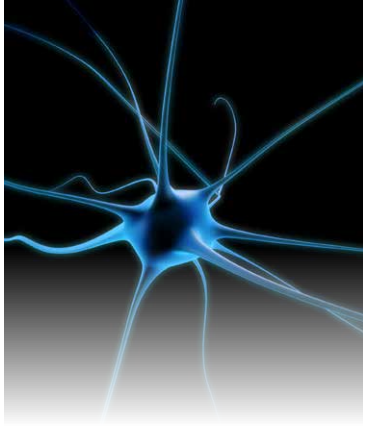
Application Note
Neuroscience



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

Alexys Neurotransmitter Analyzer for Acetylcholine and Choline

- **Fast separation on a sub-2µm UHPLC column**
- **Flexcell with easily exchangeable electrodes**
- **Detection limit down to 0.3 nmole/L ACh**
- **Total analysis time < 6 min**

Introduction

The ALEXYS® Acetylcholine analyzer featuring a FLEXCELL™ with easily exchangeable working electrode disks can be used in combination with a peroxidase kit for fast and sensitive detection of basal ACh levels in microdialysis samples.

Analysis of acetylcholine (ACh) and Choline (Ch) by HPLC-ECD is based on an ion-pairing separation, followed by post-column enzymatic conversion to hydrogen peroxide with acetylcholinesterase (AChE) and choline oxidase (ChO) [1]. Both enzymes are covalently bound to a stationary phase in an immobilized enzyme reactor (IMER). After conversion, the hydrogen peroxide can be detected electrochemically on a glassy carbon electrode coated with horseradish peroxidase (HRP).

Summary

The ALEXYS Neurotransmitter Analyzer is a modular system that can be customized for specific neurotransmitters. In this application note a fast and sensitive method is presented for the analysis of acetylcholine. Fast and efficient separation is achieved using a sub-2 µm particle UHPLC column. A post column IMER is applied to convert ACh in hydrogen peroxide which is detected. With this approach a detection limit down to 0.3 nmol/L is obtained using a total sample of 10 µL (3 fmol).

Figure 1: ALEXYS Neurotransmitter Analyzer for acetylcholine.

The ALEXYS Neurotransmitter Analyzer consists of the OR 110 degasser unit, LC 110S pump(s), the AS 110S autosampler, the DECADE II EC detector and Clarity data acquisition software. Complementary kits have been developed for common neurotransmitters such as dopamine (DA), noradrenaline (NA), serotonin (5HT) and metabolites, or acetylcholine (ACh) and choline (Ch).

Method and results

ACh and Ch are very polar molecules (Fig. 2), and are positively charged at neutral pH. They are separated using a C18 column with an ion-pairing agent in the mobile phase [2].

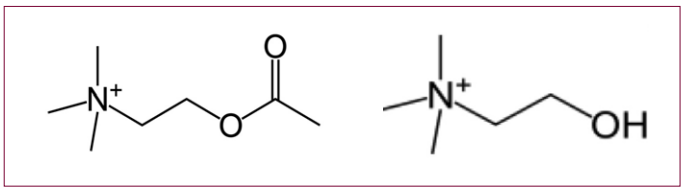


Figure 2: Structure of Acetylcholine (left) and Choline.

To convert ACh and Ch to the electrochemically detectable hydrogen peroxide (Fig. 3), an immobilized enzyme reactor (IMER) containing acetylcholine esterase and choline oxidase is connected directly behind the analytical column.

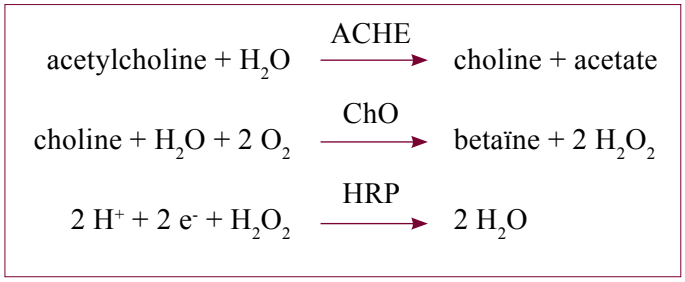


Figure 3: Enzymatic conversion of acetylcholine and choline to electrochemically detectable hydrogen peroxide in post-column immobilized enzyme reactor (IMER).

The reduction of hydrogen peroxide involves a two electron transfer per molecule. Reduction results in negative peaks in a chromatogram; this can be inverted by setting the detector polarity to 'negative'.

Electrode coating procedure

Coating a glassy carbon working electrode is done by drying a drop of 'surfactant solution' followed by a drop of 'peroxidase/polymer coating solution' from the peroxidase electrode refill kit, and letting it dry overnight [4]. It is recommended to coat two electrodes at the same time, the second electrode can serve a as a back-up or replacement electrode (shelf life: approximately 1 week in the refrigerator).

Repeatability, linearity and detection limit

The repeatability (n = 6) using a 5 nmol/L ACh in Ringer solution was found to be better than 0.20% RSD for retention time and better than 3% for peak area and height. However a significant drop in response was observed over time affecting the interday RSD's for peak area and height. This is caused by a loss in activity of the HRP enzyme coating on the working electrode which should be replaced regularly (after a few days). This highlights the importance of having short runtimes per sample and regular calibration with standards.

Table 1

Conditions for Acetylcholine analysis	
HPLC	ALEXYS Neurotransmitter Analyzer (pn 180.0091U) with AS 110 UHPLC cool 6-pv autosampler
Column	Waters Acquity UHPLC HSS T3 1.0x50 mm column, 1.8 µm
Pre-column	Acquity in-line filter kit + 6 frits
IMER	IMER, 3 mm ø x 4 mm
Mobile phase	50 mmol/L phosphoric acid adjusted to pH 7.5 with 50% NaOH, 0.5 mmol/L EDTA, 1.6 g/L octanesulfonic acid, 0.5 mmol/L tetramethylammonium chloride
Flow rate	150 µL/min
Temperature	35 °C (separation and detection)
Vinjection	10 µL, with user defined injection program
AS wash solution	Water (HPLC grade)
Flow cell	FLEXCELL with peroxidase-coated GC electrode and HyREF Pd/H2 reference electrode
Ecell	-100 mV vs. Pd/H2
Range	5 nA/V
ADF™	0.5 Hz
Icell	-50 to -10 nA
Noise	< 5 pA

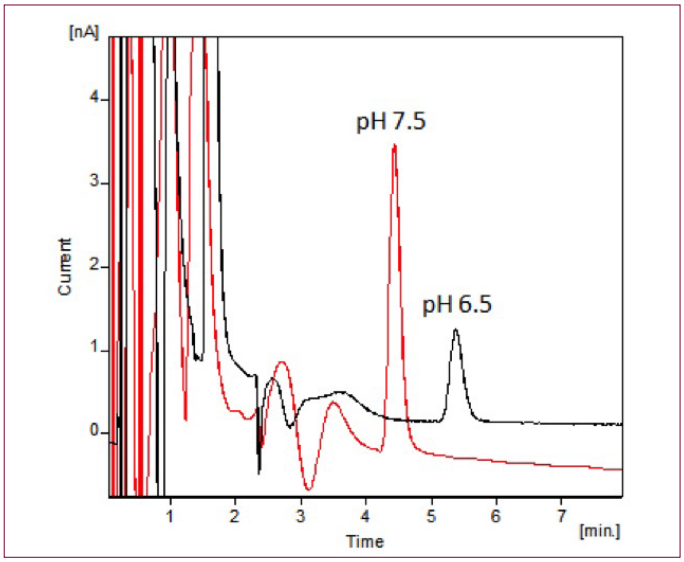


Figure 4: Mobile phase pH is affecting the IMER performance and peak height, pH 7.5 is used in this method.

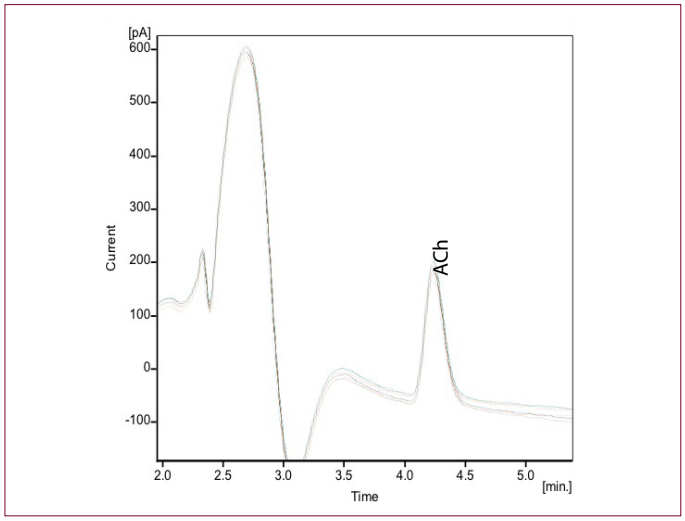


Figure 5: Overlay of 6 chromatograms of 5 nmol/L ACh in Ringer's solution. Injection with method UP101 that uses 10 µL of sample in total.

A detection limit down to 3 fmol has been obtained for ACh using a well performing IMER, extensively stabilized system with a noise level below 5 pA, making this system sensitive enough to measure basal levels of ACh in microdialysate samples.

Conclusion

The ALEXYS Neurotransmitter Analyzer utilizes the extraordinary separation power of sub-2 µm packed columns. Plate numbers, retention times and detection sensitivity have been pushed to their limits.

Due to an optimized and dedicated method using an extremely selective enzyme reactor which is also ion-pair LC compatible, an improved selectivity is obtained in combination with very short run times of < 6min. The detection limit at basal level down to 0.3 nmol/L is obtained using a total sample of 10 µL.

The ALEXYS Neurotransmitter Analyzer can be extended with several options and kits for any combination of other neurotransmitters, Monoamines and Metabolites as well as Amino Acids.

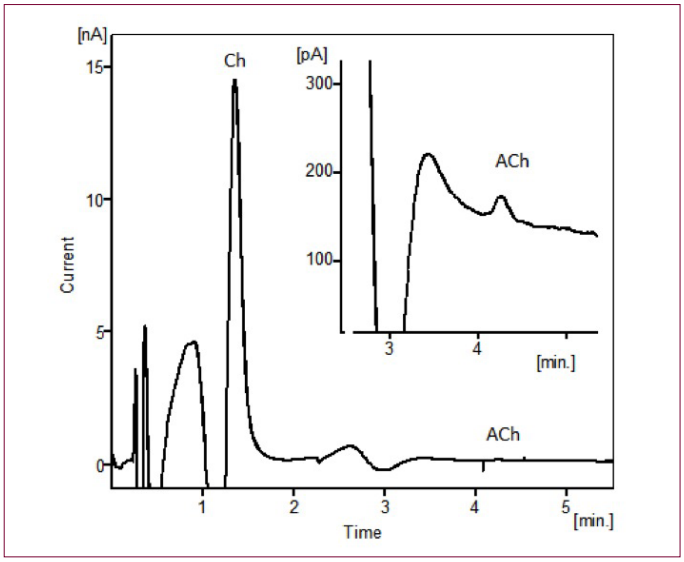


Figure 6: Chromatogram of a basal level rat microdialysate sample. The acetylcholine concentration was calculated to be 1 nmole/L.

Ion-pair vs. ion-exchange chromatography

Instead of ion-pairing chromatography sometimes ion-exchange chromatography is used (see f.e. ref [8]) for analysis of Ach and Ch. With ion exchange the elution order of the peaks is reversed. In such case often the small ACh peak and large Ch peak are not very well separated, and a large late eluting peak is present in the chromatogram (at about 25-35 min) resulting in long runtimes. Therefore, the method presented in this application note is preferred in that respect.

Ordering number

ALEXYS Neurotransmitter Analyzer for Acetylcholine	
180.0091U	ALEXYS neurotransmitters
191.0035U	AS 110 autosampler UHPLC cool 6p
180.0506	Acetylcholine SCC kit
250.3531	AChE/ChOx post column-IMER

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 acetylcholin-esterase inhibitors.* Cur. Separations 2002, 20, 11-16.
3. De Bundel, D.; Sarre, S., Van Eeckhaut, A; Smolders, I.; Michotte, Y. *Critical evaluation of acetylcholine determination in rat brain microdialysates using ion-pair liquid chromatography with amperometric detection.* Sensors 2008, 8, 5171-5185.
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5. *Flexcell user manual.* Antec (102.0010a)
6. Ichikawa, J., Dai, J., Meltzer, H.Y. *Acetylcholinesterase inhibitors are neither necessary nor desirable for microdialysis studies of brain acetylcholine.* Cur. Separations 2000, 19, 37-43.
7. Kehr, J, Dechent, P., Kato, T., Ögren, S. *Simultaneous determination of acetylcholine, choline and physostigmine in microdialysis samples from rat hippocampus by microbore liquid chromatography/electrochemistry on peroxidase redox polymer coated electrodes.* Journal of Neuroscience Methods 83 (1998) 143–150
8. *Acetylcholine and Choline.* Application note, Antec (213-022)

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