Bioanalytical Quantitation Method for Analysis of 1, 25-Dihydroxyvitamin D3 (DHVD3) in Human Plasma Using Amplifex™ Reagent Kit on AB SCIEX Triple Quad™ 5500 System

A high-throughput bioanalytical method for low level quantitation of 1, 25-Dihydroxyvitamin D3 (DHVD3) in human plasma using Amplifex™ Reagent Kit on AB SCIEX Triple Quad™ 5500 LC/MS/MS System and UHPLC Chromatography

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Key challenges of 1, 25-Dihydroxyvitamin D3 Quantitation

- **Need for High Selectivity** - Low-level detection of DHVD3 is a big challenge due to the presence of co-eluting epimers and analogues (epimer 3-epi-25OHD3 and isobars (1-α-hydroxyvitamin-D3, and 7-α-hydroxy-4-cholesten-3-one).
- **Poor Ionization –** 1, 25-dihydroxy-vitamin D3 (DHVD3) comprises a group of secosteroids with low propensity to be ionized, low-level quantitation is a big challenge with secosteroids.

Key benefits of Amplifex™ Reagent Kit for 1, 25-Dihydroxyvitamin D3 Quantitation

- **High Sensitivity** – Low level Quantitation in human plasma (at pg/mL concentrations) is enabled by intelligent chemistry supported by **New, patented QJet® 2 Ion Guide** for ultimate sensitivity.
- **Excellent Precision And Accuracy** – Data quality (LLOQ QC, LQC, MQC and HQC levels) met USFDA bioanalytical method validation criteria.
- **Simple fast (30 min) and robust** reactions using Amplifex reagent

Figure 1: AB SCIEX Triple Quad™ 5500 System.

Figure 2: Amplifex™ Diene Reagent

Unique features of the Amplifex™ Diene Reagent for low-level detection

- Intelligent design boosts ionization efficiency and improves fragmentation.
- Substantial reduction in sample volume and analysis time is enabled.
- Generic derivatization technique can be used with any molecule that has a cis-diene system (e.g., a broader range of vitamin D3 and vitamin D2 analogs that are natural or synthetic).

Introduction

Vitamin D comprises a group of secosteroids, and mainly refers to two physiologically inactive fat soluble prohormones: vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol). Vitamin D3 is photochemically synthesized from 7-dehydrocholesterol in the epidermal layer of the skin of vertebrates under ultraviolet (UV) B light (270–290 nm) radiation. Vitamin D2 is derived from fungal and plant sterol when ergosterol is exposed to UV radiation. Vitamin D2 is not produced by the human body and is much less effective than vitamin D3 in humans.

Levels of vitamin D3 and its metabolites, 25 hydroxyvitamin D3 and 1, 25-dihydroxyvitamin D3, are usually clinical indicators of nutritional vitamin D deficiency in humans. Based on FDA guidance for bioavailability and bioequivalence studies for orally administered drug products, the moieties to be measured in biological fluids collected are either the API or its active moiety in the administered dosage form (parent drug) and, when appropriate, its active metabolites.For bioequivalence studies, measurement of the concentration–time profile of the parent drug released from the dosage form or the active metabolite is generally recommended. DHVD3 is the active metabolite of the Vitamin D3.

Measurement of a metabolite is preferred when parent drug levels are too low to allow reliable analytical measurement in blood, plasma or serum for an adequate length of time, or when a metabolite may be formed as a result of gut wall or other presystemic metabolism, or metabolite is active. Therefore, it is of more clinical importance to directly monitor DHVD3 levels in the human body when a vitamin D drug or a supplement is taken. LC-MS is the most commonly used technology for measuring low level analytes and metabolites in biological matrices. However, for such studies involving direct measurement of DHVD3, most LC-MS based approaches failed to meet the sensitivity requirements. In this study, we report the benefits of using an optimal solution based approach with sample preparation and LC-MS that addresses the sensitivity requirements of DHVD3 in human plasma.

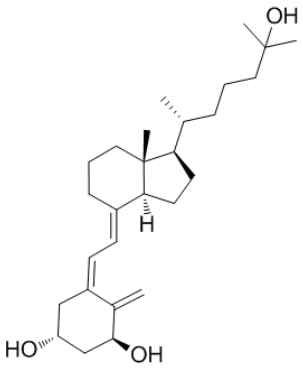

Figure 3: 1,25-Dihydroxyvitamin D3 (DHVD3)

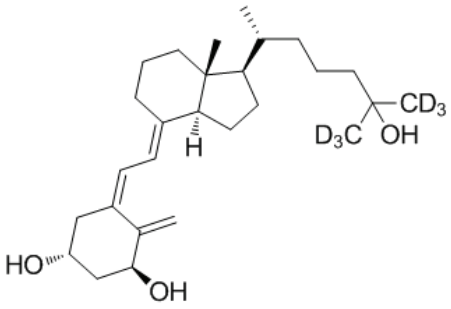

Figure 4: 1,25-Dihydroxyvitamin D3–d6 (DHVD3-d6)

Materials and Methods

Sample Preparation

- 475 µL of Blank pooled plasma was transferred in pre-labeled polypropylene tubes.
- 25 µL of Analyte was spiked in respective pre-labeled polypropylene tubes containing 475 µL of blank plasma.
- All the polypropylene tubes were vortexed for about 30 seconds.
- 50 µL of DHVD3-D6 Spiking Solution (1.00 ng/mL) was spiked in all polypropylene tubes (except in the Blank pp tube) and vortexed for about 30 seconds.
- 1, 25 dihydroxy affinity slurry (Alpco Diagnostics) was added to the above sample and mixed gently.
- Sample was loaded on reverse phase C18 cartridge.
- Contaminants were removed by washing with water followed by 10% methanol in water.
- Samples were eluted using methanol.
- Elute was evaporated to dryness under N_2 at 40°C.

Derivatization Using Amplifex™ Reagent

- Amplifex™ Diene reagent is available from SCIEX, 500 Old Connecticut Path, Framingham, MA 01701, USA
- 75 µL of Amplifex™ Diene reagent solution (1:1 Diluted with diluent provided in the kit) was added to dried residue and vortexed for 30 sec and leave the samples at ambient for 30 min for the derivatization to take place.
- Add 50 µL of water for quenching (stop the reaction). Vortex for 30 sec.
- Inject 20 µL of samples.

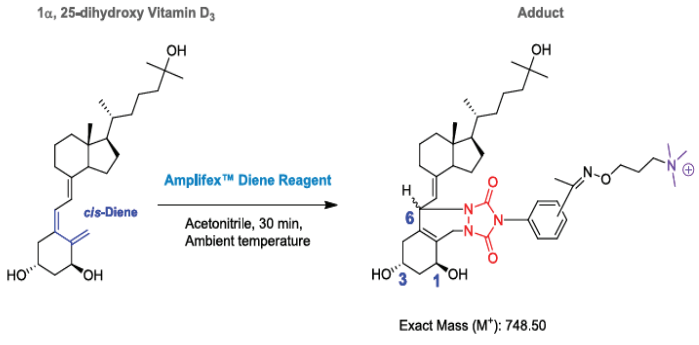

Figure 5: Chemistry of Diene Derivatisation

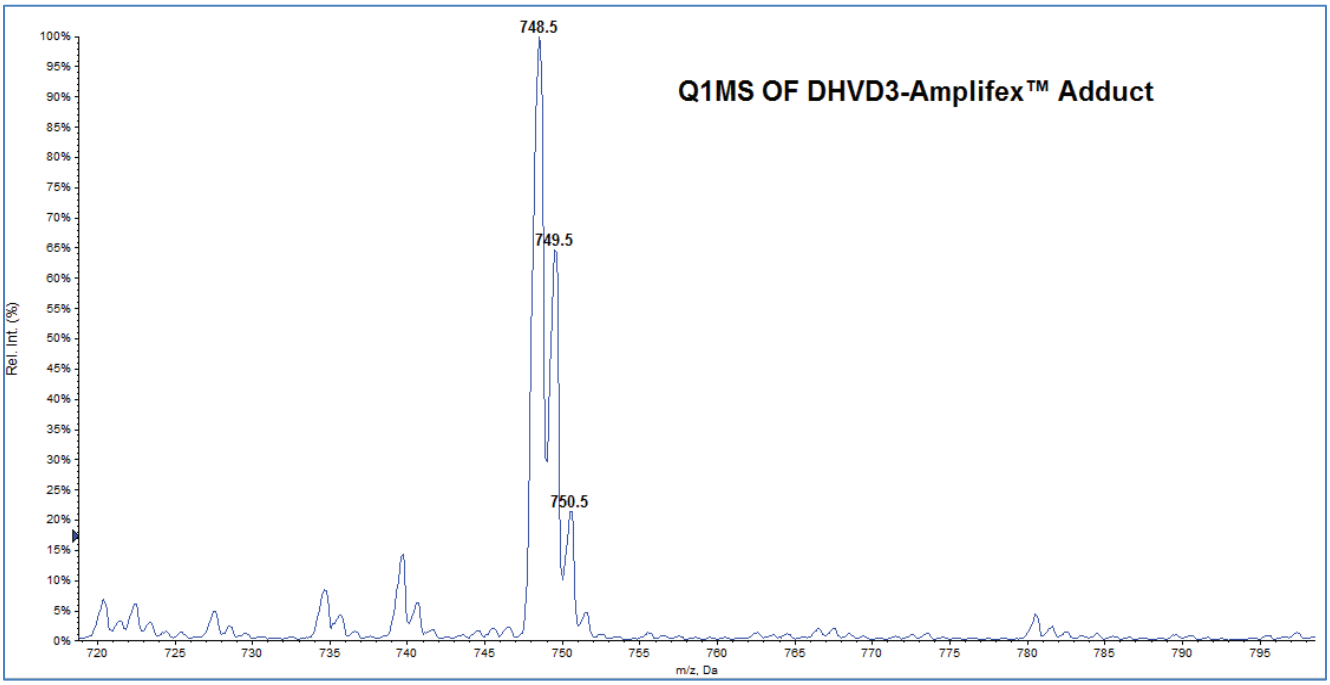

Figure 6: Q1MS Spectra of DHVD3-Amplifex™ Adduct

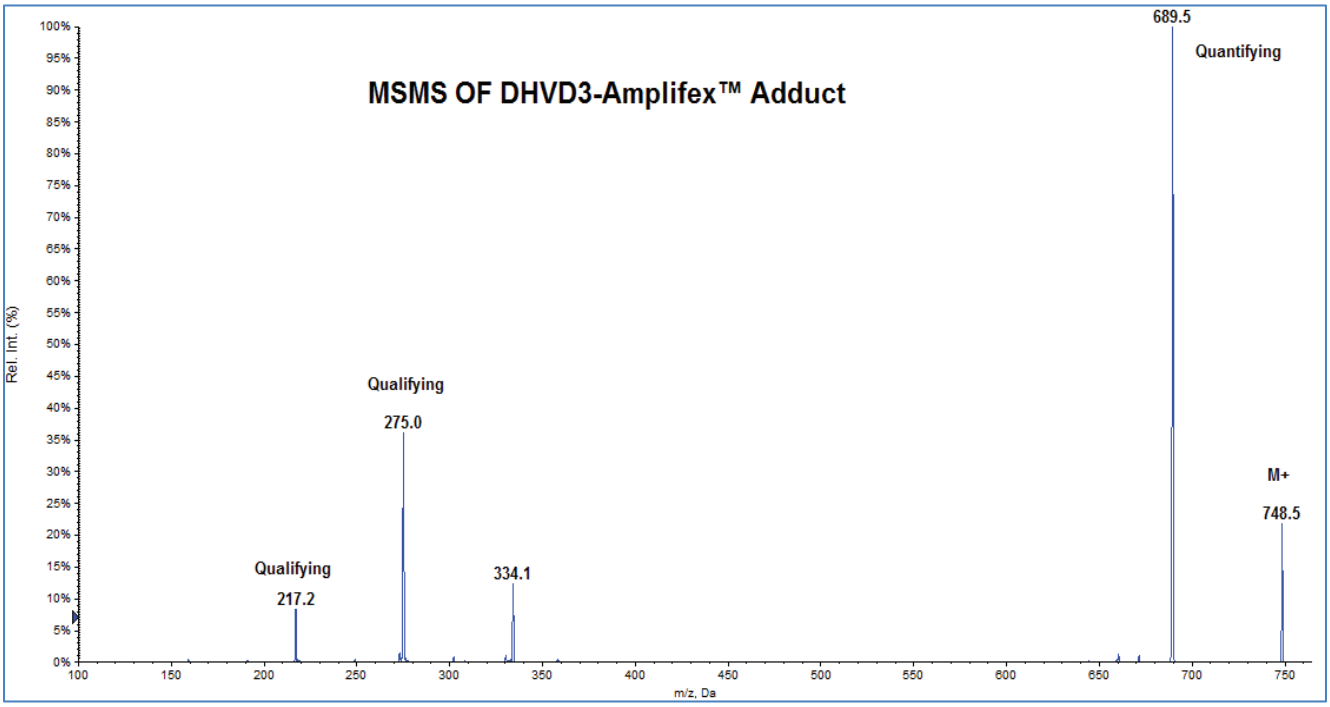

Figure 7: MSMS Spectra of DHVD3-Amplifex™ Adduct

Chromatography

LC System: Shimadzu Nexera with 30AC auto sampler
Column: Kinetics C18, 100 mm × 2.1 mm, 1.7 µ
Column Temp.: 40 °C
Injection Volume: 20 µL
Flow Rate: 0.350 mL/min
Mobile phases: A) 0.1% v/v Formic Acid in Water
B) 0.1% v/v Formic Acid in Acetonitrile

Gradient:

Time (min)	Module	Events	Parameter (%)
0.01	Pumps	Pump B Conc.	5.00
0.50	Pumps	Pump B Conc.	5.00
1.00	Pumps	Pump B Conc.	35.00
5.40	Pumps	Pump B Conc.	54.00
5.60	Pumps	Pump B Conc.	98.00
6.25	Pumps	Pump B Conc.	98.00
6.40	Pumps	Pump B Conc.	5.00
8.00	System Controller	Stop	

Table 1: Gradient Elution Method

Mass Spectrometry

Analysis of Amplifex™ Diene adducts of DHVD3 and DHVD3-d6 was carried out in ESI mode and required different mass spectrometric settings. The MRM transitions monitored for DHVD3 adduct was 748.5/689.4 and 754.7/695.5 for DHVD3-d6 adduct.

Dwell time: 250 ms.

Data System: AB SCIEX Triple Quad™ 5500 System

Interface: IonDrive™ Turbo V Source in positive ion mode

Instrument Parameter	Amplifex™ DHVD3	Amplifex™ DHVD3-d6
DP	50	66
EP	11	10
CE	40	38
CXP	20	31
CUR	40	
TEM	600 ºC	
IS	5500	
CAD	8	
GS1	50	
GS2	60	

Table 2: Mass spectrometry conditions

Data processing

Data acquired on the AB SCIEX Triple Quad™ 5500 System was processed using the quantitation tools within Analyst® 1.6 Software. The concentration curves were analyzed using a linear fit with a $1/x^2$ weighting.

Results and Discussion

Method Analysis and Data Quality

The DHVD3 quantitative assay was validated by using DHVD3-d6 as an internal standard spiked into human plasma. Figure 8 and Figure 9 shows representative chromatograms for plasma blank extract and plasma spiked lower limit of quantification with 2.59 pg/mL DHVD3 in plasma. Linearity range covered plasma concentrations varied from 2.59 pg/mL to 159.56 pg/mL in plasma. Lower limit of quantitation (LLOQ) in human plasma of 2.59 pg/mL resulted in an excellent signal to noise ratio of 31.3 showed in the Figure 9. Reproducibility of the assay was assessed by multiple replicate injections of quality control samples (Three Precision Accuracy batches, n=18) at LLOQ QC, LQC, MQC and HQC concentration levels. The calibration curve in plasma extracted samples showed excellent linearity with r value of >0.99 for the linearity range from 2.59 pg/mL to 159.56 pg/mL in extracted plasma samples (Fig. 11).

Fig.10 shows the chromatogram of the internal standard (DHVD3-d6) and Fig. 12 showing the chromatograms of 6 plasma extracted LLOQ QC samples from Precision Accuracy Batch 01 with good reproducibility.

Three precision accuracy batches were processed and data was compared, Table 3 displaying the data for between batch accuracy and precision for quality control samples. All the three batches were within the acceptance criteria of %CV ±20% at LLOQ level and ±15% at other levels.

Percent recovery of the DHVD3 from the extracted samples were calculated at three different levels (LQC, MQC and HQC) using peak area and compared with the 6 replicates of the unextracted samples at all the three levels. Percent recovery of the DHVD3-d6 internal standard was calculated using 18 samples from all concentration levels. Extracted sample data from PA Batch 03 was used for the recovery calculations. Percent recovery for DHVD3 was found to be 102% and for DHVD3-d6 it was 102%. Table 4 shows the recovery calculations for DHVD3 and DHVD3-d6.

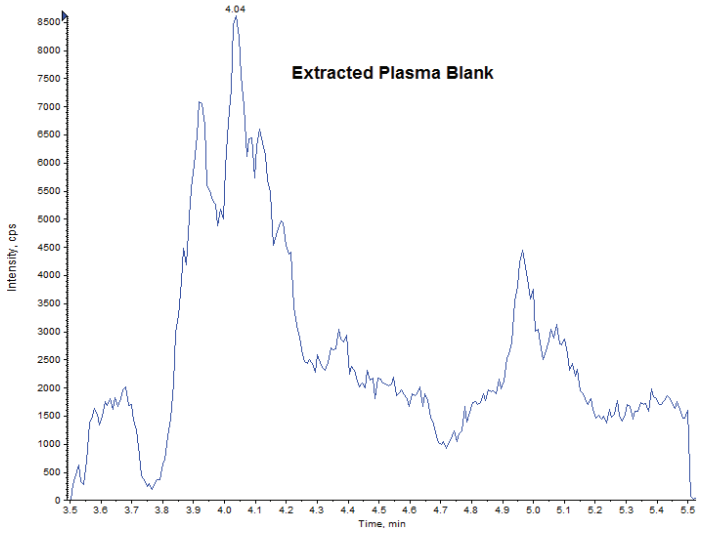

Figure 8: Chromatogram of Extracted Plasma Blank

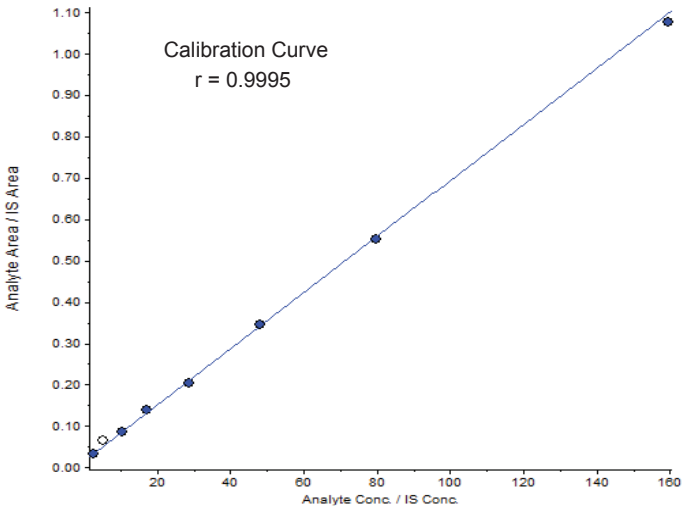

Figure 11: Calibration Curve (PA - 01) showing linearity range from 2.59 pg/mL to 159.56 pg/mL with r value of 0.9995

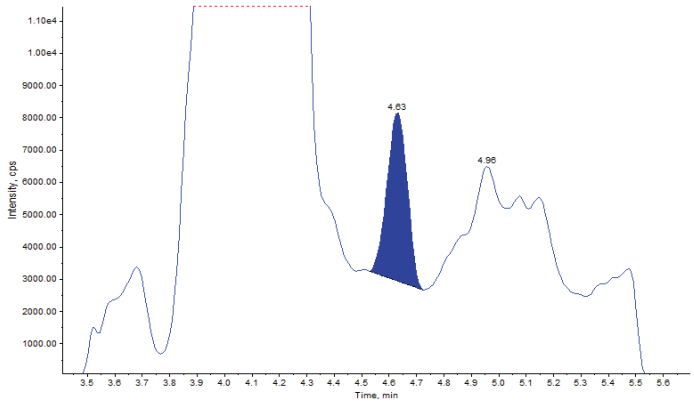

Figure 9: Chromatogram of Extracted Plasma LLOQ 2.59 pg/mL (103 fg on Column) with Signal to Noise (S/N): 31.3

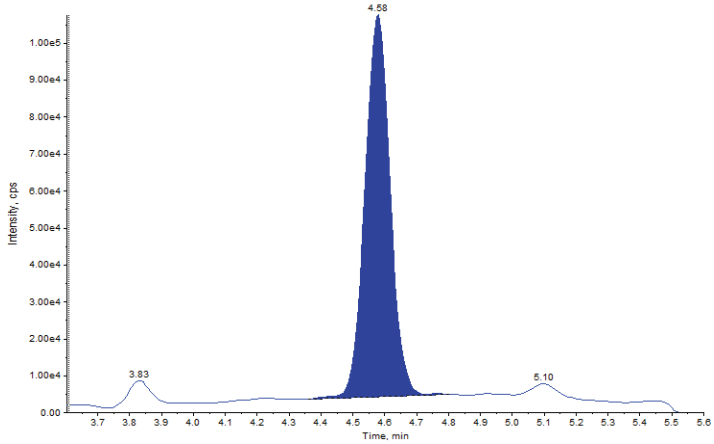

Figure 10: Chromatogram of DHVD3-d6 (Internal Standard)

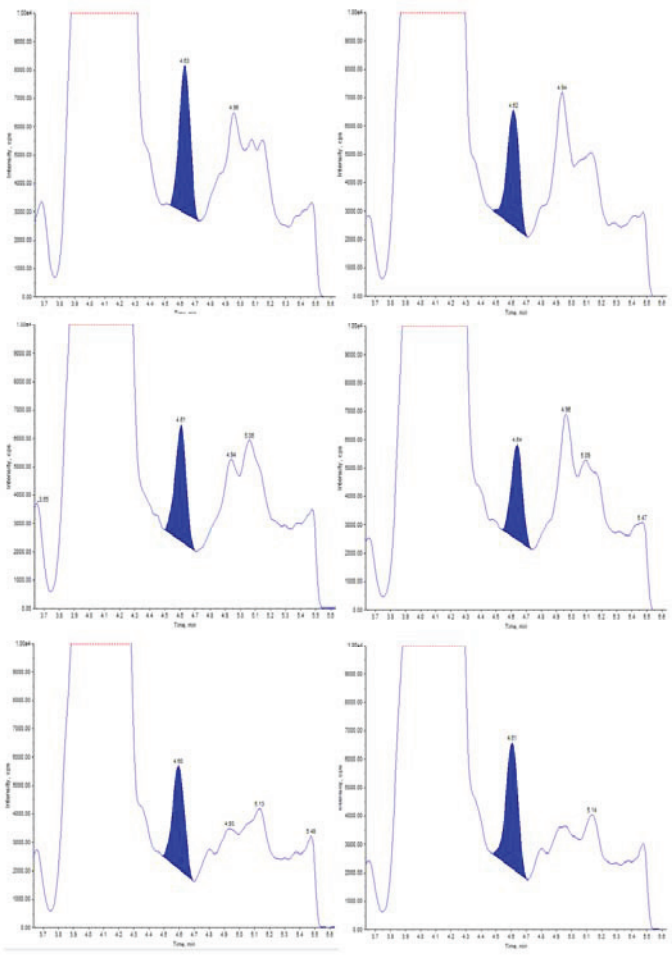

Figure 12: Chromatograms of six LLOQ QC samples

Sample	LLOQ QC	LQC	MQC	HQC
Nominal Concentration (pg/mL)	**2.75**	**8.10**	**82.97**	**127.65**
PA 01	2.67	7.19	84.34	132.28
	2.74	7.62	85.37	155.26
	3.03	7.53	89.01	129.5
	2.18	7.24	83.49	128.22
	2.72	7.93	86.43	137.46
	2.75	8.18	88.04	136.01
PA 02	2.61	9.43	88.56	131.42
	2.53	8.16	89.81	134.57
	3.24	8.33	86.20	136.15
	3.05	8.10	88.59	134.44
	3.13	8.77	90.48	136.68
	2.92	8.51	88.90	134.13
PA 03	2.64	8.44	80.13	120.67
	2.65	8.12	82.00	127.86
	2.67	8.40	81.84	124.81
	2.73	8.28	81.95	124.86
	2.77	8.41	82.17	125.61
	2.62	8.06	82.36	123.77
Mean	**2.758**	**8.150**	**85.537**	**131.872**
N	**18**	**18**	**18**	**18**
C.V. (%)	**8.91**	**6.58**	**3.88**	**5.88**
% Nominal	**100.30**	**100.62**	**103.09**	**103.31**

Table 3: Between Batch Precision and Accuracy

% Recovery								
Statistics	**DHVD3**						**DHVD3-d6**	
	LQC Response		**MQC Response**		**HQC Response**		**Internal Standard**	
	Extracted	**Unextracted**	**Extracted**	**Unextracted**	**Extracted**	**Unextracted**	**Extracted**	**Unextracted**
N	6	6	6	6	6	6	18	18
Mean	73292.0	71570.3	585805.0	575465.0	903956.0	885009.17	337999.22	330265.28
S.D	1447.01	2714.38	9273.28	9418.13	24347.87	31926.26	3087.55	3813.53
% C.V	1.97	3.79	1.58	1.64	2.69	3.61	0.91	1.15
% Recovery	**102.41**		**101.80**		**102.14**		**102.34**	
Mean % Recovery	**102.12**							

Table 4: % Recovery Table of DHVD3 and DHVD3-d6 at Three Concentration Levels

Conclusions

- A highly selective, sensitive and high-throughput bioanalytical method was developed and validated for the detection of low levels of the DHVD3, in human plasma on the AB SCIEX Triple Quad™ 5500 LC/MS/MS System.
- Amplifex reagent showed exceptional ability to derivatize DHVD3 and increased the sensitivity to many folds and showed good reproducibility.
- Method sensitivity for DHVD3 detection was exceptional (2.59 pg/mL in plasma), and demonstrated high-reproducibility and time effectiveness with good precision and accuracy
- Analyte recovery is 102.12% for DHVD3 and 102.34% for DHVD3-d6

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