Sample Preparation for HPLC

Summary

This protocol was developed to measure monoamines in fresh or frozen samples from brain tissue, cultured cells, or body fluids and purines from cultured cells. Application to tissue was described in Egami et al, Neurobiol Dis, 26: 396-407, 2007. Application to cultured cells was described in Lewers et al, Neuroscience, 152:761-772, 2008: Shirley et al, J Neurochem, 101, 841-853, 2007.

Sample processing

1. Sample collection
    a. Brain tissue should be dissected rapidly in the cold and the tissue samples frozen in microfuge tubes immediately on dry ice.
    b. Cultured cells may be harvested by scraping or trypsinization following removal of overlying culture medium. Any excess culture medium should be removed following centrifugation at 300 × g for 5 minutes.
    c. Body fluid samples, such as microdialysate, CSF, urine samples should be collected and aliquoted.
    d. Plasma samples should be collected in EDTA coated tube and centrifuged at 3000 × rpm for 15 minutes at 4°C to remove blood cells.
    e. Samples may be stored indefinitely at -80ºC
2. Sample deproteinzation/acidification by perchloric acid
    a. For frozen sample, add approximately 10 X of the sample weight of cold 0.1 M perchloric acid directly to the frozen sample and homogenize immediately.
    b. For large specimens such as whole mouse brain, the volume typically is about 5 ml and requires homogenization by polytron for 10 seconds on setting 70
    c. For small specimens such as striatum, the volume typically is about 400 µL and the sample can be disrupted by probe sonication for 10 seconds on setting 3 with a 30% duty cycle.
    d. For a single 10 cm plate of cells, the volume is about 200 µL and the sample is disrupted by probe sonication.
    e. For body fluid samples, such as microdialysate, CSF, urine samples, cold 4 M perchloric acid is added to sample to bring the final concentration to 0.1 M and mixed thoroughly.
    f. For plasma samples, cold 4 M perchloric acid is added to sample to bring the final concentration to 0.4 M and mixed thoroughly.
    g. The instructions on how to prepare perchloric acid solution: https://www.sigmaaldrich.com/chemistry/stockroom-reagents/learning-center/technical-library/molarity-calculator.html
3. HPLC analysis for monoamines on ESA instrument
    a. Centrifuge the homogenates at 10,000 × g for 10 minutes at 4ºC.
    b. Collect the supernatant into a new tube, being cautious to avoid collecting any of the pellet. Reserve the pellet for measurement of protein concentration. Either sample may be re-frozen and stored at -80 ºC prior to analysis.

c. Filter any remaining particulate matter from the supernatants individually by spinning in a 0.45 µM PVDF microcentrifuge filter tube at 5000 × rpm for 5 minutes at 4ºC.
d. Load filtrate directly into HPLC vials for analysis on the ESA instrument.

4. HPLC analysis for purines on Waters instrument
   a. Adjust pH of supernatant to 7.0 by adding a small volume of 2.5% 3.5 M K_2CO_3 and store on ice for 10-15 minutes to precipitate potassium perchlorate.
   b. Centrifuge the homogenates at 10,000 × g for 10 minutes at 4ºC.
   c. Filter any remaining particulate matter from the supernatants individually by spinning in a 0.45 µM PVDF microcentrifuge filter tube at 5000 × rpm for 5 minutes at 4ºC.
   d. Load filtrate directly into HPLC vials for analysis on the Waters instrument.
   e. If samples are refrozen, they must be filtered through new microcentrifuge filter tubes prior to HPLC analysis.

5. Protein analysis
   a. Remove all residual supernatant.
   b. Resuspend pellet in 500 µL of 2% SDS by gentle pipetting. Dissolving the pellet fully may require sonication for 10 seconds along with overnight incubation at 37ºC.
   c. Dilute 5-15 µL of sample protein in additional 2% SDS to a total volume of 50 µL for the assay.
   d. Prepare a protein standard curve that ranges from 0 to 2 mg/ml of BSA, also in 2% SDS.
   e. Add 200 µL of Pierce BCA Protein Assay reagent (mix 50 parts reagent A to 1 Part of reagent B) to each 10 µL of sample and vortex mix.
   f. Incubate 30 minutes at 37ºC.
   g. Read on the microplate spectrophotometer at 562 nm