Determination of HVA in Urine

The ortho-dihydroxyphenyl (catechol) compounds derived from tyrosine, foremost among these being the catecholamines, are metabolized by enzymatic pathways leading to a variety of neutral and acidic products. Measurement of these compounds as well as their catecholamine precursors is highly desirable in biomedical and clinical research.

One of the acidic catabolites of clinical interest is 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA). Altered concentrations of catecholamine metabolites in urine are indicative of a number of abnormal physiological and pathological conditions. For example, HVA determinations are useful in confirming Parkinsonism, monitoring therapeutic response to L-dopa treatment, and in combination with VMA determinations, diagnosing persons afflicted with neuroblastoma and other "neural crest" tumors.

The following outlines an analytical protocol for the determination of HVA in urine. The protocol utilizes liquid chromatography with electrochemical detection. The use of Clin-Elut extraction tubes permits large numbers of samples to be prepared rapidly.

Materials

Reagents

2 N Hydrochloric Acid: Add 83 mL concentrated HCl to a 500 mL volumetric flask. Dilute to the mark with deionized, distilled water.

0.2 M Acetate buffer, pH 4.2: Add 11.5 mL glacial acetic acid to a 1 liter volumetric flask. Dilute to the mark with deionized, distilled water. Adjust the pH to 4.2 with sodium hydroxide.

Synthetic Sample: 0.01 M acetate buffer, pH 4.5 containing 6 µg HVA/mL. This should be made fresh daily.

Extraction Solvent: 9:1 methylene chloride: isopropanol.

Apparatus

Liquid Chromatograph. Bioanalytical Systems LC-154T with a TL-5 glassy carbon working electrode.

Nitrogen Evaporator. .

Recommended Accessories

Clin Elut Extraction Columns. Model CE 1001-M, 0.3 mL total capacity (P/N 5062) with Models UR 100M and LR 1716 upper and lower racks.

Centrifuge tubes. 10-12 mL capacity conical centrifuge tubes (glass or polypropylene).

Volumetric or Mechanical Pipets. 3 mL, 2 mL, and 100 µL sizes.

Vortex Mixer.

Experimental

Conditions

Liquid Chromatograph: LC-154T (Bioanalytical Systems)
Mobile Phase: 0.2 M, pH 4.2 acetate buffer with 15% (v/v) MeOH.
Flow Rate: 1.5 mL/min
Stationary Phase: Biophase ODS (BAS P/N 6016), 10 µm particle size.
Temperature: 30°C
Amount Injected: 100 µL
Detector: LC-4A/17 electrochemical detector package, using a TL-5 glassy carbon working electrode (Bioanalytical Systems)
Potential: +0.80 V (vs. Ag/AgCl reference electrode).
Controller (LC-4A) Sensitivity (current amplification): 10 or 20 nA/V and the 2 or 5 sec. output time constant.

Procedure

1. Place Clin Elut columns in appropriate rack with centrifuge tubes positioned under each column.

2. Add 100 µL of 2 N HCl to each Clin Elut column.

3. Add 100 µL of the raw urine sample(s) or synthetic sample(s) to each Clin Elut column. Wait approximately 3 minutes. Note: At least two synthetic samples should be run with each batch (10-15) of urine samples.

4. Add 2 x 3 mL of the Extraction Solvent (9:1, methylene chloride: isopropanol). Wait approximately 5 minutes for complete elution of solvent.

5. Remove the centrifuge tubes containing the extraction solvent and place in an evaporator. Exaporate to dryness under a stream of nitrogen.

6. Add 2 mL of the mobile phase solution to each centrifuge tube and vortex mix for approximately 1 minute.

7. Inject 100 µL of this aqueous solution into the chromatograph. A typical chromatogram from a urine sample is shown in Figure 1.

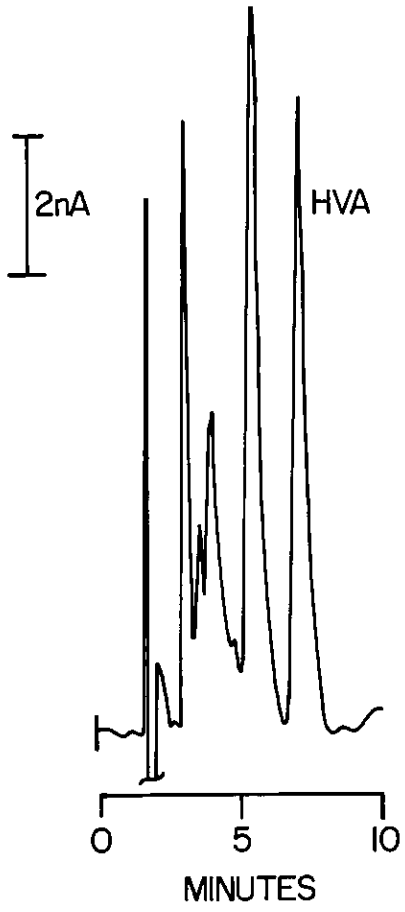

Figure 1. Typical urine sample chromatogram following the procedure outlined in text. 3.4 µg HVA/mL.

Sample Calcuations

The amount of HVA in each sample is determined by comparison of the chromatographic peak height in nanoamps of the unknown urine sample to the peak height in nanoamps of the synthetic sample containing a known amount of HVA. Using the following equation the HVA concentration in each urine sample can be determined.

$$[HVA]_{unk} \ (\mu g/mL) = \frac{(Pk.\ Ht., HVA)_{unk}\ (nA) \times [HVA]_{syn}\ (\mu g/mL)}{(Pk.\ Ht., HVA)_{syn}\ (nA)}$$

Example: A synthetic sample contains 6 µg HVA/mL (as is suggested above) and this sample gave a peak height of 10 nAmps after going through the extraction procedure and final separation and quantitation on the LCEC instrumentation, and if a urine sample going through this sample procedure gave a peak height of 5 nAmps, the concentration of HVA in the urine sample is

$$[HVA]_{\text{unknown urine sample}}\ (\mu g/mL) = \frac{5\ nAmps \times 6\ \mu g\ HVA/mL}{10\ nAmps} =$$

3 µg HVA/mL