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Noradrenaline, dopamine and serotonin in microdialysates

Introduction

An ALEXYS® 100 system solution has been developed for the trace analysis of noradrenaline (NA), dopamine (DA) and serotonin (5-HT) in microdialysates. By using column switching, the NA peak can be well resolved from the solvent front over two serially connected columns, while 5-HT elutes over one column only. This reduces the retention of the late eluting 5-HT considerably, resulting in a total analysis time of 15 minutes only.
Sample volumes as small as 3 µL were analysed, with a detection limit of 100 pmol/L (300 attomol on column). Injecting 6 µL resulted in a detection limit of 50 pmol/L (also 300 attomol).

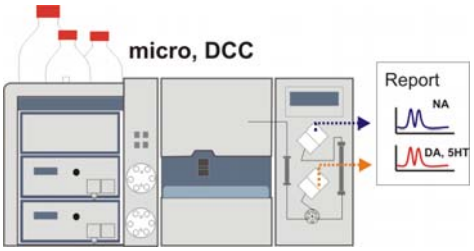


Fig. 1. Configuration for the analysis of microdialysates using a DECADE II™ DCC with two cells. NA is resolved from the solvent front using 2 columns in series, while retention time of 5-HT is strongly reduced by the use of column switching.

Experimental

The ALEXYS 100 LC-EC system configuration is shown in figure 1. A DECADE II electrochemical detector equipped with two micro VT-03 electrochemical flow cells with 0.7 mm diameter glassy carbon electrode and 25 µm spacer were used for all experiments. A potential of 150 mV (cell 1) and 300 mV (cell 2) versus in situ Ag/AgCl reference electrode (8 mM KCl in mobile phase) was applied in all experiments.

Table 1. Experimental conditions

HPLC system	ALEXYS 100 micro LC-EC system (180.0081)
Flow rate	50 µL/min
T_{oven}	35 °C (separation and detection)
$V_{injection}$	5 µL (unless stated otherwise)
Cell 1, 2	VT-03 cell with 0.7 mm GC electrode, ISAAC™ reference electrode, 25 µm spacer
Range	500 pA/V
ADF™ 1, 2	0.1 Hz, 0.05 Hz

The concept of column switching is shown in Fig. 2. NA elutes over a 5 and 15 cm column connected in series. When NA arrives on column 2, the valve switches and redirects the flow from column 1 directly to flow cell 2, thus by-passing the second column.

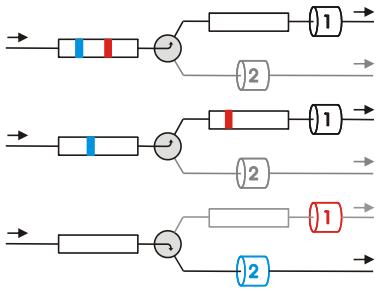


Fig. 2. NA (red) is resolved from the front of the chromatogram and detected in cell 1. The run time of late eluting 5-HT (blue) is shortened by column switching followed by detection in cell 2. For more details see Fig. 3.

Column switching

The system consists of 2 pumps, 2 columns, 2 cells and a switching valve (Fig. 3). To avoid large pressure drops after valve switching, two restrictors are applied. In fact restrictor 105 balances the pressure drop over column 105, and the same holds for column and restrictor 115. Both pumps have the same mobile phase and run at the same flow rate (50 µL/min). A similar set-up has been published elsewhere [3]. Balancing the pressure in the system is not only necessary to improve the column lifetime. It also improves the baseline stability and reproducibility of the analysis. To balance the pressure of the different flow channels the following procedure is executed:

1. Measurement of the back pressure of both LC channels (pump 1 and 2) with the CS valve in position A.
2. Measurement of the back pressure of both LC channels with the CS valve in position B.
3. Calculation of the length of tubing to be removed of restrictor 105 and 115 to balance the back pressure over the different LC channels.
4. Subsequently the calculated length of tubing is removed from the pressure restrictors using a tubing cutter. The resulting maximum pressure drop during column switching should not exceed 1 bar.

A detailed description of the pressure balancing procedure can be found in manual 180.7018 [4] .

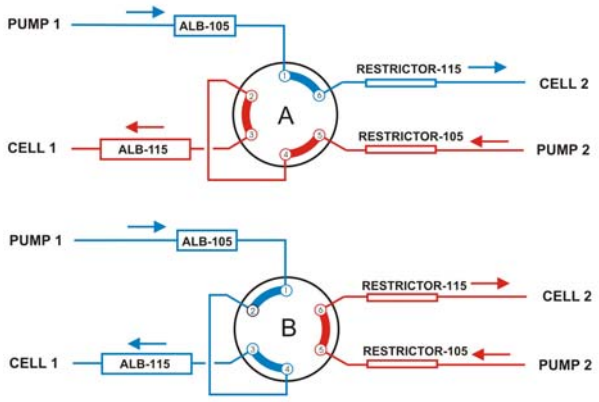


Fig. 3. The two valve positions for column switching. Position A: 5 and 15 cm column in parallel, Position B: 5 + 15 cm column in series, (P_{red} = P_{blue}).

Results

Not only detection sensitivity but also limited sample volume and temporal resolution are key issues in trace analysis of microdialysates. Concentration of neurotransmitters in microdialysates are often below the nanomolar and sometimes even below picomolar concentration range. Available sample volume is typically 5 – 50 µL depending on the microdialysis flow rate and required temporal resolution.
Optimisation of the system was done in a two step approach.

- Firstly, the system was optimised with respect to detector settings, minimum injection volume and detection limits using a single column (Table 2).
- Secondly, the column switching method was optimised using a dual column set-up with respect to resolution, analysis time, detection parameters, reproducibility and detection limit.

Table 2. Experimental conditions with single column set-up for system optimisation.

HPLC system	ALEXYS 100 micro LC-EC system (180.0081)
Flow rate	50 µL/min
T_{oven}	35 °C (separation and detection)
$V_{injection}$	5 µL
Cell	VT-03 cell with 0.7 mm GC electrode, ISAAC, 25 µm spacer
Range	500 pA/V
ADF	0.1 Hz

Voltammogram of standards

A hydrodynamic voltammogram has been constructed to find the optimum working potential for the detection of NA, DA and 5-HT. In the dual column setup also two flow cells are applied. Typically, one cell is used for the detection of 5-HT and the other for NA. An additional advantage of this setup is that a different optimum detection potential can be applied for each cell. The IE curve for NA, DA and 5-HT shows differences in optimal working potential. At 150 mV both NA and DA show a signal at respectively 60 and 80% of the maximum. The peak of 5-HT however, is not visible at this potential and reaches a maximum at 300 mV.

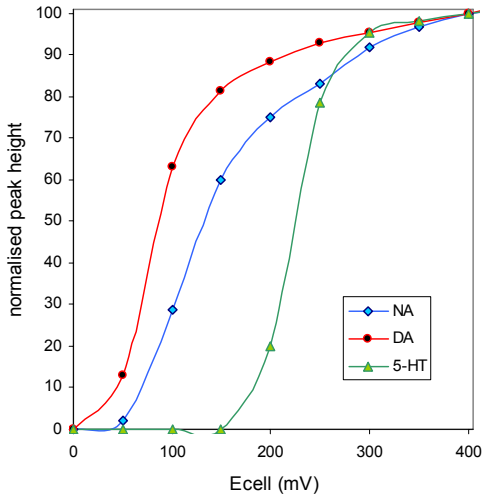


Fig. 4. Voltammograms of 10 nM NA, DA and 5-HT. For conditions see Table 1.

Based on the information in Fig. 4 the optimum potential for NA and DA is 200-250 mV, and 300-350 mV for 5-HT.
However, the data were obtained from standards. Additional information regarding selectivity is required for the analysis of real,samples with complex matrices.

Voltammogram of microdialysates

There is no sensitivity without selectivity. In other words, a pure standard can be analysed at 100 pmol/L detection limit, but when the peak co-elutes with another interfering peak in the sample it will be impossible to quantify at this low concentration in real samples. Improving the selectivity by optimising the working potential can be realised in cases where the half-wave potential (E½) of the interfering peak is higher than the E½ of the analyte of interest. In that case the selectivity can be improved by selecting a lower working potential.

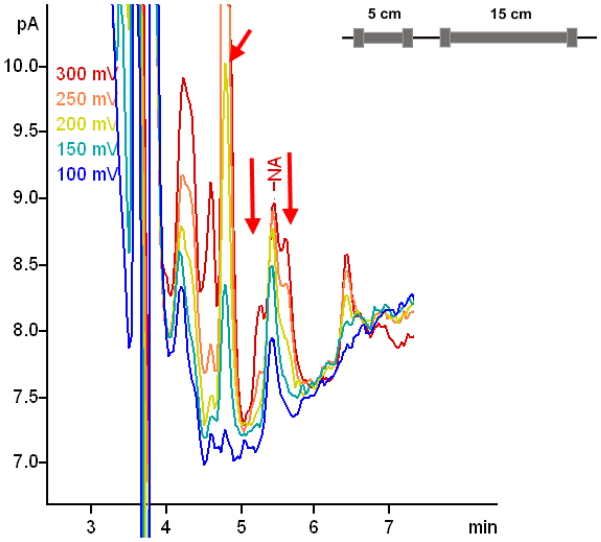


Fig. 5. Microdialysate analysed at different working potentials. Peaks to the left and right of NA disappear at lower working potential (arrows), thus improving the detection limit.

Improvement of selectivity for NA is evident in Fig. 5. Decreasing the working potential to 150 mV resulted in a 30% decrease in peak height for NA, but selectivity and separation from co-eluting peaks improved considerably. Also several peaks in the front of the chromatogram are considerably smaller, which creates a possibility to decrease total analysis time by adding a higher modifier percentage.
The signal of 5-HT showed a significant decrease between 250 and 200 mV (Fig. 4 and Fig. 6). Therefore, the working potential of this flow cell should be kept at 300 mV.

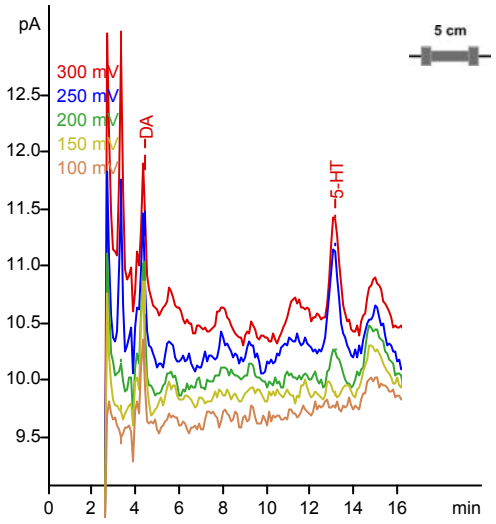


Fig. 6. Microdialysate spiked with DA and 5-HT analysed at different working potentials. S/N ratio for 5-HT does not improve at lower potential and is best at 300 mV.

Loadability

One of the parameters affecting the detection limit is the injection volume. Typically, increasing the injection volume will result in higher peaks and a lower concentration detection limit. Increasing the injection volume further will eventually lead to a poor separation: peaks only get wider, not higher. Assuming that a 5% increase in peak width is acceptable the maximum injection volume can be estimated based on the retention time, flow rate and peak efficiency [2]:

$$Vi = 1.1\ Vr / \sqrt{N} = 1.1 * tr * F / \sqrt{N}$$

For example, with a flow rate of 50 µL/min, a retention time of 5 min and a plate number of 4500, the estimated maximum allowable injection volume is 4 µL.
In Fig. 7 the loadability is shown for the 150 x 1 mm ID column with 2-10 uL injections. As can be seen, peak area and peak height increase linearly with injection volume without any indication of overloading.
We found that the improved loadability is related to the mobile phase composition (Fig. 9). When adding MeOH with OSA to the mobile phase the loadability is considerably higher (up to 20 µL) than expected. Without these additives we found a loadability of about 4 µL which is in correspondence with the calculated value.

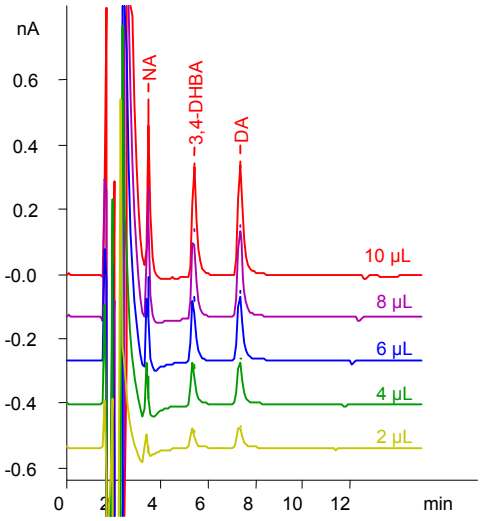


Fig. 7. Analysis of 10 nM NA, 3,4-DHBA and DA standards in 0.01 M PCA using a 150 x 1 mm column. Injection volume varied from 2 µL (bottom) to 10 µL (top).

The peak height and plate numbers of the chromatograms in Fig. 7 are shown in Fig. 8. The results show that the loadability of the 1 mm ID columns is sufficient for the analysis of microdialysis samples. In principle, up to 10 µL sample can be injected without significant peak broadening. Provided that selectivity remains the same, these results also show that not only peak heights but also the minimum detectable concentration (MDC) will improve linearly with injection volume.
In other words, an MDC of 100 pmol/L (300 attomol on column) with 3 µL injection volume will improve to an MDC of 50 pmol/L (also 300 attomol) when injecting 6 µL. In practice however, the amount of sample available in microdialysates will be the limiting factor.

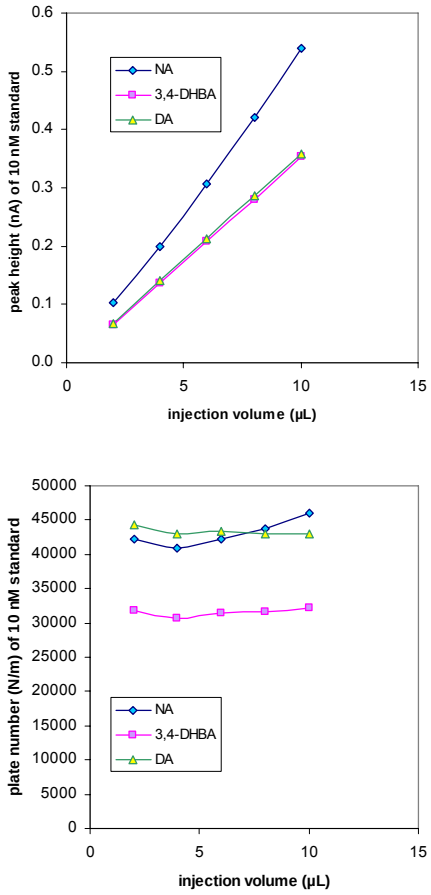


Fig. 8. Loadability of 10 nM NA, 3,4-DHBA and DA on a 150 x 1 mm column. Peak height (upper graph) and plate number versus injection volume.

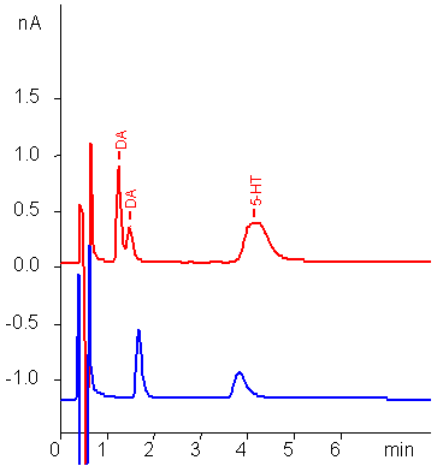


Fig. 9. Chromatograms of 6 µL injections of 100 nM DA and 5-HT, using 10% MeOH and 100 mg/mL OSA (bottom trace) and without MeOH or OSA (upper trace). Note that DA has two peaks because of overloading (upper trace).

Optimisation of injection method

In microdialysate analysis often only a small sample volume is available for injection. Therefore, a user program (UP) injection method was developed and evaluated to minimize the total sample loss per injection.

Method programming with the AS 100 is used to pick-up a well-defined sample volume and to transport this to the sample loop quantitatively with minimum peak broadening.
By switching the valve to Load the diluted front is cut off. The non-diluted sample segment is loaded into the loop and injected onto the column. This method is described in detail elsewhere [1].
For example a method designated as UP514, means that 5 uL sample is aspirated into the sample needle, with the valve in the inject position. This step is followed by the aspiration of1 uL transport liquid. Subsequently, the valve is switched into the load position, and another 4 µL transport liquid is aspirated. So effectively 4 µL of sample enters the loop and will be injected onto the column.
In Fig. 10, the results are shown for the UP51x and UP52x method. It is evident from Fig 11 that the peak heights for the 52x method are somewhat higher for injection volumes smaller then 4 µL. This is caused by the fact that the diluted solvent front passes the valve for 1 more µL compared to the 51x method. As a result a more concentrated part of the sample is injected. However, at injection volumes of 4 µL and higher the diluted tail of the sample plug enters the loop resulting in sample dilution and a decrease in peak height. Furthermore, increasing the injection volume leads to a smaller plate number. Method UP514 can be considered an optimal compromise between peak height and plate number.

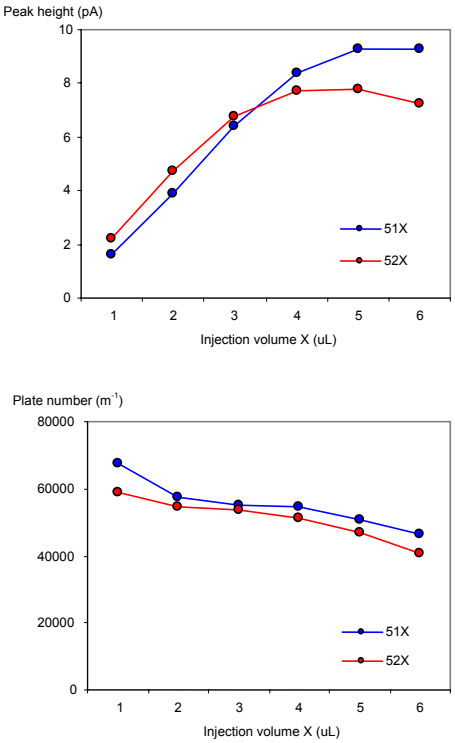


Fig. 10. Injection with AS 100 user program. Method UP514 is an optimum, a compromise between peak height and plate number. Details are described elsewhere [1].

Valve switching

To resolve NA from the front peaks, it must be analysed with two columns in series to obtain sufficient retention. For the rapid analysis of 5-HT, retention over the short 5 cm column is

required. DA however can be eluted over either the 1 or the 2 column configuration (see Fig. 11), depending on the moment of valve switching.
The choice must be made based not only on analysis time, but also on chromatographic resolution and detection performance. The single column configuration uses a cell at 300 mV (required for 5-HT), and the smaller column results in less peak dilution and a better detection limit for DA. The serial configuration uses a cell at 150 mV and will result in more retention for DA. This can be used as alternative in case of interfering peaks in the other configuration.
Note that valve switching disturbs the baseline in both cells, though at cell 1 the disturbance is very subtle. Therefore, it is not recommended to let the valve switching moment coincide with the time that NA elutes to avoid peak distortion. The chromatograms show that the baseline in Cell 2 is stable within half a minute.

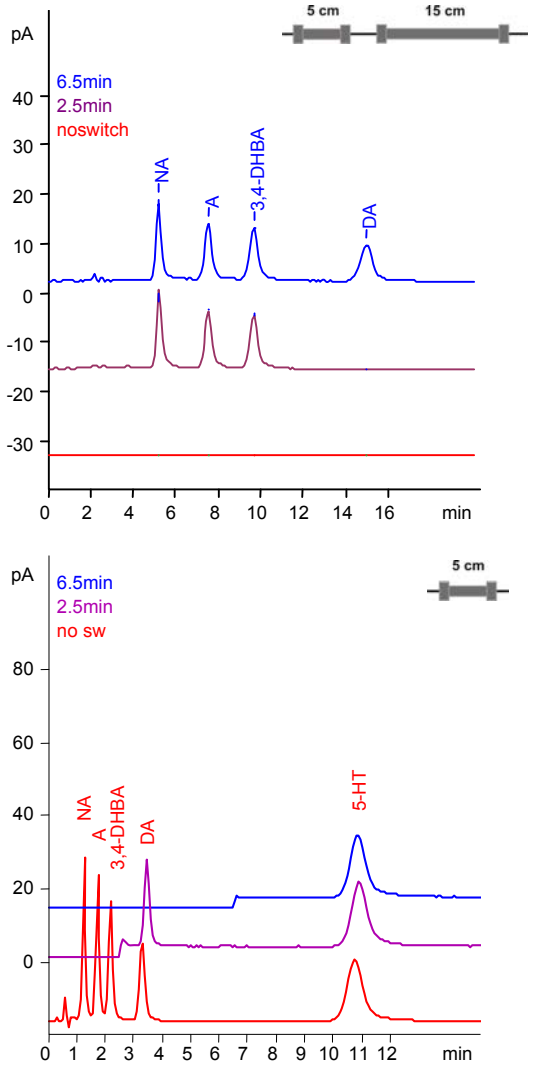


Fig. 11. Analysis of 10 nM of NA, A, 3,4-DHBA, DA and 5-HT in mobile phase using column switching and 2 flow cells (cell 1 top, cell 2 lower chromatogram). Traces: no valve switching (lower, red traces), switching at t=2.5 min (middle, purple traces) and switching at t=6.5 min (upper, blue traces).

Reproducibility and LOD

The reproducibility of the column switching method has been analysed by multiple injections (n=9) of a standard solution of 1 nM NA, DA, and 5-HT. Injection method 'UP523' was used, which injects 3 µL sample on column with a total sample use of 5 µL.

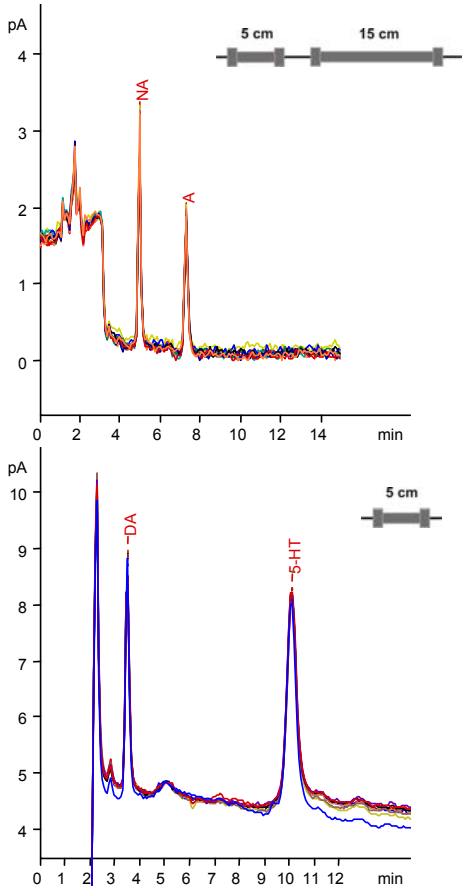


Fig. 12. Analysis of 1 nM of NA, A, DA and 5-HT in mobile phase using column switching. Conditions according to Table 1.

Reproducibility results are given in Table 3. RSD for peak heights are about 1 - 2 %, for peak areas about 2 - 3 % and for retention times 0.1 % or better. With an injection volume of 3 uL, the detection limit is about 100 pM for NA, DA and 5-HT.

Table 3. Reproducibility (n=9) of 3 µL injections of a 1 nM of NA, A, DA and 5-HT solution using method 'UP523'.

	H		A		tr	
	(pA)	%RSD	(pA*sec)	%RSD	(min)	%RSD
NA	3.0	1.2	27	3.1	4.96	0.05
A	1.8	1.9	22	2.8	7.28	0.05
DA	4.2	0.7	44	1.8	3.50	0.10
5-HT	3.8	1.1	104	3.2	10.10	0.08

Microdialysate analysis

Microdialysates collected from rat brain were analysed using micro-vials. User program UP523 was used for injection.

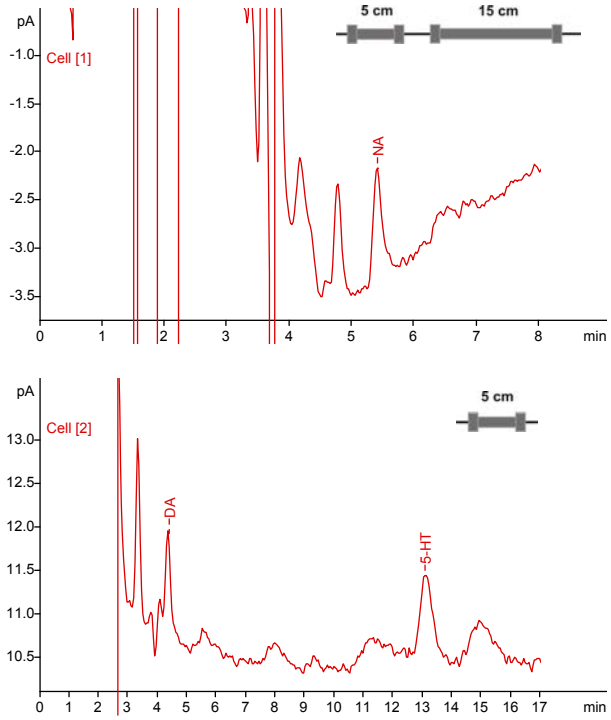
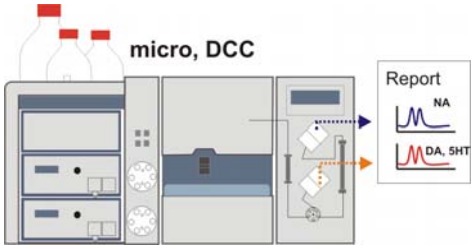


Fig. 13. Analysis of 3 uL microdialysate out of a total sample aspiration volume of 5 uL. Concentrations are 400 pmol/L for NA (base level), and 250 pmol/L for DA and 5-HT (spiked).

Part numbers and configuration

Part no.	Description
180.0081	ALEXYS 100 'Microdialysis VII' noradrenaline, dopamine and serotonin
250.1096	ALB-115, 150x1 mm ID, 3 µm, C18
250.1097	ALB-105, 50x1 mm ID, 3 µm, C18



Conclusion

ALEXYS 100 'Microdialysis VII' has been developed for the analysis of noradrenaline, dopamine and serotonin in microdialysates. Sample volumes as small as 3 µL were analysed with a detection limit of 100 pmol/L (300 attomol). Injecting 6 µL resulted in a detection limit of 50 pmol/L (also 300 attomol).

References

1. Antec Leyden technical note 220_011, *Micro volume injection with the ALEXYS AS 100*
2. *Liquid Chromatography for the Analyst*, by Raymond P. W. Scott, Marcel Dekker (January 17, 1994)
3. Antec Leyden application note 213_019, *GABA and glutamate*
4. Antec Leyden, *Installation guide LC connection kit DCC I-I CS, document* 180.7018, (2006) 23 - 29