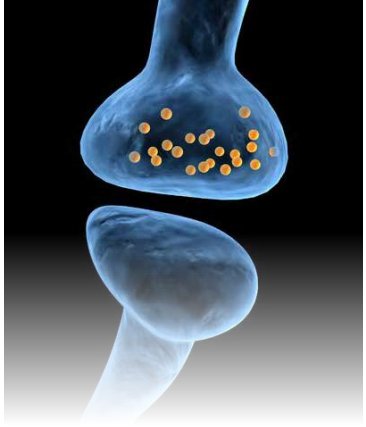
Application Note
Neuroscience



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 Monoamines and Metabolites

- **Fast separation on a sub-2µm UHPLC column**
- **Wall-jet flow cell for best detection limit**
- **Small injection volume, for better time resolution**
- **Optimized separation for multi-component analysis**
- **Dual channel option for parallel separations**

Introduction

Microdialysis of neurotransmitters *in vivo* has become an invaluable tool to study neurotransmission in living brain. Cerebrospinal fluid of the brain is sampled trough a microdialysis device and analyzed by HPLC with electrochemical detection [1-7].

A neurotransmitter analyzer has been developed with features that meet the most demanding requirements. The required injection volume is small, as a smaller volume means a better time resolution in microdialysis.

The method has a low limit of detection, as some neurotransmitters have a concentration below 100 picomole/L. Separation and selectivity are optimized for multicomponent analysis, to get as much information as possible from a drop of dialysate. Where possible the analysis time has been shortened by any means such as by using UHPLC.

Summary

In this application note a fast and sensitive method is presented for the analysis of monoamines and metabolites. A microbore UHPLC column is applied in combination with electrochemical detection using a high sensitivity wall jet flow cell. Detection limits down to 50 pmol/L have been achieved for dopamine. Analysis times vary between 1-15 minutes depending on the complexity of the sample matrix and the number of substances of interest. The system can be equipped with an additional channel for simultaneous analysis of (for example) the metabolites.

Figure 1: ALEXYS Neurotransmitters Analyzer.

The ALEXYS Neurotransmitter Analyzer consists of a DECADE II electrochemical detector, an OR 110 degasser unit and LC 110S pump(s), an AS 110S autosampler and Clarity data acquisition software. Complementary kits for analysis of noradrenaline, dopamine, serotonin and metabolites, GABA and glutamate or acetylcholine and choline are available.

Method and results

In method development for the analysis of monoamines and metabolites a number of parameters are optimized to meet the requirements for detection limits, the use of small samples, and short analysis times of multiple components.

Small sample volume and low detection limits

In a previous communication the optimization for best possible detection limit using small samples has been described [8]. Briefly, a wall-jet micro flow cell has been used which is fully compatible with microbore HPLC. It is well known that small samples are best analyzed using microbore LC with less peak dilution, resulting in more signal. Miniaturization using micro electrodes results in smaller noise. With this combination an improved signal-to-noise ratio with detection limits down to 50 pmol/L (dopamine) have been achieved for the analysis of standards (Fig. 2).

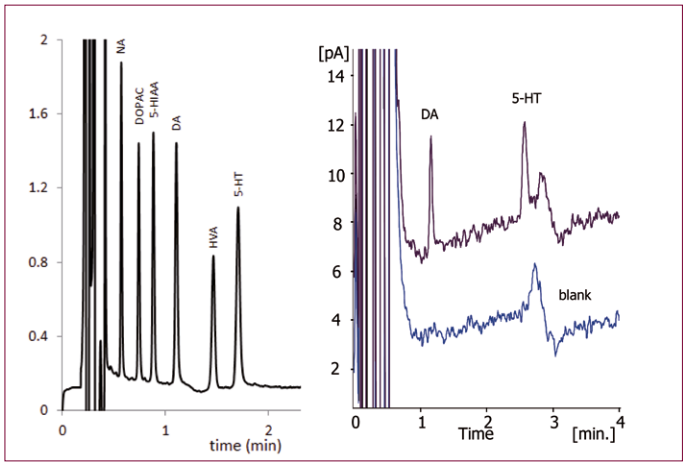


Figure 2: Analysis of monoamines and metabolites (standards) showing a separation within 2 (left) and a 5 µL injection of 100 pmol/L DA and 5HT in Ringer with 10 mmol/L HAc (right). A LOD of 50 pmol/L for DA and 65 pmol/L for 5HT is obtained. There is a trade-off in speed of analysis vs. detection limit and resolution in biological samples.

Separation and speed of analysis

To meet the requirement for fast analysis of multiple components is a matter of selecting a suitable column and optimizing the mobile phase. An analysis time less than 2 minutes is feasible for standards using UHPLC (Fig. 2), however we found there is a trade-off in analysis time vs. detection limit and resolution when analyzing biological samples.

Speeding up a standard HPLC analysis with a factor 2 – 4 using UHPLC columns is feasible, depending on sample matrix and injection volume. At higher velocities a few things are limiting the performance. One of the most critical was the analysis of NA which is close to the solvent front (see figure 4). At high flow rates the peaks overlapped or disappeared in the front. Assumingly this is due to non-ideal behavior of high concentration matrix components eluting in the front peak.

The method and results presented describe an approach, not a fixed set of conditions. It is very well possible or even required to tune the method for a different matrix composition (f.e. measuring in a different brain area) or a different set of neurotransmitters or metabolites of interest. In other words, the ALEXYS system is a flexible system which is not limited to a few applications. There is range of columns available to assist you in optimizing the chromatography to your specific application needs.

Secondary HPLC channel for metabolites

To extend the possibilities a secondary channel can be added to the ALEXYS Neurotransmitter system (Fig. 1). The dual channel system contains one additional pump, a column, and uses an autosampler with a 10 port valve and a dual channel detector.

Both channels share the same autosampler and electrochemical detector (Fig. 3). Detection parameters and HPLC conditions are optimized for each channel depending on the substances of interest. Channel 1 is optimized for NA, DA and 5-HT. Channel 2 is for the acidic metabolites 5-hydroxyindole acetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA).

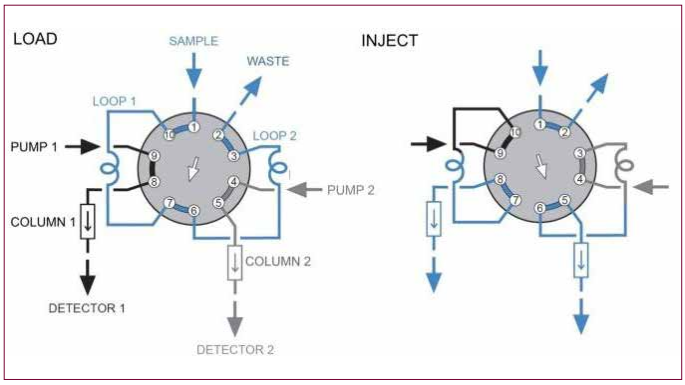


Figure 3: Schematic configuration of sampling 2 parallel systems with a 10-port valve.

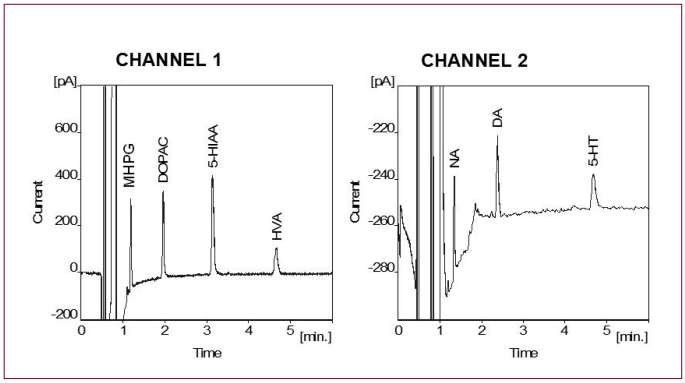


Figure 4: Analysis of standards 5 nmol/L acidic metabolites and 0.5 nmol/L monoamines in Ringer solution with 10 mmol/L acetic acid.

Linearity and repeatability

Using a dual channel configuration the acidic metabolites and the monoamines are measured (Fig. 4). They are loaded simultane-ously on both HPLC's in a single injection and analyzed under completely different and optimized conditions.

The relative standard deviation (RSD) has been investigated for 8 injections of a mix of metabolites (100 nmol/L) and monoamines (10 nmol/L). The RSD in retention times is better than 0.5%. The RSD of peak areas and heights is better than 2% for the metabo-lites and better than 1.5 % for the mono-amines. Linearity shows a correlation coefficient better than 0.998 in the range of 2 to 100 nmol/L.

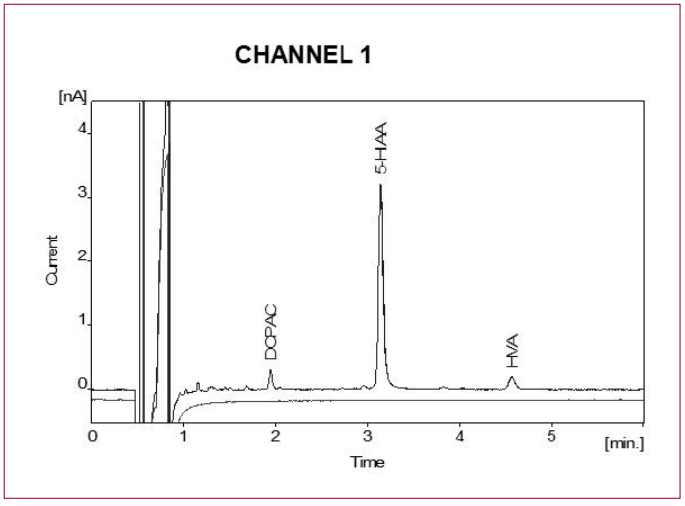


Figure 5: Analysis from a single injection of basal level rat Prefrontal Cortex (PFC) microdialysate. In both chromatograms the bottom trace is an injection of blank (Ringer solution). Samples kindly provided by Mrs. Gerdien Korte-Bouws, Department of Psychopharmacology, University of Utrecht, The Netherlands.

Table 1

Conditions for analysis of monoamines (NA,DA, 5-HT) and metabolites	
HPLC	ALEXYS Neurotransmitter Analyzer
Oven temperature	38 °C (separation and detection)
Injection method	5 µL per column
ASSAY 1 (MPHG DOPAC, 5-HIAA, HVA)	
Flow rate	100 µL/min, pressure about 420 bar
Flow cell	Sencell 2 mm GC, sb, spacing 0.5
Column	Acquity HSS T3 1 x100 mm, 1.8 um + pre-filter
ADF™	off
Range	10 nA/V
Ecell	800 mV
Icell	0.9 nA
Mobile phase	50 mM phosphoric acid, 0.1 mM EDTA, pH 3.0, 10 % ACN
ASSAY 2 (NA, DA and 5-HT)	
Flow rate	100 µL/min, pressure about 420 bar
Flow cell	Sencell 2 mm GC, sb, spacing 0.5
Column	Acquity HSS T3 1.0x100 mm, 1.8 um
ADF™	Off
Range	1 nA/V
Ecell	460 mV
Icell	0.6 nA
Mobile phase	200 mM Acetic Acid, 0.1 mM EDTA, 300 mg/L DSA, pH 5.5, 14 % ACN

LOD: 80 pM NA (0.40 fmol), 70 pM DA (0.35 fmol), 100 pM 5-HT (0.50 fmol), 169 pM DOPAC (0.85 fmol), 69 pM 5-HIAA (0.34 fmol), 208 pM HVA (1.04 fmol)

Analysis of microdialysis samples

The matrix of microdialysate samples typically consist of the Ringer solution or artificial cerebrospinal fluid (aCSF) that is used for dialysis. The main constituent of these solutions is NaCl in a concentration of almost 0.15 mol/L.

Monoamines and some metabolites are not stabile in these solu-tions and break down rapidly. Adding a few microliters of concen-trated preservative mix or acid to each collected fraction can prevent this. Care must be taken that the added preservative is not interfering with the chromatographic analysis later on.

Good results have been obtained using 1:4 addition of 0.1 mol/L acetic acid to samples in Ringer. Adding a high concentration of perchloric acid (PCA) sometimes interferes with chromatography and causes deformation of peaks.

The results shown in this note are based on the analysis of stand-ards in Ringer solution, acidified with acetic acid (final concentra-tion 10 mmol/L HAc) unless mentioned otherwise. In case of UHPLC additional care must be taken that the sample does not contain small particles. Centrifugation or filtration is required in such case to avoid clogging of the column. To illustrate the applicability of the method a microdialysis fraction of basal level rat Prefrontal Cortex (PFC) has been analyzed (Fig. 5). Depending on the sample composition a little tuning might be required to get a bit more retention for NA. In case only DA and 5HT are of interest, the time of analysis can be decreased further.

The concentrations of monoamines in the microdialysis fraction are 0.24 (NA), 0.21 (DA), and 0.12 nmol/L (5HT). The concentra-tions of metabolites are 2.6 (DOPAC), 36.1 (5-HIAA), and 7.8 nmol/L (HVA).

Under these conditions, the calculated detection limits in pmol/L are (brackets: amounts in fmol) for monoamines NA 80 (0.40), DA 70 (0.35), 5-HT 100 (0.50) and for the metabolites DOPAC 169 (0.85), 5-HIAA 69 (0.34), HVA 208 (1.04).

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.

The application for Monoamines and their Metabolites in microdialysates is robust and suitable for routine based analyzes. Optimized method files are developed e.g. to prevent sample loss, to minimize retention times without compromises on the sensitivity and to inject samples volumes of 2-10 uL. Typical detection limits of 50 pmol/L are feasible.

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

Alexys Neurotransmitter Analyzer for Monoamines and Metabolites

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8. Antec application note: 220_001 - Attomole detection limits

Ordering number

ALEXYS Neurotransmitter Analyzer for Monoamines single channel	
180.0091U	ALEXYS neurotransmitters, 1 ch
191.0035U	AS 110 autosampler UHPLC cool 6p
180.0502	ALEXYS Monoamines SSC kit
dual channel	
180.0092U	ALEXYS neurotransmitters, 2 ch
191.0041U	AS 110 autosampler UHPLC cool 10p
180.0502	ALEXYS Monoamines SSC kit (2x)

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