Vitamin C HPLC Kit

For the determination of Vitamin C in Li-heparine plasma

Valid from 23.03.2011
1. INTENDED USE

The Immundiagnostik HPLC application is a chromatographic procedure intended for the quantitative determination of Vitamin C in plasma. This application is designed for in vitro diagnostic use only.

2. SUMMARY AND EXPLANATION

In the 15th to 17th century more sailors died of scurvy than of any other disease. The food provided on board contained nearly no Vitamin C. In the 16th century the importance of Vitamin C supplied by citrus fruits in healing scurvy was discovered.

Ascorbic acid (Vitamin C) is a strong reducing substance. The oxidation of Vitamin C leads over a radical intermediate to dehydroascorbic acid in vivo. The three forms mentioned constitute a reversible redox-system.

Ascorbic acid plays an important role in hydroxylation reactions, i.e. in the synthesis of collagen. So it is rather important for the de novo synthesis of bone, cartilage and tooth, and for the healing of wounds. Vitamin C is needed for the production of noradrenalin. Another important role of Vitamin C is its antioxidant capability, e.g. protection of other substances from oxidative damage. Ascorbic acid promotes the resorption of iron in the intestine. In addition, it reduces the production of nitrosamines which might cause cancer.

The primary unspecific signals of a lack of Vitamin C are: tiredness, physical and mental weakness and increased susceptibility for infections. Psychic disturbances like depressions or hysteria are possible.

The advantage of the HPLC method lies in the simultaneous handling of many analytes in a single test. The HPLC system enables even laboratories without experience in high performance liquid chromatography to use this technique for clinical routine determination in a quick and precise manner. Unlike immuno assays with up to six calibrators per test, a one-point calibration is mostly sufficient to calibrate the test system. It is possible to automate the sample application and calculation of the results so that even higher sample numbers of can be handled nearly without control.

**Indication**

- Determination of Vitamin C status
3. PRINCIPLE OF THE TEST

The application of HPLC for Vitamin C analysis allows its quantitation in an easy, fast, and precise way. The kit contains all reagents necessary for sample preparation and separation in ready-to-use form except the column.

The first step in the Vitamin C determination is precipitation of the higher molecular components. After their removal by centrifugation, the supernatant is injected into the HPLC system.

The Vitamin C analysis via HPLC follows an isocratic method at 30°C using a reversed phase column. One run lasts 12 minutes. The chromatograms are recorded by a UV-detector. The quantification is performed with the delivered calibrator. The concentration is calculated via integration of the peak areas by the external standard method.

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>KC2900LM</td>
<td>MOPHA Mobile phase</td>
<td>Mobile phase</td>
<td>1000 ml</td>
</tr>
<tr>
<td>KC2900KA</td>
<td>CAL calibrator (0,25 ml; lyoph., the concentration is given on the label)</td>
<td>CAL calibrator (0,25 ml; lyoph., the concentration is given on the label)</td>
<td>8 vials</td>
</tr>
<tr>
<td>KC2900FR</td>
<td>PREC Precipitating reagent (lyoph.)</td>
<td>Precipitating reagent (lyoph.)</td>
<td>1 vial</td>
</tr>
<tr>
<td>KC2900RE</td>
<td>RECSOL Reconstitution solution</td>
<td>Reconstitution solution</td>
<td>27 ml</td>
</tr>
<tr>
<td>KC2900KO</td>
<td>CTRL1 CTRL2 Control 1 and 2; 250 μl lyophilized (concentration, see product data sheet)</td>
<td>Control 1 and 2; 250 μl lyophilized (concentration, see product data sheet)</td>
<td>2 x 3 vials</td>
</tr>
</tbody>
</table>

HPLC column (KC 2900RP) 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

- Vortex- mixer
- Centrifugation tubes (1.5 ml)
- High precision pipettes
- HPLC pump with UV-detector
- reversed phase C\textsubscript{18}-column
- Centrifuge

6. PREPARATION AND STORAGE OF REAGENTS

- Reconstitute the calibrator (CAL) with 250 μl aqua bidest. The reconstituted standard solution is not stable and cannot be stored. The concentration is given on the label.
- Reconstitute the controls (CTRL1, CTRL2) in 250 μl aqua bidest.
- Reconstitute the precipitating reagent (PREC) in 25 ml reconstitution solution (RECSOL) in an ultrasonic bath for approx. 10 min. The precipitation solution (PREC) is stable for 3 months at 2-8 °C.
- All reagents are stable at 2-8 °C, calibrator (CAL) and controls (CTRL1, CTRL2) at –20 °C up to the date of expiry (see label of the test package).

7. PRECAUTIONS

- For in vitro diagnostic use only.
- Human materials used in kit components were tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, for safety reasons, all kit components should be treated as potentially infectious.
- The precipitating reagent (PREC) contains acid. Even diluted, it still must be handled with care. It can cause acid burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spills should be wiped out immediately with copious quantities of water. Do not breathe vapor and avoid inhalation.
- Reagents should not be used beyond the expiration date shown on kit label.
8. SPECIMEN COLLECTION AND PREPARATION

Venous fasting blood is suitable for this test system. We recommend Lithium-heparine plasma because of the better vitamin C stability. Therefore, commercial available sample tubes (e.g. Sarstedt S-Monovette LH) should be used. The best stability is achieved when a 7.5 ml tube is filled with 2 ml of blood.

Vitamin C is highly sensitive against oxidation; therefore samples should be stabilized immediately after arrival in the laboratory. For stabilisation, the precipitating reagent must be added (see 9. Assay procedure).

Plasma, containing the precipitating reagent is stable for 24 h at 2-8°C. The supernatant after centrifugation is stable for 3 month at -20°C.

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 can therefore not be held responsible for any damage resulting from wrong use.
- The assay should always be performed according the enclosed manual.

Sample preparation

Add into 1.5 ml reaction tubes:

200 μl patient sample, calibrator (CAL) and controls (CTRL1, CTRL2)

+ 200 μl precipitating reagent (PREC)

Mix well. Incubate the tubes for 10 minutes at 2-8°C and then centrifuge at 10,000 x g for 10 min.

Inject 20 μl of the supernatant into the HPLC

*The supernatant is stable at least for 24 hours at room temperature, if kept in the dark.
HPLC-Analytik Vitamin C

Chromatographic conditions

**Column material:** Bischoff Prontosil AQ; 5 μm

**Column dimension:** 125 x 4 mm

**Flow rate:** 0.75 ml/min

**UV-Detection:** 254 nm

**Temperature:** 30 °C

**Injection volume:** 20 μl

**Running time/sample:** 12 min

10. TREATMENT OF THE COLUMN

After each run, the column should be washed with 30 ml aqua bidest (1 ml/min) and stored in 50% methanol (v/v in aqua bidest., 30 ml), flow rate 0.7 ml/min. Before use, the system should be equilibrated with ca. 30 ml mobile phase (MOPHA).

11. RESULTS

Calculation

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

EDTA-blood is not suitable for this test system and should not be used.

13. QUALITY CONTROL

Expected values

4 to 20 mg/l (mean ± 2 SD).

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 plasma pools should be analyzed with each run of calibrators and patient samples. Results, generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the patient samples may not be valid, if within the same assay one or more values of the quality control samples are outside the acceptable limits.

14. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Intraassay CV: 5.6 % (4.4 mg/l) [n=6]
               4.1 % (18.8 mg/l) [n=6]

Interassay CV: 8.8 % (4.4 mg/l) [n=8]
               5.9 % (18.6 mg/l) [n=8]

Linearity
up to 250 mg/l

Detection limit
0.58 mg/l

15. DISPOSAL

The mobile phase (MOPHA) and the precipitation solution (PREC) can be neutralized to neutral pH with NaOH and disposed as a salt solution.

Important: Reaction will produce heat, be careful!
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>Double peaks</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>Auto sampler 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


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.

- Human materials used in kit components were tested and found to be negative for HIV, Hepatitis B and Hepatitis C and Australia antigen. However, for safety reasons, all kit components should be treated as potentially infectious.

- Reagents of the test package contain sodium azide as a bactericide. Contact with skin or mucous membranes must be avoided.

- All reagents in the test package are for in-vitro diagnostics 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.
Used symbols:

- Temperature limitation
- Catalogue Number
- In Vitro Diagnostic Medical Device
- Contains sufficient for <n> tests
- Manufacturer
- Use by
- Lot number

For reference use
For research use only