

## ACETYLCHOLINE IN MICRODIALYSATES

THE SMARTEST LC-EC APPLICATIONS FOR  
NEUROSCIENCE ANALYSIS  
EVER DEVELOPED

## Monoamines and the 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  
4-aminobutyrate (GABA)  
Glutamate (Glu)

## Choline and Acetylcholine

Choline (Ch)  
Acetylcholine (ACh)

## Markers for oxidative stress

3-nitro-L-Tyrosine  
8-OH-DPAT

## Glutathione and other thiols

## INTRODUCTION

Analysis of acetylcholine (ACh) and Choline (Ch) by LC-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 immobilised enzyme reactor (IMER). After conversion, the hydrogen peroxide can be detected electrochemically on a glassy carbon electrode coated with horseradish peroxidase (HRP).

The ALEXYS<sup>®</sup> Acetylcholine analyzer featuring a FLEXCELL<sup>™</sup> 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 microdialysate samples of for instance mice and rats.

- Selectivity by enzyme reactor
- Fast and easy coating procedure for working electrode
- Flexcell with easily exchangeable electrodes
- Detection limit: about 0.3 nmole/L ACh
- Total sample use: 10  $\mu$ L (can be decreased further)
- Total analysis time < 6 min

## Summary

## ACh analysis with ALEXYS Neurotransmitter Analyzer

The ALEXYS Neurotransmitter analyzer is a modular LC-ECD system that can be combined with different applications kits for the analysis of various Neurotransmitters. For the fast and sensitive analysis of Acetylcholine in microdialysate samples, the ALEXYS Neurotransmitter analyzer can be employed with the Acetylcholine application kit. Using the principle of ion-pairing chromatography on a sub-2 micron microbore column, a detection limit of about 0.3 nmol/L ACh can be reached using a total sample of 10 only  $\mu$ L (3 fmol on column), in a 6 minute run per sample.

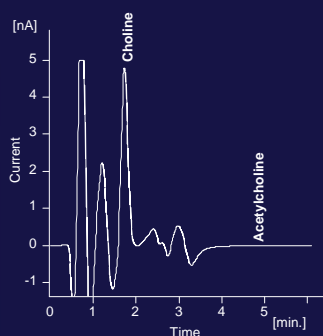


Fig. 1. ALEXYS Neurotransmitter Analyzer.

## Results

### Separation

ACh and Ch are very polar molecules (Fig. 2), and contain a tertiary nitrogen moiety that is positively charged at neutral pH. This feature can be used to generate retention and separation using a C<sub>18</sub> column and ion-pairing agent in the mobile phase (Fig. 3) [2].

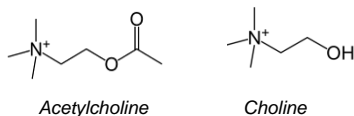


Fig. 2.

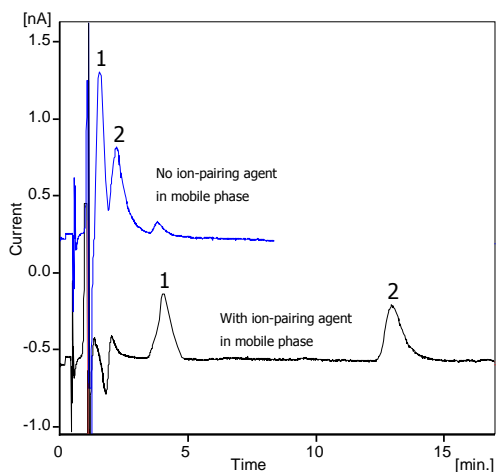


Fig. 3. The combination of a C<sub>18</sub> column and mobile phase with ion-pairing agent makes it possible to separate Ch (1) and ACh (2).

The mobile phase composition that was used for separation was adapted from reference [3]. It is basically a pH 6.5 phosphate buffered aqueous solution containing a bactericide, an ion-pairing agent, octanesulfonic acid (OSA), and a silanol blocking agent that improves peak shape (Fig. 4).

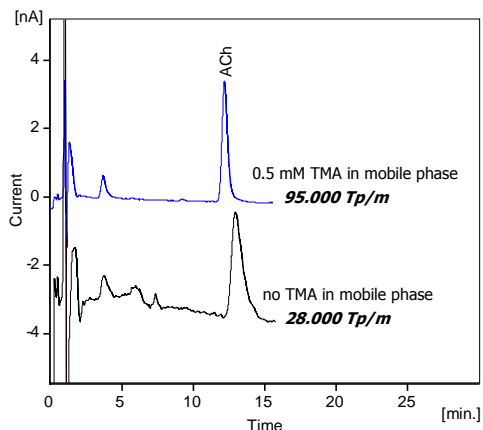


Fig. 4. The silanol blocking agent tetramethylammonium chloride (TMA) improves peak shape for the reversed phase ion-pairing separation of ACh (1  $\mu$ L injections).

The amount of ion-pairing agent in the mobile phase strongly affects the baseline pattern. As ACh was not well enough separated from the front peak at a level of 400 mg/L OSA, the concentration was increased further. The OSA level had to be increased to 1600 mg/L for ACh to elute at a time period where the baseline is optimized for quantitation (Fig. 5 and Fig. 6).

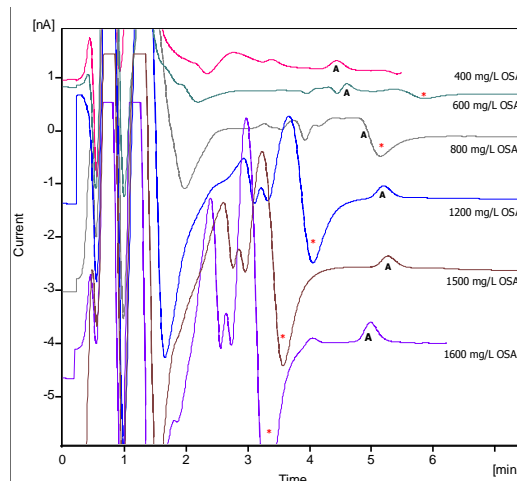


Fig. 5. Effect of different concentrations of the ion pairing agent octane sulphonic acid (OSA) on chromatograms of 10 nM ACh (A) in Ringer's solution (10  $\mu$ L total sample use, about 8  $\mu$ L injections). The red star highlights the location of a problematic baseline disturbance.

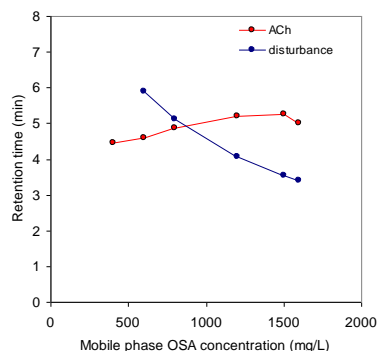


Fig. 6. Effect of OSA on retention times of ACh and the highlighted baseline disturbance in Fig. 5.

### Enzymatic conversion

To convert ACh and Ch to the electrochemically detectable hydrogen peroxide (Fig. 7), an immobilised enzyme reactor (IMER) containing acetylcholine esterase and choline oxidase is connected directly behind the analytical column.

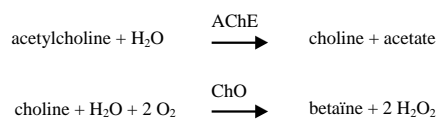


Fig. 7. Enzymatic conversion of acetylcholine and choline to electrochemically detectable hydrogen peroxide in post-column immobilised enzyme reactor (IMER).

### Electrochemical detection

Traditionally, platinum electrodes were used for their selectivity to hydrogen peroxide in an electrochemical cell. Due to relatively longer stabilisation time, lower sensitivity, and faster decrease in response when platinum electrodes are used, the use of the horseradish peroxidase (HRP) osmium-redox polymer coated glassy carbon (GC) working electrodes [4] became more popular. HRP coated electrodes detect the peroxide according to the following reaction:

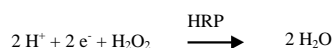


Fig. 8. Reduction of hydrogen peroxide on HRP-GC working electrode.

The reduction of hydrogen peroxide involves a two electron transfer per molecule (Fig. 8), and therefore results in negative peaks in a chromatogram; this can be reversed to positive peaks by setting the detector polarity to 'negative'.

For troubleshooting purposes, the ACh kit for the ALEXYS Neurotransmitter analyzer also contains a platinum working electrode.

### 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 (BASi, MF-2096), and letting it dry overnight [4]. It is recommended to coat two electrodes at the same time, the second electrode being stand-by in the refrigerator for 1 week.

### UHPLC hardware

The ALEXYS Neurotransmitter Analyzer has a maximum pressure rating of 700 bar and can be used with sub-2 micron particle columns. This supports the application of higher flow rates to speed up analysis time, and the high plate count of sub-2 micron particle columns results in sharper/higher peaks thus providing better sensitivity. Using a micro-bore version of such columns further enhances sensitivity.

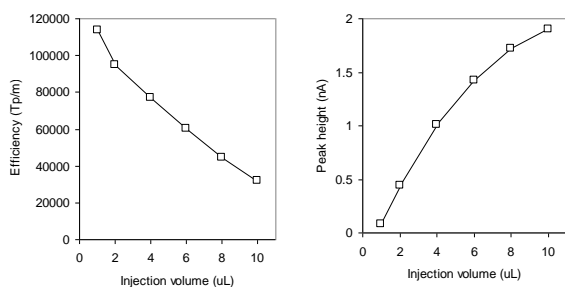


Fig. 9. Injection volume affects plate count: loadability is limited but response to an injection increases (thus sensitivity).

Loadability of micro-bore columns is limited, and it is normal that injection of volumes larger than 1 uL will decrease plate count (Fig. 9). However, plate counts may be sacrificed by injecting larger volumes of sample if there is enough separation and a need

for low concentration detection limits as with the analysis of Acetylcholine in microdialysates. This is the rationale behind practically overloading the column with a large injection volume of almost 10 uL.

Applying higher flow rates results in faster analysis, and narrower peaks. Very narrow peaks tend to be quenched in case a filter has to be applied to obtain best sensitivity. Therefore, the analysis of ACh is performed at only 50% of maximum system pressure, thus combining sensitivity and short analysis times. Apart from a high pressure rating, the system also has minimised dead volumes, which further preserves sensitivity.

### Injection method for small samples

Since the collection time for microdialysate samples are usually as short as possible to have good time resolution between subsequent data points, the available sample volumes are also small. However, low basal concentrations of ACh (0.17-10 fmol/uL, depending on experimental set-up [6]) demands an as large as possible on-column injection volume to be able to accurately detect ACh. Time resolution and detectability have to be balanced.

Programming a user-defined injection method for the autosampler of the ALEXYS analyzer in the Clarity data acquisition software makes it possible to inject as much as possible from a small sample. Moreover, the ALEXYS autosampler is a micro-version, with minimised dead volume and low dispersion effects. To perform the analyses described in this note, injection method 'UP101' was used. This method will take 10 uL of sample from a vial, and inject about 8 uL on column. In case the analysis of smaller samples is required, the method can easily be adapted; however, it should be noted that the concentration detection limit will increase then.

### Starting up the analysis

When new, the system should be flushed with 15% HNO<sub>3</sub> during the installation to kill bacteria and to passivate the metal parts. In principle, this is only necessary once but can be done again in case of serious contamination.

New unused columns are filled with a storage solution consisting of a mixture of water/organics (see the documentation that comes with the column). As the enzymes in the IMER and on the working electrode will lose their activity when in contact with organics, this solvent must be flushed out of the column with water (30 bed-volumes). Subsequently, the system and column can be flushed with mobile phase. When the column is conditioned with mobile phase, the IMER can be connected and flushed, and then the cell can be connected and turned on as well.

The system will need a few hours before the ion-pairing chromatography and baseline have fully stabilised (Fig. 10). However, the baseline can already be evaluated soon after start up (use the auto zero button when running off-scale) to get an indication if the system is performing. Fig. 11 shows a baseline with normal noise

level during stabilisation (<1nA), and after 18 min the baseline of a leaking cell. In this case mobile phase was creeping under the spacer.

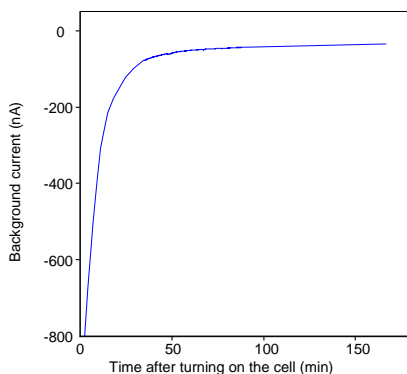


Fig. 10. Stabilisation pattern of the background current ( $I_{cell}$ ) after turning on the electrochemical flow cell. Typically, the  $I_{cell}$  stabilises within a few hours to a level between -50 nA and -10 nA.

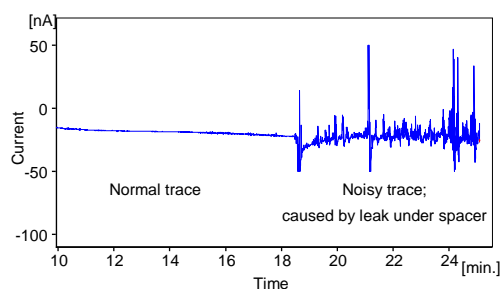


Fig. 11. During stabilisation of the signal, a visual estimation of the noise level can already indicate if everything is all right. Using a range setting of 50 nA/V and ADF filter off, a noise level below 1 nA is normal. In the example trace with high noise (> 1 nA), the problem was caused by a leak inside the flow cell, and in that particular case the problem was solved by replacing the (damaged) spacer.

### Signal response

To check if the area response is more or less as expected, the ACh peak area of a test injection has to be recalculated to relate with the on-column load. This is done by multiplying the peak area (in nA\*sec) with a factor of 1000, and dividing it by the product of injection volume (in  $\mu$ L) and the concentration of the standard (in nmole/L). An area response between 50-150 pA\*sec/fmol ACh is reasonable, and - depending on the quality of the column and injection method - a height response of 2-10 pA/fmol ACh is also reasonable.

If the response is significantly lower, the first parameters to check are leaks, the injection procedure, the quality of the IMER, the quality and thickness of the coat on the electrode, and the quality of the coating solutions.

### Noise levels

During start up, the noise will decrease gradually and stabilise at a level close to or below 10 pA (with DECADE II settings of 5 nA/V range, 0.01 Hz ADF filter, and 5 Hz data rate).

## Method evaluation

### Repeatability

Using a standard of 10 nM (S/N~30) ACh in Ringer solution and performing the analysis 6 subsequent times (Fig. 12), repeatability was found to be better than 0.25 %RSD for retention time, and better than 4% RSD for peak area (Table 1). The inter-day repeatability from 3 subsequent days was almost 3% RSD for retention time, and 25% RSD for peak area. A steady decline in response significantly affects the inter-day RSD value for peak area. This can be caused by a loss of enzyme activity on the electrode (which should be replaced after a few days of use), but also by a loss of enzyme activity in the IMER. Such steady loss of signal can be a typical feature of the ACh analysis when using enzymes, and an average loss of about 10% signal per day (and fastest loss on the first day) has been reported earlier [6]. This highlights the importance of having short runtimes per sample and regular calibration of the system (recalibration after every 20 samples is suggested).

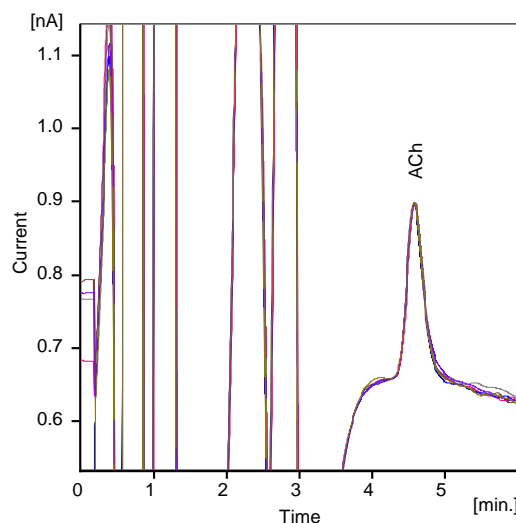


Fig. 12. Overlay of 6 chromatograms of 10 nM ACh in Ringer's solution, injected on column with method UP101 that uses 10  $\mu$ L of sample in total.

Table 1

Repeatability of ACh analysis (n=6)						
	Retention		Height		Area	
	Avg (min)	RSD (%)	Avg (nA)	RSD (%)	Avg (nA.s)	RSD (%)
Day 1	4.58	0.13	0.24	0.8	3.67	1.2
Day 2	4.55	0.16	0.19	0.7	2.82	1.5
Day 3	4.79	0.22	0.15	2.4	2.21	3.8
Inter-day (n=3)	4.64	2.8	1.19	23	2.90	25

### Linearity

The linearity of the method was determined in the concentration range of 0.5-2.5 nM ACh and for 0.5-2.5  $\mu$ M Ch in Ringer solution. The method shows good linearity for both ACh and Ch (Fig. 13 and Fig. 14) with correlation coefficients better than 0.998.

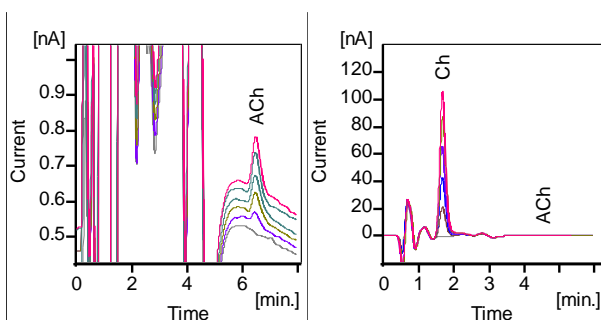


Fig. 13. Overlay of 0-2.5 nM ACh (L) and 0-2.5  $\mu$ M Ch (R) in Ringer's solution, injected on column with method UP101 that uses 10  $\mu$ L of sample in total. The application of a lower flow rate resulted in somewhat longer analysis time in this case.

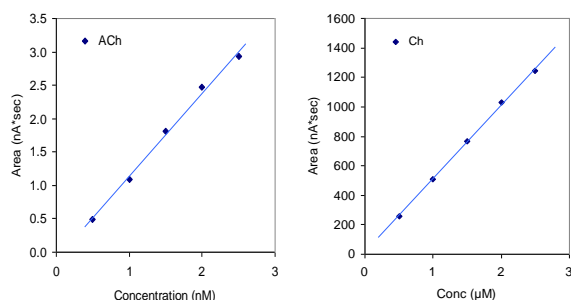


Fig. 14. Calibration plots based on chromatograms shown in Fig. 13.

### Detection limit

Detection limit (3 times peak-to-peak noise) was calculated to be about 2 fmol on column for ACh in the best case (well performing IMER, extensively stabilized system, noise level below 5 pA). Having a system with a detection limit better than 5 fmol is reasonable. In combination with the injection method that uses only 10  $\mu$ L of sample in total, this corresponds with a concentration detection limit between 0.3-0.6 nM ACh, making this system sensitive enough to measure basal levels of ACh in microdialysate samples.

### Basal level microdialysates

#### Rat

Basal rat brain microdialysate ACh concentration was measured to be about 1 nM (Fig. 15).

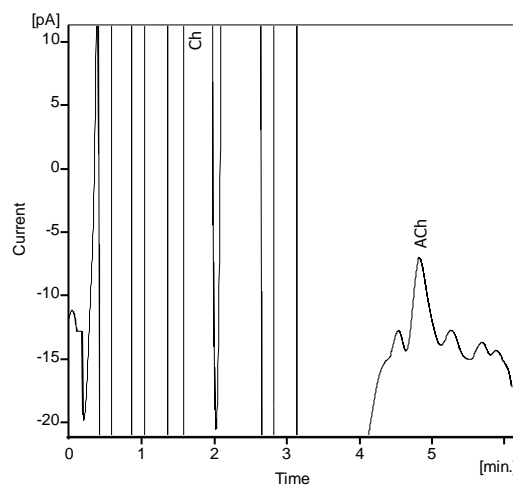


Fig. 15. Chromatogram of a basal level rat microdialysate sample. The acetylcholine concentration was calculated to be 1 nmole/L.

#### Mouse

Basal mouse brain microdialysate ACh concentration was measured to be about 0.4 nM (Fig. 16), which was about a factor 2 above the detection limit.

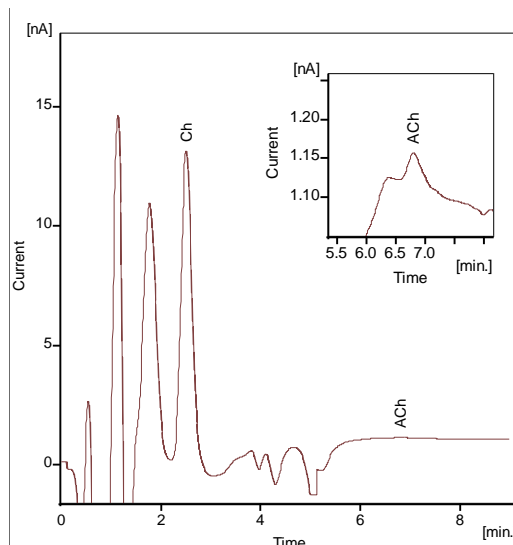


Fig. 16. Chromatogram of a basal level mouse microdialysate sample. The acetylcholine concentration was calculated to be 0.4 nmole/L. The inset shows a zoom in on the ACh peak. The application of a lower flow rate resulted in somewhat longer analysis time in this case.

### Alternative separation method: ion-exchange

The traditional and alternative separation method to the ion-pairing chromatography is the use of ion-exchange chromatography, previously described in an Antec note [8]. In that case, the column has a strong cation-exchange polymer-based resin (BASi microbore column for ACh analysis; MF-8904), and the mobile phase does not contain an ion-pairing agent. With this method, the elution order of the peaks is reversed compared to the use of ion-pairing chromatography on a C<sub>18</sub> column. Drawbacks of the ion-exchange separation method are that the small ACh peak and

large Ch peak are not very well separated, and a large late eluting peak is often present in the chromatogram (at about 25-35 min), thus imposing long runtimes to prevent this peak from disturbing the following chromatograms.

Traditionally, ion-exchange has been the separation method of choice, but since the emergence of good micro-bore C<sub>18</sub> columns with high efficiency, the use of ion-pairing separation is now also a serious option with the added benefit of shorter run times.

## CONCLUSION

**The ALEXYS Neurotransmitters base system with Acetylcholine application kit is a dedicated solution for the analysis of ACh in microdialysates. The ion-pairing separation method, enzymatic ACh conversion and BASi peroxidase kit result in sensitive, linear, and reproducible detection of ACh.**

## References

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## PART NUMBERS

180.0091U	ALEXYS neurotransmitters system
180.0508	Acetylcholine application kit (containing peroxidase kit, IMER, flow cell and column)



Fig. 17. ALEXYS Neurotransmitter Analyzer.