Application Note

Neuroscience

ALEXYS Analyzer for Highest Sensitivity in Neurotransmitter Analysis

Monoamines and Metabolites
Noradrenaline
Dopamine
Serotonin
5-hydroxyindole acetic acid (5-HIAA)
3,4-dihydroxyphenylacetic acid (DOPAC)
homovanillic acid (HVA)

OPA derivatized amines and amino acids
GABA and Glutamate
Histamine (LNAAs)
4-aminobutyrate (GABA)
Glutamate (Glu)
LNAAs

Choline and Acetylcholine
Choline (Ch)
Acetylcholine (ACh)

Markers for oxidative stress
3-nitro-L-Tyrosine
8-OH-DPAT

Glutathione and other thiols

Acetylcholine and Choline

- **Choline well separated from acetylcholine**
- **SenCell with programmable cleaning cycle**
- **Detection limit 0.5 nmol/L ACh (5 µL injection)**
- **Small total sample use of 10 µL**
- **Total analysis time < 10 min**

Summary

The ALEXYS Neurotransmitter Analyzer is a modular system for UHPLC with electrochemical detection (ECD) of neurotransmitters. This application note shows the use of the system to sensitively measure acetylcholine (ACh) in brain microdialysates. Such samples are challenging due to low levels of ACh and small available sample volumes (typically <20µL). Efficient separation of ACh is achieved on a reversed phase microbore column under ion-pairing conditions. Conversion of ACh to the electrochemically detectable hydrogen peroxide takes place in a post column IMER. A SenCell with platinum working electrode is used for detection. With this approach, the detection limit is about 0.5 nmol/L (2.5 fmol on column; 5 µL injection). Basal ACh levels are shown to be detectable in real microdialysis samples.

Introduction

Acetylcholine (ACh) is a neurotransmitter that activates muscles at the neuromuscular junction, and in brain it is a neuromodulator involved in processes like arousal, attention and motivation. In-vivo studies of the changes in ACh levels in the brain often use the well-established sampling technique of microdialysis [1]. This results in small samples (typically <20µL) with low nanomolar levels of ACh. A sensitive detection method is needed to quantify the ACh levels.

This note describes ACh measurements in brain microdialysates using the ALEXYS Neurotransmitter Analyzer (Figure 1). The ACh analysis is based on ion-pairing HPLC separation, followed by on-line enzymatic conversion of ACh to hydrogen peroxide, and detection on a Pt working electrode.

Method

ALEXYS Neurotransmitter Analyzer

The ALEXYS Neurotransmitter Analyzer consists of a P6.1L pump with degasser, a DECADE Elite EC detector, an AS 110 autosampler and Clarity data acquisition software. The additional ACh kit contains the column and a SenCell with Pt working electrode. Other kits are available for neurotransmitters and amino acids such as dopamine, noradrenaline, serotonin, metabolites, GABA and glutamate [5].

Figure 1: ALEXYS Neurotransmitter Analyzer for acetylcholine

Separation

Acetylcholine (Figure 2) is positively charged and is therefore separated on a C18 column with an ion-pairing agent in the mobile phase [2]. This new ion-pairing chromatography method is superior to the traditionally applied ion-exchange chromatography (see for example ref [2]). The new method shows a reversed elution order of Ch and ACh peaks with much better separation. A large late eluting peak at about 25- 35 min observed in ion exchange LC, was not observed in the applied ion-pairing method

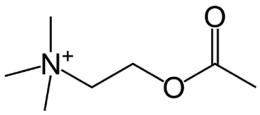


Figure 2: Structure of acetylcholine (ACh)

Enzymatic conversion

After separation, ACh passes through an immobilized enzyme reactor (IMER) before entering the detector. The IMER contains covalently bound acetylcholine esterase (AChE) and choline oxidase (ChOx), which convert ACh to the electrochemically detectable hydrogen peroxide (Figure 3).

The AChE/ChOx IMER has some requirements for efficient enzymatic conversion:

- Mobile phase 100% aqueous, as enzymes denature in contact with organic solvents.
- Mobile phase with pH close to the enzyme's optimal pH of 8.2
- A low LC flow rate, as conversion efficiency and flow rate are inversely related.
- A thermostated oven at the optimal temperature of 35 °C

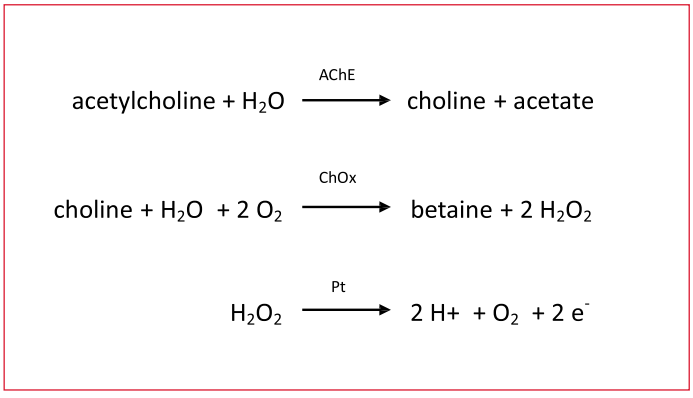
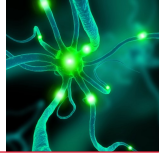


Figure 3: Enzymatic conversion of acetylcholine to electrochemically detectable hydrogen peroxide with the enzymes acetylcholine esterase (AChE) and choline oxidase (ChOx). These enzymes are covalently bound in an immobilized enzyme reactor (IMER) that is placed between the separation column and detector.



Detection

Hydrogen peroxide is electrochemically detected on a Pt working electrode. The working potential of the detector has to be set as low as possible to ensure selectivity, but high enough to generate a clear response. A potential of 0.2V is chosen as optimum (Figure 4). Applying a higher potential unnecessarily decreased the selectivity of the method by showing more and larger interfering peaks (Figure 5).

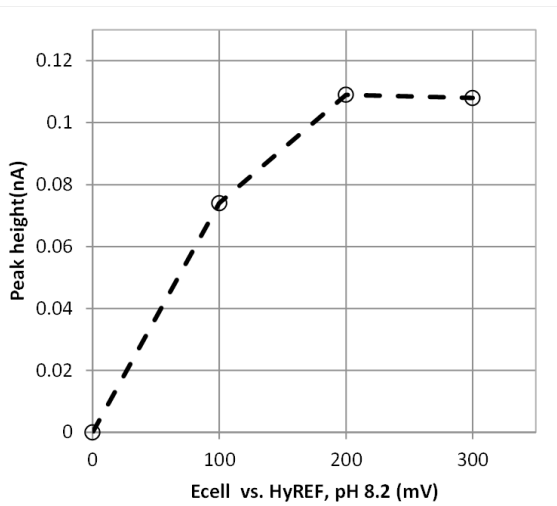


Figure 4: Plot of peak height vs. potential for the analysis of acetylcholine after conversion to hydrogen peroxide. The system was left to stabilize for 2.5 hours before each measurement.

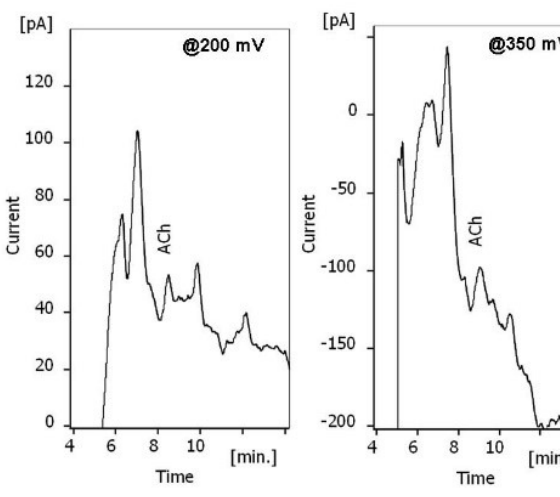


Figure 5: Chromatograms of brain microdialysate sample detected with potential settings of 200 mV and 350 mV. Notice the relatively higher response of non-relevant signals at the higher working potential.

Table 1

Recommended conditions and settings for ACh analysis

UHPLC	ALEXYS Neurotransmitter Analyzer
Column*	Acquity UPLC HSS T3 50 x 1 mm ø 1.8 µm (Waters)
Pre-column filter	Acquity in-line filter kit (Waters)
IMER	4 x 1 mm ø
Mobile phase	50 mmol/L sodium dihydrogen phosphate, 0.5 mmol/L EDTA.Na_2, pH 7.5 set with 50% NaOH, 1.6 g/L octanesulfonic acid sodium salt, 0.5 mM tetramethylammonium chloride
Flow rate	50 µL/mL
Temperature	35 °C for separation and detection
Backpressure	100 - 150 bar
$V_{injection}$	5 µL max
Sample loop	5 µL
Injection method	Dedicated user program with 10 µL total sample use
Needle wash	Water (HPLC grade, refresh at least weekly)
Pump piston wash	Water (HPLC grade, refresh at least weekly)
Flow cell	SenCell™ with 2 mm Pt WE, AST setting 1
Ecell	200 mV vs. HyREF™ reference electrode
Range	50 nA/V; change to 1 nA/V around ACh peak
ADF	0.02 Hz
I-cell	5 - 15 nA
Standards	Acetylcholine in Ringer solution (147 mM NaCl, 3 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$)

* Revised conditions; chromatograms may differ slightly.

Electrode (re)activation

A new flow cell with Pt electrode requires a one-time initialization step. After installation of the UHPLC/ECD system, it is necessary to apply an activation pulse. The detector is set to PAD mode (E1=+1.0V, E2=-1.0V, t1=1000ms, t2=1000ms, ts=20ms), and the pump is set to the standard flow rate and mobile phase. After 10 minutes [3] the signal is stabilized in DC mode at 0.2V for at least 30 min. The background current should drop below 25 nA in less than 30 min. This activation procedure can be programmed in the DECADE Elite detector and Clarity software. The pulse mode is not available in the SDC or Lite versions of the detector.

An inherent feature of the Pt electrode is a gradual decrease of responsiveness over time due to formation of Pt oxide (Figure 6). Noise level has a slight tendency to decrease as well, but overall sensitivity decreases over time as well. Regular 2-hourly recalibration of the response is advised, and this “calibration bracketing” is an automated feature in Clarity.

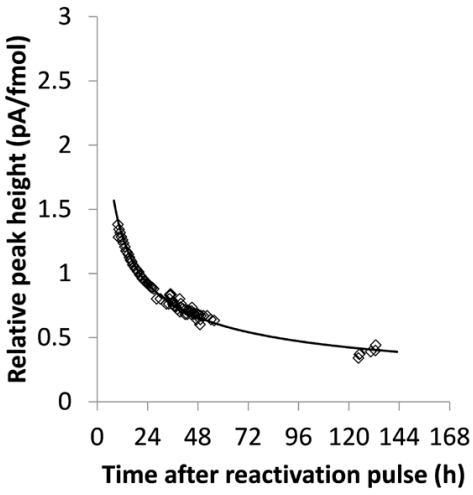


Figure 6: Response over time of ACh standard in Ringer solution, detected on a Pt working electrode at 0.2V vs HyREF. At t=0, a short reactivation pulse was applied (E=-0.5V for 12 s and E=0.8V for 12s).

To bring back the response (Figure 7), a short reactivation pulse is advised every 2 days: E=-0.5V for 12 s followed by E=0.8V for 12 s. The background current should drop below 25 nA in less than 10 min after this pulse.

Sample use

The maximum volume that can be injected onto the column is 5 µL. The injection of larger volumes only results in the ACh peak becoming wider; not higher. A dedicated injection program was applied to inject the maximum volume of 5 µL while only using a total of 10 µL sample [4].

Results

Repeatability, linearity and detection limit

The repeatability was evaluated with a 100 nmol/L ACh standard in Ringer solution, analyzed in a sequence with a time frame of about 1 hour per 6 runs. A repeatability of peak area better than 3% RSD (n = 6) was found, 16 h after a reactivation pulse. The long term drop in response affects the inter-day RSD for peak area and height. Retention time of the ACh peak shows an RSD better than 0.2%.

Working with an application that shows a steady decrease of responsiveness makes it important to regularly calibrate the system with standards (calibration bracketing). A 5-point linear calibration in the range between 0-10 nmol/L ACh showed a correlation coefficient better than 0.998.

A detection limit down to 2.5 fmol ACh was measured using a well performing IMER and a noise level below 2 pA. Best detection limits are obtained within a few hours after the reactivation pulse. Figure 8 shows the chromatogram of a near-LOD concentration of ACh.

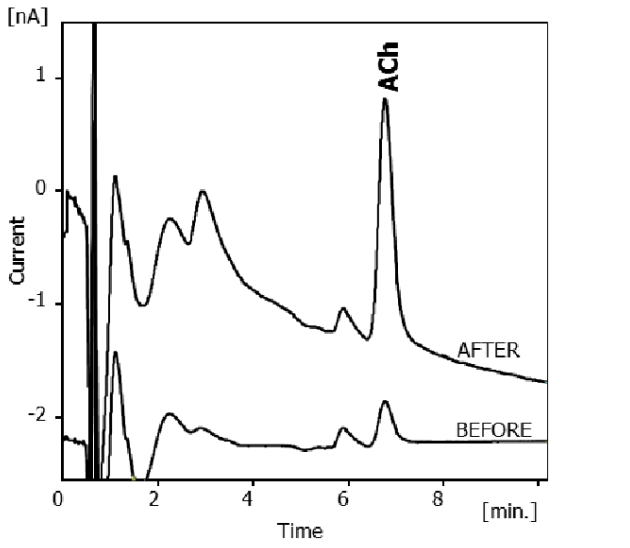


Figure 7: Overlay of LC-ECD chromatograms of an ACh standard in Ringer solution, recorded with a Pt working electrode before and 10 min after an electrochemical reactivation pulse of the electrode.

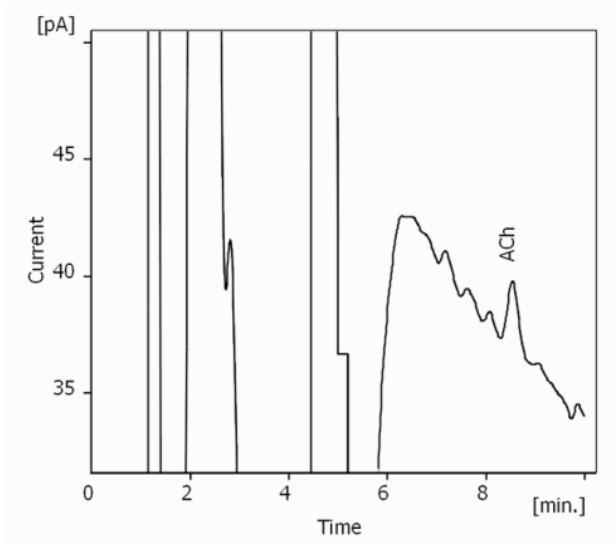


Figure 8: Chromatogram of 0.5 nM acetylcholine in Ringer solution. This concentration is near the LOD.

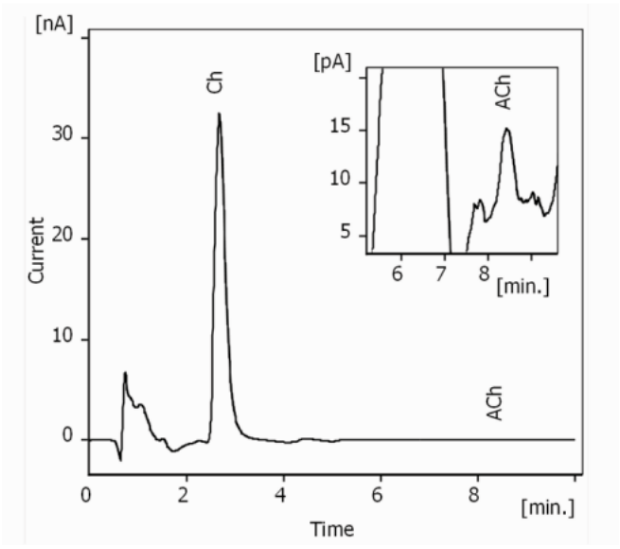


Figure 9: Chromatogram of basal level microdialysate sample from medial prefrontal cortex of a male Wistar rat. Inset is zoomed-in part of chromatogram. Concentrations were quantified as 1 nM acetylcholine and 40 µM choline. Sample origin: 30 µL samples were collected by dialysis at a flow rate of 1.25 µL/min using aCSF (166.5 mM NaCl, 27.5 mM $NaHCO_3$, 2.4 mM KCl, 1.2 mM $CaCl_2$, 0.5 mM KH_2PO_4 and 1.0 mM glucose (pH 7.1)). Samples were kept frozen at -20°C until analysis. Samples were kindly provided by Sarah Beggiato, Maryland Psychiatric Research Center, Baltimore (MD), USA

Brain microdialysate samples

Acetylcholine can be detected in basal brain microdialysate samples using the presented method (Figure 9 and 10). The large peak of choline (the metabolite of ACh) is also detectable in the chromatogram, but it does not interfere with the detection of ACh as both peaks are well separated from each other using this method.

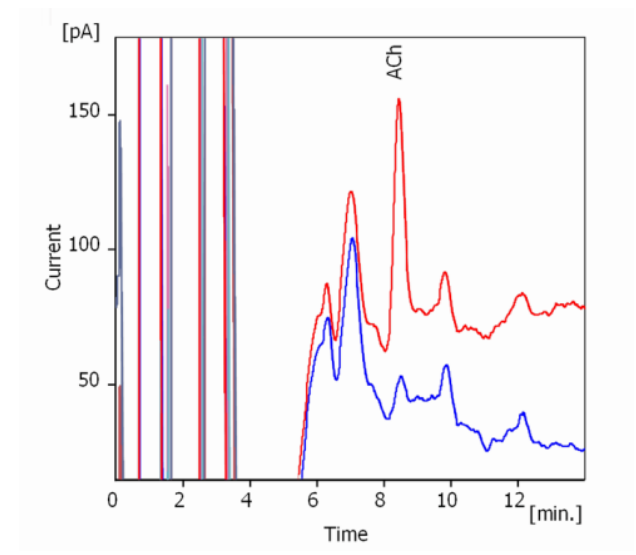
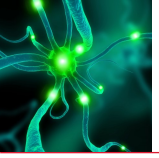


Figure 10: Overlay of chromatograms from pooled rat medial Prefrontal Cortex (mPFC) microdialysate sample, spiked with 10 nM acetylcholine (red trace) and not-spiked (blue trace).

Conclusion

The ALEXYS Neurotransmitter Analyzer for Acetylcholine results in a fast and sensitive detection method of ACh. A detection limit of 2.5 fmol is obtained which facilitates basal level ACh detection without the need for Acetylcholinesterase inhibitors. The UHPLC ion-pair separation is superior to existing ion-exchange methods, by reversing the elution order of Ch and ACh, resulting in a better separation and no late eluting peaks



References

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2. Sotoyama, H.; Zhu, Y.; Gitzen, J.; Xie, F.; Kissinger, P. Feasibility of ion-pair reversed-phase liquid chromatography/electrochemistry detection for determination of acetylcholine in microdialysates collected without acetylcholinesterase inhibitors. Cur. Separations 2002, 20, 11-16.
3. Sencell user manual, Antec Scientific document 116_0010.
4. Micro volume injections, Antec Scientific technical note 220_011.
5. Analysis of Glutamate, GABA, Noradrenaline, Dopamine, Serotonin and Metabolites using microbore UHPLC with Electrochemical Detection, Reinhoud NJ, Brouwer HJ, van Heerwaarden LM, Korte-Bouws GA.; ACS Chem Neurosci. 2013, 4:888–894

Ordering information

ALEXYS Neurotransmitter Analyzer for analysis of ACh and Ch	
180.0091UW	ALEXYS Neurotransmitters SCC base
180.0505W	Add-on parts for ACh/Ch analysis
116.4322	SenCell 2 mm Pt HyREF
250.1165*	Acquity UPLC in-line filter kit + 6 frits (205000343)
250.1160*	Acquity UPLC C18 HSS T3, 1x50 mm 1.8 µm (186003535)
250.3532	AChE/ChOx IMER, 1mm

*) Columns are products of Waters Corporation (Milford, USA). The Waters part numbers are given between parenthesis for reordering purposes.

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