NORADRENALINE, DOPAMINE, SEROTONIN AND METABOLITES IN MICRODIALYSATE

THE SMARTEST LC-EC APPLICATIONS FOR NEUROSCIENCE ANALYSIS EVER MASTERMINDED

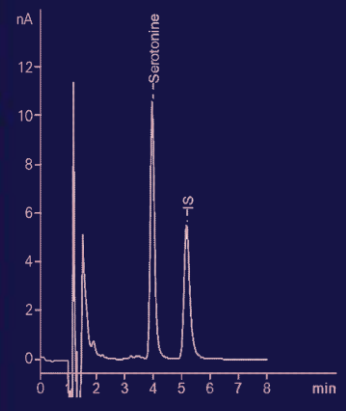
Monoamines and the metabolites
- *Noradrenaline*
- *Dopamine*
- *Serotonin*
- *5-hydroxyindole acetic acid (5-HIAA)*
- *3,4-dihydroxyphenylacetic acid (DOPAC)*
- *homovanillic acid (HVA)*

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

Microdialysis of neurotransmitters in vivo has become an invaluable tool to study neurotransmission in living brain. Cerebrospinal fluid of the brain of conscious animals is sampled trough a microdialysis device and analyzed by HPLC with electrochemical detection. Neurotransmitter concentrations are often below the nanomolar and sometimes even below picomolar range. The sample volume is limited by the dialysis flow rate (0.5 – 2 µL/min) and the temporal resolution required. Multiple components analysis without compromising speed of analysis, and picomolar detection limits out of a few microliter sample are the requirements that have been met in the Monoamines Analyzer.

- One injection, two chromatograms
- Two HPLC's for best selectivity of amines and acids
- Dual channel ECD for lowest detection limits
- Multi components analysis, more data with less rodents
- Increased sample throughput

Summary

ALEXYS Monoamines Analyzer

A method is presented for analysis of monoamines and metabolites in microdialysates and tissue homogenates. The method is optimised for small sample volumes (3 or 5 µL injection) with a detection limit below 50 pmol/L for the biogenic amines.

By means of a dual loop 10-port valve a single injection is loaded on two LC-flow paths simultaneously. This concept was applied to the analysis of noradrenaline (NA), dopamine (DA), serotonin (5-HT) and their metabolites. Chromatography is optimized for fast eluting NA, as well as late eluting 5-HT and acidic metabolites. Optimizing ECD parameters in each flow path resulted in best selectivity and sensitivity for the different components. Especially for the volatile 5-HT speeding up the analysis time was necessary to obtain acceptable detection limits.

Filling two sample loops during one injection event minimizes sample use and running two analyses in parallel results in an increased sample throughput.

Fig. 1. ALEXYS Monoamines Analyzer

Method

Method development for monoamine analysis is facing a number of challenging and sometimes contradictory requirements. Detection limit for NA, DA and 5-HT needs to be less than 100 pmol/L and the available sample volume is limited because of the low flow rate and collection time in microdialysis.
Multiple components need to be analysed without compromising speed of analysis. In particular the combination of the strongly retained 5-HT and the almost unretained NA makes quantitative analysis in a reasonable time frame almost impossible.
To meet all requirements for sensitivity, selectivity and speed of analysis a two dimensional system has been configured. The system consists of two completely independent HPLC's that share the same dual loop autosampler and a dual channel electrochemical detector. Detection parameters and HPLC conditions were all optimized for each channel individually.
Not only the modifier percentage and detection potential but also the concentration ion-pair reagent and pH are crucial parameters that were investigated for optimisation of selectivity and detection limits.
Careful adjustment of these parameters is required, as it will be impossible to obtain picomolar sensitivity in dialysis samples without prior optimisation of selectivity.
Assay 1 has been optimized for DA and 5-HT, and Assay 2 for NA and the acidic metabolites 5-hydroxyindole acetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA).

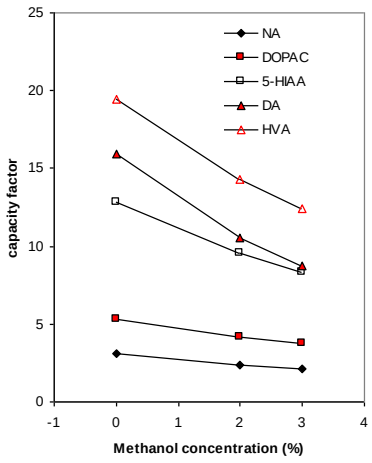


Fig. 2. Capacity factors of all substances are affected by methanol percentage.

Modifier

The percentage modifier will typically affect retention of all sample components. Increasing the percentage modifier will decrease retention times (Fig.1). Acetonitrile [3] and methanol [1-4] are often used, sometimes in combination. We have a preference for methanol as in our experience certain qualities of acetonitrile may result in electrode contamination. The use of tetrahydrofuran (THF) is not recommended because it will lead to an unacceptable high background current.

Ion-pair reagent

An ion pairing reagent, such as hexyl-, octyl- or dodecylsulfate is often added to the mobile phase to selectively increase retention of amines. The apolar carbon chain from the ion paring reagent will adsorb strongly to a stationary reversed phase. The sulfonic acid group is forming a pseudo-stationary cation exchange phase. Below pH 7 the amine group of the catecholamines will be fully protonated and retained by complex formation with the sulfonic acid (Fig. 3). The neutral and acid metabolites however are not affected [1].

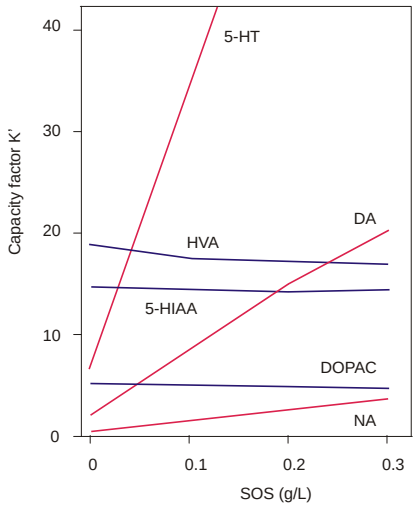


Fig. 3. Capacity factors at different ion pair concentrations of sodium octylsulfate (SOS).

Although in ion chromatography the concentration of the counter ion (Na^+) will affect the retention [1], it is not commonly used as parameter for optimisation in ion pair chromatography of biogenic amines.

pH

The retention times of acid metabolites HVA, 5-HIAA and DOPAC decrease at high pH (Fig. 4). In fact, at pH 6 these metabolites elute in the unretained front peak.

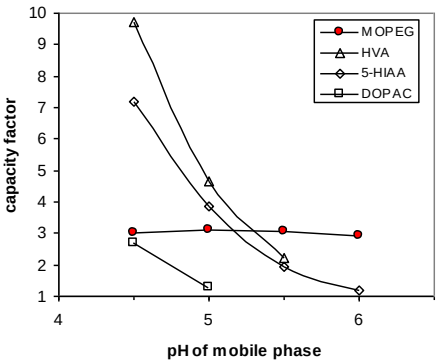


Fig. 4. Capacity factors of acid metabolites strongly decrease at high pH.

Retention of catecholamines is not affected by pH in the range 3-6, however above pH 6 the retention times of amines increase.

Detection potential

Optimisation of detection potential is one of the first steps in method development (Fig. 5). A number of excellent papers are available reporting voltammetric behaviour of relevant biogenic amines and metabolites [1, 3, 4]. Nagao and Tanimura [3] classified the biogenic amines in four groups depending on their electrochemical behaviour, using a mobile phase at pH 3.6 and a salt bridge AgAgCl reference electrode. These four groups are catechol compounds such as the catecholamines, DOPAC and DOPA (E½ = 380-500 mV), indoles such as 5-HT and 5-HIAA (E½ = 480-520 mV), vanillic compounds such as VMA, HVA and MHPG (E½ = 640-680 mV) and monohydroxyphenols such as tryptophan and tyrosine (E½ = 870 mV). It should be noted that the given values are dependent on pH (about 60 mV per pH unit), mobile phase composition and differences in glassy carbon working electrode material.

Although absolute values are different (smaller) at pH 6 we found the expected relative differences in E½ for DA, 5-HT and HVA.

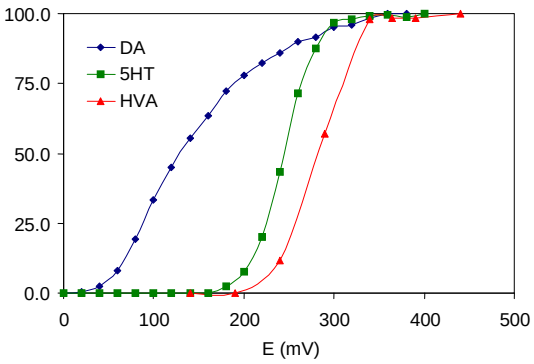


Fig. 5. Hydrodynamic voltammograms of DA, 5-HT and HVA at pH 6.0, reference electrode ISAAC™ (8 mM KCl).

An example how the potential setting can be optimised for best selectivity is shown in Fig. 6. In this example only NA was the target compound of interest therefore pH 6 is used. At 300 mV NA is measured with excellent signal to noise ratio in standard solutions. However, in samples from certain brain areas shouldering peaks appear. By decreasing the working potential to 150 mV these peaks disappear, while NA is still easily measured.

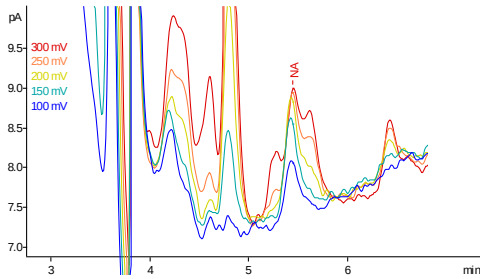


Fig. 6. Optimization of working potential, shouldering peaks near NA disappear below 200 mV. This potential can be applied if only NA is of interest and not the acidic metabolites which require a higher potential.

This also underlines the strength of a dual HPLC approach. Analysis of DA and 5-HT, which requires a higher working potential is not affected as it is done on the other channel.

Matrix effect

When microdialysates are collected for analysis at a later time, precautions are taken to preserve the target compounds. For that reason the samples are usually acidified or an anti-oxidant is added. 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 M acetic acid to samples in Ringer, and addition of less than 1 mM antioxidant [6], final pH 3.2. High concentrations of perchloric acid (PCA) may interfere with chromatography and cause deformation of peaks.

ALEXYS Monoamines Analyzer

A DECADE II™ with dual cell control (DCC) and an autosampler with dual loop 10-port valve is applied. Loading a sample loop will always require a certain flush volume to fill the sampling needle and to discard the diluted front of the aspired sample plug. In case of parallel analysis a single flush volume is used while 2 loops are filled simultaneously. Using 2 pumps and flow cells, the described assays can be run in parallel starting with a single injection.

The acquired data of both channels is stored each in a different location. Also methods files and reprocessing of data is completely separated and optimised for both channels individually.

Minimizing the injected sample consumption is further improved by developing a customized injection. Traditionally, a full loop injection requires that about 3 times the flow path volume is aspired. One injection using 2 injection loops of 3 µL in series and a

needle with 5 µL internal volume would require the aspiration of at least 30 µL sample.

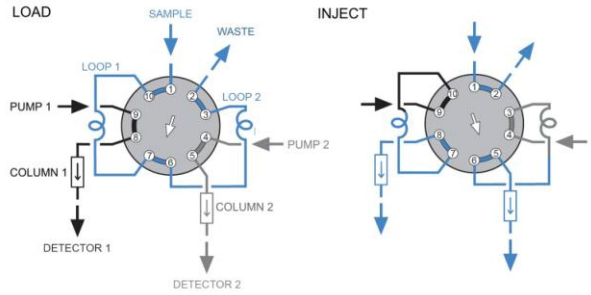


Fig. 7. Schematic configuration of sampling 2 parallel systems with a 10-port valve.

However, by careful tuning of the injection events the consumption of actual sample is minimized, 14 µL of sample is enough to fill both loops of 5 µL. In case 3 µL loops are used, only 8 µL of sample is aspirated. After aspirating this small sample volume, another vial is selected containing water. The plug of sample is carried into the loop by aspirating water, the so called 'transport fluid'. This injection principle is shown in Fig. 8 for a single loop injection.

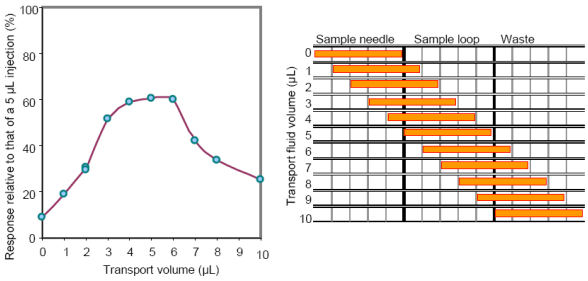


Fig. 8. Left: aspirating 5 µL sample followed by increasing volumes of transport fluid. Right: the aspirated volume of transport liquid determines the position of sample in the loop.

Table 1

Conditions for DA and 5-HT analysis	
HPLC	ALEXYS Monoamine Analyzer
Oven temperature	35 °C (column and detection)
Flow rate	50 µL/min
Flow cell	Micro VT-03 GC / ISAAC
ADF™	0.02 Hz
Range	1 nA/V

Assay 1: DA and 5-HT

For the analysis of DA and 5-HT a mobile phase has been selected with ion-pair reagent and methanol. To avoid interfering peaks from acid metabolites a pH of 6 has been chosen. A column of 50 x 1 mm has been selected for short analysis times and best detection limits. The total run time is less than 7 min. Detection limits are calculated as the concentration resulting in a signal that is 3 times the peak-to-peak noise of the baseline. The limit of detection (LOD) is better than 0.2 fmol for DA and 0.4 fmol for 5-HT. This corresponds to respectively 40 and 80 pmol/L for 5 µL injection.

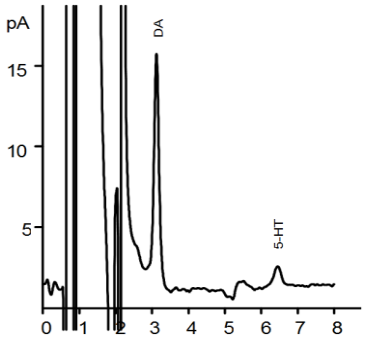


Fig. 9. Analysis of mouse prefrontal cortex microdialysate. With courtesy of Mrs. Gerdien Korte-Bouws, Psychopharmacology, University of Utrecht, The Netherlands.

Assay 2: NA and metabolites

For the analysis of NA, HVA, 5-HIAA and DOPAC a 15 cm column is used. Especially for NA, which elutes close to the front peak it is important to have enough capacity. Also it must be taken into account that peaks of the acidic metabolites are usually much higher than the NA peak. Similar to the DA and 5-HT assay the LOD is better than 0.3 fmol for NA.

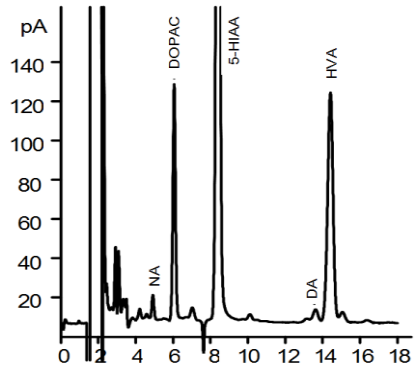


Fig. 10. Analysis of mouse prefrontal cortex microdialysate. With courtesy of Mrs. Gerdien Korte-Bouws, Psychopharmacology, University of Utrecht, The Netherlands.

Table 2

Conditions for the analysis of NA, HVA, 5-HIAA and DOPAC.

HPLC	ALEXYS Monoamine Analyzer
Oven temperature	35 °C (column and detection)
Flow rate	50 µL/min
Flow cell	Micro VT-03 GC / ISAAC
ADF™	0.02 Hz
Range	1 nA/V

Reproducibility and linearity

Reproducibility was studied for eight injections of a 1 nmol/L NA and DA standard. Relative standard deviation (RSD) in retention time is 0.1%, and RSD in peak height is 3% on both assays.
At a concentration of 10 nmol/L RSD values for peak height and area are 1.5% or better.
The linearity of DA and NA was investigated in the range of 0.25 - 2 nmol/L (0.25 nmol/L steps). Correlation coefficients of 0.998 – 0.999 were found for peak heights and areas in both assays.

Customization

The ALEXYS Monoamines Analyzer has proven to be a flexible approach and has been customized depending on user requirements. An example is shown in Fig. 11: with slightly different conditions also L-DOPA, DOPAC and 3-OMD could be analyzed in assay 2.

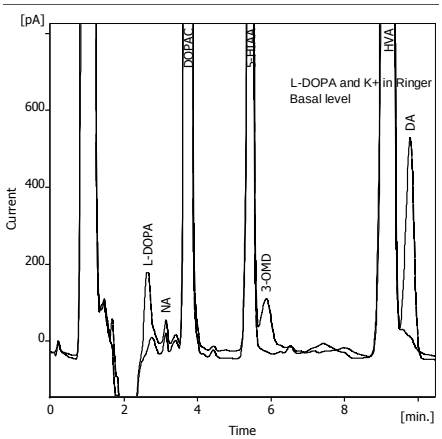


Fig. 11. Analysis of microdialysates with L-DOPA, DOPAC and 3-OMD in addition to the other target compounds.

For the analysis of brain homogenates also MHPG is analysed, together with 3, 4-DHBA and 5-HMT as internal standards. DHBA is also seen in assay 1 (Fig. 12).

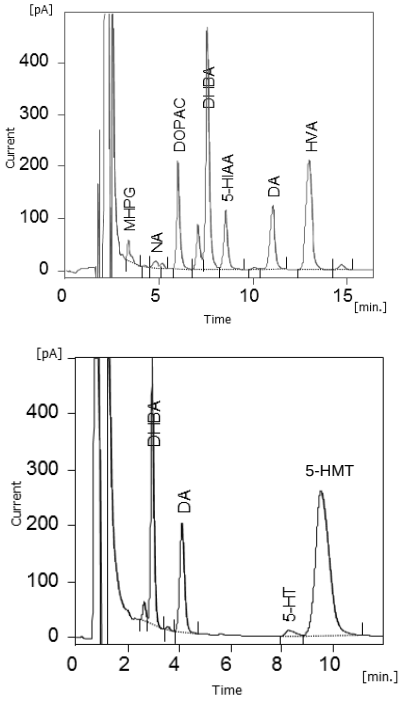


Fig. 12. Analysis of brain homogenates including MHPG and DHBA (internal standard).

In case there is no interest in the acidic metabolites, but only the set of NA, DA and 5-HT, Assay 2 can be adjusted for the selective analysis of NA only. In that case, the total analysis time per injection is 10 min (Fig. 13).

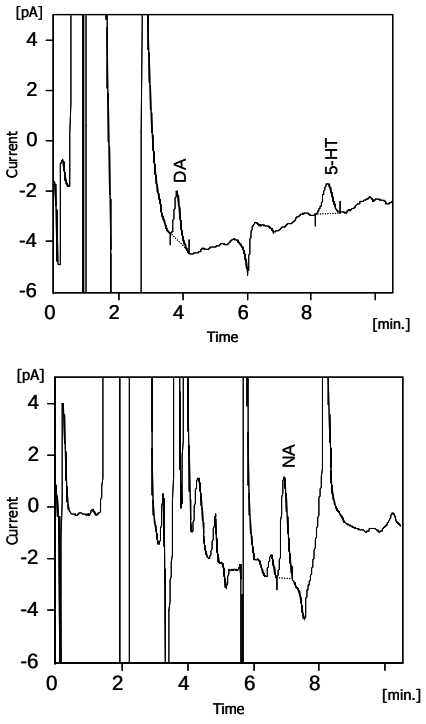


Fig. 13. Analysis of microdialysates in rat prefrontal cortex. Only NA, DA and 5-HT were target compounds.

CONCLUSION

ALEXYS monoamine analyzer using a multi-channel approach is a proven concept for neurotransmitter analysis.
Unique features of the ALEXYS Analyzers are:

- **Fast analysis times, without compromising chromatographic resolution.**
- **Superior detection sensitivity, optimized conditions for each channel separately.**
- **Optimized for small samples, using customized injection programming.**
- **Efficient system solution, using less rodents to get more information.**

References

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Fig. 14. ALEXYS Monoamines Analyzer.

PART NUMBERS AND CONFIGURATIONS	
180.0088B	ALEXYS Monoamines Analyzer