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# Determination of free and protein-bound DA and NE and their metabolites and oxidation products by UPLC-MS/MS method

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ABSTRACT

Protocol for the determination of free and protein-bound DA and NE and their metabolites and oxidation products by UPLC-MS/MS method

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MATERIALS TEXT

**External standards**

**DA, DOPA, NE, DOPAC, DOMA, DOPE, VMA** --> up to 3000 nM in 25 mM FA in water
**3MT, AC, VMA, 5-SCDA, 5-SCD** --> up to 1000 nM in 25 mM FA in water

**Internal standard (IS)**

**DA-4d** --> 500 nM in 25 mM FA in water

## Preparation of the aminochrome (AC) external standard 1m

1 Mix **500 µL** of **1 millimolar (mM) dopamine (DA)** with **500 µL** of **2 millimolar (mM) KIO4** disolved in **100 micromolar (µM)** aqueous ammonium acetate buffer **pH 5.8** at RT with vigorous shaking for 1 min.

Following oxidation, aminochromes are placed on ice to prevent further decomposition. Significant degradation of all aminochromes occurs at both RT and 4 ◦C within 24 h and -20ºC at 24-48h (Ochs 2005; Lemos-Amado 2001).

## Preparation of calibration curves 1h

2 Prepare a stock solution of the IS in **25 millimolar (mM) FA** and store it at −80 °C.

Prepare fresh solutions of each metabolite in **25 millimolar (mM) FA** and use them to make three mixtures: MIX1 (DA, L-DOPA, NE, 3MT, AC), MIX2 (DOMA, DOPE, DOPAC) and MIX3 (5SCD and 5SCDA).

3 Serially dilute mixtures with **25 millimolar (mM) FA** to obtain the concentration series used in calibration curves.

Tipically, final calibration levels cover a range of 1.72–3000 nM for DA, NE, and MIX2 and



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0.39–1000 nM for L-DOPA, 3MT, AC and MIX3.

4 Homogenize control samples (i.e brain, intestines, heart, blood serum, cells...) in the appropriate volume of [M]**250 millimolar (mM) FA**

5 Distribute the sample into **90 µL** aliquots prior to the addition of **30 µL** of the appropriate working mixture (MIX1, MIX2 or MIX3), **96 µL** of [M]**25 millimolar (mM) FA** and **24 µL** of [M]**8 micromolar (µM) IS** .

6 **20000 rcf, 4°C, 00:10:00** 10m

7 Transfer supernatant to an Ostro protein precipitation and phospholipid removal plate (Waters, USA) to filter it.

Save the pellet for protein-bound determinations (see below)

8 Finally, inject **7 µL** into the UPLC-MS/MS system.

## Sample preparation 2h

9 Add **300 µL** of [M]**250 millimolar (mM) FA** to each brain, intestine, heart or cell pellet sample prior homogenization. Dilute blood serum samples 1:10

Due to the poor stability of aminochrome, usually a maximum of 50 samples can be analyzed at a time

10 Take a **20 µL** 20 µl sample for protein determination (diluted 1/5 in [M]**25 millimolar (mM) FA** )

11 Take **240 µL** for metabolite determination and add **26 µL** of IS



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Important!!: ensure the concentration of IS is exactly the same in both calibration curves and samples

12 **20000 rcf, 4°C, 00:10:00** 10m

The supernatant is used to determine free neurotransmitters and metabolites (that is, those present in the deproteinated supernatant) while the pellet is used for protein-bound determinations (that is, those present in the acid-Insoluble pellet and released by HCl hydrolysis)

13 Transfer supernatant to an Ostro protein precipitation and phospholipid removal plate (Waters, USA) to be filtered.

14 Inject **7 µL** of filtered supernatant samples into the UPLC-MS/MS system

## Reductive HCl hydrolysis of resulting pellets 18h

15

Work in fume hood during all the procedure

After removal of the supernatant, wash the pellet (from both calibration curves and samples) with **1 mL** of chloroform: methanol (1: 1, v/v) by vortex mixing

16 **20000 rcf, 4°C, 00:10:00** 10m

17 Transfer the resulting pellets to a sealed-capped tube with **[M]6 Molarity (M) HCl** containing **[M]5 % volume thioglycolic acid** and **[M]1 Mass Percent phenol**



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- **Calibration curves** --> add **280 µL** of the mixture and **40 µL** of the corresponding calibration curve working mixture

- **Samples** --> add **288 µL** of the mixture and **32 µL** of IS

18 Purge tubes with a stream of nitrogen, seal them and heat them at **110 °C** for **16:00:00** 16h

19 Let tubes cool at **4 °C** for at least **00:30:00** 30m

20 **20000 rcf, 4°C, 00:10:00** 10m

21 Treat the supernatant with with acid-washed alumina to extract catecholic compounds

## Alumina extraction of catecholic compounds 1h

22 Transfer a **100 µL** aliquot of each hydrolysate into a new Eppendorf tube containing **50 mg** of acid-washed alumina and **200 µL** of **[M]1 Mass Percent Na2S2O5** - **[M]1 Mass Percent EDTA.2Na**

23 Add **500 µL** of **[M]2.7 Molarity (M) Tris. HCl** - **[M]2 Mass Percent EDTA.2Na** **pH9** to the mixture

24 **1100 rpm, 22°C, 00:05:00 on a microtube mixer** 5m

25 **20000 rcf, 00:10:00** 10m



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26 Remove the aqueous layer by aspiration and was alumina with **1 mL** of Milli-Q water

27 **20000 rcf, 00:10:00** 10m

28 Remove the aqueous layer by aspiration and was alumina with **1 mL** of Milli-Q water

29 **20000 rcf, 00:10:00** 10m

30 Remove the aqueous layer by aspiration and was alumina with **1 mL** of Milli-Q water

31 **20000 rcf, 00:10:00** 10m

32 Remove the aqueous layer by aspiration

33 Elute catechols from alumina with **100 µL** of **[M]0.4 Molarity (M) HClO4** by shaking for 2 min 2m

34 Collect all liquid into the injection plate without taking alumina

Alumina is discarded after extraction

35 Finally, inject **7 µL** into the UPLC-MS/MS system.



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## UPLC-MS/MS analysis

36 A Waters Acquity™ UPLC system is coupled with a Xevo TQ-S triple quadrupole mass spectrometer with electrospray ionization interface (Waters). Instrument control, data acquisition, and analysis is performed using MassLynx V4.1 (Waters).

Chromatographic separation of samples is performed on a Waters Acquity™ HSS T3 (1.8µm; 2.1x100mm) column coupled to an Acquity™ HSS T3 VanGuard (100Å, 1.8 µm, 2.1 mm X 5 mm) pre-column (Waters). Column temperature is set at 45 ºC and samples are maintained at 6 ºC in the thermostatic autosampler.

37 The mobile phase consisted of solvent A (methanol 100%) and solvent B (25 mM FA in MQ water) at a flow of 0.4 mL/min with the following gradient profiles (depending on the MIX):

MIX1 and MIX2:

0.5% B maintained for 0.5 min, 5% B at 0.9 min and maintained for 2.1min, 50% B at 2.8 min and maintained for 1.2 min, 0.5% B at 4.1 min followed by 0.2 min of equilibration. Total run time 4.3 min.

MIX3:

0.5% B maintained for 0.5 min, 8% B at 2.6 min, 50% B at 2.9 min and maintained for 0.6 min, 0.5% B at 3.7 min. Total run time 3.7 min

38 The mass spectrometer detector operates under the following parameters: source temperature 150 ºC, desolvation temperature 450 ºC, cone gas flow 50 L/hr, desolvation gas flow 1100 L/hr and collision gas flow 0.15 mL/min. Argon is used as the collision gas. The capillary voltage is set at 0.5 kV for MIX1 and MIX3, and at 2 kV for MIX2 detection. The electrospray ionization (ESI) source was operated in both positive and negative modes, depending on the analyte.



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