Recent improvement for the analysis of **acetylcholine** levels in brains

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Introduction

Acetylcholine analysis in microdialysates

Acetylcholine (ACh) is an important neurotransmitter that plays a crucial role i.e. in memory and movement. The quantification of ACh in the brains of freely moving test animals requires the use of microdialysis probes for sampling. The quantification of ACh in microdialysis samples is challenging due to the following conditions:

- Small sample volumes (typically 5- 20 µL)
- Low ACh concentration levels (nanomolar range)
- High Choline (Ch) concentration levels (micromolar range)

The analytical method of choice that can handle all these requirements is microbore ultra-high performance liquid chromatography with electrochemical detection (UHPLC-ECD) in combination with a post-column Immobilized Enzyme Reactor (IMER) for conversion of ACh into hydrogen peroxide (H_2O_2). H_2O_2 is electrochemically active and can be detected on a platinum (Pt) electrode with good sensitivity. The detection limit for ACh is low enough to measure the low basal levels of ACh present in microdialysis samples of small rodents. This is demonstrated with a couple of example chromatograms of striatum samples of mice from a study that evaluated the effect of the psychoactive drug pyrovalerone [1].

Pyrovalerone affects ACh levels in the striatum

Pyrovalerone and its derivatives (α-pyrrolidinophenones) are very potent dopamine (DA) and norepinephrine transporter inhibitors (DAT and NET, respectively) acting as monoamine reuptake inhibitors [2]. In the CNS, the cholinergic system includes both cholinergic projection neurons as well as cholinergic interneurons. The cholinergic interneurons are located mainly in the striatum and nucleus accumbens. DA and ACh seem to be in mutual balance in the striatum. The increased extracellular levels of ACh in the mouse striatum observed in this study may be explained by the stimulatory effects of DA through excitatory dopamine D1-like receptors localized on cholinergic interneurons.

Materials & methods

Method for analysis of ACh in microdialysates

The fast and sensitive method for ACh analysis is based on the use of the versatile ALEXYS Neurotransmitter Analyzer (Fig. 1), which is a UHPLC-ECD system equipped with the DECADE Elite detector and SenCell. The highlights of the method (Table 1) are listed below:

- Small sample use per analysis: 10 µL (injection volume 5 µL)
- Fast and efficient separation using sub-2 µm particle column
- A short total runtime of 14 min.
- Typical detection limit of about 0.5 nM for ACh (2.5 fmol on-column)
- Reproducible (RSD area<3%) and linear (r>0.998) responses in the relevant concentration ranges

Ion-pairing separation makes the large Ch peak elute first, and it is well separated from the smaller ACh peak, and there are no late eluting disturbances in the baseline. These benefits stand in contrast with the application of ion exchange chromatography, where the elution order is fully reversed and separation between Ch and ACh is challenging.

Table 1. Conditions for ACh analysis

LC-ECD system	ALEXYS Neurotransmitter Analyzer (Antec Scientific)
Temperature	35°C (separation and detection)
Reversed-phase UHPLC separation	
Column	Acquity UPLC HSS T3, 1x50 mm, 1.8 µm (Waters™)
Immobilized enzyme reactor (IMER)	AC-ENZYM II, 1 × 4 mm (Eicom)
Mobile phase	50 mM monosodium phosphate set to pH 7.5, 1.6 g.L^{-1} 1-octanesulfonic acid sodium salt, 0.5 mM tetramethylammonium chloride, 0.5 mM Na_2EDTA,
Flow rate	50 µL/min
Backpressure	About 130 bar
$V_{injection}$	5 µL (on the basis of 10 µL sample use)
Electrochemical detection	
Detector	DECADE Elite (Antec Scientific)
Flow cell	SenCell™ with 2 mm Pt working electrode and HyREF reference electrode, AST 1
Potential (E_{cell})	200 mV vs. HyREF
Range	50 nA (Ch peak), 5 nA (ACh peak)
ADF™	0.02 Hz

Fig. 1 The ALEXYS Neurotransmitter Analyzer.

Fig. 2 Representation of the UHPLC-ECD flow path.

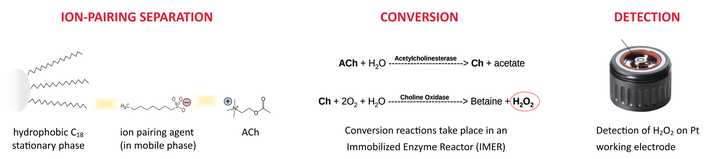

Fig. 3 Principle of acetylcholine analysis. The analysis of acetylcholine is based on ion-pairing separation, followed by an enzymatic conversion and electrochemical detection of hydrogen peroxide on a platinum working electrode.

Microdialysis

- Vertical microdialysis probe (MAB 10.8.2. Cu, AgnTho's AB Sweden) implanted into the striatum (AP: +1.0, L +1.8, V –3.8, from the dura)
- Perfusion: artificial cerebrospinal fluid (aCSF) with composition 147 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$, 1 mM $MgCl_2$) at a flow rate of 1.5 µL/min.
- Basal level sample collection: after an initial wash out period of 1 h, baseline samples were collected every 40 minutes.
- Sample collection following treatment: after intraperitoneal (ip) treatment with 10 mg.kg^{-1} pyrovalerone, fractions of 40 minutes were collected for the next 160 minutes.

Animals

Adult male C57BL/6J inbred mice were obtained from Charles River Laboratories and experimental studies were carried out at approx. 12 weeks of age. Housing conditions and procedures were in accordance with the European Union legal regulation (Directive 2010/63/EU for animal experiments regarding the care and use of laboratory animals) and approved by Local Ethics Commission for Experiments on Animals (Maj Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland).

Results

The treatment of male mice with 10 mg.kg^{-1} pyrovalerone resulted in a 9-fold increase in extracellular ACh levels in the striatum. The chromatograms from the microdialysate samples clearly show the signal of ACh in the sample of a basal level as well as after treatment.

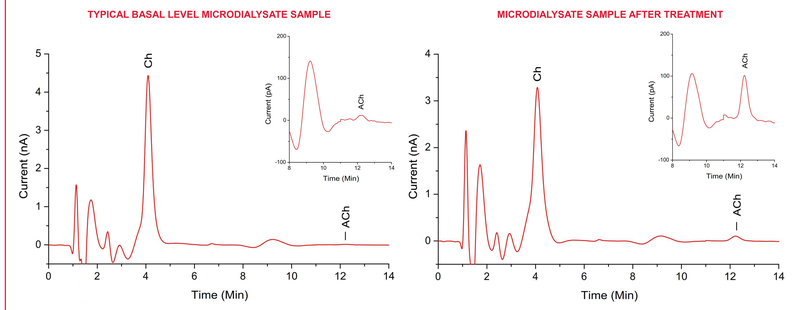

Fig. 4 Chromatograms from the analysis of ACh in basal level male mice striatum microdialysate samples. The Ch peak is also identified. Left: sample from the basal level showing 0.1 nM ACh (S/N = 4). Right: sample from 1 h after treatment with pyrovalerone showing 0.9 nM ACh (S/N = 34). Inserts: zoom-in on ACh peak. Analytical conditions according to Table 1.

Conclusions

A fast and sensitive UHPLC-ECD method is presented for the analysis of acetylcholine in microdialysates using the ALEXYS Neurotransmitter Analyzer. The system combines good chromatographic performance with ease of use. The method was able to detect basal levels of extracellular ACh in the microdialysis sample of mice striatum down to 0.1 nmol/L in this study.

References

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2. J. Wojcieszak, D. Andrzejczak, A. Wojtas, K. Gołembiowska, J.B. Zawilska, *Forensic Toxicol.* **36** (2018), 334–350