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Determination of free and protein-bound DA and NE, 5HT and Ach 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, 5HT and Ach and their metabolites and oxidation products by UPLC-MS/MS method

Materials

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

Note

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 -20°C at 24-48h (Ochs 2005; Lemos-Amado 2001).

Preparation of calibration curves

1h

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

Prepare fresh solutions of each metabolite in [M] 25 millimolar (mM) FA in water and use them to make four mixtures: MIX1 [dopamine (DA), noradrenaline (NA), 3-methoxytyramine (3MT), 3,4-dihydroxyphenylalanine (L-DOPA) and aminochrome (AC)], MIX2 [3,4-Dihydroxyphenylacetic
acid (DOPAC), 3,4-dihydroxymandelic acid (DOMA) and vanillylmandelic acid (VMA)], MIX3 [5SCD and 5SCDA] and MIX4 [serotonin(5-HT), tryptophan (Trp) and 5-hydroxyindole-3-acetic acid (5-HIAA)].

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

Note

Tipically, final calibration levels cover a range of 1.72−3000 nM for DA, NE, and MIX2 and 0.39−1000 nM for L-DOPA, 3MT, AC and MIX3.

3.1 Serially dilute acetylcholine (ACh) standard with [M] 0.1 % volume FA in acetonitrile (ACN) to obtain a calibration curve covering a range of 0.2-1000 nM

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, MIX3 or MIX4), 96 µL of [M] 25 millimolar (mM) FA and 24 µL of [M] 8 micromolar (µM) IS (DA-4d + 5HT-4d) .

5.1 For ACh calibration curve, distribute the sample into 90 µL aliquots prior to the addition of 30 µL of the working mixture and 120 µL of [M] 200 nanomolar (nM) IS (ACh-4d) in 0.1% FA in ACN.

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

> **Note**
>
> 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 55 µL of sample for ACh determination: dilute 1:4 with [M] 0.1 % volume FA in ACN containing a final concentration of 100 nM of ACh-4d as IS.

12 Take 216 µL for catecholaminergic and serotonergic determination and add 24 µL of [M] 8 micromolar (µM) IS (DA-4d + 5HT-4d) .

Note

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

13 20000 rcf, 4°C, 00:10:00 10m

Note

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)

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

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

Reductive HCl hydrolysis of resulting pellets 18h

16

Safety information

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

17 20000 rcf, 4°C, 00:10:00 10m

18 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

Note

- **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

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

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

21 20000 rcf, 4°C, 00:10:00 10m

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

Alumina extraction of catecholic compounds 1h

23 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

24 Add 500 µL of [M] 2.7 Molarity (M) Tris. HCl - [M] 2 Mass Percent EDTA.2Na pH 9 to the mixture

25 1100 rpm, 22°C, 00:05:00 on a microtube mixer 5m

26 20000 rcf, 00:10:00 10m

27 Remove the aqueous layer by aspiration and wash alumina with 1 mL of Milli-Q water

28 20000 rcf, 00:10:00 10m

29 Remove the aqueous layer by aspiration and wash alumina with 1 mL of Milli-Q water

30 20000 rcf, 00:10:00 10m

31 Remove the aqueous layer by aspiration and wash alumina with 1 mL of Milli-Q water

32 20000 rcf, 00:10:00 10m

33 Remove the aqueous layer by aspiration

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

35 Collect all liquid into the injection plate without taking alumina

Note

Alumina is discarded after extraction

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

UPLC-MS/MS analysis for catecholaminergic, serotonergic and cholinergic determination

37 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).

37.1 An Acquity HSS T3 (1.8 µm, 2.1 mm × 100 mm) column coupled to an Acquity HSS T3 VanGuard (100 Å, 1.8 µm, 2.1 mm × 5 mm) pre-column is used to detect MIX1-3 analytes. Column temperature is set at 45 ºC and samples are maintained at 6 ºC in the thermostatic autosampler.

37.2 An Acquity UPLC BEH C18 (1.7µm, 2.1x100mm) column coupled to a Acquity BEH C18 1.7µM VanGuard pre-column is used to detect MIX4. Column temperature is set at 55 ºC and samples are maintained at 6ºC in the thermostatic autosampler.

37.3 A Cortecs UPLC HILIC (1.6 µm; 2,1x75 mm) column coupled to a Cortecs UPLC HILIC VanGuard pre-column (Waters) is used for ACh determination. Column temperature is set at 50 ºC and samples
are maintained at 6 ºC in the thermostatic autosampler.

38 The mobile phase for MIX1-4 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.
0.5

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 maintained 0.2 min for equilibration. Total run time 3.7 min

MIX4:
1%B maintained for 0.5 min, 25 % B at 3 min, 50 % B at 3.1 min and maintained for 0.5 min, 1 % B at 3.6 min maintained 0.4 min for equilibration.

38.1 The mobile phase for ACh determination consisted of solvent A (0.1% FA in ACN) and solvent B (10 mM ammonium acetate in MilliQ water) at a flow of 0.5 mL/min with isocratic 70% A- 30% B conditions during 2.2 min.

39 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 was set at: 0.5 kV (MIX1, MIX3 and MIX3-PB), 2 kV (MIX2) or 3kV (MIX4, ACh). The electrospray ionization (ESI) source was operated in both positive and negative modes, depending on the analyte.

40 Multiple Reaction Monitoring (MRM) acquisition settings for the targeted metabolites are summarised in the following Table

A	B	C	D	E	F	G
Table 1. MRM acquisition settings a						
Analyte	**MRM transition (m/z)**	**MIX**	**RT (min)**	**CV (V)**	**CE (eV)**	**CpV (kV)**
ACh	145,98 > 86,80	ACh	1,5	10	15	3
ACh-d4	150 > 91	ACh	1,5	28	12	3
NE b	151,75 > 106,94	1	0,69	15	20	0,5
DA-d4 (IS)	157,83 > 94,8	1,2,3	1,44	10	20	0,5
DA	153,93 > 90,57	1	1,46	10	20	0,5
L-DOPA	198,1 > 152,1	1	1,48	15	15	0,5
3MT b	150,7 > 90,96	1	3,09	35	20	0,5
AC	149,61 > 121,91	1	3,36	25	25	0,5
DOMA c	182,86 > 136,85	2	1,62	20	14	2

	A	B	C	D	E	F	G
	VMA c	197 > 136,9	2	3,61	20	20	2
	DOPAC c	166,99 > 122,82	2	3,72	18	22	2
	5SCDA	273,1 > 166,9	3	1,73	20	20	0,5
	5SCD	317 > 154,86	3	2,01	24	30	0,5
	5HT	177 > 160	4	0,97	10	5	3
	5HT-d4 (IS)	181 >164	4	0,97	10	5	3
	5HIAA	192 > 146	4	1,5	25	20	3
	Trp	205 > 188	4	2,1	15	10	3
	a RT, retention time; D, dwell time; CV, cone voltage; CE, collision energy; CpV, capillary voltage.						
	b Parent mass after loss of water.						
	c Detected in negative mode.						

Data analysis and representation

41 Samples with a concentration between the limit of detection (LOD) and limit of quantification (LOQ) or bigger than LOQ are considered acceptable; samples with a concentration lower than LOD are considered
as the LOD value.

42 Catechol oxidation is measured using the formula AC+5SCDA+5SCDA-PB/DA + 5SCD+5SCD-PB/L-DOPA

43 DA synthesis is measured using the formula DA+NE+DOMA+VMA+ 3MT+DOPAC /L- DOPA

44 DA degradation is measured using the formula DOPAC+3MT/DA

45 Data is normalised by the protein concentration (determined by BCA) and presented as the percentage of the wt concentration or ratio.