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**Research Article****Open Access**

# Simultaneous Quantification of Vitamin D-2, Vitamin D-3, and their 25-Hydroxy Metabolites in Human Plasma by High Performance Liquid Chromatography

Syed N Alvi<sup>1</sup>, Ahmed Yusuf<sup>1</sup> and Muhammad M Hammami<sup>1,2\*</sup><sup>1</sup>Department of Clinical Studies and Empirical Ethics, King Faisal Specialist Hospital & Research Center, MBC-03, Riyadh 11211, Kingdom of Saudi Arabia<sup>2</sup>Alfaisal University College of Medicine, Riyadh, Kingdom of Saudi Arabia**Abstract**

A simple and reliable High Performance Liquid Chromatography (HPLC) method for simultaneous determination of vitamin D-2 (VD-2), vitamin D-3 (VD-3), 25-hydroxyvitamin D-2 [25 (OH) VD-2], and 25-hydroxyvitamin D-3 [25 (OH) VD-3] in human plasma was developed and validated. Plasma samples were deproteinized with a mixture of methanol and 2-propanol, and extracted with hexane. After evaporation, the residue was dissolved in methanol: water (9.6:0.4, v/v), centrifuged, and then clear solution was injected onto Zorbax C18 column. The mobile phase (gradient elution mode) consists of methanol, acetonitrile, and water (pH = 3.0); the eluents were monitored by photodiode array detector (wavelength set at 265 nm). The relationship between the concentration of VD-2, VD-3, 25(OH) VD-2, 25(OH) VD-3 in plasma and their peak area ratio to the IS was linear over the range of 5 - 100 ng/mL. The coefficients of variation for inter-day and intra-day assay were all  $\leq 9.7\%$  and biases  $\leq 13.1\%$ . Mean extraction recoveries of VD-2, VD-3, 25(OH) VD-2, and 25(OH) VD-3 from plasma were all over 80%. The method was applied for the determination of vitamin D levels in plasma obtained from healthy subjects. Further, it was used to assess the stability of VD-2, VD-3, 25(OH) VD-2, and 25(OH) VD-3 in plasma under various conditions encountered in the clinical laboratory.

**Keywords:** 25-Hydroxyvitamin D-2; 25-Hydroxyvitamin D-3; Vitamin D-2; Vitamin D-3; HPLC

**Introduction**

Vitamin D plays an important role in protecting from bone and potentially other metabolic diseases in humans [1-2]. Vitamin D exists in two forms: vitamin D-3 (cholecalciferol) synthesized from 7-dehydrocholesterol in the skin by the action of UVB radiation and vitamin D-2 (ergocalciferol) derived from plant/yeast by irradiation of ergosterol. Vitamin D made in the skin or supplied in the diet is metabolized in the liver to 25-hydroxyvitamin D, which is the major circulating form of vitamin D and best indicates vitamin D status. 25-hydroxyvitamin D is further metabolized in the kidney to 1, 25 dihydroxyvitamin D the active metabolite [3,4].

Deficiency of vitamin D is common with inadequate sun exposure. Recent studies reported that 30-50% of children and adults in many countries had 25 hydroxyvitamin D levels below 20 ng/mL [5].

25-Hydroxyvitamin D-3 [25(OH) VD-3] and 25-hydroxyvitamin D-2 [25(OH) VD-2] levels in human plasma have been determined by radioimmunoassay or most commonly by liquid chromatography or liquid chromatography/tandem-mass spectrometry [6-12]. HPLC method for simultaneous determination of 25- [25(OH) VD-3], [25(OH) VD-2], and VD-3 in plasma has been reported by Aksnes [13]. However, because of an interfering peak, the method was not suitable for measurement of VD-2 simultaneously. For the simultaneous measurement of vitamin D-2 (VD-2), vitamin D-3 (VD-3) 25-hydroxyvitamin D-2 [25(OH) VD-2], and 25-hydroxyvitamin D-3 [25(OH) VD-3] (Figure1) no method has been reported.

The objective of the current study was to develop, validate, and apply a simple HPLC assay for simultaneous quantification of VD-2, VD-3, 25(OH) VD-2, and 25(OH) VD-3 in human plasma using a readily available internal standard. In addition, the study addressed

the stability of these compounds under various clinical laboratory conditions.

**Materials and Methods****Apparatus**

Chromatography was performed on Waters Alliance HPLC 2695 (Waters Associates Inc, Milford, MA, USA) consisting of quaternary pump, auto sampler, column thermostat, and photodiode array detector set at 265 nm. A reversed-phase Zorbax C18 (4.6 x 250 mm, 5- $\mu$ m) column protected by a guard pak pre-column C18 (4.6 x 20 mm, 5 $\mu$ m) insert was used for separation. The data were collected with a Pentium IV computer using Empower Chromatography Manager Software.

**Chemicals and reagents**

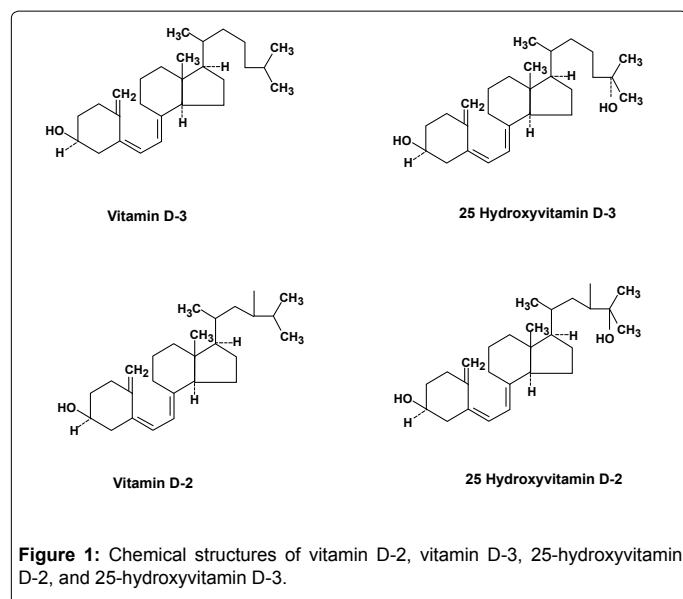
All chemicals were of analytical grade unless stated otherwise. Vitamin D-2 and vitamin D-3 were purchased from Acros Organics, New Jersey, USA, whereas 25(OH) VD-2 and 25(OH) VD-3 were purchased from Sigma-Aldrich, USA. Benzopyrene (100  $\mu$ g/mL solution in dichloromethane) was purchased from Ultra scientific, Kingstown, RI, USA. 2-Propanol, methanol, hexane, and acetonitrile

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were purchased from Fisher Scientific, Fairlawn, NJ, USA. Water for HPLC was prepared by reverse osmosis and further purified by using synergy water purification system (Millipore, Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia, after approval of the institution Research Ethics Committee.

### Chromatographic conditions

The mobile phase consisted of solvent-A: acetonitrile, methanol, and water (35:50:15, v/v) and solvent-B: acetonitrile: methanol: water (pH=3.0 adjusted with phosphoric acid) (10:80:10, v/v) and was used in a gradient elution mode. The gradient started with solvent-A at a flow rate of 1.2 ml/min for 19 minutes and switched immediately to solvent B at a flow rate of 1.5 ml/min for 25 minutes.

### Standard and control solutions

VD-2, VD-3, 25(OH) VD-2, and 25(OH) VD-3 stock solutions were prepared in methanol (100 µg/mL) and stored at -20°C. Working solutions were prepared in human plasma (100 ng/mL). Eight calibration standards were prepared in human plasma in the range of 5-100 ng/mL. Benzopyrene (IS) working solution was prepared in methanol (0.25 µg/ml). Three quality control samples were prepared: 15(low), 50 (middle), and 90 (high) ng/mL. The solutions were vortexed for one minute, and then 1.5 ml aliquots were transferred into 7 mL glass culture tubes and stored at 4°C until used.

### Sample preparation

100 µL of the IS working solution was added to 1.5 ml of plasma samples, calibration standards, or quality control samples in a 7 mL culture tubes and vortexed. A mixture of methanol and 2-propanol (9:1, v/v) was added. After further vortexing, the samples were extracted twice with 3 mL hexane and centrifuged at 4000 rpm (8°C) for 10 min., and the clear supernatant layer was transferred to a clean culture tube and dried under gentle steam of nitrogen at 40°C. The residue was reconstituted in 150 µL methanol containing water 4.0%. 100 µL of the clear solution was injected into the HPLC system.

### Calculation

In order to correct for endogenous 25-hydroxyvitamin D-3 level

in “blank” plasma, we used the difference in peak area ratios between each consecutive concentration as the response (rather than the peak area ratio). The difference in peak ratio was plotted against the concentration. For the other three compounds the peak area ratios was plotted against concentration. Bias (%) was calculated as the difference between measured and nominal concentration divided by nominal level times 100, whereas coefficient of variation (100%) was calculated as standard deviation divided by mean concentration times 100.

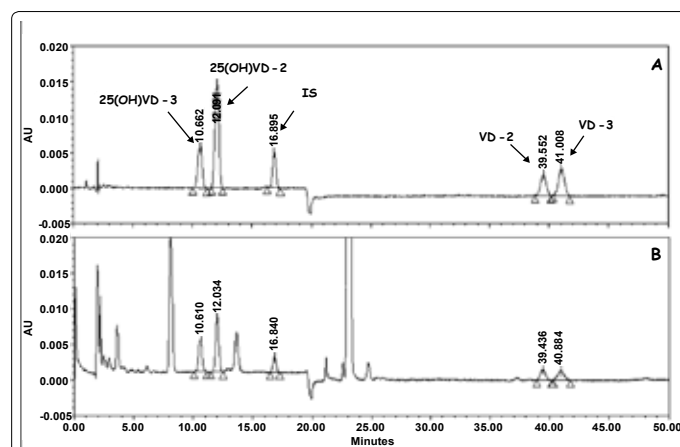
## Results

### Chromatographic separation

Under the chromatographic condition described, VD-2, VD-3, 25(OH) VD-2, and 25(OH) VD-3, and the internal standard peaks were well resolved. The retention times of 25(OH) VD-3, 25(OH) VD-2, IS, VD-2, and VD-3 were around 10.6, 12.0, 16.8, 39.4 and 40.9 minutes, respectively. Except for 25(OH) VD-3, no interfering peaks from endogenous plasma components were observed. Eight commonly used drugs (acetaminophen, caffeine, diclofenac sodium, omeprazole, nicotinic acid, ascorbic acid, ibuprofen, and ranitidine) were screened for potential interference; none co-eluted with the components of interest. Typical chromatograms of methanol and human plasma spiked with VD-2, VD-3, 25(OH) VD-2, 25(OH) VD-3, and the IS are shown in Figure 2.

### Method validation

The method was validated according to US FDA guidelines for industrial bio analytical method validation [14]. As shown in Table 1, the calibration curves for the determination of VD-2, VD-3, 25(OH) VD-2, and 25(OH) VD-3 levels in human plasma were linear over the range 5-100 ng/mL. The linearity of this method was statistically confirmed. For each calibration standard, the concentrations were back-calculated from the equation of the linear regression. Precision and bias were determined in five replicates of three concentrations (15, 50 and 90 ng/mL). The results are presented in Table 2. The largest coefficient of variation and bias of the assays were 9.7% and 13.1%, respectively. The analytical recoveries at three different levels (1.5, 50 and 90 ng/mL) were determined by comparing peak areas of VD-2, VD-3, 25(OH) VD-2, 25(OH) VD-3, and the IS prepared in methanol



**Figure 2:** Representative chromatograms of (A) methanol and (B) human plasma spiked with vitamin D-2, vitamin D-3, 25-hydroxyvitamin D-2, and 25-hydroxyvitamin D-3.

Parameter	VD-2	VD-3	25(OH)VD-2	25(OH)VD-3
No. of concentrations/curve	8	8	8	8
Range (ng/mL)	5-100	5-100	5-100	5-100
*Mean slope (SD)	0.0104 (0.0018)	0.0120 (0.0023)	0.0192 (0.0047)	0.0154 (0.0022)
*Mean intercept (SD)	-0.0167 (0.0149)	-0.0123 (0.0180)	-0.0171 (0.0406)	-0.0130 (0.0229)
*Mean regression coefficient (SD)	0.9907 (0.0070)	0.9858 (0.0129)	0.9903 (0.0064)	0.9890 (0.0066)
Limit of detection (ng/mL)	3.0	3.0	2.0	2.0
Limit of quantification (ng/mL)	5.0	5.0	5.0	5.0

\*Each of eight replicates.

**Table 1:** Regression analysis of calibration curves of assays of vitamin D-2, vitamin D-3, and their 25-hydroxy metabolites.

*Nominal Level	*Measured Level of VD-2				*Measured Level of VD-3			
	Mean	SD	CV (%)	Bias (%)	Mean	SD	CV (%)	Bias (%)
Intra-run								
15	15.3	1.3	8.4	2.0	16.0	0.9	5.7	6.6
50	55.3	1.0	1.8	10.6	50.9	3.8	7.4	1.8
90	83.1	3.7	4.4	-7.6	97.3	1.1	1.4	8.1
Inter-run								
15	14.8	1.2	8.2	-1.3	15.8	1.0	6.1	5.3
50	52.5	4.1	7.8	5.0	48.8	4.5	9.2	-2.4
90	86.0	5.6	6.5	-4.4	78.2	2.0	2.6	-13.1
*Nominal Level	*Measured Level of 25(OH)VD-2				*Measured Level of 25(OH)VD-3			
	Mean	SD	CV (%)	Bias (%)	Mean	SD	CV (%)	Bias (%)
Intra-run								
15	16.1	0.9	5.7	7.3	14.3	1.4	9.7	-4.6
50	53.9	2.1	3.9	7.8	54.9	2.7	4.8	9.7
90	97.4	6.1	6.2	8.2	97.3	4.4	4.5	8.4
Inter-run								
15	16.4	0.7	4.0	9.3	14.7	1.3	8.6	-1.9
50	52.7	4.0	7.5	5.4	54.8	2.4	4.4	9.6
90	94.4	8.1	8.6	4.8	94.1	7.1	7.6	4.5

\*nanogram/mL. SD, standard deviation, CV, standard deviation divided by mean measured concentration x 100. Bias, measured level - nominal level divided by nominal level x 100.

**Table 2:** Intra and inter-run precision and bias.

and to those prepared in plasma. Mean recoveries were 97%, 91%, 80%, and 81% for 25(OH) VD-3, 25(OH) VD-2, VD-2, VD-3, respectively, and 65% for the IS. The limit of detection was 2 ng/mL for 25(OH) VD-3, and 25(OH) VD-2, and 3 ng/mL for VD-2 and VD-3, whereas the limit of quantification was 5 ng/mL for all components.

## Stability

Table 3 summarizes the results of stability studies of the two forms of vitamin D and their metabolites.

**Freeze and thaw stability:** Stability of VD-2, VD-3, 25(OH) VD-2, and 25(OH)VD-3 was determined over three freeze and thaw cycles. Fifteen aliquots of each of two QC samples (15 ng/mL and 90 ng/mL) for the four compounds were stored at -20°C. After 24 hours, all aliquots were left to thaw unassisted at room temperature. When completely thawed, 5 aliquots of each QC sample were analyzed. The other aliquots were returned to -20°C and kept for 24 hours. The same procedure was repeated three times. The concentrations in freeze-thaw samples were compared with the concentration of freshly prepared and analyzed samples.

**Stability in processed samples:** Fifteen aliquots of each of two QC samples (15 ng/mL and 90 ng/mL) for the four compounds were processed. Five aliquots of each QC sample were analyzed immediately.

The other aliquots were analyzed after being stored at room temperature for 24 hours or at -20°C for 48 hours.

**Stability in unprocessed samples:** Thirty aliquots of each of two QC samples (15 ng/mL and 90 ng/mL) for the four compounds were prepared. Five aliquots of each QC sample were analyzed immediately. Five aliquots of each QC sample were allowed to stand on the bench-top for 24 hours at room temperature before extraction. Five aliquots of each QC sample were stored at -20°C for 1, 3, 5, or 10 weeks before analysis.

## Application to volunteer samples

Plasma samples from volunteers in a clinical trial were processed according to the method and subjected to HPLC analysis. Figure 3 depicts a chromatogram of sample collected from a representative volunteer. Over 200 plasma samples were analyzed by this method without any significant loss of resolution. No change in column efficiency or increase in backpressure was observed.

## Discussion

The aim of the study was to develop a reliable method for simultaneous analysis of the two forms of vitamin D and their metabolites in human plasma. The method developed is well suited

Stability (%)									
	*Plasma samples				**Stock Solution				
Nominal Level (ng/ mL)	Unprocessed		Processed		Freeze –Thaw Cycle				
	24 hrs RT	10 wks -20 °C	24 hrs RT	48 hrs -20 °C	1	2	3	24 hrs RT	10 wks -20 °C
VD-2									
15	89	102	104	101	98	96	97	98	96
90	108	94	101	101	99	97	97		
VD-3									
15	107	98	98	104	106	104	101	98	96
90	108	96	101	100	100	98	99		
25(OH)VD-2									
15	102	101	103	101	98	95	98	97	99
90	110	98	99	97	99	97	97		
25(OH)VD-3									
15	91	103	112	101	98	95	97	98	103
90	101	96	101	98	99	97	97		

Stability (%) = mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100. \*Spiked plasma samples were processed and analyzed immediately (baseline, data not shown), after 24 hours at room temperature (24 hrs RT), after freezing at -20 °C for 10 weeks (10 wks -20 °C), or after 1 to 3 cycles of freezing at -20 °C and thawing at room temperature (freeze-thaw), or processed and then analyzed after storing for 24 hours at room temperature (24 hrs RT) or 48 hours at -20 °C (48 hrs -20 °C). \*\* Stock solution, 100 µg/mL in methanol.

**Table 3:** Stability of vitamin D-2, vitaminD-3, and their 25-hydroxy metabolites.

for routine application in the clinical laboratory because of a simple extraction procedure and easy to handle readily available LC system. We used benzopyrene as IS, despite the fact that it does not have a similar structure to the analytes because it is well separated from endogenous components and potential interfering plasma substances and is characterized by consistent recovery under applied conditions. In regard to stability, the results showed that VD-2, VD-3, 25(OH)VD-2, and 25(OH)VD-3 are stable at least for 10 weeks at -20°C (94-103%) and after three cycles of freeze at -20°C and thaw at RT (95- 106%).

Since the level of 25(OH) VD-2 and 25(OH)VD-3 in human plasma is considered an important parameter in determining health status, many manufacturers developed commercial kits. However, for mass-scale analysis, such kits may not be affordable. The described assay offers simultaneously determine the levels of the two forms of vitamin D and their 25-hydroxy metabolites in human plasma/serum simultaneously in an affordable way.

## Conclusions

The described HPLC method is simple, precise, and accurate for the simultaneous measurement of vitamin D-2, vitamin D-3,

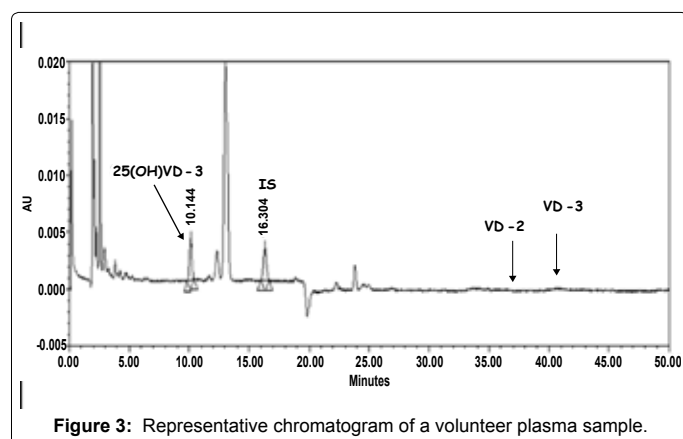
and their 25-hydroxy metabolites in human plasma. The assay was applied to monitor stability of these compounds under various condition encountered in the clinical laboratory. Further, the assay was successfully applied to determine the levels of these compounds in plasma samples from volunteers for clinical study.

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**Figure 3:** Representative chromatogram of a volunteer plasma sample.

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