplement in tablet form. In the body, vitamin D3 undergoes metabolism to 25-hydroxyvitamin D3 in the liver, and then is further metabolised to 1α,25-dihydroxyvitamin D3 in the kidney. 1α,25-dihydroxyvitamin D3 is the biological active form of vitamin D3 and is useful for evaluation of several diseases including chronic renal failure, sarcoidosis, hypoparathyroidism and rickets.

Measurement of 1α,25-dihydroxyvitamin D3 is very challenging due to the presence of interfering substances in plasma. It has been historically measured by radio receptor assay and radio immunoassay, which require extensive time consuming sample preparation to remove interfering substances and matrix effects.

Methods

Sample Preparation

Thermo Scientific HyperSep Retain PEP 30 mg/mL 96-well plate
Experimental SLE 200mg/mL 96-well plate
Sample pretreatment: Aliquot 475 µL of blank plasma or sample into a clean tube. Add 25 µL of standard spiking solution in methanol, for blanks and samples add methanol. Add 20 µL of internal standard spiking solution in methanol, for blanks add methanol. Add 100 µL of 10% ammonia solution and mix well.
Subject all samples to SLE followed by SPE methodology.

Table 1. Extraction Method

	SLE	HyperSep Retain PEP
Bed Weight	200 mg/mL 96-well plate	30 mg/mL 96-well plate
Conditioning	N/A	1 mL of methanol. 1 mL of water
Load	Load with a pulse of vacuum and wait 5 minutes	Load reconstituted SLE sample, load very slowly over ~3 mins
Wash	N/A	2 x 1 mL 40:60 methanol: water
Elute	Elute with 8 x 500 µL MTBE. Evaporate to dryness under nitrogen. Reconstitute in 1mL 60:40 methanol: water. Gently vortex mix and sonicate for 5 minutes	2 x 500 µL methanol, elute very slowly over ~ 2mins. Evaporate to dryness under nitrogen. Reconstitute in 1 mL 70:30 methanol: water. Gently vortex mix and sonicate for 5 minutes

Separation Conditions

Instrumentation: Thermo CTC Autosampler, Thermo Scientific Accela 600 pump
Column: Thermo Scientific Hypersil GOLD Phenyl 1.9µm, 50 x 2.1mm
Mobile Phase A: 70:30 methanol: water + 0.5% formic acid
Mobile Phase B: methanol + 0.5% formic acid
Gradient: 0 min 100% A, 0.5 min 0%A, 1min 0%A, 1.5 min 100% A, 4min 100% A.
Flow rate: 0.5 mL/min; Column temperature: 40°C; Injection volume: 25 µL

MS/MS Conditions

Instrumentation: Thermo Scientific TSQ Vantage
Ionization conditions: APCI Positive

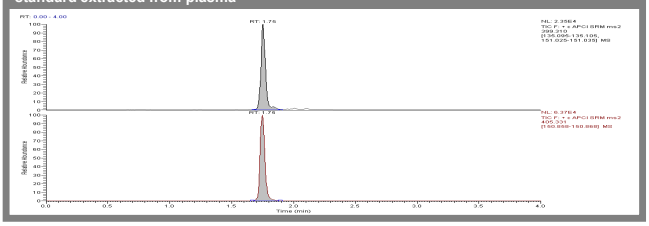
Table 2. MS/MS ions monitored

Precursor	Product	Collision Energy (eV)	S-lens (Arb units)
399.310	135.100	25	100
399.310	151.030	20	100
405.331	150.863	20	100

Results

Figure 1 displays chromatograms of extracted calcitriol standard at 70 ng/mL in plasma and deuterated calcitrol internal standard at 125 ng/mL. These chromatograms exemplify the extraction procedure in action showing a strong signal for calcitriol and low background from the matrix interferences.

FIGURE 1. Chromatograms of calcitriol standard and deuterated calcitriol internal standard extracted from plasma



Carryover

Table 3 shows the low carryover observed with this methodology. The assay gave a maximum carryover of 17% of the LLOQ.

Table 3. Carryover

	Calcitriol	Deuterated Calcitriol
Std 1 peak area=	2948	65525
20% Std 1 peak area =	589	13105
Blank after Std 8 peak area=	519	70
Carryover pass/fail (pass = <20% S1)	Pass	Pass

Linearity, accuracy and precision

Figure 2 shows the linear response of calcitriol using an internal standard of deuterated calcitriol. A dynamic range of 1 ng/mL to 100 ng/mL was demonstrated to achieve an r^2 value of 0.9993.
Table 4 shows the eight standards that were run over the dynamic range, each showing an accuracy within 11% of its specified concentration.
Table 5 shows the four sets of quality control samples that were evaluated at 1 ng/mL, 5 ng/mL, 20 ng/mL and 70 ng/mL, six replicates of each produced CV's of 6.8, 9.6, 3.3 and 6.8 % respectively.

FIGURE 2. Calibration curve to demonstrate linearity at 1ng/mL to 100ng/mL calcitriol in plasma

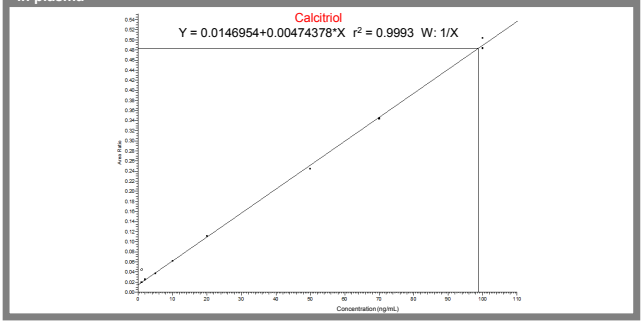


Table 4. Accuracy of calibration standards

Sample Name	Response Ratio	%Diff	Specified Concentration (ng/mL)	Calculated Concentration (ng/mL)
Std 1 (average of 2 replicates)	0.02	-3.95	1	0.961
Std 2	0.03	10.8	2	2.22
Std 3	0.04	-6.87	5	4.66
Std 4	0.06	-0.04	10	1.00
Std 5	0.11	1.99	20	20.4
Std 6	0.24	-3.02	50	48.5
Std 7	0.34	-0.78	70	69.5
Std 8 (average of 2 replicates)	0.29	-1.90	60	59.0

Table 5. Precision of quality control samples (averages of 6 replicates)

Sample Name	Response Ratio	%Diff	Specified Concentration (ng/mL)	Calculated Concentration (ng/mL)	%CV
QCLLOQ	0.02	-2.52	1	0.970	6.83
QCLOW	0.04	-2.55	5	4.87	9.60
QCMED	0.12	7.83	20	21.6	3.26
QCHIGH	0.32	7.28	70	75.1	6.81

Conclusions

• Supported liquid extraction coupled with solid phase extraction offers an alternative to radio immunoassay as a sample preparation technique for the analysis of calcitriol in plasma.

• We have demonstrated that this assay gives good linearity, accuracy and precision and low levels of carryover for a simple and efficient analysis, that is required by the growing clinical interest in calcitriol.

References:

[1] Guidance for Industry: Bioanalytical Method Validation, FDA, May 2001

Additional Information

For additional information, please visit our Chromatography Resource Centre which can be found at: www.thermoscientific.com/CRC

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