HVA ELISA Kit

Cat. No.: DEIA2251
Pkg. Size: 96T

**Intended use**

The HVA KIT is designed for quantitative measurement of homovanillic acid (HVA) concentration in patients' urine.

**General Description**

Historically, colorimetric analysis of HVA utilized the reaction of 1-nitroso-2-naphthol with biogenic amines, then the method was improved by substituting 1-nitroso-2-naphthol-4-sulfonic acid. However, the colorimetric methods are not specific for HVA due to known interference by many compounds.

Thin layer chromatography (TLC) on silica gel was another approach to determine HVA. This method is slow and requires special equipment. Using a flame-ionization detector and electron-capture detection, a gas chromatographic (GC) method was also developed, but has not been widely adapted due to the relatively poor sensitivity. Furthermore, gas chromatography-mass spectrometry was also used for quantitating of HVA in both serum and urine.

Finally, high-performance liquid chromatography (HPLC) methods involving initial separation of the biogenic amines by anion-exchange chromatography, and final separation by reverse-phase (C18) chromatography have become most common, and preferred methods. These methods are reasonably rapid (although requires sample pretreatment), have excellent sensitivity and little interference from other endogenous compounds or exogenous drugs and foods.

**Principle Of The Test**

The HVA ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the competition between HVA coated on a microtiter well and that in urine for the monoclonal antibody. Outlined steps are:

1. **Sampling and reaction:** The samples are incubated in the wells with horseradish peroxidase conjugated anti-HVA monoclonal antibody.
2. **Washing:** Unbound HVA and the antibody bound to urinary HVA are removed by washing with 0.9% NaCl solution.
3. **Enzyme Reaction (Color Development):** The amount of bound peroxidase is inversely proportional to the concentration of the HVA present in the urine sample. Upon addition of the substrate (TMB), a blue color is developed, then it is changed to yellow by adding Stopping Solution. The intensity of this is inversely proportional to the concentration of HVA in the Calibrators or urine samples.
4. **Absorbance Detection:** After addition of Stopping Solution, absorbance is measured at 450 nm. And the readings are converted into the concentrations from the Calibration curve.

**Reagents And Materials Provided**

1. HVA Coated Microwell Plate: 1 HVA coated 96-microwell plate.
2. Anti-HVA-Enzyme Conjugate: Horseradish peroxidase conjugated to an anti-HVA monoclonal antibody, 0.3 ml.
3. VMA/HVA Conjugate Diluent Solution (12 ml)
4. VMA/HVA Color Developing Reagent: Tetramethylbenzidine (TMB) solution (20 ml)
5. VMA/HVA Stopping Solution: 2N Sulfuric acid (20 ml)
6. HVA Calibrator Set: HVA solutions of 0, 0.125, 0.25, 0.5, 2, 4, 8 and 16 µg/ml in phosphate buffered saline, 0.01M, pH 7.4 (1.0 ml each).

**Materials Required But Not Supplied**

1. Plate reader with 450 nm filter
2. pH meter of pH paper with the range of 5.0-10.0
3. Pipettor with tips for 10, 50, and 100 µl
4. Pipettor with tips for 50 and 100 µl
5. Volumetric cylinders, 10 and 100 ml
6. Volumetric and serological pipettes, 10 ml
7. Disposable test tubes or vials
8. 5N NaOH solution
9. 5N HCl solution
10. Phosphate buffered saline, 0.01 M, pH 7.4
11. Plate washer (optional)
12. Plate shaker (optional)
13. NaCl, or Saline buffer
14. 0.01 M phosphate buffered saline, pH 7.4

Storage

1. Store the kits at 2-8°C in refrigerator.
2. Keep microwell plates in dry bag with desiccants. Open the bag only when needed.
3. Expiration dates of the reagents are stated on their labels.
4. Color Developing Reagent should be colorless.
5. Protect the reagents and reaction mixture from exposure to direct sunlight.

Specimen Collection And Handling

24-hour urine specimen should be collected with 10 ml of 6 M HCl as a preservative.
Overnight or randomly collected urine should be acidified to a pH between 2 and 3 immediately after collection.
Record the total volume and save 1-5 ml for the analysis of HVA and total creatinine.
All samples should be refrigerated until tested. Centrifuge turbid urine samples containing crystals or sediment.
1. Take 1.0 ml of acidified urine and transfer to a disposable tube in which the pH of urine sample can be readjusted.
2. Bring pH of all samples within the range of 6 and 9 by stepwise addition of small amounts of 5N NaOH (e.g. 5 µl) while monitoring the pH either with a pH meter or using pH paper.
3. Dilute pH re-adjusted samples at a 1:10 ratio with phosphate buffered saline. The pH for diluted samples should be between 7.0 and 8.0.

Reagent Preparation

Enzyme Conjugate Solution:
Predilute Enzyme Conjugate by: Adding 50 ml of Anti-HVA Enzyme Conjugate to 5.5 ml of VMA/HVA Conjugate Diluent.

Washing Solution:
Dissolve 9 grams of NaCl in 1 liter of deionized distilled water. Commercially available normal saline can also be used.

Assay Steps

General Remarks
1. Prepare 0.01 M phosphate buffered saline, pH 7.4.
   This solution is used to dilute all unknown urine samples prior to analysis.
2. Before beginning the test, bring all urine samples and reagents to room temperature (15-30°C) and mix well.
3. Set all reagents and urine samples ready before the assay. The entire test procedure must be performed without any interruption in order to get the most reliable and consistent results.

Assay Procedure
1. Make work sheet with Calibrators and sample identification.

2. Sampling:
   a. Dispense 50 µl of HVA Calibrators into appropriately designated wells.
   b. Dispense 50 µl diluted (1:10) controls or samples to respective wells.

3. Addition of the Anti-HVA-Enzyme Conjugate:
   Dispense 50 µl of prediluted (see Preparation of Reagents) Anti-HVA-Enzyme Conjugate to each well, using a pipettor.

4. Antigen-Antibody Reaction:
   Mix the plate by moving it back and forth slow horizontal movements for a minute. A plate shaker can be used for this purpose also. Allow the plate to stand at 15-30°C, room temperature for 1 hour.

5. Washing:
   Wash only once. Removing incubation mixture by decanting the plate into a sink and blotting the plate on absorbant paper. Washing can also be done on a plate washer.

6. Enzyme Reaction:
   Dispense 100 µl of VMA/HVA Color Developing Reagent to the well and allow it to stand at 15-30°C, room temperature for 25 minutes.

7. Stopping Color Development:
   Dispense 100 µl of VMA/HVA Stopping Solution to the wells.

8. Absorbance Measurement:
   Any microwell reader capable of detecting absorbance at 450nm may be used.

**Calculation**

1. Using semi-log linear paper or log-logit paper the Calibration curve is generated by plotting HVA concentrations on the abscissa and the absorbance on the ordinate. HVA concentration for each unknown sample is obtained from the Calibration curve.

   For example:
   
   This is a representative curve should not be used to calculate results for unknow samples.

   The results obtained above indicate HVA concentration in µg/ml. When the total HVA in 24-hour urine sample is required; HVA (µg/ml) * urine volume (ml) / 1000 = HVA mg/24 hours

   or, when HVA/Creatinine value is required;

   HVA (µg/ml)/ Creatinine (mg/dl) / 100 = HVA µg/ml Creatinine or = HVA mg/g Creatinine

2. Calculation can be made with a computer set so as to draw calibration curves based on 4 coefficient log-logit.

<table>
<thead>
<tr>
<th>Description</th>
<th>Absorbance (450 nm)</th>
<th>Average of B/Bo(%)</th>
<th>HVA (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HVA Calibrator</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 µg/ml</td>
<td>1.092</td>
<td>1.096</td>
<td>100.0</td>
</tr>
<tr>
<td>0.125 “</td>
<td>0.900</td>
<td>0.881</td>
<td>81.4</td>
</tr>
<tr>
<td>0.25 “</td>
<td>0.780</td>
<td>0.792</td>
<td>71.8</td>
</tr>
<tr>
<td>0.5 “</td>
<td>0.648</td>
<td>0.631</td>
<td>58.5</td>
</tr>
<tr>
<td>2 “</td>
<td>0.335</td>
<td>0.324</td>
<td>30.1</td>
</tr>
<tr>
<td>4 “</td>
<td>0.232</td>
<td>0.220</td>
<td>20.7</td>
</tr>
<tr>
<td>8 “</td>
<td>0.169</td>
<td>0.114</td>
<td>12.9</td>
</tr>
<tr>
<td>16 “</td>
<td>0.108</td>
<td>0.108</td>
<td>9.9</td>
</tr>
<tr>
<td>Sample A</td>
<td>0.966</td>
<td>0.950</td>
<td>87.6</td>
</tr>
<tr>
<td>Sample B</td>
<td>0.225</td>
<td>0.222</td>
<td>20.4</td>
</tr>
<tr>
<td>Sample C</td>
<td>0.555</td>
<td>0.518</td>
<td>49.0</td>
</tr>
</tbody>
</table>

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Reference Values

Each laboratory should determine a normal range to conform with the characteristics of the population being tested. The range given here was determined from 24-hour urine collections on 280 subjects. Urinary creatinine was measured on Astra to assess the completeness of each collection, and mg HVA per gram creatinine was calculated.

<table>
<thead>
<tr>
<th>Number of samples (n)</th>
<th>HVA mg/day</th>
<th>HVA mg/g Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Value (x)</td>
<td>277</td>
<td>277</td>
</tr>
<tr>
<td>±2 S.S. range</td>
<td>4.35</td>
<td>3.63</td>
</tr>
<tr>
<td>Reference Range</td>
<td>0.37 – 8.33</td>
<td>0 – 7.51</td>
</tr>
<tr>
<td></td>
<td>up to 8.30</td>
<td>up to 7.50</td>
</tr>
</tbody>
</table>

Sensitivity

The sensitivity of this test is higher than 0.125 µg/ml. The minimal concentration of HVA is estimated to be 0.035 µg/ml. The minimal detectable concentration is defined as the concentration of HVA which corresponds to the absorbance that is two standard deviations from the mean absorbance of 20 determinations of zero dose HVA.

Specificity

The following substances were tested for cross-reactivity of the assay. Cross-reactivity is expressed in terms of percentage of the concentration of each substance that produced 50% displacement to the HVA concentration resulting in 50% displacement.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross-reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homovanillic Acid</td>
<td>100</td>
</tr>
<tr>
<td>Vanillylmandelic Acid</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DL-3,4-Dihydroxymandelic Acid</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3,4-Dihydroxyphenylacetic Acid</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Metanephine</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Vanillypyruvic Acid</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Vanillyl acid</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Dopamine</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>5-Hydroxy-3-indolacetic Acid</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Vanillylactic Acid</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3-methoxy-4-hydroxyphenyl Glycol</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Reproducibility

Intra-assay coefficient of variation was evaluated in three urine samples at different HVA concentrations. Inter-assay coefficient of variation was evaluated at three different concentrations, by analyzing the samples in 16 to 19 separate occasions.
Precautions

1. The components in this kit are intended for use as an integral unit.
2. The components of different lots should not be mixed.
3. Do not use the HVA Calibrators in this kit for other purposes (e.g. HPLC).
4. Use a new pipette tip for each Calibrator or urine sample to avoid cross-contamination.
5. It is very important to wash the microwells thoroughly, yet uniformly and remove any residual liquid from the wells to achieve optimal results.
6. Pipette Calibrators or urine samples into the bottom of each well. Vortex-mixing or shaking of wells after each pipetting is not required.
7. Absorbance is a function of the incubation time and temperature. It is, therefore, recommended to ensure the equally elapsed time for each pipetting without interruption.

Limitations

1. This HVA Kit is designed for the quantitative determination of HVA in urine only.
2. All samples with HVA concentrations greater than 16 µl/ml should be repeated on much larger dilution(s), e.g. 1:20 or more.
3. Interference by Sodium Azide: As Sodium Azide inhibits the enzyme reaction, urine or any buffer used to dilute urine samples containing sodium azide as an antiseptic can not be used.

REFERENCES