Adenosin

For the determination of adenosine in urine

Valid from 06.05.2008
1. INTENDED USE

The present HPLC kit is designed for quantitative determination of adenosine in urine. It is for in vitro diagnostic use only.

2. INTRODUCTION

Adenosine is a ubiquitous, biologically important nucleoside which is a precursor of other biologically active molecules as well as a component of some co-factors. Moreover, it also has its own distinct physiological functions in the central nervous system, the cardiovascular system, the skeletal muscle and the immune system.

One of the principal intracellular actions of adenosine is inhibition of the enzyme phosphodiesterase. Extracellular adenosine has specific neuro-modulatory actions on dopamine and glutamate.

Adenosine can act either as a hormone by binding to adenosine receptors or as an intracellular modulator after its translocation into the cell by membrane transport proteins. Four adenosine receptor subtypes have been identified. Notably, adenosine receptors are most widely expressed in the brain and the cardiovascular system, but they also are found in the most of the other tissues: respiratory tract, intestine, kidney, skeletal muscle, pituitary gland, uterus and gonads. Adenosine modulates several physiological effects by stimulating specific cell surface receptors. In addition, adenosine acts as an endogenous regulator of immune and inflammatory processes.

Adenosine exerts multifaceted effects on the heart and blood vessels and is involved in the regulation of the renal function. It works as a universal protective agent against hypoxia, ischemia, excitotoxicity, toxicities induced by other substances and trauma. It is also an effective and safe therapeutic medicine for paroxysmal tachycardias in adult and pediatric patients, with basic electrophysiologic properties of slowing conduction in atrioventricular nodes.

The measurement of urinary adenosine can contribute to evaluation of renal injury, metabolic disease or severe respiratory failure, as it was found that unfavorable pathophysiologic conditions are associated with appreciable elevation of adenosine.

Indications
- Evaluation of renal injury
- Metabolic diseases
3. Principle of the Test

The HPLC separation of adenosine is performed on a reversed phase column by the application of a gradient formed from solvent A and solvent B at 30°C. The duration of one run is about 35 minutes. The chromatograms are recorded by a UV-detector. The quantification is based on integration of the peak heights of the sample and the provided calibrator as an external standard. Parallel measurements of the urinary creatinine concentration are required to normalize the HPLC-adenosine results.

Summary

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

The analytical column (KC8000RP), pre-column (KC8000VS) as well as the adenosine extraction kit (KC8100) necessary for adenosine extraction from urine can be ordered separately from Immundiagnostik. The extraction kit contains ready-to-use reagents and consumables for sample preparation of 48 urine samples (extraction plate, extraction buffer, elution solution). Note: to use the complete capacity of the adenosine HPLC kit, which is designed for 96 samples, 2 extraction kits are needed.

The present HPLC kit enables even laboratories without experience in high performance liquid chromatography to use this technique for clinical routine determination in a quick and precise manner. In addition, mostly one-point is sufficient to calibrate the test system. 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.
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>KC8000LA</td>
<td>MOPHAA</td>
<td>Mobile phase A</td>
<td>4 x 1000 ml</td>
</tr>
<tr>
<td>KC8000LB</td>
<td>MOPHAB</td>
<td>Mobile phase B</td>
<td>1 x 1000 ml</td>
</tr>
<tr>
<td>KC8000ST</td>
<td>STAB</td>
<td>Stabilizing reagent</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>KC8000RE</td>
<td>RECSOL</td>
<td>Reconstitution solution</td>
<td>20 ml</td>
</tr>
<tr>
<td>KC8000KA</td>
<td>CAL</td>
<td>Calibrator (6 ml; lyophilized)</td>
<td>2 vials</td>
</tr>
<tr>
<td>KC8000KO</td>
<td>CTRL 1, CTRL 2</td>
<td>Control 1 and 2 (lyophilized, 600 µl; concentration, see product data sheet)</td>
<td>2 x 3 vials</td>
</tr>
</tbody>
</table>

The analytical column (KC8000RP), pre-column (KC8000VS) as well as the adenosine extraction kit (KC8100) necessary for adenosine extraction from urine can be ordered separately from Immundiagnostik. The extraction kit contains ready-to-use reagents and consumables for sample preparation of 48 urine samples (extraction plate, extraction buffer, elution solution). **Note:** to use the complete capacity of the adenosine HPLC kit, which is designed for 96 samples, **2 extraction kits are needed.**

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- Vortex- mixer
- 1.5 ml centrifugation tubes (e.g. Eppendorf)
- Various pipettes
- Aqua bidist.
- Horizontal Shaker
- HPLC gradient pump system with UV-detector
- Centrifuge
- Reversed phase column Nucleosil 100 C\textsubscript{18} 125 x 4 mm, 10 µm (KC8000RP)
- Pre-column cartridges Nucleosil 100 C\textsubscript{18} 10 x 4 mm, 10 µm (KC8000VS)
- Adenosine extraction kit (KC8100) for adenosine extraction from urine
6. PREPARATION AND STORAGE OF REAGENTS

- 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).

- Reconstitute the calibrator (CAL) with 6 ml of the reconstitution solution (RECOSOL) from the kit or with aqua bidist. The reconstituted standard solution is stable at -20 °C up to the date of expiry (see label of the test package).

- Reconstitute the controls (CTRL1, CTRL2) with 600 µl of the reconstitution solution (RECOSOL) from the kit or with aqua bidist.

- The calibrator (CAL) and the controls (CTRL1, CTRL2) contain already stabilizing reagent and do not need any further stabilization.

- All other reagents are provided in ready-to-use form.

7. PRECAUTIONS

- For in vitro diagnostic use only.

- Reagents should not be used beyond the expiration date shown on kit label.

8. SAMPLE COLLECTION AND PREPARATION

Morning urine is suited for this test. Immediately after urine collection, 10 µl STAB (stabilizing reagent) should be added to 500 µl of urine sample. The addition of the stabilizing reagent can cause precipitation. The samples should be centrifuged before analysis (see Assay procedure). The resulting supernatant is used in the test. The stabilized samples are stable for at least 6 months at -20°C.

The analysis should be performed immediately after the samples have been defrosted.

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.
Assay procedure

The stabilized samples, the CAL (calibrator) and the CTRL 1, 2 (controls 1, 2) should be centrifuged for 10 minutes at 10000 rpm before analysis. The resulting supernatants are used in the test.

Add in each well of the extraction plate:

450 µl of sample, CAL (calibrator) or CTRL 1, 2 (controls 1, 2) + 50 µl EXBUF (extraction buffer)

Cover the extraction plate with a foil and incubate for 30 minutes on a horizontal shaker at room temperature.

Remove the foil, aspirate the contents of the wells and tap the microtiter plate on absorbent paper towels to remove any residual solvent.

Add 1 ml of aqua bidist. in each well of the extraction plate. Cover the extraction plate with a foil and incubate for 5 minutes on a horizontal shaker at room temperature.

Remove the foil, aspirate the contents of the wells and tap the microtiter plate on absorbent paper towels to remove any residual solvent.

Add 200 µl of ELUSOL (elution solution) in each well of the extraction plate. Cover the extraction plate with a foil and incubate for 10 minutes on a horizontal shaker at room temperature.

Remove the foil, transfer the complete content of each well in a separate 1,5 ml centrifugation tube and centrifuge for 10 minutes at 10000 rpm.

The resulting supernatant is stable for at least 24 hours at 4 °C. Inject 100µl of the supernatant into the HPLC.
**Chromatographic conditions**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column material:</td>
<td>Nucleosil 100 C18, 10 µm</td>
</tr>
<tr>
<td>Column dimension:</td>
<td>125 x 4 mm</td>
</tr>
<tr>
<td>Flow rate:</td>
<td>1.0 ml/min</td>
</tr>
<tr>
<td>UV-Detection:</td>
<td>254 nm</td>
</tr>
<tr>
<td>Temperature:</td>
<td>30 °C</td>
</tr>
<tr>
<td>Injection volume:</td>
<td>100 µl</td>
</tr>
<tr>
<td>Running time/sample:</td>
<td>35 min</td>
</tr>
<tr>
<td>Gradient</td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>(100 % A / 0 % B)</td>
</tr>
<tr>
<td>12 min</td>
<td>(100 % A / 0 % B)</td>
</tr>
<tr>
<td>24 min</td>
<td>(60 % A / 40 % B)</td>
</tr>
<tr>
<td>26 min</td>
<td>(0 % A / 100 % B)</td>
</tr>
<tr>
<td>28 min</td>
<td>(0 % A / 100 % B)</td>
</tr>
<tr>
<td>29 min</td>
<td>(100 % A / 0 % B)</td>
</tr>
<tr>
<td>35 min</td>
<td>(100 % A / 0 % B)</td>
</tr>
</tbody>
</table>

*We recommend strongly the use of a pre-column to prolong the life of the analytical column.*

## 10. Treatment of the Column

After each run, the column should be washed with ca. **50 ml** of aqua bidist. at a flow rate of 1 ml/min. Afterwards, the column is stored in 85% Acetonitril / 15% aqua bidist. (ca. 30 ml, flow rate 1.0 ml/min).

Before use, the system should be equilibrated with ca. **30 ml** mobile phase A (MOPHA).
**11. RESULTS**

Calculation

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

**Typical chromatogram**

The HPLC-adenosine results are normalized to the creatinine concentration in the urine.

\[
\text{Concentration}_{\text{Sample, [µM/mm Creatinine]}} = \frac{\text{Adenosine Concentration}_{\text{Sample, [µM]}}}{\text{Creatinine Concentration}_{\text{Sample, [mM]}}}
\]

**12. LIMITATIONS**

Blood is not suited for this test system and should not be used.
13. QUALITY CONTROL

Expected values

Normal range in urine 0.78 – 6.84 µM (average value: 2.51µM)
Adenosine-to-creatinine ratio 0.07 – 0.63 µM/mM creatinine
(average value: 0.26 µM/mM creatinine)

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

Controls 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

Intra-Assay CV: 4.7 % [n = 6]
Inter-Assay CV: 12.8 % [n = 6]

Linearity

Up to 1000 µM

Detection limit

0.42 µM

15. DISPOSAL

The mobile phases (MOPHA and MOPHB) can be disposed as halogen free spent solvents.
### 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 research use only