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Serotonin, noradrenaline, dopamine and metabolites in microdialysates

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 analysed by HPLC with electrochemical detection. Neurotransmitter concentrations are often below the nanomolar and sometimes even below picomolar concentration range. The sample volume is usually limited by the dialysis flow rate (0.5 – 2 µL/min) and the temporal resolution required. Available sample volume is typically between 1 – 15 µL.
Specific requirements for neurotransmitter analysis are investigated and relevant assay parameters for HPLC-ECD method development are presented.

Method

The method was optimised for analysis of noradrenaline (NA), dopamine (DA), serotonin (5-HT) and the metabolites 5-hydroxyindole acetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA).
In particular the combination of the strongly retained 5-HT and noradrenaline, which elutes closely to the front peak, makes quantitative analysis in a reasonable timeframe almost impossible. Therefore, samples were split and analysed using two different methods. One method for **dopamine and serotonin (I)**, and another method for **noradrenaline, dopamine and metabolites (II).** Not only the modifier content 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.

Modifier

The percentage modifier will typically affect retention of all sample components. Increasing the percentage modifier will decrease retention times (Fig. 1). Acetonitrile [1] and methanol [2, 3] 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.

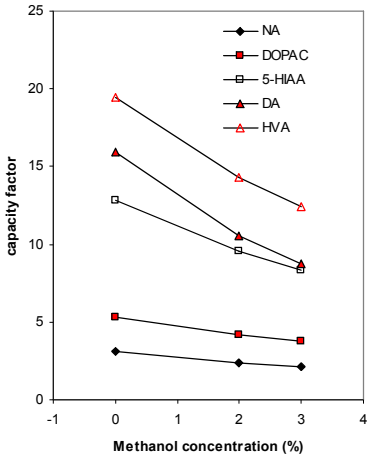

Fig. 1. Capacity factors of all substances are affected by methanol percentage.

Ion-pair reagent

An ion pairing reagent, such as hexyl-, octyl- or dodecylsulfate is often added to the mobile phase to increase retention of amines. The apolar carbon chain will adsorb strongly to a reversed phase. The sulfonic acid group is forming a pseudo-stationary cation exchange phase. Below pH 7 the amine of the catecholamines will be fully protonated and retained by complex formation with the sulfonic acid (Fig. 2). The neutral and acid metabolites however are not affected [3].

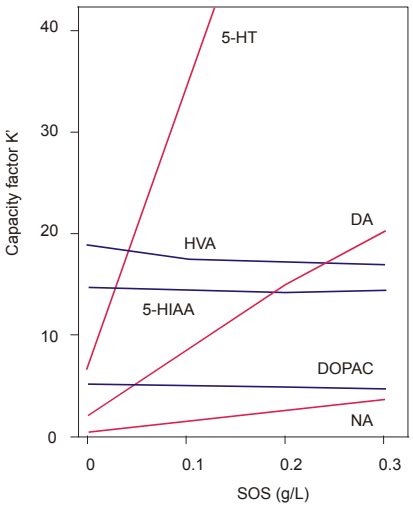

Fig. 2. Capacity factors at different ion pair concentrations of sodium octylsulfate (SOS).

Although in ion chromatography the concentration of the counter ion (Na^+) will affect the retention [5], 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. 3). In fact, at pH 6 these metabolites elute in the un-retained front peak.

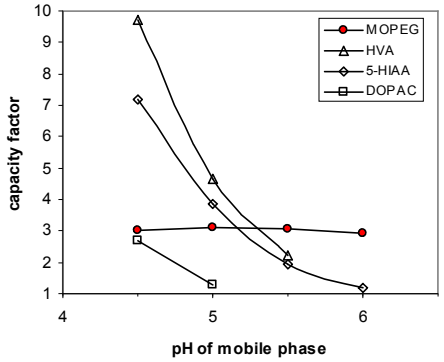

Fig. 3. Capacity factors of acid metabolites strongly decrease at high pH.

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

Detection potential

Optimisation of detection potential is one of the first steps in method development (Fig. 4). A number of excellent papers are available reporting voltammetric behaviour of relevant biogenic amines and metabolites [1-3]. Nagao and Tanimura [1] 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 monohydroxy-phenols 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.

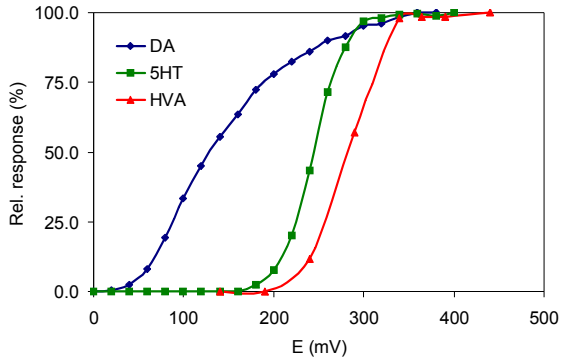

Fig. 4. Hydrodynamic voltammograms of DA and 5-HT. pH of mobile phase: 6.0, reference electrode ISAAC™ (8 mM KCl).

I. Dopamine and serotonin

For the analysis of dopamine and serotonin a simple mobile phase has been selected without ion-pair reagent or methanol (Table 1). To avoid interfering peaks from acid metabolites a pH of 6 has been chosen. No ion pairing reagent was used which further discriminates peaks of amine compounds. Both substances of interest could be separated easily with enough retention. Column length is 5 cm for short analysis times. The total run time is less than 5 min.

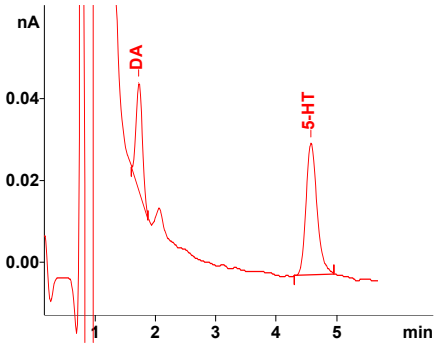

Fig. 5. Chromatogram of 5 µL injection of 1 nM DA and 5-HT standards in 0.01 M PCA. Conditions: Table 1.

Table 1. Conditions for DA and 5-HT analysis, without ion-pair reagent.

HPLC	ALEXYS® 100 LC EC system
Oven temperature	35 °C (column and detection)
Flow rate	200 µL/min
Flow cell	VT-03 GC 2 mm / ISAAC
ADF™	0.02 Hz
Range	1 nA/V

If the selectivity of the method is not sufficient, another set of HPLC conditions can be used resulting in more retention (Table 2). Ion pairing reagent and methanol must be added to optimise the separation. Under these conditions capacity factors changed from 1.3 and 8 to 2.6 and 16 for DA and 5-HT, respectively.

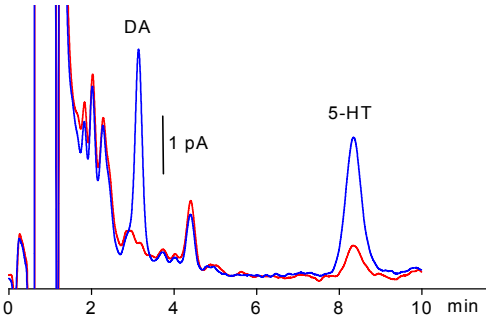

Fig. 6. Pooled frontal cortex microdialysate (red) and microdialysate spiked with 2 nM DA and 5-HT (blue). Conditions: Table 2.

Detection limits were calculated as the concentration resulting in a signal that is 3 times the peak-to-peak noise of the baseline. For DA a Limit of Detection (LOD) of 0.2 fmol (0.1 nmol/L)

was found. For 5-HT the LOD was 0.4 fmol (0.2 nmol/L) respectively.

Table 2. Conditions for DA and 5-HT analysis, with ion-pair reagent.

HPLC	ALEXYS 100 LC EC system
Oven temperature	35 °C (column and detection)
Flow rate	200 µL/min
Flow cell	VT-03 GC 2 mm / ISAAC
ADF	0.02 Hz
Range	0.5 nA/V

Using a 15 cm column will further resolve the DA peak, but will also result in a longer retention time for 5-HT (Fig. 7).

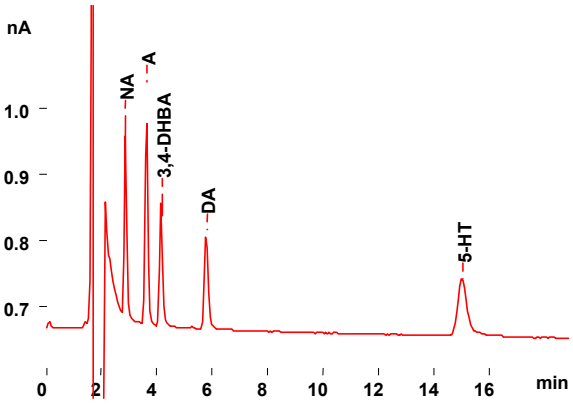

Fig. 7. Analysis of 10 nmol/L NA, A, DHBA, DA, 5-HT standards. Conditions: Table 2, but using a 15 cm column and 5% methanol applied.

II. Noradrenaline, dopamine and metabolites

For the analysis of noradrenaline, dopamine, HVA, 5-HIAA and DOPAC a 15 cm column was used. Especially for noradrenaline, which elutes close to the front peak it is important to have enough selectivity. Also it must be taken into account that peaks of the acid metabolites are usually much higher than NA and DA peaks (see figure 9).

Table 3. Conditions for the analysis of noradrenaline, dopamine, HVA, 5-HIAA and DOPAC.

HPLC	ALEXYS 100 LC EC system
Oven temperature	35 °C (column and detection)
Flow rate	200 µL/min
Flow cell	VT-03 GC 2 mm / ISAAC
ADF	0.02 Hz
Range	1 nA/V

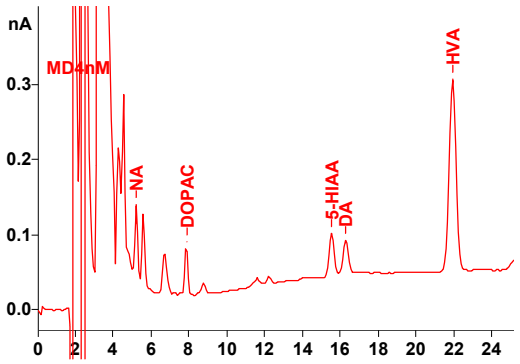

Fig. 8. Analysis of 5 µL frontal cortex microdialysate spiked with 4 nM NA, DOPAC, 5-HIAA, DA and HVA. For conditions see Table 3.

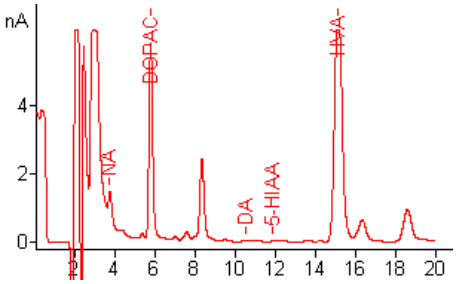

Fig. 9. Analysis of 2 µL microdialysate (striatum). DA and 5-HIAA were not detectable. For conditions see Table 3 with slightly modified methanol % and SOS concentration.

Detection limits were calculated as the concentration resulting in a signal that is 3 times the peak-to-peak noise of the baseline. Similar to the DA and 5-HT assay a concentration detection limit of 0.1 and 0.4 nmol/L was found for all substances. Reproducibility was studied for eight injections of 2 µL of a 1 nM NA and DA standard. Relative standard deviation (RSD) in retention time is 0.1%, and RSD in peak height is 3%.
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.

III. DCC for parallel “2D” analysis

Application of a DECADE II™ with dual cell control (DCC) will improve the sample throughput and minimize sample loss. By using a 10-port valve in combination with 2 pumps and flow cells, the described assays can be run in parallel starting with a single injection (Fig. 10).
Compared to sequential analysis only one additional cell, a LC 100 pump and a 10-port valve (instead of a 6-port valve) is required.

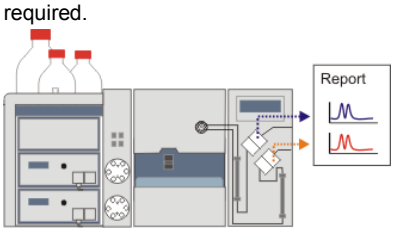

Fig. 10. Schematic representation of DECADE II DCC for simultaneously running both assays.

Not only sample throughput will improve, but also loss of sample will be minimized when using a 10-port valve. Loading the sample loop using the AS 100 autosampler will always require a certain flush volume to fill the sampling needle. In case of parallel analysis the same flush volume is used while 2 loops are filled simultaneously. A schematic configuration is shown in Fig. 11

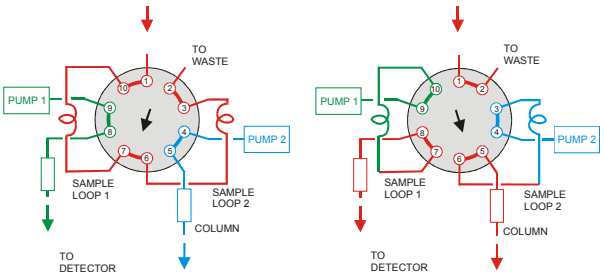

Fig. 11. Schematic configuration of sampling 2 parallel systems with a 10-port valve. Valve position: left-side is 'Load', right-side is 'Inject'.

In the ALEXYS data system the acquired data of both channels can be directed separately to different location. Also methods files and reprocessing of data can be completely separated and optimised for both channels individually.

Conclusions

For the analysis of noradrenaline, dopamine, serotonin and acid metabolites two assays are recommended. One method for **dopamine and serotonin (I),** and another method for **noradrenaline, dopamine and metabolites (II)**.
The effect of modifier content, ion-pair reagent concentration, mobile phase pH, and electrode potential on the analysis of these substances are presented. Detection limits are typically in the 0.1 – 0.4 nmolar range.
To improve the sample throughput and minimise sample loss a DECADE II DCC for parallel "2D" analysis (III) is recommended. Both methods I and II can be run simultaneously by using an autosampler with a 10-port valve.

References

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5. J. P. Crombeen, J. C. Kraak en H. Poppe, *Reversed-phase systems for the analysis of catecholamines and related compounds by HPLC*, J. Chromatogr., **167** (1978) 219-230

Part numbers and configuration

Part no.	Description
180.0060	ALEXYS 100 'Microdialysis I' dopamine and serotonin
180.0062	ALEXYS 100 'Microdialysis II' noradrenaline, dopamine, HVA, 5-HIAA and DOPAC
180.0066	ALEXYS 100 'Microdialysis IV' serotonin, noradrenaline, dopamine, HVA, 5-HIAA and DOPAC using parallel 2D HPLC

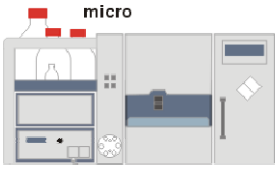

*Fig. 12. **ALEXYS 100 'Microdialysis I'** with 50 x 2 mm column and VT cell. The system has a micro LC 100 pump and micro AS 100 autosampler. **ALEXYS 100 'Microdialysis II'** has a 150 x 2 mm column.*

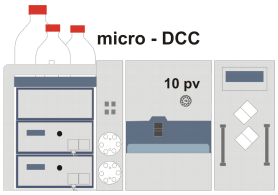

*Fig. 13. **ALEXYS 100 'Microdialysis IV'** with 2 columns and flow cells (5 and 15 cm, see Fig. 12). The system has 2 micro LC 100 pumps, a dual channel OR 100 and a micro AS 100 autosampler with 10-port valve.*