A new bioanalytical tool for neurotransmitter monitoring in central nervous system diseases

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Contextualization

- **Noradrenaline** (NA), adrenaline (AD), **dopamine** (DA) and **serotonin** (5-HT) are essential neurotransmitters for normal neuronal function[1];
- Neurotransmitter homeostasis is compromised by aging and neurological diseases such as **Alzheimer's disease** and **depression**[2,3];
- The dynamic interaction between the monoamine pathways sustains their simultaneous monitorization[3,4,5];
- Monitoring the levels of neurotransmitters, their metabolites, namely DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and their precursors, such 5-HT precursor tryptophan (TRYP), in biological matrices presents high relevance particularly to assess disease severity and progression[1].

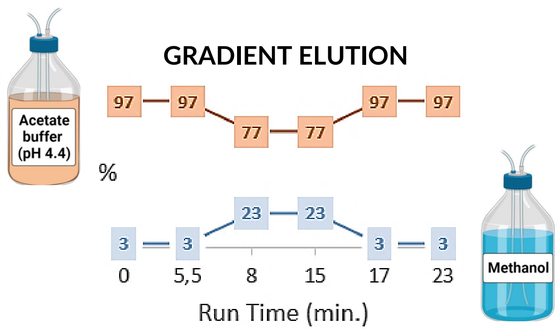
Objectives

- **To develop** an accurate and precise High-Performance Liquid Chromatography (**HPLC**) **method to quantify NA, AD, DA, DOPAC, HVA, TRYP and 5-HT** in mouse brain tissues;
- **To validate** the HPLC method according to the **ICH guideline** M10 on Bioanalytical Method Validation and study sample analysis;
- **To demonstrate its application** in preclinical *in vivo* studies.

Method development

Chromatography conditions

Anhydrous sodium acetate (0.1 M)
EDTA (0.3 mM)
Octane sulfonic acid sodium salt (0.5 mM)
Acetic acid (0.05 M)



Column temperature: 27°C

Flow rate: 1.0 mL/min

Total analysis time: 23 min

Grace Apollo C_{18} column
(250 x 4.6 mm, 5 µm)

Shimadzu HPLC system coupled to a fluorescence detector
λ_{Exc} = 279 nm, λ_{Em} = 320 nm

Tissue preparation

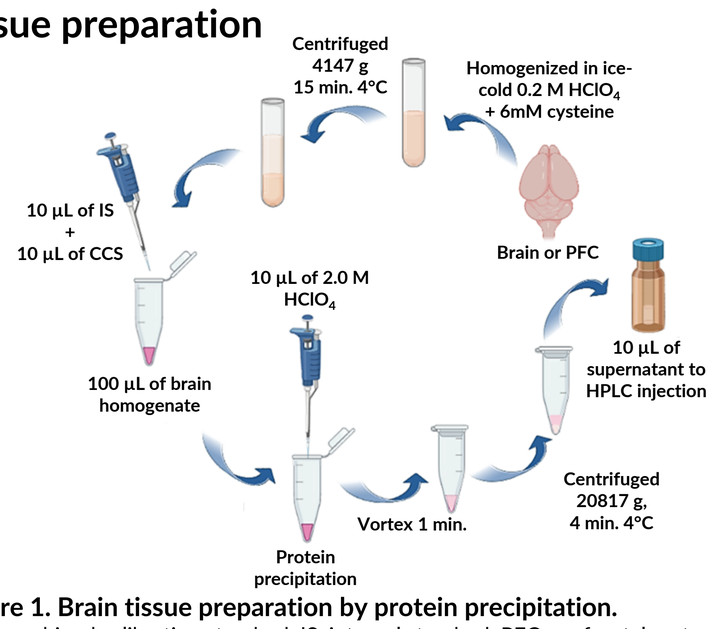


Figure 1. Brain tissue preparation by protein precipitation.
CCS: combined calibration standard; IS: internal standard; PFC: prefrontal cortex.

Method validation

ICH guideline requirements for method validation [6]:

1. Selectivity
2. Specificity
3. Carry-over
4. Calibration curve range and linearity
5. Accuracy, precision and recovery
6. Stability

1 NA, AD, DOPAC, DA, HVA, 5-HT and TRYP → Analytes that are also Endogenous Compounds

Background Subtraction Approach
(ICH guideline for bioanalytical method validation)[6]

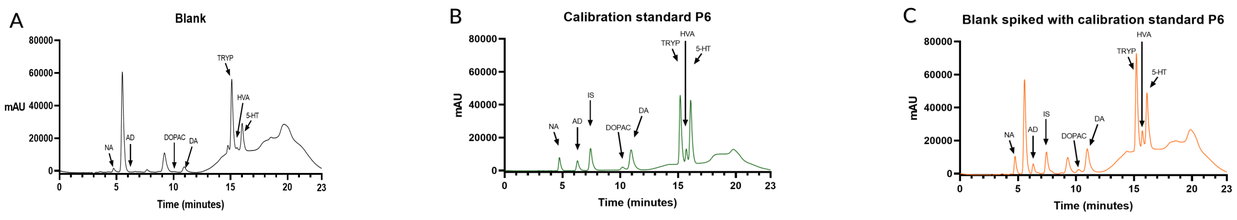


Figure 2. Chromatogram representative of a blank sample of mice brain homogenate (A), the calibration standard P6 (B) and the blank spiked with the calibration standard P6 (C).
5-HT: serotonin; AD: adrenaline; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; NA: noradrenaline; TRYP: tryptophan

2 No interference of:

- Norfloxacin
- Ofloxacin
- Prulifloxacin
- Gatifloxacin
- Pazufloxacin
- Amitriptyline
- Antipyrine
- Pitavastatin
- Atorvastatin
- Metformin
- Clinofloxacin
- Lacosamide
- Levetiracetam
- Zonisamide,
- Perampanel
- Cannabidivarin
- Cannabigerol
- Cannabichromene
- Cannabinol
- Cannabidiol

3 Not observed

4 Table 1. Validation parameters of the analytes.

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	Calibration curves range (ng/mL)	Linearity (R-squared²)	Accuracy (%)	Precision (CV)	Recovery (%)
NA	10-400	0.997	-8.72 – 1.56	1.37 – 11.39	101.80 ± 11.39
AD	10-200	0.988	-7.62 – 14.71	1.01 – 7.98	70.88 ± 6.17
DOPAC	50-300	0.998	-9.97 – 5.90	4.36 – 15.29*	67.73 ± 12.84
DA	10-800	0.997	-7.60 – 18.87*	1.19 – 16.22*	89.52 ± 11,69
HVA	55-300	0.997	-8.69 – 5.58	0.95 – 3.81	84.53 ± 8.20
TRYP	35-1000	0.997	-12.73 – 6.03	-12.73 – 14.56	80.86 ± 11.59
5-HT	35-300	0.999	-2.60 – 14.34	1.29 – 8.56	86.95 ± 8.48

Representation of the maximum ranges and values in inter- and intra-day validation (n=5); Accuracy (%) = 100 × [(Measured concentration of spiked sample − endogenous concentration) − Nominal concentration] /Nominal concentration; CV, coefficient of variation (%) = 100 x (Standard Deviation/Mean); Recovery (%) = Mean ± Standard Deviation.
*Values obtained at the lower limit of quantification (LLOQ) quality sample.

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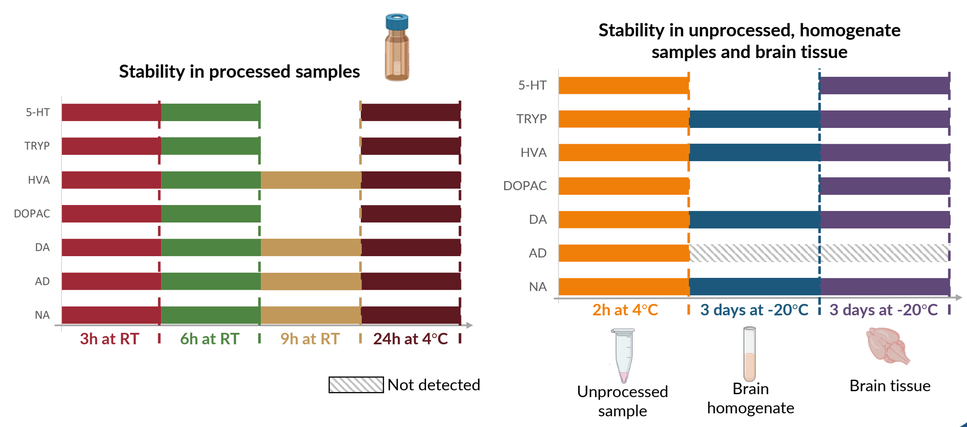

Method application

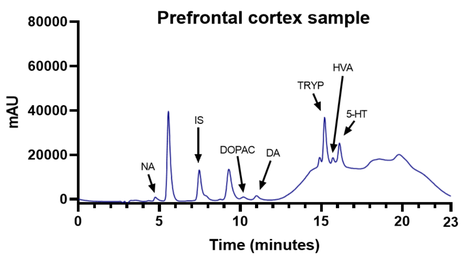

Figure 3. Chromatogram of a prefrontal cortex sample collected from mice treated with 55 µmol/Kg of ketamine intraperitoneally.
5-HT: serotonin; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; IS: Internal Standard; NA: noradrenaline; TRYP: tryptophan.

→ **Applied to study the chronic antidepressant effect of sertraline** (DOI:10.1016/j.ejpb.2023.12.002)

→ **Applied in a genetic mouse model of autism spectrum disorders**

→ **Applied to study the acute antidepressant effect of ketamine**

Final remarks

- **The method was successfully developed and validated** according to the **ICH M10 guideline** for bioanalytical method validation;
- It resorts to **native fluorescence** of the analytes, avoiding unspecific detections;
- It enables a **sustainable sample preparation**, saving time and resources;
- **It allows simultaneous quantification** of 7 compounds (4 neurotransmitters, 2 metabolites and 1 precursor) in mouse brain and prefrontal cortex homogenates;
- **It has demonstrated reproducible application** in *in vivo* preclinical studies.
- **It can be further applied in several animal models of neurological diseases.**

References:

[1] G. E. De Benedetto et al., (2014). J. Pharm. Biomed. Anal., 98, 266–270. **[2]** M. Mather, (2021). Semin. Cell Dev. Biol., 116(May), 108–124. **[3]** C. Kraus, et al., (2017). Neurosci. Biobehav. Rev., 77, 317–326. **[4]** Correia et al., Int. J. Mol. Sci., 2022. **[5]** Kanova et al., Int.J. Mol. Sci., 2021. **[6]** International Council Harmonised Guideline (2019). Bioanalytical Method Validation M10.

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