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25-OH Vitamin D₃ HPLC Kit

Zur Bestimmung von 25-OH Vitamin D₃ in Plasma und Serum

For the determination of 25-OH Vitamin D₃ in plasma and serum

Gültig ab/valid from 25.08.2005

Artikelnummer/Catalogue no.: KC 3400

Packungsgröße/Package size: 100 Tests/100 determinations

Lagerung/Storage: 2-8 °C, Kalibrator, Kontrollen und ethanol. Standard bei -20 °C
2-8 °C, Calibrator, controls and ethanol. standard at -20 °C



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1. INTENDED USE

The *Immundiagnostik* assay is intended for the quantitative determination of 25 (OH) Vitamin D in serum and plasma. This assay is designed for in vitro diagnostic use only.

2. SUMMARY AND EXPLANATION OF THE TEST

D-vitamins and calciferols arise from provitamins by the UV radiation of sunlight catalysed splitting of the B-ring of the steran backbone. The two most important D-vitamins are vitamin D₃ and vitamin D₂. In the contrary to vitamin D₂ which has to be added via food, vitamin D₃ can be produced in the liver.

In the skin formed or together with vitamin D₂ by food ingested 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-D. More than 95% of 25-OH-D is 25-OH-D₃. 25-OH-D₂ is only detectable in patients with medication of vitamin D₂.

3. PRINCIPLE OF THE TEST

For the determination of 25-OH vitamin D₃ samples have to be prepared as follows. To get rid of high molecular weight substances the samples are precipitated and solid phase extracted with C₁₈-cartridges. The eluat is evaporated with nitrogen, suspended in mobile phase and injected into the HPLC system.

The HPLC separation works with an isocratic method at 35 °C with a "normal phase" column. Chromatograms are detected by an UV-detector. The separation takes 20 minutes for each run. Results are quantified by the delivered serum calibrator and calculated by the "external standard-method" by integration of the peak area. The ethanol solution of standards is used for the recognition of the peaks.

Summary:

This application for the determination of 25-Hydroxy-Vitamin D₃ is a specific, reliable method for the determination of this parameter without usage of radioactive substances.

This complete kit includes all reagents for analytical HPLC separation and for the extraction of the samples but no columns for the preparation of the samples.

As with many other parameters the advantage of HPLC analytic is the simultaneous handling of many analytes in one test. The HPLC complete system enables even laboratories without experience in high performance liquid chromatography to use this technique for clinical-chemical routines quickly and without difficulties. Mostly a one-point calibration is sufficient for calibrating the test system - unlike immuno assays with up to 6 calibrators per test. It is possible to automate the sample application and calculation of the results, so that even higher numbers of samples can be handled nearly without control. (With short test series the one-point-calibration is much more economic than 6-point-calibration for immuno assays.)

4. MATERIAL SUPPLIED

Catalogue No	Kit Components	Quantity
KC3400LM	Mobile Phase	1 x 1000 ml
KC3400KA	Calibrator (lyoph. 6 ml; Concentration is given on the label)	2 vials
KC3400ST	ethanol . Standard (0.5 µg/ml = 1250 nmol/l)	1 x 1 ml
KC3400RE	Reconstitution solution	1 x 20 ml
KC3400FR	Precipitation reagent (contains acetonitril)	1 x 50 ml
KC3400WL	Washing solution	1 x 300 ml
KC3400EL	Elution solution (contains acetonitril)	1 x 400 ml
KC3400RL	Regeneration solution for C18-Cartridges	1 x 300 ml
KC3400KO	Control 1 and 2; 0.6 ml lyophilized	2 x 3 vials

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- 1.5 ml reaction tubes (Eppendorf)
- Centrifuge
- Vortex mixer
- Various pipettes
- pointed-bottomed glass tubes
- HPLC with UV-detector
- Silica column, 4 µm, 125 x 4 mm
- Solid phase extraction unit
- Methanol p.A.
- Sep-Pack C18 cartridges, available by Immundiagnostik. (Product Code: Wato54945)

6. PREPARATION AND STORAGE OF REAGENTS

- Reconstitute the **calibrator** in 6 ml and controls in 600 µl reconstitution solution, divide the calibrator in several portions 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.
- Reconstitute the **controls** in 0.6 ml aqua bidest.
- All other test reagents are stable at 2-8 °C, up to the date of expiry stated on the label.

7. PRECAUTIONS

- For in vitro diagnostic use only.
- This product contains human source material which was tested and found to be non-reactive to HBsAg, anti-HIV-1/2, and anti-HCV. Since no method can offer complete assurance that hepatitis B virus, HIV-1/2, HVC or other infectious agents are absent, these reagents should be handled as if potentially infectious.

- The precipitating reagent and elution solution contain acetonitril and have to be handled carefully. Acetonitril 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 breath vapor and avoid inhalation. In case of an accident or indisposition contact immediately a physician
- The mobile phase contains n-hexan. Hexan 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. Do not breath vapor and avoid inhalation. In case of an accident or indisposition contact immediately a physician
- Reagents should not be used beyond the expiration date shown on kit label.

8. SPECIMEN COLLECTION AND PREPARATION

Serum and plasma could be used. The samples should be stored at -20°C until testing.

9. ASSAY PROCEDURE

Procedural notes

- The quality control guidelines should be observed.
- Incubation time, incubation temperature and pipetting volumes of the different components are defined by the producer. Any variations of the test procedure, that are not coordinated with the producer, may influence the test results. Immundiagnostik can therefore not be held reliable for any damage resulting from this.
- The assay should always be performed due to the manual which is given in the kit.

Preparation of the SPE-cartridges

The cartridge is rinsed with 5 ml methanol and 5 ml aqua dest.

Regeneration of the SPE-cartridges

Rinse the cartridges immediately after the sample preparation with 3 ml of regeneration solution. Store the cartridges in a closed plastic sack. Before the next sample preparation, the cartridges should be prepared as mentioned above. One cartridge can be used five times.

Sample and standard preparation

Due to the affinity of 25-OH vitamin D3 to plastic all steps should be performed in glass tubes, preferable pointed-bottomed glass tubes.

- Add 0.5 ml precipitation reagent to 0.5 ml serum or calibrator (**attention - with acetonitril always work in a hood**).
- Vortex, incubate for 10 min at 4 °C and then centrifuge with 10.000 g for 3 minutes.
- Pipette supernatant on the prepared cartridges and let it soak in.
- Rinse with 3 ml washing solution.
- Elute with 4 ml elution solution. **The elution must last at least 60 seconds, because otherwise an optimal refunding cannot be guaranteed.**
- Evaporate the elute with nitrogen (**attention - with acetonitril always work in a hood**) or with a vacuum centrifuge.
- Dissolve evaporated sample in 150 µl running mobile phase and mix well. Incubate for **10 min** at 2-8°C and centrifuge for **10 min** at 10.000 g. The prepared sample is now stable for min. 6 days at 2-8°C.
- Inject **100 µl** of the supernatant into the HPLC system.

Chromatographic conditions

Material of the column	:	Silica column, 4 μm
Column	:	dimension: 125 mm x 4 mm
Temperature	:	35 $^{\circ}\text{C}$
UV-Detector	:	264 nm
flowing velocity	:	1-1,5 ml / min
Volume of injection	:	100 μl
running time/chromatogram	:	ca. 20 min.

For the determination of retention time a run with the ethanolic standard is recommended before each test. For this reason 100 μl of ethanolic standard are evaporated under nitrogen or in the vacuum centrifuge, resuspended in mobile phase and injected into the HPLC-system.

Notice: The mobile phase can be recirculated. After the analysis of 100 samples the mobile phase should be renewed.

Immundiagnostik recommends to use a guard-column to enlarge lifetime of the column.

10. TREATMENT OF THE COLUMN

The HPLC column is filled with silica (normal-phase). You **must** not use water in the system, because water damages silica columns. After the analysis, the column should be stored in HPLC-Eluent.

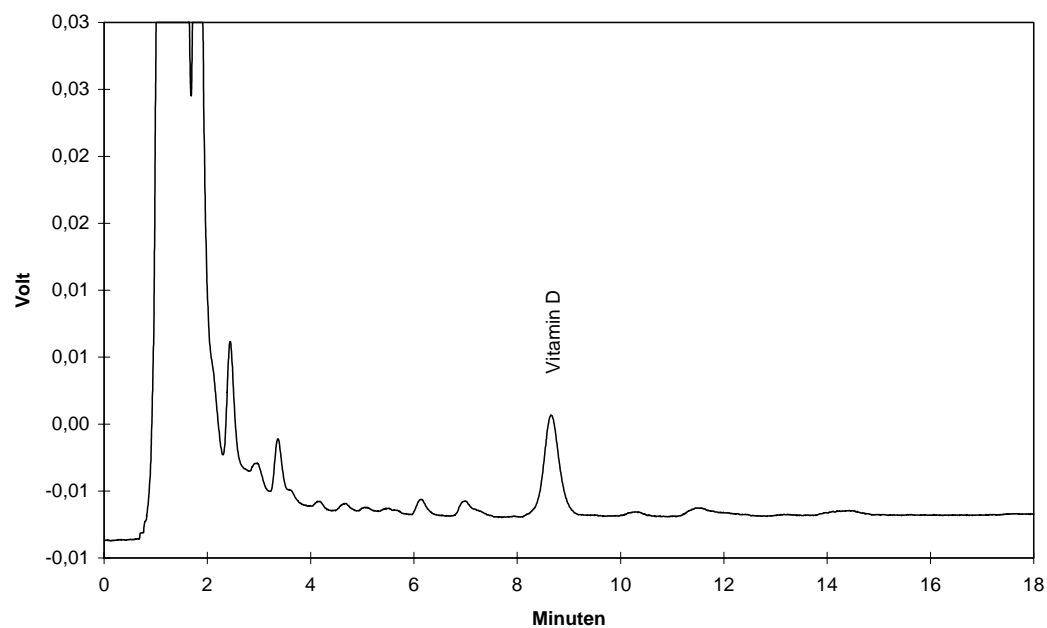
11. RESULTS

Calculation

$$\text{conc. patient sample} = \frac{\text{Peak height patient} \times \text{conc. calibrator}}{\text{Peak height calibrator}}$$

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

Typical chromatogram



12. LIMITATIONS

Don't use whole blood.

13. QUALITY CONTROL

Expected values

15-150 nmol/l

The values are reduced in winter and in elderly patients.

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

Controls

Control samples or serum pools should be analyzed with each run of calibrators and patient samples. Results generated from the analysis of the control samples should be evaluated for acceptability using appropriate statistical methods. In assays in which one or more of the quality control sample values lie outside the acceptable limits, the results for the patient sample may not be valid.

14. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Intra-Assay CV: 5.2 % (50 nmol/l) [n = 6]

Inter-Assay CV: 8.4 % (50 nmol/l) [n = 6]

Linearity

up to 1250 nmol/l

Detection limit

4 nmol/l

Recovery

85.5% ± 3%

15. DISPOSAL

The mobile phase, ethanolic standard, precipitation solution, washing solution, elution solution and regeneration solution must be disposed as non-halogenated solvent.

Please refer to the appropriate national guidelines.

16. TROUBLESHOOTING

Problem	Possible reason	Solution
No signal	No or defect connection to evaluation system	Check signalcord and connection
	Detectorlamp is altered	Change lamp
No peaks	Injector is congested	Check Injector
Doublepeaks	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
	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

17. 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.

18. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and put on the market according to the IVD guidelines of 98/79/EC.
- The test components contain organic solvents. Contact with skin or mucous membranes has to be avoided.
- All reagents in the test package are to be used for research only.
- The reagents should not be used after the date of expiry stated on the label.
- Single components with different lot numbers should not be mixed or exchanged.
- The guidelines for medical laboratories should be observed.
- Incubation time, incubation temperature and pipetting volumes of the different components are defined by the producer. Any

variations of the test procedure, that are not coordinated with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held reliable for any damage resulting from this.