25-OH Vitamin D₃ HPLC Kit

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

Valid from 28.08.2008
1. INTENDED USE

The Immundiagnostik assay is intended for the quantitative determination of 25-(OH) Vitamin D_3 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 D_3 and vitamin D_2. In the contrary to vitamin D_2 which has to be added via food, vitamin D_3 can be produced in the liver.

In the skin formed or together with vitamin D_2 by food ingested vitamin D_3 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-D_3. 25-OH-D_2 is only detectable in patients with medication of vitamin D_2.

3. PRINCIPLE OF THE TEST

For the determination of 25-OH vitamin D_3 samples have to be prepared as follows. To get rid of high molecular weight substances the samples are precipitated and solid phase extracted with C_18-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 30 °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

<table>
<thead>
<tr>
<th>Cat. No</th>
<th>Content</th>
<th>Kit Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>KC3400LM</td>
<td>MOPHA</td>
<td>Mobile Phase</td>
<td>1000 ml</td>
</tr>
<tr>
<td>KC3400KA</td>
<td>CAL</td>
<td>Calibrator (lyoph. 6 ml; Concentration is given on the label)</td>
<td>2 vials</td>
</tr>
<tr>
<td>KC3400ST</td>
<td>STD</td>
<td>Ethanolic Standard (0.5 µg/ml =1250 nmol/l)</td>
<td>1 ml</td>
</tr>
<tr>
<td>KC3400RE</td>
<td>RECSOL</td>
<td>Reconstitution solution</td>
<td>20 ml</td>
</tr>
<tr>
<td>KC3400FR</td>
<td>PREC</td>
<td>Precipitation reagent (contains acetonitril)</td>
<td>50 ml</td>
</tr>
<tr>
<td>KC3400WL</td>
<td>WASHSOL</td>
<td>Washing solution</td>
<td>300 ml</td>
</tr>
<tr>
<td>KC3400EL</td>
<td>ELUSOL</td>
<td>Elution solution (contains acetonitril)</td>
<td>400 ml</td>
</tr>
<tr>
<td>KC3400RL</td>
<td>REGSOL</td>
<td>Regeneration solution for C18-Cartridges</td>
<td>300 ml</td>
</tr>
<tr>
<td>KC3400KO</td>
<td>CTRL1 CTRL2</td>
<td>Control 1 and 2; 0.6 ml lyophilized</td>
<td>2 x 3 vials</td>
</tr>
</tbody>
</table>

HPLC column (KC 3400RP) as well as individual components can be ordered separately from Immundiagnostik. Please ask for the price list of the individual components.
5. MATERIAL REQUIRED BUT NOT SUPPLIED

- Centrifuge
- Vortex mixer
- Pointed-bottomed glass tubes
- 1.5 ml reaction tubes (Eppendorf)
- Various pipettes
- HPLC with UV-detector
- Sample evaporation unit
- Solid phase extraction unit
- Methanol p.A.
- Silica column, 4 µm, 125 x 4 mm
- Sep-Pack C$_{18}$ cartridges, available by Immundiagnostik. (Product Code: KC3400CK)

6. PREPARATION AND STORAGE OF REAGENTS

- Reconstitute calibrator (CAL) in 6 ml, and controls (CTRL1, CTRL2) in 600 µl reconstitution solution (RECSOL), divide calibrator (CAL) in several aliquots and store at -20 °C. Avoid thawing and freezing several times. The content of 25-OH vitamin D$_3$ might have minor changes from lot to lot.
- 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 (PREC) and the elution solution (ELUSOL) contain acetonitril and must be handled carefully. Acetonitril is highly flammable and toxic by inhalation 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.
• The mobile phase (MOPHA) contains n-hexane. Hexane 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 breathe 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

• Quality control guidelines should be observed.

• Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test. Immundiagnostik AG cannot therefore not be held responsible for any damage resulting from wrong use.

• The assay should always be performed according the enclosed manual.

Preparation of the SPE-cartridges

Rinse the cartridge 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 (REGSOL). Store the cartridges in a closed plastic sack. Before the next sample preparation, prepare the cartridges should as mentioned above. One cartridge can be used five times.
Sample and standard preparation

To avoid losses of 25-OH vitamin D₃ due to absorption on the walls of plastic vessels, all steps should be performed in glass tubes, preferably pointed-bottomed glass tubes.

- Add 0.5 ml precipitation reagent (PREC) to 0.5 ml serum, calibrator (CAL) or controls (CTRL1, CTRL2).
  
  **Attention - Always work with acetonitril in a fume 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 (WASHSOL).

- Elute with 4 ml elution solution (ELUSOL). **For optimal recovery, the elution must be performed for minimum 60 seconds.**

- Evaporate the elute with nitrogen or with a vacuum centrifuge. **Attention - Always work with acetonitril in a fume hood!**

- Dissolve evaporated sample in 150 µl running mobile phase (MOPHA) and mix well. Incubate for **10 min** at 2-8°C and centrifuge for **10 min** at 10.000 g. The prepared sample is stable for min. 6 days at 2-8°C.

- Inject **100 µl** of the supernatant into the HPLC system.
Chromatographic conditions

**Column material** : Silica column, 4 µm
**Column dimension** : 125 mm x 4 mm
**Temperature** : 30 °C
**UV-Detector** : 264 nm
**Flowing** : 1-1,5 ml / min
**Injection volume** : 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 (MOPHA) can be recirculated. The mobile phase (MOPHA) should be renewed after analysis of 100 samples.

It is recommended that a guard column is used to extend column life.

**10. TREATMENT OF THE COLUMN**

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

Before use, the system should be equilibrated with 50 ml eluent: Run first 20 ml without column, and then the remaining 30 ml with integrated column.
11. RESULTS

Calculation

\[
\text{Concentration sample} = \frac{\text{Peak height sample} \times \text{Concentration of the calibrator}}{\text{Peak height calibrator}}
\]

Tip: Alternatively, the peak area instead of the peak height can be used for quantification.

Typical chromatogram

12. LIMITATIONS

Do not use whole blood.
13. QUALITY CONTROL

Expected values

Normal ranges for 25-OH-Vitamin D₃

1 ng/ml = 2.5 nmol/l
1 nmol/l = 0.4 ng/ml

Information from ASBMR 2006

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deficiency (seriously deficient)</td>
<td>&lt;12 ng/ml</td>
<td>resp. &lt;30 nmol/l</td>
</tr>
<tr>
<td>Insufficiency (deficient)</td>
<td>12 - 30 ng/ml</td>
<td>resp. 30 - 75 nmol/l</td>
</tr>
<tr>
<td>Sufficiency (adequately supplied)</td>
<td>&gt;30 ng/ml</td>
<td>resp. &gt;75 nmol/l</td>
</tr>
</tbody>
</table>

Society of Osteology SACHSEN E.V.
http://osteologie-sachsen.de/aktuelles_vitamin_d.html

Note

The vitamin D production in the skin is highly variable and depends on the season- and daily time, degree of latitude, age, sun protection etc.

The normal ranges depend on the method used (e.g. vitamin-D-release from the vitamin D binding protein, DBP) and serve only as orientation.

Literature references


It is recommended that each laboratory should establish its own normal range. Above mentioned values are only for orientation and may vary from other published 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 (MOPHA), ethanolic standard (STD), precipitation solution (PREC), washing solution (WASHSOL), elution solution (ELUSOL) and regeneration solution (REGSOL) must be disposed as non-halogenated solvent.

Please refer to the appropriate national guidelines.
## 16. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>No signal</td>
<td>No or defect connection to evaluation system</td>
<td>Check signal cord and connection</td>
</tr>
<tr>
<td></td>
<td>Detector lamp is altered</td>
<td>Change lamp</td>
</tr>
<tr>
<td>No peaks</td>
<td>Injector is congested</td>
<td>Check Injector</td>
</tr>
<tr>
<td>Doublepeaks</td>
<td>Dead volume in fittings and / or column</td>
<td>Renew fittings and / or column</td>
</tr>
<tr>
<td>Contaminating peaks</td>
<td>Injector dirty</td>
<td>Clean injector</td>
</tr>
<tr>
<td></td>
<td>Contamination at the head of the column</td>
<td>Change direction of the column and rinse for 30 min at low flow rate (0.2 ml/min) with mobile phase</td>
</tr>
<tr>
<td></td>
<td>Air in the system</td>
<td>Degas pump</td>
</tr>
<tr>
<td></td>
<td>Autosampler vials contaminated</td>
<td>Use new vials or clean them with methanol</td>
</tr>
<tr>
<td>Broad peaks, tailing</td>
<td>Precolumn / column exhausted</td>
<td>Use new precolumn / column</td>
</tr>
<tr>
<td>Variable retention times</td>
<td>Drift in temperature</td>
<td>Use a column oven</td>
</tr>
<tr>
<td></td>
<td>Pump delivers imprecise</td>
<td>Check pump, degas the system</td>
</tr>
<tr>
<td></td>
<td>System is not in steady state yet</td>
<td>Rinse system mobile phase for 15 min</td>
</tr>
<tr>
<td>Baseline is drifting</td>
<td>Detector lamp did not reach working temperature yet</td>
<td>Wait</td>
</tr>
<tr>
<td></td>
<td>Detector lamp is too old</td>
<td>Renew lamp</td>
</tr>
<tr>
<td></td>
<td>System is not in steady state yet</td>
<td>Rinse system mobile phase for 15 min</td>
</tr>
<tr>
<td></td>
<td>Pump delivers imprecise</td>
<td>Check pump, degas the system</td>
</tr>
<tr>
<td>Baseline is not smooth</td>
<td>Pump delivers imprecise</td>
<td>Check pump, degas the system</td>
</tr>
<tr>
<td></td>
<td>Detector flow cell is dirty</td>
<td>Clean flow cell</td>
</tr>
</tbody>
</table>
17. REFERENCES

Wicherts IS et al. (2007) J Clin Endocrinol Metab 92(6):2058-65

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 must be avoided.
- All reagents in the test package are for in-vitro diagnostic use only.
- Reagents should not be used beyond the expiration date shown on the kit label.
- Do not interchange different lot numbers of any kit component within the same assay.
- Quality control guidelines should be observed.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from wrong use.