



EAGLE
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25-OH Vitamin D₃ HPLC Assay

Catalog Number: VD331-H100

100 Tests

For Research Use Only. Not for use in diagnostic procedures.

v. 1.0

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1. Intended purpose

The Eagle Biosciences 25-OH Vitamin D₃ HPLC Assay kit is intended for the quantitative determination of 25-OH Vitamin D₃ in plasma and serum. The 25-OH Vitamin D₃ HPLC Assay kit is for research use only and not to be used in diagnostic procedures.

2. Introduction

Vitamin D is one of the most important essential bioregulators of the Ca²⁺- and phosphate metabolism in higher animals. Together with the peptide hormones parathormone and calcitonin it is responsible to obtain the Ca²⁺- and phosphate homeostasis. The most important physiological functions of vitamin D are the stimulation of the intestinal resorption of Ca²⁺ and phosphate and their incorporation in the bone matrix.

D-vitamins or calciferols arise from provitamins by the UV radiation of sunlight catalyzed splitting of the B-ring of the steran backbone. In the skin, formed vitamin D₃ is bound to a vitamin D binding-protein in the plasma, transported into the liver and hydroxylated in position 25 to form 25-OH vitamin D₃. Another hydroxylation is performed in the kidney to yield 1,25 (OH)₂ vitamin D₃ and 24,25 (OH)₂ vitamin D₃. 1,25 (OH)₂ vitamin D₃ is the metabolic most active form of vitamin D. Nevertheless, more than 95% of 25-OH vitamin D is 25-OH vitamin D₃. A lack of vitamin D leads in growing humans and animals can lead to rickets, hypocalcaemia and secondary hyperparathyroidism and in adults to osteomalacia.

The Eagle Biosciences 25-OH Vitamin D₃ HPLC Assay kit makes it possible to determine the vitamin in an easy, fast and precise method. The kit includes all reagents in ready to use form for preparation and separation of the samples with exception of the column (IC3401rp) and the controls (IC3401ko). Both can be supplied by Eagle Biosciences. Beside the complete test kit, it is possible to order all components separately. Please request our single component price list.

3. Warnings and precautions

- All reagents of the 25-OH Vitamin D₃ HPLC Assay kit are strictly intended for research use only and are not to be used for diagnostic procedures.
- Test kit and column are concerted. Using alternative columns might cause in insufficient separation, resulting in false high results. The given test characteristics might not be fulfilled.
- Do not interchange 25-OH Vitamin D₃ HPLC Assay kit components from different lots.
- Calibrator and controls contain human blood. It was tested and found negative for HBsAg, anti-HIV-1/2, and anti-HCV. No test can guarantee the absence of HBsAg or HIV, and so all human serum based reagents in this kit must be handled as though capable of transmitting infection.
- The internal standard, precipitating reagent and mobile phase contain acetonitrile and have to be handled carefully. Acetonitrile is highly flammable and toxic if inhaled or



contact the skin. It should be handled with gloves, eye protection, and appropriate protective clothing in a hood. Any spill should be wiped out immediately with copious quantities of water. Do not breathe vapor and avoid inhalation. In case of an accident or indisposition contact immediately a physician.

- Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.
- Calibrator and controls contain sodium azide. Sodium azide is highly toxic and reactive in pure form. At the product concentration, thought not hazardous.
- Do not pipette by mouth.
- Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled.
- Reagents of the 25-OH Vitamin D₃ HPLC Assay kit should not be used beyond the expiration date shown on kit label.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. During handling of all kit reagents, controls and serum samples observe the existing legal regulations.

4. Materials Provided

Article no.	Component	Designation	Amount
IC3401lm	ELU	Mobile Phase (contains acetonitrile)	1 x 1000 ml
IC3401ka	CAL	Calibrator (Concentration is given on the label)	1 x 6 ml
IC3401is	IS	Internal standard (contains acetonitrile)	1 x 40 ml
IC3401fr	PREC	Precipitation reagent (contains acetonitrile)	1 x 50 ml



5. Additional special equipment

- 1.5 ml reaction tubes (Eppendorf)
- Centrifuge
- Various pipettes
- HPLC with UV-detector
- HPLC column 25-OH Vitamin D₃ (IC3401rp)
- Vortex mixer

6. Reagent preparation

- Aliquot the calibrator and the controls and store at -20 °C. Avoid several times thawing and freezing. The content of 25-OH vitamin D₃ might have minor changes from lot to lot.
- Before use the mobile phase (ELU) should have room temperature.
- The internal standard should be stored at -20°C. All other test reagents of the 25-OH Vitamin D₃ HPLC Assay kit are stable at 2-8°C up to the date of expiry stated on the label.

7. Specimen

- Serum and plasma could be used. The samples could be transported (max 48 h) at 20-25 °C. Afterwards they should be stored at -20°C until testing.
- Using gel vials for blood collection might cause disturbed chromatograms caused by ingredients of the gel. We recommend using EDTA plasma or serum vials without gel.

8. Procedure

Principle of the method

For the determination of 25-OH vitamin D₃ a sample preparation has to be performed. Therefore a precipitation step is combined with an extraction. After centrifugation the upper layer is injected into the HPLC system. The HPLC separation works with an isocratic method at 30 °C with a "reversed phase" column. Chromatograms are detected by an UV-detector. The separation takes 15 minutes for each run depending on the column used. Results are quantified by the delivered serum calibrator and calculated by the "internal standard-method" by integration of the peak areas or heights.



Sample preparation

1. Pipette into 1.5 ml reaction tubes:
 400 µl sample, CAL or CTRL
 +
 400 µl IS ice cold
 (**Important:** for better precipitation use ice cold IS)
2. Mix briefly on a vortex mixer.
3. Add **500 µl** PREC
4. Mix for **2 min** on a vortex mixer. Leave the tubes for **15 minutes at 2-8°C** and centrifuge afterwards at 10.000g for 5 minutes.
 Crucial step: After centrifugation the sample shows 2 liquid phases and a solid disc (precipitated proteins). For injection only use the upper liquid phase.
5. Inject **50 µl** of the supernatant for chromatography into the HPLC-system

Chromatographic settings

Column material:	Column (IC3401rp)
Column dimension:	125 mm x 4 mm
Flow rate:	1.0 ml/min
UV-detection:	264 nm
Injection volume:	50 µl
Running time:	15 min
Temperature:	30 °C

The mobile phase can be re-circulated and lasts for 100 samples.



Treatment of the HPLC-column

After the analysis the column should be flushed with 15 ml deionized water (1 ml/min) and stored in 85% acetonitrile in deionized water (approx. 15 ml, flow 1.0 ml/min). Before use, the system should be equilibrated with approx. 30 ml Eluent.

9. Calculation of analytical results

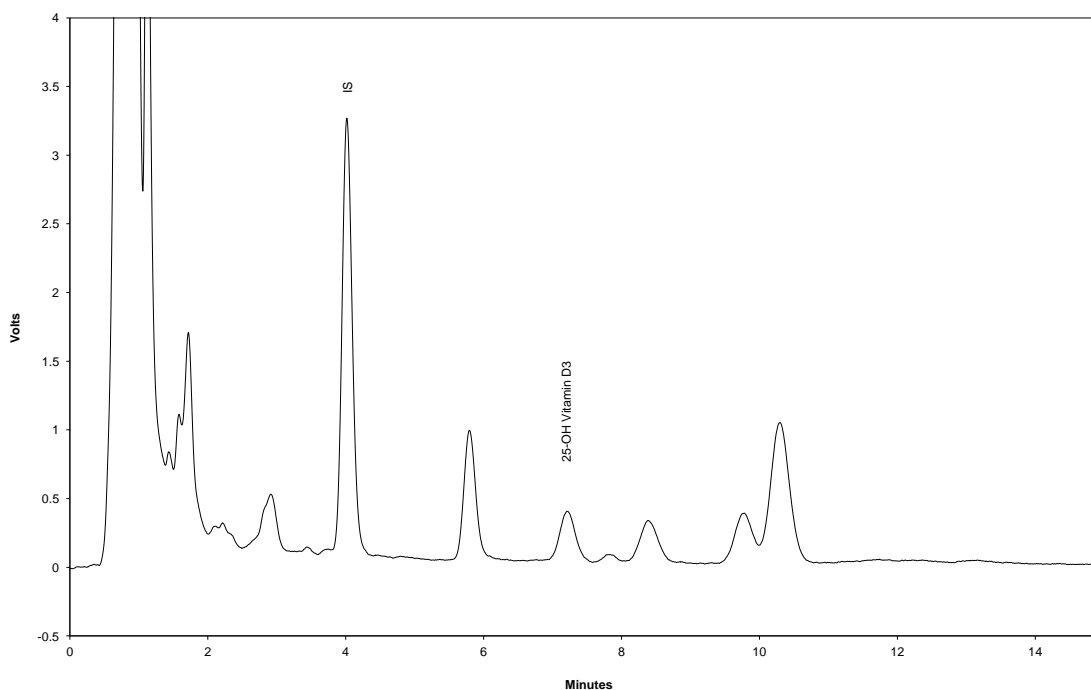
Calculation

$$\frac{\text{peak area patient} \cdot \text{concentration of the standard}}{\text{peak area IS patient}} * F = \text{concentration patient sample}$$

$$F = \frac{\text{Peak area IS of the calibrator}}{\text{Peak area analyte of the calibrator}}$$

Alternatively to the peak height it is possible to take the peak area for calculation.

Typical chromatogram





10. Internal Quality Control

Reference intervals

Summer	50-300 nmol/l (20-120 µg/l)
Winter	25-150 nmol/l (10-60 µg/l)

(H. Greiling, A.M. Gressner, Lehrbuch der klinischen Chemie und Pathobiochemie, 3. Auflage, 1995, Schattauer Verlagsgesellschaft mbH, ISBN 3-7945-1548-X)

We recommend that each laboratory should develop their own normal range. The values mentioned above are only for orientation and can deviate from other published data.

11. Validation data

Precision and reproducibility

Intra-Assay CV:	2.6 % (56.5 nmol/l)	[n = 6]
	1.5 % (104.8 nmol/l)	[n = 6]
Inter-Assay CV:	4.0 % (54.1 nmol/l)	[n = 6]
	3.6 % (105.4 nmol/l)	[n = 6]

Linearity

up to 1250 nmol/l

Detection limit

5.8 nmol/l

Recovery

97.8 %



12. Limitations of the method

- Whole blood is not suited for the 25-OH Vitamin D₃ HPLC Assay kit.
- Using gel vials for blood collection might cause disturbed chromatograms caused by ingredients of the gel. We recommend using EDTA plasma or serum vials without gel.

13. Disposal

The mobile phase (ELU), internal standard (IS) and precipitation reagent (PREC) must be disposed as non-halogenated solvent. Please refer to the appropriate national guidelines.

14. Troubleshooting

Problem	Possible reason	Solution
No signal	No or defect connection to evaluation system	Check signal cord and connection
	Detector lamp is altered	Change lamp
No peaks	Injector is congested	Check Injector
Double peaks	Dead volume in fittings and / or column	Renew fittings and / or column
Contaminating peaks	Injector dirty	Clean injector
	Contamination at the head of the column	Change direction of the column and rinse for 30 min at low flow rate (0.2 ml/min) with mobile phase
	Air in the system	Degas pump
	Autosampler vials contaminated	Use new vials or clean them with methanol
Broad peaks, tailing	Precolumn / column exhausted	Use new precolumn / column
Variable retention times	Drift in temperature	Use a column oven
	Pump delivers imprecise	Check pump, degas the system
	System is not in steady state yet	Rinse system mobile phase for 15 min



Problem	Possible reason	Solution
Baseline is drifting	Detector lamp did not reach working temperature yet	Wait
	Detector lamp is too old	Renew lamp
Continue baseline is drifting	System is not in steady state yet	Rinse system mobile phase for 15 min
	Pump delivers imprecise	Check pump, degas the system
Baseline is not smooth	Pump delivers imprecise	Check pump, degas the system
	Detector flowcell is dirty	Clean flow cell

15. Literature references

- Merke et al. (1986). Med. Wochenschr, Vol. 9, 345-349.
- Reichel et al. (1989). New Engl. J. Med., Vol. 320, 980-991.
- Schmidt-Gayk et al. (1991). Klin. Lab., Vol. 37, 219.

For further information about this kit, its application or the procedures in this kit insert, please contact the Technical Service Team at Eagle Biosciences at info@eaglebio.com or at 866-411-8023.