# Method Comparison of Underivatized 1,25-Dihydroxyvitamin $D_2$ and $D_3$ Quantitative Analysis in Serum by LC-MS/MS Utilizing Ion Funnel Technology

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## Introduction

1,25-dihydroxyvitamin D exists in two forms – $D_2$ and $D_3$ – and while they are not isobaric to each other, both have several isobaric compounds which can be separated through liquid chromatography to achieve accurate quantitation using triple quadrupole mass spectrometry. Whereas it is common in positive ESI to identify analytes by their protonated species, 1,25-$(OH)_2$D is more commonly measured as the ammonium or lithium adduct. The work in this poster looks at these two adducts and assess the methods for sensitivity and robustness.

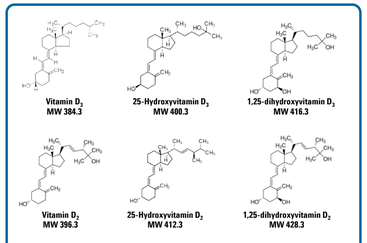



**Figure 1.** Structures

## Experimental

### Samples

1,25-$(OH)_2D_3$ standards were prepared neat in 50% MeOH and spiked into charcoal stripped serum at various concentrations

### Sample Preparation

*Ammonium adduct method*

Proteins were precipitated from 500 µL of serum with acetonitrile. Samples were vortexed, incubated at room temperature and centrifuged. The supernatant was transferred to autosampler vial for injection to the LC-MS/MS.

*Lithium adduct method*

Proteins were precipitated from 500 µL of serum with acetonitrile. Samples were vortexed, incubated at room temperature and centrifuged. The supernatant was evaporated to dryness under $N_2$ and reconstituted in 110uL of $H_2O$.

## Experimental

### LC Method

Agilent 1290 Infinity UHPLC series binary pump, 1260 binary pump, well plate sampler, temperature-controlled column compartment, 2 position/6 port switching valve

| Parameter | Value |
|---|---|
| Trapping Column | Eclipse Plus C18, 4.6x12.5mm 3.5mm |
| Analytical Column | Eclipse Plus C18, 2.1x50mm 1.8mm |
| Column Temp | 50°C |
| Injection Volume | 100 µl |
| Autosampler Temp | 4°C |
| Needle Wash | Flush port for 10 seconds |
| Mobile Phase A | $NH_4$ Formate or Lithium Acetate in $H_2O$ |
| Mobile Phase B | $NH_4$ Formate or Lithium Acetate in MeOH |

**Table 1.** LC Parameters

### MS Method

Agilent 6490 triple quadrupole mass spectrometer with Agilent JetStream in ion positive mode (ESI).

| Parameter | $NH_4$ Adduct | Li Adduct |
|---|---|---|
| Drying Gas Temp | 130°C | 130°C |
| Drying Gas ($N_2$) | 20 L/min | 17 L/min |
| Nebulizer Gas ($N_2$) | 15 psi | 60 psi |
| Sheath Gas Temp | 200°C | 100°C |
| Sheath Gas ($N_2$) | 11 L/min | 7 L/min |
| Capillary Voltage | 3500 V | 5000 V |
| Nozzle Voltage | 300 V | 2000 V |
| Q1/Q2 Resolution | 1.2/0.7 | 1.2/0.7 |
| Delta EMV | 400 V | 400 V |
| Collision Accel | 2 V | 2 V |

**Table 2.** MS Parameters

| Compound | Prec Ion | Prod Ion | Dwell | CE (V) |
|---|---|---|---|---|
| $([M+Li]-[H_2O])^+$ | 423.3 | 405.3 | 100 | 22 |
| $([M+Li]-[2H_2O])^+$ | 423.3 | 387.3 | 100 | 22 |
| $([M+Li]-[3H_2O])^+$ | 423.3 | 369.3 | 100 | 28 |
| $([M+NH_4]-[H_2O+NH_3])^+$ | 434.3 | 399.3 | 60 | 6 |
| $([M+NH_4]-[2H_2O+NH_3])^+$ | 434.3 | 381.3 | 60 | 8 |
| $([M+NH_4]-[3H_2O+NH_3])^+$ | 434.3 | 363.3 | 60 | 8 |

**Table 3.** MRM table

## Experimental

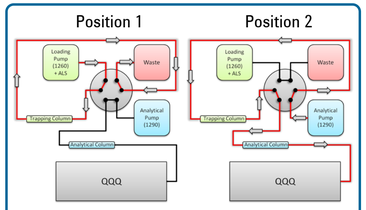



**Figure 2.** Back-Flush LC configuration for online sample cleanup

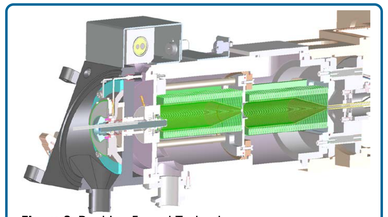


**Figure 3.** Dual Ion Funnel Technology

## Results and Discussion

### Analysis of 1,25-dihydroxyvitamin $D_3$

One of the challenges of this assay is that in many biological samples, 1,25-Diydroxyvitamin D is present at low picogram per milliliters levels and most instruments are unable to reach this level of sensitivity without the use of extensive sample preparation. There are several existing methods that employ chemical derivatization. However, in this work, development of a simple method without derivatization was explored. The proposed method utilizes state of the art ion funnel technology (Fig.3) integrated into the Triple Quadrupole Mass Spectrometer (QQQ) in conjunction with best in class, Ultra High Performance Chromatography (UHPLC) system, taking advantage of the chromatographic separation power of the column packed with sub two micron particles to achieve the limit of quantification (LOQ) of 10 pg/mL for 1,25-dihydroxyvitamin $D_3$ in extracted biological matrix.

### Signal selection for quantitation

Both ammonium and lithium adducts loose 1,2 and 3 water molecules under CID. The most intense product ion for both adducts is the first water loss – 434.3 $([M+NH_4]-[H_2O+NH_3])^+$ and 423.4 $([M+Li]-[H_2O])^+$. However, these fragments suffer from an interfering background resulting in poor specificity. The m/z 381 $([M+NH_4]-[2H_2O+NH_3])^+$ fragment was about 2 times smaller then m/z 399 fragment but showed no interfering peaks. The m/z 405.3 $([M+Li]-[H_2O])^+$, and 387.1 $([M+Li]-[2H_2O])^+$ suffer from interferences but the 3rd water loss product ion at 369.1 $([M+Li]-[3H_2O])^+$ does not and provides the required sensitivity.

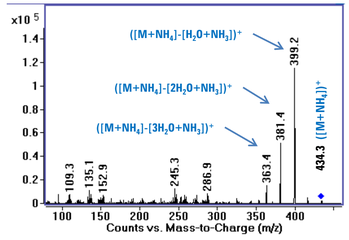



**Figure 4.** Ammonium adduct product ion spectra

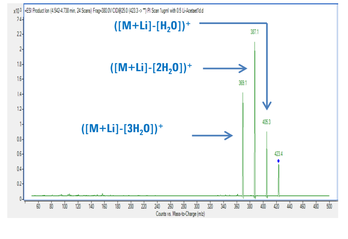
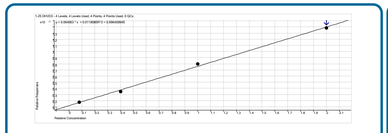


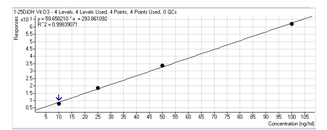

**Figure 5.** Lithium adduct product ion spectra

## Results and Discussion



| Level | Exp. Conc. | RT | Resp. | Conc. | Accuracy |
|---|---|---|---|---|---|
| 1 | 10 | 10.67 | 276.6 | 9.26 | 92.63 |
| 2 | 40 | 10.68 | 526.9 | 41.22 | 103.05 |
| 3 | 100 | 10.67 | 857.8 | 98.17 | 98.17 |
| 4 | 200 | 10.67 | 1548.2 | 200.31 | 100.16 |

**Figure 6.** Results for 1,25-dihydroxyvitamin $D_3$ ammonium adduct



| Level | Exp. Conc. | RT | Resp. | Conc. | Accuracy |
|---|---|---|---|---|---|
| 1 | 10 | 12.67 | 782.8 | 8.20 | 81.97 |
| 2 | 25 | 12.67 | 1855.9 | 26.19 | 104.75 |
| 3 | 50 | 12.60 | 3363.6 | 51.46 | 102.93 |
| 4 | 100 | 12.64 | 6208.2 | 99.15 | 99.15 |

**Figure 7.** Results for 1,25-dihydroxyvitamin $D_3$ lithium adduct

### Limit of Quantification (LOQ)

The LOQ for ammonium and lithium adducts was observed at 10pg/ml with 100 µL injection (1 pg on column) (Fig.8b and d respectively). Calibration plots for both the ammonium and lithium adducts demonstrate excellent linearity with an $R^2 > 0.996$, (Fig. 6 & 7).

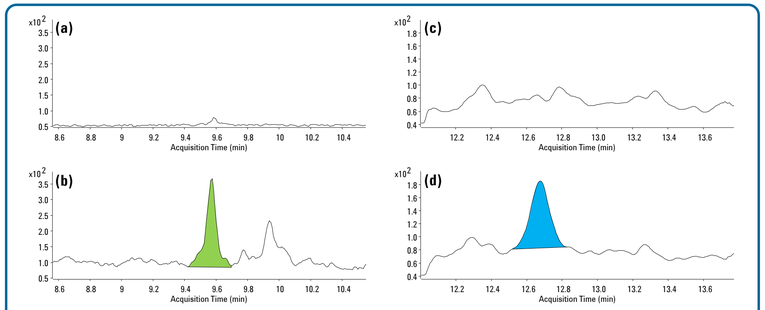



**Figure 8.** Extracted charcoal stripped serum containing 0pg/ml (a, c) and 10pg/ml (b, d) of 1,25-$(OH)_2$-$D_3$ monitoring transitions for ammonium adducts (a, b) and lithium adducts (c, d).

## Conclusions

The limit of detection for underivatized 1,25-dihydroxvitamin $D_3$ was observed at 10 pg/mL (1 pg on column) for both ammonium and lithium adducts when spiked into charcoal stripped serum. Linearity of calibration curves for both samples were found to have $R^2$ better then 0.996. However, the use of lithium acetate proved to be less robust compared to ammonium formate. While using lithium acetate, the source required more frequent cleaning, making the application less suitable.

## References

1. A-M. Kissmeyer, K. Sonne, *J. of Chrom. A* 935 (2001) 93-103
2. Z. Maunsell, D.J. Wright, S.J. Rainbow, *Clinical Chem.* 51:9 (2005) 1683-1690
3. R.J. Singh, R.L. Taylor, G.S. Reddy, S.K. Grebe, *J. Clin. Endocrinol. Metab.* 91(8) (2006) 3055-3061