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

Reductive detection of monoamines for improved selectivity in HPLC-ECD

- First cell: oxidation of monoamines and their metabolites
- Second cell: reductive detection at +250 mV
- Selective: only reversible reactions/reactants detectable at the 2nd cell

Summary

Monoamines and their metabolites are detectable by LC-ECD with oxidative amperometric detection. The substances that contain a catechol moiety (which has reversible redox reaction properties), are subsequently detected by reductive amperometric detection in a 2nd cell. A cell potential of only +250 mV at the 2nd cell results in enhanced selectivity for catecholamines. This is particularly helpful for complex samples.

Introduction

Monoamines and their metabolites are directly detectable by LC-ECD with oxidative amperometric detection. Molecules with a catechol-moiety typically have reversible redox reaction properties (Fig. 1) and this feature can be used as a selection parameter in their analysis [1]. They can be first oxidized, and subsequently detected by reductive amperometric detection in a 2nd cell. This is particularly helpful for complex samples. The Dual Cell Control (DCC) option for the DECADE Elite™ electrochemical detector makes it possible to control two cells and use both cells for data acquisition (Fig. 2).

In this note we describe the serial ox-red configuration, and the optimization of the ECD settings for the ox-red analysis of catecholamines and their metabolites in microdialysates.

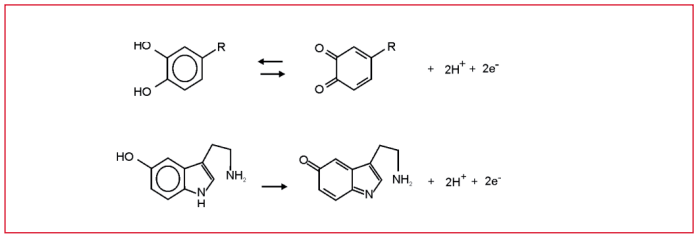


Figure 1: Reaction scheme of the reversible red-ox reaction of catecholamines (top) and irreversible oxidation reaction of isoindoles like serotonin and 5-HIAA.

Method

The ALEXYS Neurotransmitter Analyzer is an HPLC-ECD system with all parts optimized and dedicated to the robust and reproducible analysis of small samples with low levels of neurotransmitters (Fig. 10). The DECADE Elite electrochemical detector of the ALEXYS system was outfitted with the optional Dual Cell Control (DCC) to facilitate ox-red analyses with two amperometric flow cells. The 1st cell in series was the FlexCell: this cell has a very small internal cell volume of less than 1 µL and therefore it should not contribute significantly to band broadening in the 2nd cell. The 2nd cell is the SenCell, which was chosen for its superior sensitivity.

Conversion in the 1st cell

In serial flow cell configurations it is important that the 1st cell has a high conversion efficiency to favor detection in the 2nd cell. In an amperometric flow cell only a fraction of the analyte that enters the cell is actually converted at the working electrode (Fig. 3). The conversion percentage in a cell is the quantity of electrons (in moles) as a percentage of the total quantity of available electrons in the analyte that passes through the cell after an injection:

Conversion = [quantity detected / quantity injected] * 100 %

The number of electrons detected (in moles) is derived from the total electric charge that has been transferred to the working electrode. In amperometric detection peak heights are measured as electric current given in nA. The total electric charge is expressed in Coulomb (nA.s), and is given by the peak area which has the same units (nA.s).

Conversion = A / [w * n * F] * 100%

where A is peak area (nA.s), w is sample load (nmol), n is the number of electrons involved in the electrochemical reaction mechanism (2 in case of catecholamines) and F is Faraday's number (96485 Coulomb/mol). There are a few ways to increase the conversion efficiency

- Using a smaller spacer thickness will result in a smaller diffusion layer and makes it easier for an analyte to reach the electrode surface.

Figure 2: ALEXYS Neurotransmitter Analyzer (l) and detail of the DECADE Elite DCC with opened door, showing the oven compartment with the separation column and two cells in series

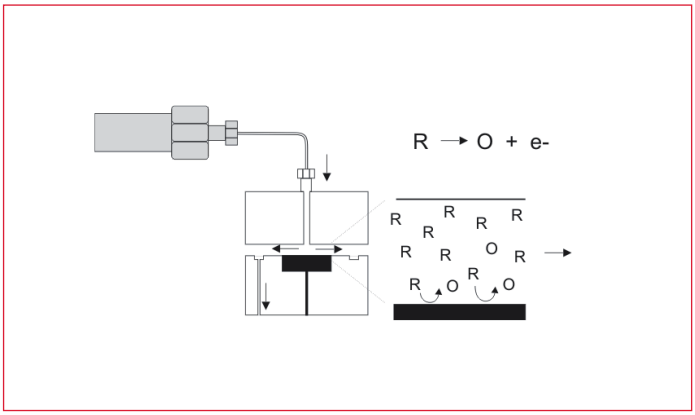


Figure 3: In an amperometric flow cell only a fraction of the analyte is oxidized, as not all analytes reach the electrode surface. At lower flow rates a higher percentage of the analytes reach the surface.

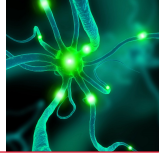


Table 1

Conditions	
LC system	ALEXYS Neurotransmitter Analyzer for ox-red, including DECADE Elite DCC
Column*	HyPURITY™ AQUASTAR™ HPLC column, 150 x 1.0 mm ID, 3 µm (Thermo Scientific™)
Mobile phase*	50 mmol/L phosphoric acid, 50 mmol/L citric acid, set to pH 3.8 with NaOH solution, 8 mM KCl, 0.1 mM EDTA.Na_2, 400 mg/L octane sulfonic acid (sodium salt hydrate)
Flow rate	50 µL/min
Injection	2 µL
Temperature	35 °C (separation and detection)
Flow cell 1 (ox)	GC FlexCell, HyREF, 50 µm spacer
$E_{cell\ 1}$	+0.54 V
Flow cell 2 (red)#	2 mm GC SenCell sb, AST 1
$E_{cell\ 2}$	+0.25 V (and polarity of signal inverted)
I-cell	about nA
ADF	0.02 Hz (both channels)
Range	1 nA/V (both channels)

* These separation conditions are not endorsed in particular. See ref 2,3 for alternatives.
# Original work done with 2 mm GC VT-03, sb, 25 µm spacer

- A larger electrode area will result in a higher conversion.
- When decreasing the flow rate, the conversion goes up.

At flow rates associated with standard-bore HPLC (about 1 mL/min) this is typically 5 - 10%, but at lower flow rated this percentage can increase considerably up to 30 - 80% or even 100% at very low flow rate (< 10 µL/min). As column dimensions dictate the applied flow rate, a decrease in flow rate can only be realized by using a smaller bore HPLC column. The use of microbore HPLC with 1 mm ID columns was thus chosen for two particular reasons:

- Microbore HPLC is compatible with small sample volumes (typical for neurotransmitter analyses) and does not compromise detection sensitivity [2].
- Microbore HPLC runs at lower flow rates, and this will help to increase the oxidation ratio in the 1st cell. For a 1 mm column with a flow rate of 50 µL/min we found a conversion efficiency of about 80%.

The applied LC-ECD conditions are summarized in Table 1 and applied unless stated otherwise.

Results

Peak broadening is limited

When using two cells in series it is important that the contribution to dead volume of the 1st cell is minimized. For this purpose, the FlexCell with an internal volume of less than 1µL was applied [3], and the effect on peak shape is minimal (Fig. 4).

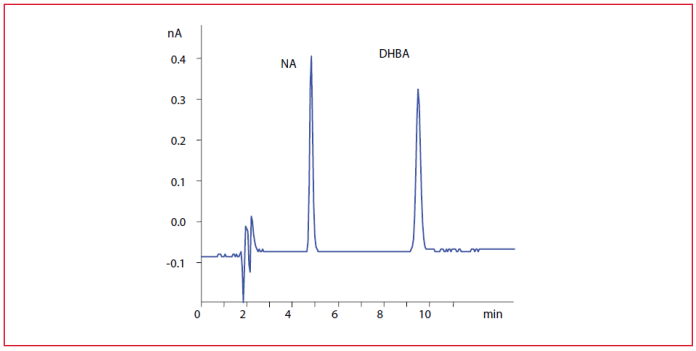


Figure 4: Chromatogram of 1 µL of 100 nM NA and DHBA, detected at the 2nd flow cell. Plate numbers are 44 713 m^{-1} (NA) and 73 394 m^{-1} (DHBA). Comparable plate numbers were found at the 1st flow cell, indicating that the contribution to peak broadening is minimal.

Voltammograms

Optimization of cell potential in ox-red configuration was done in two steps. First, the oxidative voltammogram is constructed for the 1st cell. The oxidation of noradrenaline and dopamine becomes diffusion-limited at a potential of about 500 – 600 mV (Fig. 5). This observation is as expected for detection at pH 3.0 and the used of a HyREF as the reference electrode.

Secondly, the potential of the 2nd cell was varied from +400 mV down to +100 mV for reduction. The 1st (oxidative) cell was kept at a constant potential of 650 mV during these experiments. Note that the polarity of the output has to be switched to '-' to invert the signal; otherwise the chromatogram shows negative peaks (as expected). Figure 4 shows that oxidized catecholamines can be reduced back in the 2nd cell with a working potential set below 400 mV (vs. sb).

A rather broad peak between 4 – 8 min was observed in the chromatograms from the 2nd (reductive) cell trace when constructing the voltammogram (Fig. 6). The peak was larger at more reductive potentials and it probably originates from the reduction of dissolved oxygen that is injected.

Based on these observations, the optimal ECD settings for cell 1 and 2 were chosen to be +540 mV and +250 mV respectively .

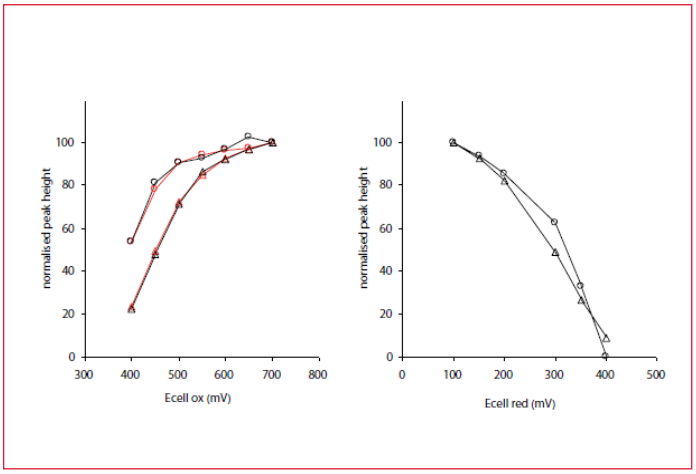


Figure 5: Hydrodynamic voltammograms for NA (Δ) and DA (O) measured in the 1st cell under oxidative conditions (left), and measured in the serially connected 2nd cell under reductive conditions (right). The reductive voltammogram (right) was constructed with the 1st cell set to 650 mV. Conditions according Table 1.

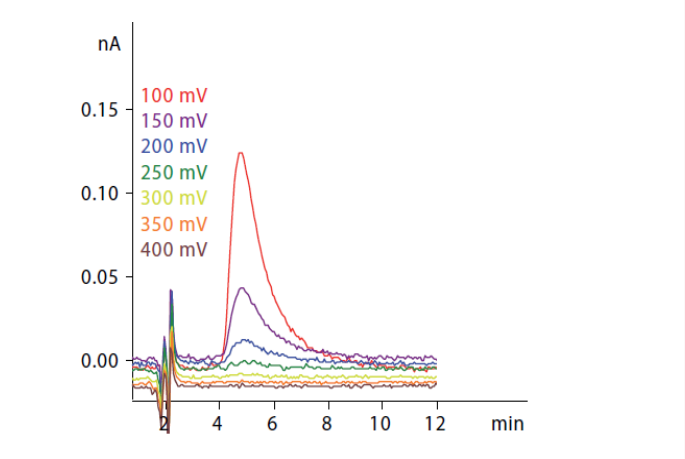


Figure 6: Overlay of blank chromatograms as recorded at the 2nd (reductive) cell showing an interfering peak below 250 mV (vs. Ag/AgCl). Conditions according Table 1.

A short test with a mobile phase at pH 6 revealed that it was not possible to separate noradrenaline from the peak at 5 min. A reductive potential below -100 mV was required, but this low working potential also resulted in a large interfering disturbance between 4 and 8 min (like shown in Fig. 6). Therefore the use is limited to separations with acidic mobile phase.

Reproducibility, linearity and detection limit

Reproducibility of ox-red analysis was investigated by analysis of standards. Data from the 2nd (reductive) cell are given in Fig. 8 and Table 2. The RSD values at the 2nd cell were comparable to the ones obtained at the 1st (oxidative) cell.

Table 2

Averages and % RSD of retention time, area and height measured on the basis of 2 µL injections of 10 nM NA, DHBA and DA (n=8) in reductive mode (2nd cell). Chromatograms are shown in Fig. 7.

	Retention		Height		Area	
	tr (min)	RSD (%)	H (nA)	RSD (%)	A (nA.s)	RSD (%)
NA	3.88	0.19	0.11	0.95	1.07	1.1
DHBA	7.00	0.11	0.10	1.0	1.23	1.4
DA	10.95	0.06	0.073	0.8	1.24	1.7

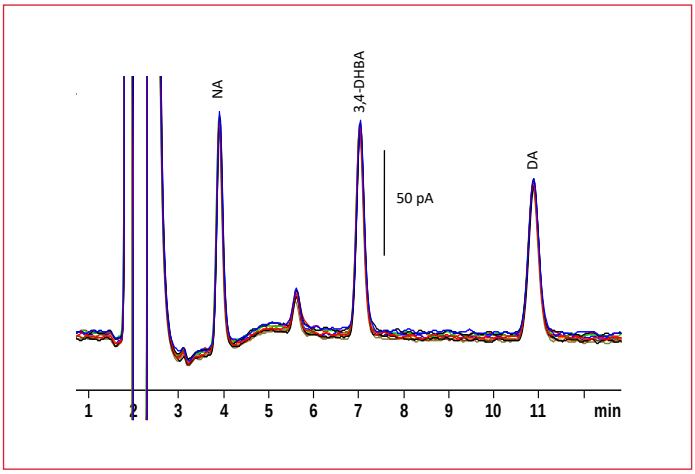
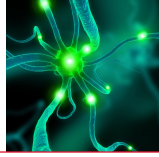


Figure 7: Overlay of 8 chromatograms recorded at the 2nd (reductive) cell with potential set at +200 mV. Other conditions as in Table 1.

The detection limits (S/N=3) of NA and DA at the 2nd (reductive) cell were 0.5 nM and 0.7 nM respectively. The detector response for NA, DHBA, DOPAC and DA is linear in the concentration range of 1-100 nM, with a correlation coefficient better than 0.999.



Ox-red analysis of catecholamines in samples

Microdialysates samples are relatively clean and can in principle be analyzed without any sample pre-treatment. However, samples from some particular brain regions can result in chromatograms that have many peaks in the 1st few minutes (Fig. 9).

The addition of a 2nd cell in reductive mode shows a considerable improvement in selectivity (Fig. 10). The chromatograms of both channels is relevant for quantification, as the isoindoles (like 5-HIAA) will only show up in the chromatogram of the 1st cell. The 2nd cell shows a much 'cleaner' chromatogram with better separated peaks.

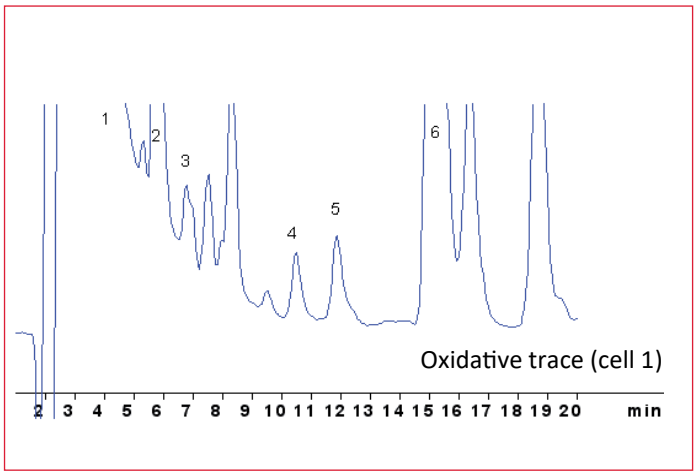


Figure 8: Chromatogram of a microdialysate sample, obtained with the 1st (oxidative) cell. A number of interfering peaks appear in the first 10 minutes. Peaks are NA(1), DOPAC (2), DHBA (3), DA (4), 5-HIAA (5), and HVA (6).

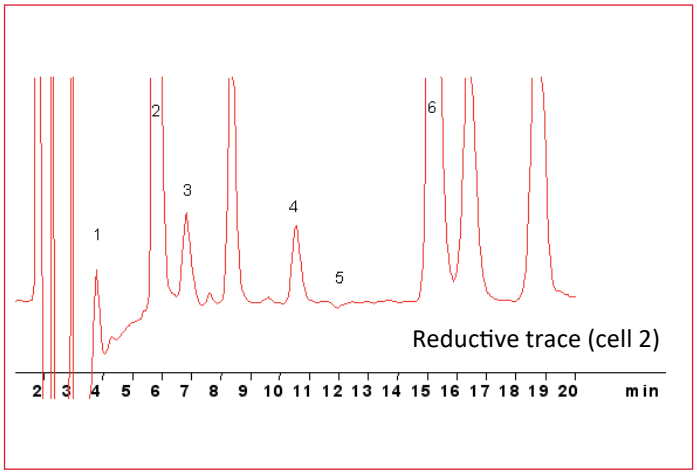


Figure 9: Chromatogram of a microdialysate sample, obtained with the 2nd cell using reductive detection at +200 mV after oxidation in the 1st cell (Fig. 8). The chromatogram shows significant improvement. Peaks: see Fig. 8.

References

1. JK Cullison, J Waraska, DJ Buttaro, IN Acworth, ML Bowers; Electrochemical detection of catecholamines at sub- 5 fg levels by redox cycling; J Pharm Biomed Anal. 1999 Feb;19(1-2):253-9.
2. Antec Scientific Application note 213-028: Monoamines and Metabolites
3. 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
4. Antec Scientific application notes: GABA and Glutamate, Histamine, Amino Acids (213_020); Acetylcholine and Choline (213_023).

Conclusion

Ox-red analysis has been applied for selectivity improvement in analysis of monoamines and their metabolites in microdialysates. Microbore HPLC is the method of choice because of the small microdialysis sample volume and higher conversion rate in the 1st cell at a low flow rate. Detection limits down to 0.5 nmol/L were found in the 2nd (reductive) cell. The application of ox-red improved selectivity.

Figure 10: Recommended instrument configuration for this application: the ALEXYS Neurotransmitter Analyzer + additional channel
The system consists of a P6.1L pump with integrated degasser, an AS110 autosampler, and the DECADE Elite electrochemical detector. The ALEXYS Neurotransmitter Analyzers can be fully controlled by the Clarity™ Chromatography Data System (CDS) software from DataApex™ (version 8.3 and up).

Ordering information

Recommended ALEXYS analyzer + parts	
180.0091UW	ALEXYS Neurotransmitters SCC base
175.0210	DECADE Elite additional channel kit
102.4305	Flexcell GC HyREF
116.4120	SenCell 2 mm GC sb
180.0234A	Flow cell interconnecting tubing
Software	
195.0035#	Clarity CDS single instr. incl LC, AS module

#) optional: Antec ECD drivers for use with Chromeleon CDS , OpenLAB CDS or OpenLAB Chemstation CDS are available.

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