Application Note: 544

Quantitative Analysis of Serum 1α,25-dihydroxyvitamin D by APPI-LC-MS/MS

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Key Words

- TSQ Vantage
- Clinical Research
- Endocrine Analysis

Introduction

Quantitation of 1α,25-dihydroxyvitamin D_2 and D_3 (1,25D) in serum is very important in clinical research but is challenging because of the low circulating serum concentration of 1,25D. Due to its high analytical specificity and sensitivity, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been used for quantitation of 1,25D.

We have previously reported the use of immunoextraction and atmospheric pressure chemical ionization (APCI) for LC-MS/MS analysis of 1,25D in human serum[1]. Immunoextraction greatly simplifies the sample preparation and efficiently removes interferences. In addition, while APCI is good for this analysis, atmospheric pressure photoionization (APPI) is a more specific ionization technique than APCI and, therefore, further improves the analytical sensitivity of 1,25D detection.

Goal

To develop a highly sensitive LC-MS/MS analytical method to quantitate 1,25D with APPI using immunoextraction that provides better sensitivity than an APCI method.[1]

Methods

Sample Preparation

Serum 1,25D was purified with an immunoextraction method using an ImmunoTube® immunoextraction tube (Immundiagnostik AG, Bensheim, Germany). Briefly, samples were mixed with immobilized 1,25D antibody slurry and incubated at room temperature for 1 hour before the 1,25D-antibody beads were washed with aqueous buffer. Then, $1,25D_2$ and $1,25D_3$ were eluted with ethanol, dried, and reconstituted for LC-MS/MS injection.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Thermo Scientific Accela UHPLC system. A Thermo Scientific Hypersil GOLD column (150 × 1 mm, 3 µm particle size) was used. The column temperature was maintained at 50 °C. Mobile phases were 70% methanol in water and methanol from Fisher Chemical brand. The LC method used a 10-minute gradient, and the LC flow was diverted to the mass spectrometer between 2 and 5 minutes.

The mass spectrometer was equipped with an APPI probe and operated in the positive ion mode. Selected reaction monitoring (SRM) transitions of $1,25D_2$, $1,25D_3$, d6-$1,25D_2$ and d6-$1,25D_3$ were monitored (see Table 1).

Table 1. SRM transitions

	Q1 *(m/z)*	Q3 *(m/z)*	CE (V)	S-Lens (V)
$1,25D_2$	411.3	135.0	19	87
		151.0	20	87
$1,25D_3$	399.2	135.0	21	90
		151.0	22	90
d6-$1,25D_2$	417.3	151.0	19	95
d6-$1,25D_3$	405.3	151.0	20	90

Validation

The validation procedure included tests for 1) recovery, linearity, and lower limit of quantitation (LLOQ) and 2) precision.

Results and Discussion

1. Sample Preparation

The immobilized 1,25D antibody used in this study was highly specific and had no cross-reactivity from other vitamin D derivatives. Serum samples processed with immunoextraction showed no matrix effects or ionization suppression.

2. Recovery, Linearity, and LLOQ

Two sets of calibrators were prepared in ethanol (solvent) and pooled human plasma sample. Human plasma contains endogenous 1,25D, so it is not an appropriate choice to be used as the matrix for calibrators. Different levels of 1,25D were spiked into both solvent and human plasma to evaluate the feasibility of using solvent as the calibrator matrix. Solvent calibrators were prepared without immunoextraction, but with drying and reconstituting steps. Endogenous concentrations of 1,25D in pooled plasma were determined with solvent calibrators first. The pooled human plasma samples were then spiked with increasing levels of 1,25D and processed with immunoextraction. Concentrations of total 1,25D (endogenous and spiked concentration) in plasma were determined against solvent

calibrators and compared to expected concentrations to calculate recovery (Table 2).

Table 2. Recovery

$1,25D_2$			$1,25D_3$		
Expected (pg/mL)	Measured (pg/mL)	Recovery (%)	Expected (pg/mL)	Measured (pg/mL)	Recovery (%)
43.7	45.0	103.0	11.1	11.1	100.0
48.7	47.3	97.2	16.1	17.4	108.5
58.7	57.0	97.1	26.1	27.7	106.5
88.7	99.5	112.2	56.1	57.6	102.7
238.7	235.9	98.8	206.1	203.2	98.6

The slopes of the calibration curves of $1,25D_2$ and D_3 in both solvent and pooled human plasma calibrators were compared and found to be nearly identical (Figures 1 and 2). This indicated that 1,25D originated from spiked solvent and 1,25D originated from human plasma behaved similarly relative to their corresponding IS during the whole process of immunoextraction and LC-MS/MS.

The method was linear between 5 and 200 pg/mL for both $1,25D_2$ and $1,25D_3$. The LLOQ was 5 pg/mL for both $1,25D_2$ and D_3. Figure 3 shows the representative SRM chromatograms of $1,25D_2$ and $1,25D_3$ of the lowest calibrator in solvent and pooled human plasma.

3. Precision

Precision was determined with spiked charcoal stripped serum at both 10 and 20 pg/mL, which are close to the LLOQ (Table 3).

Table 3. Precision

$1,25D_2$	Measured (pg/mL)	Accuracy (%)	Precision (%)
10 pg/mL	9.1	90.8	8.4
20 pg/mL	19.8	99.2	7.4
$1,25D_3$			
10 pg/mL	9.9	98.8	12.5
20 pg/mL	20.9	104.4	11.1

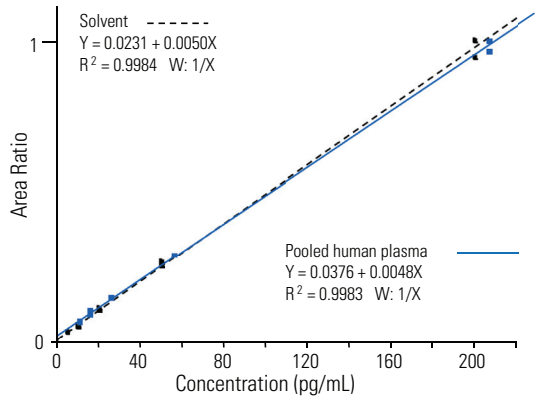

Figure 1. Calibration curves of $1,25D_2$ in solvent (dotted line, black) and pooled human plasma (solid line, blue)

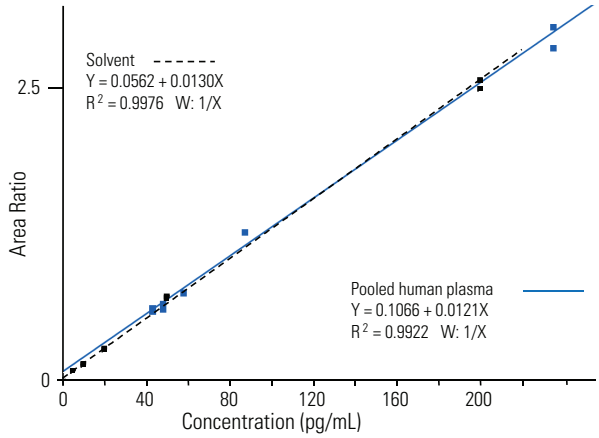

Figure 2. Calibration curves of $1,25D_3$ in solvent (dotted line, black) and pooled human plasma (solid line, blue)

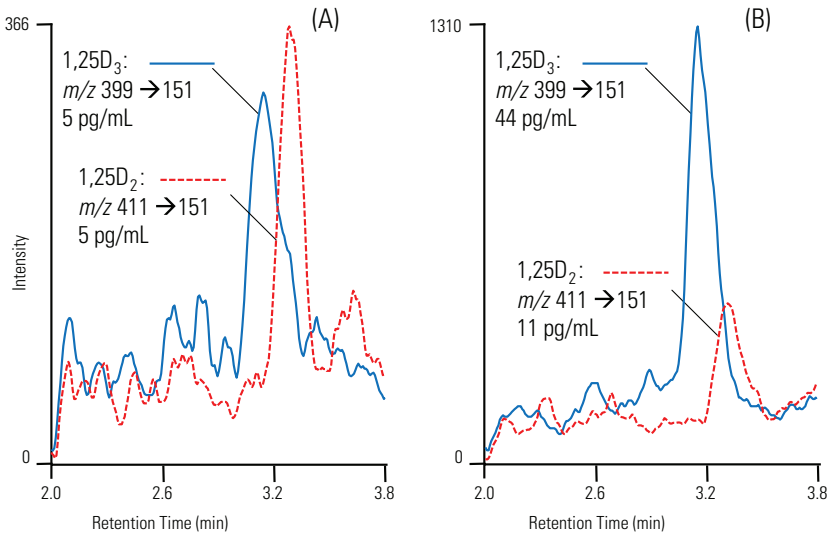

Figure 3. Representative SRM chromatograms of $1,25D_2$ and $1,25D_3$ of the lowest calibrator in solvent (A) and in pooled human plasma (B)

Conclusion

A fast and analytically sensitive LC-MS/MS method for quantitation of 1,25D in human plasma was developed for clinical research laboratories. Sample preparation was done with immunoextraction. APPI ionization was used for its ionization specificity and sensitivity. The LLOQ of this method was 5 pg/mL for both $1,25D_2$ and $1,25D_3$.

Reference

1. He, X; Damkroger, G; Kozak, M. *Quantitative Analysis of 1,25-dihydroxyvitamin D_2 and D_3 using Immunoaffinity Extraction with APCI-LC-MS/MS*, Thermo Fisher Scientific Application Note: 522.

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