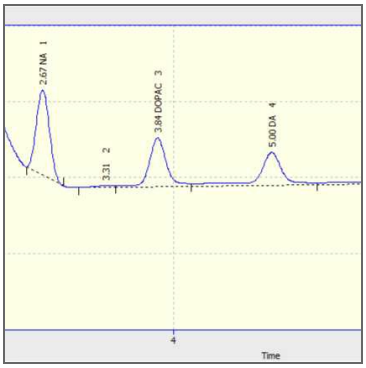

JAN 29, 2024

DOI:
dx.doi.org/10.17504/protocols.io.8epv5xkz4g1b/v1

Protocol Citation: Emanuel F Lopes, Stephanie J Cragg 2024. High-performance liquid chromatography with electrochemical detection of monoamine neurotransmitters and metabolites. **protocols.io** https://dx.doi.org/10.17504/protocols.io.8epv5xkz4g1b/v1

Protocol status: Working
We use this protocol and it's working

High-performance liquid chromatography with electrochemical detection of monoamine neurotransmitters and metabolites

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ABSTRACT

This protocol describes a method to identify and quantify the content of the monoamine neurotransmitters dopamine (DA) and norepinephrine (NE), along with several related metabolites (3,4-Dihydroxyphenylacetic acid – DOPAC; homovanillic acid – HVA; 5-Hydroxyindoleacetic Acid – 5-HIAA) from brain tissue samples using HPLC with electrochemical detection.

GUIDELINES

Adjusting different components of the mobile phase will change retention times of compounds:

- Increasing the concentration of OSA will increase the retention time of DA.
- Altering the pH of the mobile phase affects the state of the acid metabolites DOPAC, HVA and 5-HIAA. An increase in pH reduces the retention time of these acid metabolites. To increase pH of mobile phase use high concentration of NaOH, decrease pH with orthophosphoric acid. Add small amounts and wait a while to allow pH meter to respond before adding more. Remember to operate within the pH range of your column.
- Increasing the percentage of methanol reduces the retention time of all metabolites.

Created: Dec 13, 2023

Last Modified: Jan 29, 2024

PROTOCOL integer ID: 92253

Keywords: noradrenaline, dopamine, metabolites, electrochemical detection, HPLC

Funders Acknowledgement:
Aligning Science Across Parkinson's
Grant ID: ASAP-020370

MATERIALS

Reagents:

- 1-Octanesulphonic acid sodium salt (Sigma-Aldrich, CAS#5324-84-5)
- 3,4-Dihydroxyphenylacetic acid (Sigma-Aldrich, CAS#102-32-9)
- 5-Hydroxyindole-3-acetic acid (5HIAA)(Sigma-Aldrich, CAS#54-16-0)
- DL-Norepinephrine hydrochloride (Sigma-Aldrich, CAS#55-27-6)
- Dopamine hydrochloride (abcam, CAS#62-31-7)
- Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)(Sigma-Aldrich, CAS#6381-92-6)
- Homovanillic acid (HVA)(Sigma-Aldrich, CAS#306-08-1)
- Methanol (Sigma-Aldrich, CAS#67-56-1)
- Orthophosphoric acid (85%) (VWR, CAS#7664-38-2)
- Perchloric acid (60%) (PCA)(Sigma-Aldrich, CAS#7601-90-3)
- Sodium phosphate monobasic (Sigma-Aldrich, CAS#7558-80-7)
- Sodium hydroxide (Sigma-Aldrich, CAS#1310-73-2)

Equipment:

- Automatic Sample Injector AS-2057 Plus (SpectraLab, SKU#JASCO-00-0400)
- Autosampler target vial, micro insert and cap (Jaytee, SKU#JW41160, JW47000, JW43941_B)
- Corning® 1000 mL Vacuum Filter/Storage Bottle System (Corning®, SKU#431098)
- DECADE SDC Electrochemical Detector
- Eppendorf Hydrocarbon Refrigerated Centrifuge 5427R (camlab, SKU#1237269)
- Eppendorf™ Polypropylene Graduated Microtubes (ThermoFisher Scientific, SKU#10509691)
- Fisherbrand™ Model 50 Sonic Dismembrator (ThermoFisher Scientific, SKU#12961151)
- Gilson Pipetman P200L (Gilson, SKU#FA10005M)
- Harris Micro Punch (I.D. 2.0mm, 1.2mm) + cutting mat (Sigma-Aldrich, SKU#WHAWB100029, WHAWB100028, WHAWB100088)
- HPLC column Microsorb 100 - 5 C18, S150 x 4.6mm (Scientific Laboratory Supplies, SKU#CP30710)
- HPLC Pump PU-2080 Plus (SpectraLab, SKU#JASCO-01-0610)
- Mettler Toledo FiveEasy™ pH / mV bench meter (Sigma-Aldrich, SKU#MT30266626-1EA)

Preparing 0.1 M perchloric acid (PCA) solution:

1. Add 1.09 ml of 60% PCA solution in 100 ml dH_2O.

This solution can be maintained in - 4°C for several months.

Preparing Mobile Phase:

1. Add reagents:

- 13% methanol (260 mL)
- 0.12 M NaH_2PO_4 (37.44 g)
- 0.8 mM EDTA (0.596 g)
- 0.5 mM OSA (0.22 g)

2. Make up to 2 L with dH_2O

Preparing Standard Solutions:

1. Make up 10 mM (10^{-2} M) of each standard solution:

- 5HIAA (19.1 mg in 10 mL 0.1 M PCA)
- NA (16.9 mg in 10 mL 0.1 M PCA)
- DOPAC (16.8 mg in 10 mL 0.1 M PCA)
- HVA (18.2 mg in 10 mL 0.1 M PCA)
- DA (19.0 mg in 10 mL 0.1 M PCA)

BEFORE START INSTRUCTIONS

Note 1: This protocol is optimised for detecting tissue punches from acute coronal striatal slices, normally obtained after *ex vivo* fast-scan cyclic voltammetry experiments (see **Protocol: Fast-scan cyclic voltammetry to assess dopamine release in ex vivo mouse brain slices**). Tissue obtained from other preparations are suitable with modifications to this protocol, but note the analysis will have to be adjusted.

Note 2: All reagents, chemicals and solvents must be HPLC grade where possible, or of the highest purity. All solutions must use ultrapure water.

Note 3: To help increase the longevity of the equipment, we keep the HPLC pump flow rate at 0.25mL/min and the electrochemical cell turned off when the HPLC set-up is not in use. When running samples, remember to turn the cell on, and increase the flow rate to 1 mL/min.

Note 4: Do not let air bubbles enter the system. Make sure there is plenty of mobile phase available during sample runs, if the equipment is in between uses the mobile phase can be recycled. When switching to a new batch of mobile phase be sure to turn the pump off when moving the in-flow tubes to prevent air from entering the system.

Obtaining tissue punches

1 Dispense 200 µL of 0.1 M PCA solution (see **Materials**) into Eppendorf tubes. We generally use each Eppendorf to prepare material from 2 tissue punches.

2 Lay acute coronal striatal slices (slice thickness is 300 µm) flat on a cutting mat, and use a micro punch to remove a region of the striatum to analyse.

Note

We use 2 mm-diameter punches for the Caudate Putamen (CPu), and 1.2 mm for the Nucleus Accumbens (NAc).

3 Use forceps to transfer the punch(es) into the Eppendorfs, making sure the punches are submerged in the PCA solution.

4 Store punches in -80°C until ready to analyse.

Preparing mobile phase

5 Use a 2 L volumetric flask to make up the Mobile Phase (see **Materials**).

6 Check the pH of the mobile phase is ~4.65; if not, add orthophosphoric acid or sodium hydroxide.

7 Filter solution with a vacuum pump and PES membrane filter to remove larger contaminating particles.

8 A day prior to running samples, switch to new mobile phase solution. Ensure no air bubbles enter the system when switching to new mobile phase solution as this can damage the column. Leave overnight to guarantee that the new mobile phase is entirely occupying the system.

9 When running samples make sure the flow rate is 1 mL/min. Run time should be between 10-20 minutes. Injection volume is 50 µL, check this is the case on the autosampler.

10 On the DECADE II software, make sure electrochemical cell is on and at 0.7 V. Turn on auto zero.

Preparing and Running Standard Solutions

11 Make up 10 mM (10^{-2} M) of each standard solution: 5-HIAA, NA, DOPAC, HVA, and DA (see **Materials**).

12 Proceed to make serial dilutions for each standard solution to reach a final volume of 1 mL of 10^{-7} M for each standard solution. In addition, prepare a mixed 10^{-7} M solution containing all of the above compounds.

13 Run each standard separately, then the mixed standard, followed by a PCA-only sample.

14 Ascertain retention times for each compound of interest, and confirm that sample components do not co-elute.

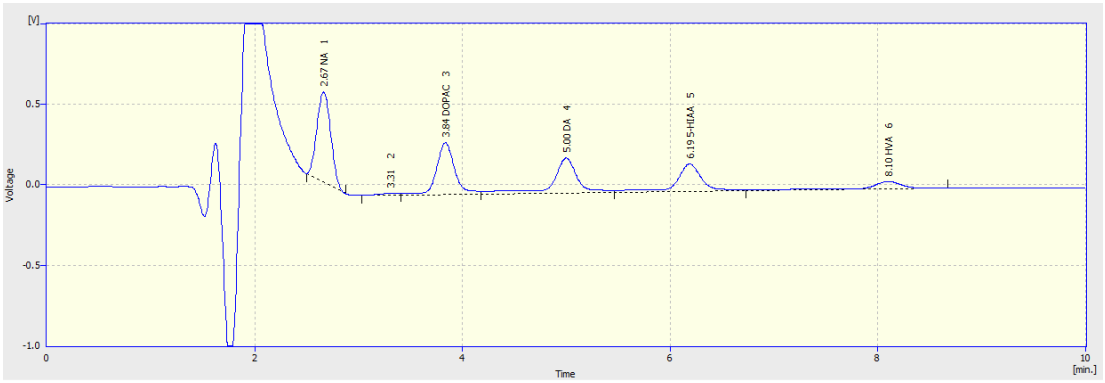

Figure 1: Example chromatogram from a 10^{-7} M mixed standard solution. Metabolites of interest have clearly distinguishable peaks and do not co-elute.

Note

Check whether there are unexpected signals from contaminates. If so, re-make the contaminated solutions. If contamination is present throughout samples + PCA, then re-make PCA. If this does not get rid of the contamination, it is likely that dH_2O or the autosampler are the issue.

If samples are co-eluting, or not present in the chromatogram, extend recording time as some metabolites may be present past the recording window, or adjust mobile phase as mentioned in the guidelines.

Preparing Samples

15 Defrost samples and homogenize them using handheld sonicating probe.

Note

Keep samples on ice as much as possible to prevent compound degradation.

Be sure to wash probe in methanol and then dH_2O between samples.

16 Centrifuge samples in 4°C at 15,000g for 15 mins, then place back on ice.

17 If using a pair of 2 mm tissue punches, dilute supernatant by 1:10 in 0.1 M PCA. A pair of 1.2 mm tissue punches can be run undiluted.

Running Samples

18 Create a new protocol on Clarity software. Make a note of column being used, mobile phase composition, flow-rate, pump pressure, and room temperature.

19 Aliquot 120 µL of each sample into labelled HPLC vials (with insert).

20 Load all samples into autosampler. Include 10^{-7} M mixed standard at the beginning, middle, and end of the run to control for degradation.

Data Analysis

21 Extract data for each sample and the mixed standards.

22 Calculate metabolite content as pmol/mm^3.

22.1 Calculate average peak values for each metabolite from each mixed standard.

22.2 For each metabolite, based on extracted peak values, calculate pmol of metabolite present in injected volume (50 µL). Convert this to metabolite present in full volume (200 µL).

22.3 If samples were diluted (see above for DLS punches in which the supernatant was diluted 1:10), account for dilution.

22.4 If using 2 tissue punches per sample, divide by 2.

22.5 This results in a value in pmol/punch, convert to pmol/mm^3.