Manual

Vitamin K₁ HPLC Kit

For the determination of Vitamin K₁ in plasma and serum

Valid from 18.03.2008
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1. INTENDED USE

The Immundiagnostik Assay is intended for the quantitative determination of Vitamin K1 in plasma and serum. This Assay is designed for in vitro diagnostic use only.

2. INTRODUCTION

Vitamin K1 is a derivative of 2-methyl-1, 4-naphtochinone and is found in green plants. Vitamin K1 is insoluble in water and readily soluble in ether, n-hexane and chloroform.

It's an essential co-factor in the posttranslational carboxylation reaction of glutamic acid residues (GLU) to \( \gamma \)-carboxyglutamic acid residues (GLA) in a number of blood clotting factors and also in some other proteins e.g. osteocalcin. The adjacent carboxyl groups of the GLA-residues provide the Vitamin K dependent proteins with characteristic calcium- and phospholipid-binding properties that are essential for their activation and function. A decrease in Vitamin K1 is reported in osteoporotic patients. Other clinical symptoms of Vitamin K1 deficiency are clotting disorders, which manifest themselves as bleeding in the skin, in the mucous membranes, in muscles and in internal organs. Symptoms of deficiencies normally appear within few days. Vitamin K1 is rapidly metabolized and only minor amounts are stored in the organism.

Applications:
- Determination of Vitamin K1 status
- Vitamin K deficiency induced by:
  - obstructive liver disease
  - obstructive icterus
  - malabsorption due to celiac disease,
  - pancreatitis, diarrhea, antibiotic abuse
- Blood clotting disorder
- Bone metabolism disorders
- Haemorrhagic disorders of newborns

3. PRINCIPLE OF THE TEST

The newly developed Vitamin K HPLC-application is the first commercially available kit. After a solid phase extraction on SPE-cartridges, serum or plasma samples are precipitated. The supernatant is then extracted with an organic solvent and evaporated. After resuspension the sample is measured in an isocratic HPLC-system. A post-column reduction reactor reduces Vitamin K and enables the measurement of Vitamin K with a fluorescence detector. An internal standard is added before the solid phase extraction step to ensure the high quality of the measurement.
Summary:
The application of Vitamin K\textsubscript{1} for HPLC makes it possible to determine the Vitamin in an easy, fast and precise way. The kits includes all reagents in ready to use form for preparation and separation of the samples with exception of the columns. As with many other parameters the advantage of HPLC measurements are the simultaneous handling of many analytes in a single test. The „complete HPLC-system“ enables even laboratories without experience in „high performance liquid chromatography“ to use this technique for clinical chemical routines quickly and precisely. 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 number of samples can be handled nearly without control. (With short test runs the one-point calibration is much more economic than 6-point calibration for immuno assays).

4. MATERIAL SUPPLIED

<table>
<thead>
<tr>
<th>Catalogue No</th>
<th>Content</th>
<th>Kit Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>KC2400LM</td>
<td>MOPHA</td>
<td>Mobile phase</td>
<td>3 x 1000 ml</td>
</tr>
<tr>
<td>KC2400KA</td>
<td>CAL</td>
<td>Calibrator, lyophilized</td>
<td>8 vials</td>
</tr>
<tr>
<td>KC2400IL</td>
<td>STD</td>
<td>Isopropanolic standard</td>
<td>10 ml</td>
</tr>
<tr>
<td>KC2400IS</td>
<td>INT STD</td>
<td>Internal Standard</td>
<td>1 ml</td>
</tr>
<tr>
<td>KC2400FR</td>
<td>PREC</td>
<td>Precipitating reagent</td>
<td>200 ml</td>
</tr>
<tr>
<td>KC2400EX</td>
<td>EXTSOL</td>
<td>Extraction solution</td>
<td>400 ml</td>
</tr>
<tr>
<td>KC2400ZI</td>
<td>ZINC</td>
<td>Zinc (Important: store under argon)</td>
<td>20 g</td>
</tr>
<tr>
<td>KC2400ZN</td>
<td>ZUB</td>
<td>Accessories for post-column reduction-reactor</td>
<td>5 pieces</td>
</tr>
<tr>
<td>KC2400KO</td>
<td>CTRL 1</td>
<td>Control 1 and 2;1.1 ml lyophilized</td>
<td>2 x 3 vials</td>
</tr>
<tr>
<td></td>
<td>CTRL 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The starter-kit contains also a post-column reduction-reactor (empty). HPLC column (KC2400RP), SPE cartridges (KC2400CK) 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

- Glass tubes for centrifugation, V-bottom (10 ml)
- SPE cartridges, C18 (KC 2400ck)
- MERCK-Catridge Holder Manu-Kart (ordering no. KC2400RK)
- Evaporator
- Centrifuge
- Various pipettes
- HPLC with Fluorescence-detector
- Reversed phase C18-column – Superspher RP 18,4 μm, 125 x 4,6 mm
- Vortex mixer

6. PREPARATION AND STORAGE OF REAGENTS

Preparation of the calibrator and controls

- Reconstitute CAL (calibrator) in aqua bidest. The volume is given on the label. One vial is for a single use only; discard the material, which have not been used. The content of Vitamin K₁ might have minor changes from lot to lot.
- Reconstitute CTRL 1 and CTRL 2 (control 1 and 2) in 1.1 ml aqua bidest.
- All other test reagents are stable at 2-8 °C, up to the date of expiry stated on the label.

Preparation of the post-column reduction reactor

For getting well detectable peaks it is necessary to exchange the zinc particles in the post-column reduction reactor each day. The zinc particles are used up after 12 h by oxidation. Filling the column is very easy and takes just 10 min of time.

For assembling the column see the following figure.

1. Cap nut
2. Stainless steel inlet
3. PTFE seal
4. Stainless steel sieve (grey)
5. Glass fiber sieve (3 pieces, white)
6. Stainless steel sieve (grey)
7. Column tube

1. Close one side of the column according to the figure above.
2. Fill in the zinc-particles with a funnel while knocking the column slightly on the table, so that the packing will not show any cavities.
3. Close the upper side of the column.
The post-column reduction reactor should be mounted in the HPLC-system as described by the following picture:

```
  Pump  Column  Reactor  Detector
```

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 supplied reagents contain different dangerous chemical reagents like methanol (mobile phase), ethanol (precipitating reagent), n-hexan (extraction solution), isopropanol and acid (mobile phase). Although diluted, it still must be handled with care and should only be handled with gloves, eye protection, and appropriate protective clothing. Do not breath vapor and avoid inhalation.
- Reagents should not be used beyond the expiration date shown on kit label.

8. SPECIMEN COLLECTION AND PREPARATION

Plasma and serum can both be used for analysis. The samples must be cooled immediately.

The samples are stable at 2-8°C for 1 week. For longer storage samples should be frozen at -20°C.

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.
## Sample and standard preparation

1. The **cartridge** is rinsed with 3 ml methanol and 3 ml aqua bidist.

2. Add **10 µl INT STD** (internal standard) to **1 ml patient sample, CAL** (calibrator) or **CTRL 1 and CTRL 2** (control 1 and 2) and pipette the mixture on the SPE cartridge. Let it soak through by vacuum.

3. **Collect** the **break-through** in a glass vial.

4. Add **2 ml PREC** (precipitation reagent), **vortex** for 1 min and **centrifuge** for 10 min at 3.500 x g.

5. Pipette the **supernatant** in a fresh glass vial and add **4 ml** of **EXTSOL** (extraction solution).

6. **Vortex** for 2 min and **centrifuge** for 5 min at 3.500 x g.

7. Pipette the **upper phase** in a new glass vial and evaporate to dryness.

8. **The dried sample is stable for 8 days at 4-8°C**

9. Connect the **post-column reduction reactor** in the HPLC-system, as described above and wait for equilibration (30-45 min).

10. **Check** the performance of the reactor by the injection of **100 µl of STD** (isopropanolic standard) and determine the **signal to noise ratio**, which should be **greater than 25**.

11. Resuspend the dried sample in **150 µl MOPHA** (mobile phase) and **inject 100 µl** in the HPLC-system.
Chromatographic conditions

For determination of the retention time, inject 100 µl of the STD (isopropanolic standard) in the HPLC-system.

- **Column material:** Superspher 100 RP 18; 4 µm
- **Column dimension:** 125 mm x 4.6 mm
- **Flow rate:** 0.9 - 1.2 ml/min
- **Fluorescence detector:**
  - ex.: 248 nm
  - em.: 418 nm
- **Temperature:** 30°C
- **Running time:** approx. 20 min

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

10. TREATMENT OF THE COLUMN

After analysis the column should be flushed with 30 ml aqua bidest (1.0 ml/min) and stored in 50% methanol in aqua bidest (approx. 30 ml, flow 0.5 ml/min). Before use, the system should be equilibrated with ca. 50 ml MOPHA (mobile phase).

*Important: Do not re-circulate the MOPHA (mobile phase) in this test system.*

11. RESULTS

Calculation

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

\[
F = \frac{\text{Peak height internal standard in the calibrator}}{\text{Peak height calibrator}}
\]
Typical chromatogram

12. LIMITATIONS

We recommend not to measure hemolytic and lipaemic patient samples.

13. QUALITY CONTROL

Reference value

0.22 - 2.28 ng/ml (Mean value = 1.29 ng/ml) (n = 19)

We recommend that each laboratory should develop its own reference range. The values mentioned above are only for orientation and can deviate 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:** 4.5% (1.7 ng/ml) [n = 6]

**Inter-Assay CV:** 5.6% (1.7 ng/ml) [n = 6]

Linearity

up to 25 ng/ml

Detection limit

0.15 ng/ml

15. DISPOSAL

*MOPHA* (mobile phase), *STD* (isopropanolic standard), *INTSTD* (internal standard), *EXTSOL* (extraction solution) and *PREC* (precipitating reagent) must be disposed as non-halogenated solvent. Please refer to the appropriate national guidelines.

16. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible reasons</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>Double peaks</td>
<td>Dead volume in fittings and / or column</td>
<td>Renew fittings and / or column</td>
</tr>
<tr>
<td>Contaminating</td>
<td>Injector dirty</td>
<td>Clean injector</td>
</tr>
<tr>
<td>peaks</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>Problem</td>
<td>Possible reasons</td>
<td>Solution</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------------------------------------</td>
<td>----------------------------------------------------</td>
</tr>
<tr>
<td>Air in the system</td>
<td>Degas pump</td>
<td></td>
</tr>
<tr>
<td>Autosampler vials contaminated</td>
<td>Use new vials or clean them with methanol</td>
<td></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

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.

Used Symbols:

- Store at
- Catalog Number
- In Vitro Diagnostic Device
- No. of tests
- Manufacturer
- Use by
- Lot number