The use of microbore UHPLC-ECD as a tool for the analysis of **monoamine neurotransmitters** and **metabolites** in neurodegenerative disease research

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Introduction

Neurodegenerative conditions such as Parkinson's and Alzheimer's disease, or, related proteinopathies (e.g. Lewy body and frontotemporal dementia) are characterized by early-onset alterations of the monoaminergic neurotransmitter system both central (brain) and peripheral (cerebrospinal fluid (CSF); blood). Such alterations contribute to severe behavioral and cognitive disturbances in patients (memory loss, aggression, depression and related neuropsychiatric symptoms). Measuring levels of monoamines, such as (nor)adrenaline, serotonin, dopamine, and, related metabolites (MHPG, 5-HIAA, DOPAC, HVA, respectively), thus provide a window of opportunities for the discovery of disease-specific CSF markers, and, comprise an important tool in overall disease monitoring and development. Simultaneous quantification of these neurotransmitters in biofluids and brain requires a highly sensitive and selective analysis method.

A fast and reliable microbore reversed-phase ultra high performance chromatography method with electrochemical detection (RP-UHPLC-ECD) is presented using the ALEXYS Neurotransmitter Analyzer for the simultaneous analysis of abovementioned 8 monoamines and metabolites in postmortem acquired frozen human brain tissue and CSF.

Materials & methods

Cerebrospinal fluid (CSF)

CSF samples (several mL) are obtained via lumbar puncture. The sample preparation consisted of a purification on Amicon® Ultra 0.5 Centrifugal Filters (cutoff 3 KDa; Millipore, Ireland), which were washed twice beforehand with 450 µL sample preparation buffer* while centrifuging (14,000×g, 25 min, 4 °C). 400 µL of CSF sample was directly transferred onto the washed Amicon® filter and centrifuged at 14,000xg for 40 minutes at 4°C. After centrifugation, one fraction of the eluate was diluted 1:4 and analyzed [1,2].

Human brain tissue

The brain sample preparation procedure prior to RP-UHPLC-ECD system analysis was simple and fast. 200 - 300 mg frozen brain tissue was weighted in 4 mL of sample preparation buffer*. The mixture was homogenized for 40s (50s in case samples weighted ≥250 mg) using a Ultra-Turrax TR 50 homogenizer (Janke & Kunkel, Ika-Werk, Staufen, Germany). The homogenate was then centrifuged (26,000xg, 20 min, 4°C) and filtered using a 0.2 µm Millipore syringe filter (Millex, Millipore, Ireland). Further elimination of proteins was accomplished using Amicon® Ultra 0.5 Centrifugal Filters (10 KDa cutoff; Millipore, Ireland) which were washed twice beforehand with buffer*. The filtrate (undiluted & diluted) was analyzed [3].

*) The sample preparation buffer used both type of samples consisted of 50 mM Citric acid, 50 mM Phosphoric acid, 389.3 mg/L OSA, 0.1 mM EDTA, 8 mM KCl, pH 3.6.

Abbreviation list

MHPG	-	3-methoxy-4-hydroxyphenylglycol
NA	-	Noradrenaline
A	-	Adrenaline
DOPAC	-	3,4-dihydroxyphenylacetic acid
5-HIAA	-	5-hydroxyindoleacetic acid
DA	-	Dopamine
HVA	-	Homovanillic acid
5-HT	-	5-hydroxytryptamine (Serotonin)
OSA	-	Octane-1-sulfonic acid
EDTA	-	Ethylenediamine tetraacetic acid
MeOH	-	Methanol

Fig 1. Left: ALEXYS Neurotransmitter Analyzer with DECADE Elite electrochemical detector. Right: SenCell 2 mm Glassy Carbon (GC) working electrode and In-Situ Ag/AgCl (ISAAC) reference electrode.

Table 1 - LC-ECD conditions

HPLC system	ALEXYS Neurotransmitter Analyzer* (Antec Scientific™)
CDS	Clarity Chromatography software (DataApex)
Column	Acquity UPLC BEH C18, 1x150 mm, 1.7µm column (Waters™)
Guard	Acquity in-line filter (Waters™)
Mobile phase	120mM phosphoric acid, 120mM citric acid, 0.1mM EDTA, 8mM KCl, 600mg/L OSA, pH 3.0, 11% MeOH
Flow rate	60 µL/min
Temperature	37°C (separation and detection)
Backpressure	About 440—450 bar
$V_{injection}$	5 µL
Flow cell	SenCell™ 2 mm GC WE, ISAAC REF, AST 1
E_{cell}	670 mV vs. Ag/AgCl
Range	5 nA/V
ADF™	0.1 Hz

*) Note that the presented data are obtained with an older version of the ALEXYS Neurotransmitter Analyzer than shown in figure 1.

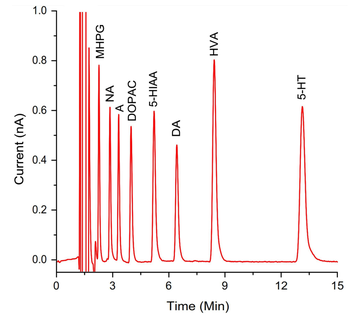

Fig 2. Chromatogram obtained with a 5 µL injection of a standard mix of MHPG, NA, A, DOPAC, 5-HIAA, DA, HVA and 5-HT in sample preparation buffer. Concentration of the individual components in the mix are in the range of 6 –16 ng/mL (30 - 85 nM).

Limit of Detection & linearity

Table 2 - Limit of Detection (LOD)

Analyte	*LOD*			
	concentration		*On-column*	
	(nmol/L)	*(pg/mL)*	*(fmol)*	*(pg)*
MHPG	0.42	77	2.1	0.4
NA	0.26	44	1.3	0.2
A	0.26	47	1.3	0.2
DOPAC	0.30	51	1.5	0.3
5-HIAA	0.25	49	1.3	0.2
DA	0.38	59	1.9	0.3
HVA	0.60	109	3.0	0.5
5-HT	0.51	90	2.6	0.4

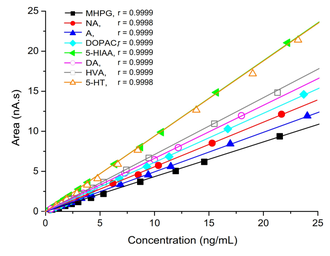

Fig 3. Calibration curves obtained with standards of MHPG, NA, A, DOPAC, 5-HIAA, DA, HVA and 5-HT dissolved sample preparation buffer.

Analysis of cerebrospinal fluid

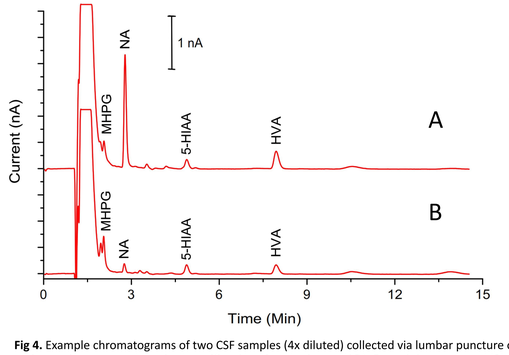

Fig 4. Example chromatograms of two CSF samples (4x diluted) collected via lumbar puncture of patients with neuropathologically verified Alzheimer's disease (AD). *Data: J. Janssens et al [2].*

Table 3 - Concentration of neurotransmitters and metabolites in CSF samples (figure 4)

Analyte	*Concentration (ng/mL)*	
	A	*B*
MHPG	13	23
NA	86	7
A	n.d.*	n.d.
DOPAC	n.d.	n.d.
5-HIAA	11	10
DA	n.d.	n.d.
HVA	31	16
5-HT	n.d.	n.d

*) Not detectable/ quantifiable (below LOD).

Analysis of human brain tissue

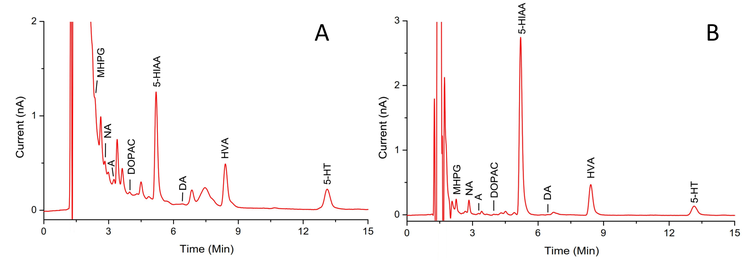

Fig 5. Example chromatograms of frozen human brain tissue samples: [A] Brain homogenate of hippocampus (HC) from a patient with neuropathologically verified Alzheimer's disease (AD), [B] Brain homogenate (7x diluted) of locus coeruleus (LC) from a non-AD control. *Data: Y. Vermeiren, unpublished results.*

Table 4 - Concentration of neurotransmitters and metabolites in human brain tissue samples (figure 5)

Sample	*Brain region*	*Disease category*	*Concentration (ng/ g tissue)*							
			MHPG	*NA*	*A*	*DOPAC*	*5-HIAA*	*DA*	*HVA*	*5-HT*
A	HC	AD	15	9	5	5	156	2	115	65
B	LC	Control	189	235	15	21	3259	9	970	341

Conclusions

The ALEXYS neurotransmitter analyzer in combination with the presented microbore RP-UHPLC-ECD method provides a reliable solution for the routine analysis of low levels of monoamines neurotransmitters and metabolites in various sample matrices encountered in this research field.

Method highlights:

- Samples were prepared in phosphate-citrate buffer at 4°C using a **simple sample preparation protocol** to assure that the analytes of interest are stable for at least 24 hours in the sample matrix without prior antioxidant additions.
- **Good LC separation** (r > 3.5) of all 8 analytes is achieved within a 15 minute run time on a 15 cm short Waters Acquity C18 BEH UPLC column (1 mm ID, particle size 1.7 µm), using optimized LC conditions and a sample injection volume of only 5 µL.
- **High detection sensitivity** with typical on-column LODs in the range of 0.2 – 0.5 pg (1 – 3 fmol) for all analytes.

References

1. J. Janssens, S.D. Atmosoerodjo, Y. Vermeiren, A. R. Absalom, P.P. De Deyn, *Neurochemistry International*, **128** (2019), 154–162
2. J. Janssens, Y. Vermeiren, M. van Faassen, C. van der Ley, I.P. Kema, P.P. De Deyn, *Neurochemical Research*, **45** (2020) 1191–1201
3. Y. Vermeiren, D. van Dam, T. Aerts, S. Engelborghs, P.P. De Deyn, *Neurobiology of Aging*, **35** (2014), 2691-2700

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