CAPSULES
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Determination Of Plasma Catecholamines By LCEC

Purpose

Offer a method for the preparation of small volume plasma samples, and detection of catecholamines including norepinephrine (NE), epinephrine (EPI), dopamine (DA), and 3- hydroxytyrosine (L-Dopa). The nature of this procedure will exclude detection of acidic metabolites which would otherwise be seen in the sample, including vanilmandelic acid (VMA), and homovanillic acid (HVA). It is preferable to determine these acidic components via different extraction/chromatographic schemes. Uric acid, a common plasma component at the ppm level, is extracted with very low efficiency and elutes prior to norepinephrine. DHBA, not a naturally occurring catecholamine, is used as an internal standard in the assay.

Reference

Reverse-phase columns containing 3 µm packing materials exhibit a broad range of applicability to the determination of catecholamines and indoleamines, and their metabolites (1). BAS has recently introduced a new ODS 3 µm column (2). The utilization of this column requires some adaptation of existing methodologies.

Equipment

200 µL mechanical pipette
1-5 mL adjustable pipette
25 µL glass syringe (Hamilton) (BAS MF-5002)
100 µL glass syringe (Hamilton) (BAS MF-5005)
5 mL conical reaction vials (BAS MF-7000)
Mobile Phase Filtration kit (BAS MF-6126)
Vortex Mixer
Reciprocal Shaker
Vacuum Aspirator
Pasteur pipettes
Small Centrifuge (BAS MF-5063)
Centrifugal Microfilters (BAS MF-5500)
Regenerated Cellulose Membrane for Microfilters (BAS MF-5658)
pH Meter

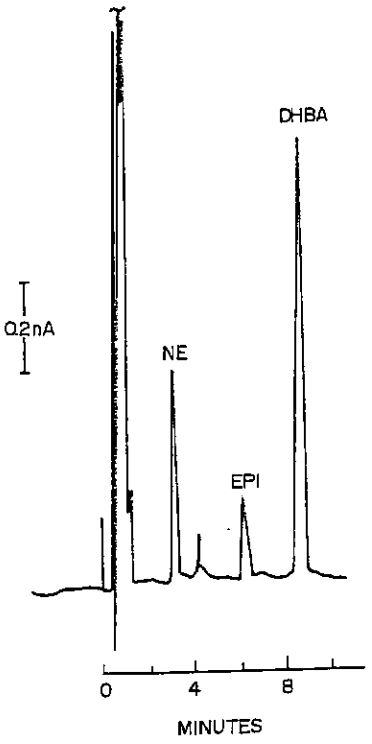

Figure 1. A representative chromatogram of a plasma sample processed by the present procedure. 30 µL of the final 0.1 M $HClO_4$ solution was injected.

Reagents

The following reagents are sufficient for processing 200 plasma samples:

0.1 M $HClO_4$: Dilute 17.1 mL of 70% $HClO_4$ to 2 L. Filter and refrigerate in a sealed bottle until ready for use.

100 ng/mL DHBA*: Dissolve 16 mg of DHBA • HBr in 100 mL of 0.1 M $HClO_4$. Mix well, then dilute 100 µL to 100 mL with 0.1 M $HClO_4$. Refrigerate.

Norepinephrine*, epinephrine* standard solution:

(75 ng/mL NE + 25 ng/mL EPI): Dissolve 14.2 mg of norepinephrine-bitartrate, and 4.6 mg of epinephrine- bitartrate in 100 mL of 0.1 M $HClO_4$. Mix this combined stock solution, then dilute a 100 µL aliquote to 100 mL with 0.1 M $HClO_4$. Refrigerate.

**Hydrochloride, bitartrate, etc., salts of these compounds may be used but concentrations should always be calculated (corrected) for the freebase.*

Tris Buffer: Combine 45 g of Tris base (Sigma T-1503) and 5 g Na_2EDTA in approximately 200 mL of distilled H_2O. Adjust to pH 8.6 by the dropwise addition of conc. HCl. Dilute to 250 mL, filter, and refrigerate.

Phosphate Buffer: Dissolve 8.64 g Na_2HPO_4, 2.36 g KH_2PO_4, and 20.0 g Na_2EDTA in a liter of distilled water (pH 7.0). Filter and refrigerate.

Acid Washed Aluminum Oxide: (AAO, BAS P/N CF-8010): After opening, keep vial closed and in a dessicator between uses.

Liquid Chromatography: The mobile phase is optimized for BAS PHASE II ODS, 3 µm columns, 100 x 3.2 mm. Other columns may require a different relative composition.

Conditions

System: BAS 400, BAS 460 or BAS 200 chromatograph.

Mobile Phase: 0.075 M MCAA, pH 3.0, 0.5 mM Na_2EDTA, 1 mM SOS, 1.5% CH_3CN. Flow rate was 1.0 mL/min.

Column: Phase II ODS 3 µm, 100 x 3.2 mm (BAS P/N MF-6213)

Electrode: Glassy carbon

Potential: + 0.65 V vs. Ag/AgCl

Injection volume: 30 µL. Small peaks may be compensated for by increasing the injection volume.

Sample Calculation

For calibration purposes, a synthetic sample consisting of 1.0 mL of phosphate buffer, 8 µL of NE/EPI standard solution, and 12 µL of DHBA solution are assayed in duplicate. The absolute and relative recoveries for both this synthetic sample and spiked plasma samples are virtually the same. Therefore, to calculate sample concentrations, peak height ratios (relative to the internal standard DHBA) for unknown plasma samples are compared to those for this synthetic standard whose original concentrations are known. For norepinephrine this would be:

$$(\text{conc. NE})_{unk} = (\text{NE/DHBA})_{unk} \times \frac{(\text{conc NE})_{known}}{(\text{NE/DHBA})_{known}}$$

Procedure

1. Into a 5 mL conical reaction vial, place the following: (a) 1.0 mL plamsa, (b) 12 µL DHBA standard, and (c) 50 mg AAO. For every set of test samples, prepare at least two additional synthetic samples as follows: (a) 1.0 mL phosphate buffer (b) 12 µL DHBA standard (c) 8 µL NE/EPI standard and (d) 50 mg AAO. These last samples will be used to calibrate the instrument.

2. To the first sample, add 0.5 mL Tris buffer, cap, and immediately vortex. Set the vortexed sample aside and treat each additional sample in a similar manner. All samples are then shaken (together) for 5 min. with the aid of a reciprocal shaker.

3. After shaking, allow the alumina to settle. Aspirate the supernatant.

4. Wash the alumina twice (with shaking) with water and aspirate to near dryness each time.

5. Add about 0.5 - 1.0 mL of water to each sample and transfer the alumina slurry, with a disposable pipette, to a Mircofilter loaded with a RC58 membrane.

6. Place the Microfilter in a centrifuge and spin the AAO at 1000 x g for 1 min. or until no fluid remains in the sample chamber.

7. Put a new receiver tube on the Microfilter and add 100 µL of 0.1 M $HClO_4$ to the sample compartment. Vortex briefly, let stand for 5 minutes, and again vortex briefly.

8. Centrifuge the Microfilter at 1000 x g for 1 min. or until no fluid remains in the sample chamber. The acidic extract in the receiver tube contains the catecholamines, ready for injection into the LC system. At this stage, the sample is relatively stable, but still should not be held for long periods of time before analysis.

Comments

The most common problems encountered in this assay are poor recovery of the catecholamines and chromatographic interferences. The following are measures which should be explored to achieve maximal results from this procedure: (1) The adsorption of catechols to the AAO is pH dependent, being optimal at pH 8.5. If the sample pH is less than this, after the addition of Tris buffer, titrate to pH 8.5 with NaOH or increase the volume of added Tris buffer. (2) The catecholamines are readily oxidized in an alkaline medium, therefore, it is important to vortex the sample immediately after the addition of the Tris buffer. (3) Shaking has a pronounced effect on the recovery, make sure samples are well mixed during the adsorption step. (4) Shaking (mixing) is also critical at the desorption step, a uniform acid concentration must contact all the catecholamine-AAO complex to ensure maximal recovery. Desorption of catechols from the AAO occurs at pH 1.0. The pH of the 0.1 M $HClO_4$ eluent should be checked. If it is less acidic, a more vigorous water wash (step 4) is required. Dissolved CO_2 will affect the pH of the water used in this step, the more acidic the water the greater the amount of catechols that could be lost. Titrating the wash water to neutrality (with NaOH) may be of benefit.

Chromatographic conditions can be fine tuned to resolve the catecholamines from interferences: (1) Increasing the concentration of the ion-pair reagent (SOS), elevating the pH, or reducing the ionic strength of the mobile phase buffer results in increased retention times (capacity factor) of the catecholamines. Decreasing ionic strength is most helpful when dealing with the close elution of uric acid and NE. (2) Addition of an organic solvent, such as methanol or acetonitrile, decreases the retention times of the analytes. When an ion-pair reagent is used the column must be fully equilibrated (mobile phase going to waste for a 100 x 3.2 mm column, at least 250 mL) prior to recycling. This will ensure reproducible chromatography. (3) An extraction of a blank should be performed (all reagents minus plasma or standards) to be assured that the reagents do not contain extractable contaminants that would produce an interference peak on the chromatogram. If an interference peak is present it should be isolated to the reagent salts, hardware (syringes, injector, reaction vials, etc.) water, or desorbing acid used, and these cleaned or replaced. (4) Interferences present in the sample, due to diet, medication, etc. will have to be handled by changing chromatography conditions (changes in mobile phase composition usually) to separate them from the analytes of interest.

We at BAS, as well as a good number of our customers, have found MCAA to be a convenient buffer; due to it's lot to lot reliability, good buffering capacity around pH 3.0, and recycling capabilities (poor medium for bacterial growth). However, some chromatographers may consider MCAA to be too toxic, and thus pose an unacceptable saftey hazard. We have found that the following mobile phase gives similar separation characteristics with the 3 µm column: 20.7 g $NaH_2PO_4 \cdot H_2O$, pH 3.1 (with H_3PO_4), 681 mg EDTA, and 413 mg SOS made to 2 L with deionized water. As above, CH_3CN can be added and the final working mobile phase filtered and degassed before use. With this mobile phase, bacterial contamination should be considered as a potential problem and it should not be recycled.

Related References

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