DUAL CELL CONTROL FOR IMPROVED SELECTIVITY

THE SMARTEST LC-EC APPLICATIONS FOR NEUROSCIENCE ANALYSIS EVER MASTERMINDED

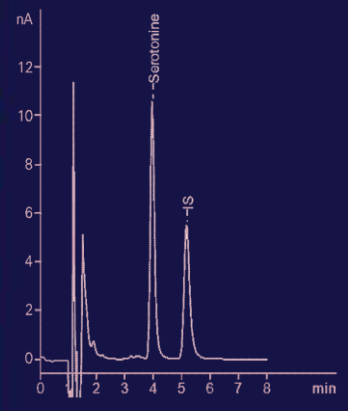
Monoamines and the metabolites
- *Noradrenalin*
- *Dopamine*
- *Serotonin*
- *5-hydroxyindole acetic acid (5-HIAA)*
- *3,4-dihydroxyphenylacetic acid (DOPAC)*
- *homovanillic acid (HVA)*

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

The Dual Cell Control (DCC) option for the DECADE II™ makes it possible to control two electrochemical cells and use both cells for data acquisition. Placing both cells in parallel can be used to improve the sample throughput and minimize sample loss. In another publication we demonstrated the use of a 10-port valve in combination with 2 pumps and flow cells, for analysing one sample in two independent assays in parallel starting with a single injection [1].

- One injection, two chromatograms
- Fully automated OPA derivatization
- Column switch: no late eluting system peaks
- Total analysis time < 25 min (including derivatization)

Summary

Here we describe a **serial** configuration for improvement of selectivity in the analysis of catecholamines and metabolites in microdialysates. Using an ALEXYS® 100 micro LC-EC system with a Reactor™ cell, catecholamines and metabolites are detected by *oxidative* amperometric detection. In addition, substances with reversible reaction properties are subsequently detected by *reductive* amperometric detection in the second cell. For reduction we use a cell potential of only +250 mV resulting in enhanced selectivity.

Fig. 1. ALEXYS GABA and Glu Analyzer

Method

Table 1

Conditions	
HPLC system	ALEXYS 100 micro LC-EC (180.0064 see p.
Flow rate	50 µL/min
Toven	35 °C (separation and detection)
Vinjection	2 uL
First cell (ox)	Reactor cell with GC electrode and Hy-REF
Range	1 nA/V
ADF™	0.02 Hz
Second cell (red)	VT-03 cell with 2 mm GC electrode and
Range	1 nA/V
ADF	0.02 Hz

Experimental

A DECADE II electrochemical detector (Antec Leyden, The Netherlands) equipped with a VT03 electrochemical flow cell (Antec Leyden) with 2 mm diameter glassy carbon electrode and 25 µm spacer was used for all experiments. Effective cell volume determined by electrode area and spacer thickness, was 71 nl. A potential of 540 mV versus Ag/AgCl reference electrode in saturated potassium chloride was applied in all experiments. A reactor cell with glassy carbon electrode and HyREF™ was applied between column and VT03 cell.

Temperature for separation and detection was 35 °C. The HPLC system consisted of a ALEXYS 100 LC-EC system (Fig. 2). Chromatographic column was a ALB-115, 150 x 1 mm (ID), particle size 3 µm. Mobile phase consisted of 50 mmol/L phosphoric acid, 50 mmol/L citric acid, 400mg/L octanesulphonic acid, 0.1 mmol/L EDTA, 8 mmol/L KCl, brought at pH 3.8 with concentrated NaOH. A flow rate of 50 µl/min was applied, injection volume was 2 µL unless specified otherwise.

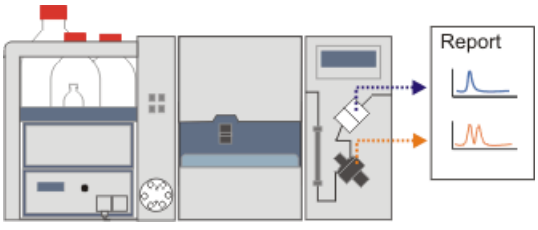


Fig. 2. Configuration for analysis of microdialysates using a DECADE II DCC with two cells in series. An oxidative reactor cell followed by a reductive VT03 cell (ox-red configuration).

Results

Not only detection sensitivity but also the limited sample volume and temporal resolution are key issues in 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 mobile phase pH, ion pairing agent and modifier concentration for separation of catecholamines and metabolites has been described in another publication [1].

In this work we have chosen for microbore HPLC with a 1 mm ID column for two reasons:

- Microbore HPLC is compatible with the small sample volume and we have demonstrated that detection sensitivity is not compromised [2].
- The electrochemical conversion of an amperometric reactor cell is higher at low flow rates which is a pre-requisite for serial red-ox or ox-red configurations.

Voltammogram

Optimisation of cell potential in ox-red configuration is done in two steps:

- First an oxidative voltammogram is constructed using a reactor cell (Fig. 3). As expected, at pH 3.0 the oxidation of noradrenaline and dopamine becomes diffusion-limited at a potential of about 500 – 600 mV (vs. HyREF).
- Secondly, the potential of the second cell is varied from +400 mV down to +100 mV for reduction. The first (oxidative) cell is set at a constant potential of 650 mV during this step.

At pH 3 catecholamines are reduced at a *positive* working potential when applied immediately after oxidation. Polarity of the output has to be switched to '-' to invert the signal, otherwise negative peaks will appear.

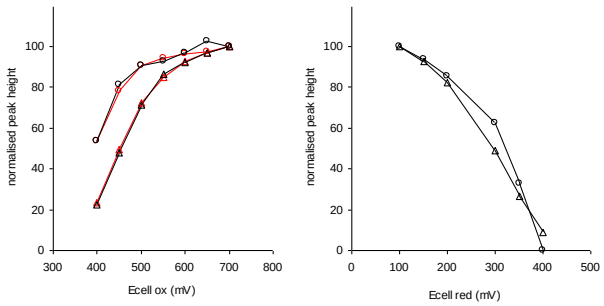


Fig. 3. Voltammogram for NA (△) and DA (○) at pH 3.0, measured in the oxidative reactor cell (left) and the reductive VT03 cell (right). The oxidative voltammogram (left) is measured in the first cell (red), and the second cell (black) and is showing corresponding results.

The optimum reactor cell potential is set at +540 mV (vs. HyREF) and a reductive potential of +250 mV (vs. Ag/AgCl in saturated KCl) for the VT03 cell.

A rather broad peak between 4 – 8 min was observed in the reductive trace when constructing a voltammogram. The peak was larger at more reductive potential (Fig. 4) and originates probably from reduction of oxygen dissolved in the sample.

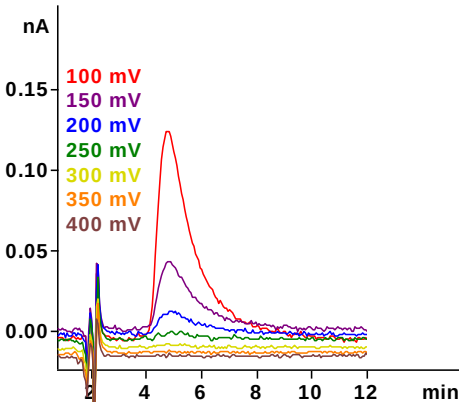


Fig. 4. Below 250 mV (vs. Ag/AgCl) an interfering peak was observed in the second (reductive) cell.

A mobile phase pH of 3 was used throughout all experiments. It was not possible to detect noradrenaline at a reductive potential at mobile phase pH 6.

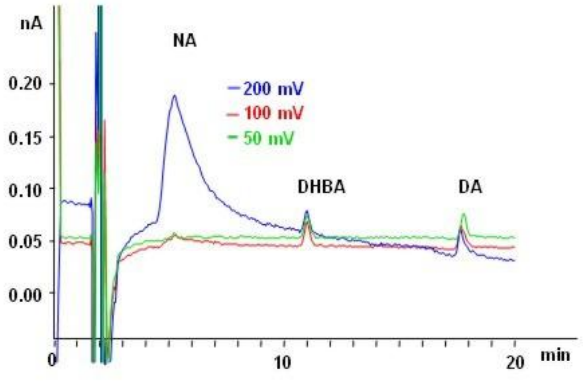


Fig. 5. At pH 6 the noradrenaline peak cannot be measured. A reductive potential below -100 mV is required which results in a large interfering oxygen peak between 4 and 8 min.

Ox-red analysis of catecholamines

Microdialysates samples are relatively clean and can be analysed without any sample pre-treatment. However, peaks in the first few minutes of a chromatogram are difficult to analyse as a number of interfering peaks can be expected (Fig. 6). Using an additional cell in series shows a considerable improvement in selectivity (Fig. 7).

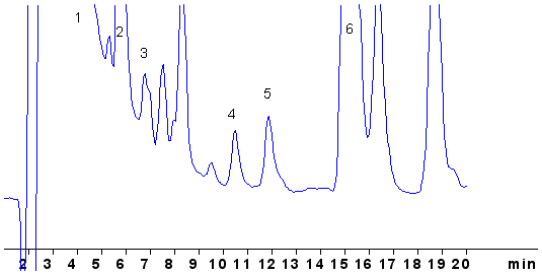


Fig. 6. Chromatogram obtained with the first oxidative cell. A number of interfering peaks appear in the first 10 minutes. Peaks are NA(1), DOPAC (2), DHBA (3), DA (4), 5-HIAA (5), and HVA (6).

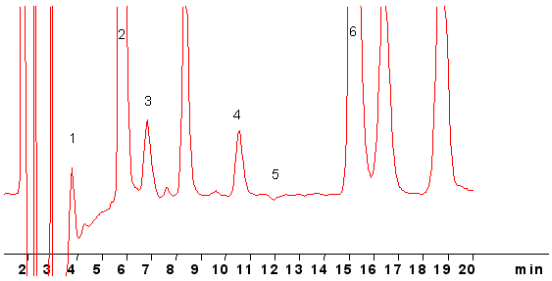


Fig. 7. Chromatogram obtained with the second cell using reductive detection at +200 mV. A number of interfering peaks are no longer detectable. Peaks: see Fig. 6.

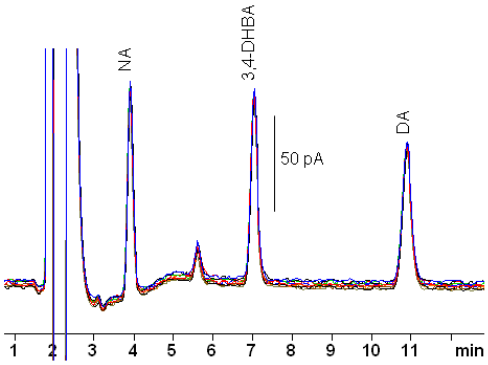


Fig. 8. Overlay of 10 injections (2 µL) of a 10 nM NA, 3,4-DHBA, DA standard mixture detected with cell 2 (Red).

Reproducibility of ox-red analysis has been investigated by analysis of standards (n=10). Data obtained with the second (reductive) cell are used and shown in Fig. 8. The RSD in peak area and peak height is respectively 1.7% and 1% or better. These RSD values are comparable to the ones obtained with the first (oxidative) cell. Retention times are reproducible with an RSD of 0.2% or better for both cells.

Table 2. Averages and % RSD of retention time, peak area and peak height of 2 µL injections of 10 nM NA, DHBA and DA, measured with the second (reductive) cell. Chromatograms are shown in Fig. 8).

Table 3

Reproducibility (n=8)				
	Height, nA		Area, nA*sec	
	Mean	%RSD	mean	%RSD
NA	0.11	0.95	1.07	1.1
DHBA	0.10	1.0	1.23	1.4
DA	0.073	0.8	1.24	1.7

	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

We define detection limit as the concentration that gives a signal that is 3 times the peak-to-peak noise of the baseline. The detection limits of NA and DA are 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.

Dead volume and plate numbers

A micro HPLC needs to be optimised not only with respect to mobile phase and detection parameters, but also minimization of dead volume in connectors and tubing. This is illustrated in Fig. 9 for the separation of catecholamine metabolites. In the upper trace a 55 cm tubing with 0.064 mm ID has been used between column and injector. Plate numbers for DOPAC, MOPEG, 5HIAA and HVA are respectively 77,000, 73,000, 81,000 and 81,000 (m^{-1}). In the lower trace the same length, but 0.25 mm ID tubing has been applied. Plate numbers are 27,000, 26,000, 54,000 and 76,000 (m^{-1}).

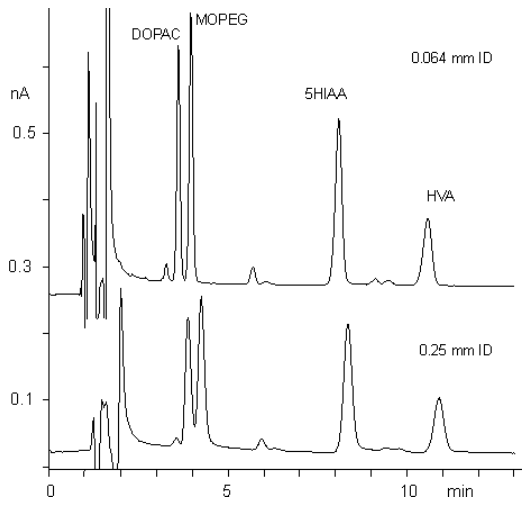


Fig. 9. Separation of metabolites using micro HPLC with 0.064 mm (upper trace) or 0.25 mm ID (lower trace) x 0.55 m tubing between injector and column.

The use of a standard bore in-line filter, valve or connectors can also have a dramatic effect on plate numbers and peak shape. When using two cells in series it is important that the first cell does not introduce a dead volume. For this purpose, the *reactor cell* with an internal volume of less than 1µL has been developed [3]. The effect of this reactor cell on peak shape is minimal.

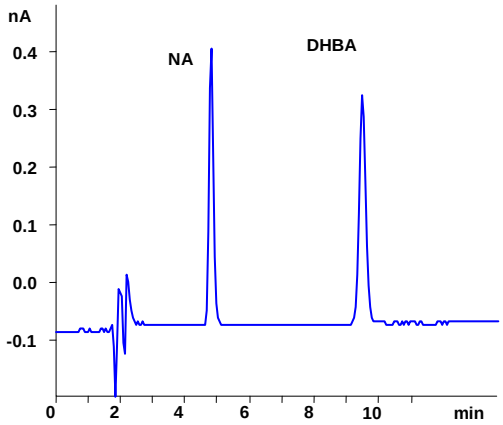


Fig. 10. Reductive analysis of 1µL of 100 nM NA and DHBA in the second flow cell. Plate numbers are 44713 m^{-1} (NA) and 73394 m^{-1} (DHBA). Similar plate numbers are measured in the first flow cell, indicating that the reactor cell does not contribute to peak broadening.

Conversion

In an amperometric flow cell only a fraction of the analyte that enters the cell is actually oxidised at the working electrode. In standard HPLC this is about 5 – 10%, at microbore flow rates this percentage can be considerably higher 30 – 80% or even 100% at very low flow rate (< 10 µL/min). In serial flow cell configuration it is important that the first cell has as high as possible conversion in favour of detection limits in the second cell.

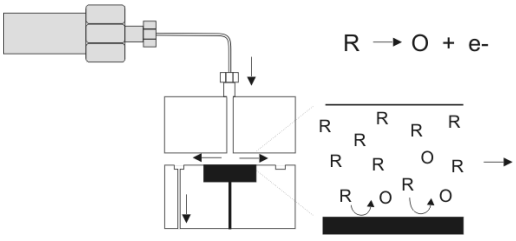


Fig. 11. In an amperometric flow cell only a fraction of the analyte reaches the electrode surface and is oxidised. In micro HPLC the percentage conversion is considerably higher.

To be able to quantify the percentage conversion the quantity of measured electrons (in moles) is expressed as a percentage of the total quantity of analyte loaded on the column.

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 number of electrons involved in the electrochemical reaction mechanism (2 in case of catecholamines) and F is Faraday's number (96485 Coulomb/mol).
In data acquisition the peak area is usually given in V.min or V.s. In those cases the area must be multiplied with the detector range setting (in nA/V) and time converted to seconds.

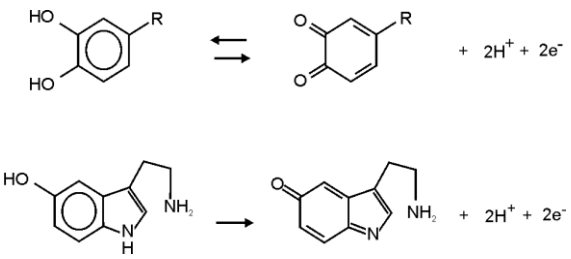


Fig. 12. Reversible reaction of catecholamines (top) and irreversible oxidation reaction of serotonin. In both cases 2 electrons are involved.

There are a few ways to increase the percentage conversion. Using a *smaller spacer thickness* will result in a smaller diffusion layer and makes it easier for an analyte to reach the electrode surface. Also a larger *electrode area* will result in more signal and conversion. However, when applied in standard HPLC changing these parameters may result in a higher conversion, but it will be difficult to get more than 20% in total.
A third factor resulting in a higher conversion is using *a smaller flow rate*. When decreasing the flow rate, the conversion may go up to 100%. As column dimensions dictate the applied flow rate, a decrease in flow rate can only be realised by using a smaller bore HPLC column. For a 1 mm column with a flow rate of 50 µL/min we found a conversion of about 80%.

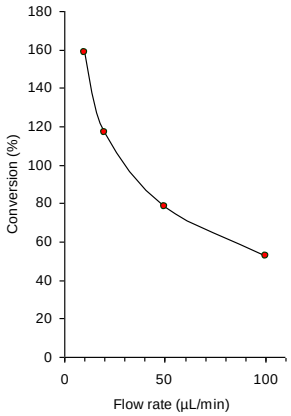


Fig. 13. Conversion for dopamine at different micro flow rates. Conversion was calculated from peak area obtained in flow injection analysis. When using a 1 mm ID column with a flow rate of 50 µL/min, the conversion is about 80%.

Decreasing the flow rate further to 20 or 10 µL/min a conversion of more than 100% has been observed. It is assumed by several authors that this is a result of so-called 'redox-cycling' [4]. A phenomena that occurs at low flow rate where a substance is oxidised, falls back into the reduced form and is oxidised again, resulting in a conversion of more than 100 %.

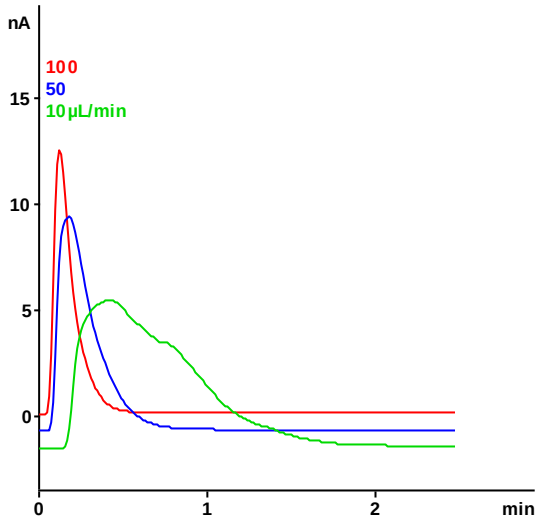


Fig. 14. Flow injection analysis of dopamine at different flow rates. Peak area and percentage conversion at 10 µL/min is about 3 times higher than measured at 100 µL/min.

ALEXYS data system

The ALEXYS data system has a number of features that support DCC. Organisation and storage of data is controlled by the method file. Therefore it is recommended to create at least two 'recorders' in a system, one for each data acquisition channel. If auxiliary channels are monitored (for pressure, temperature etc) those can all be acquired using a third recorder.

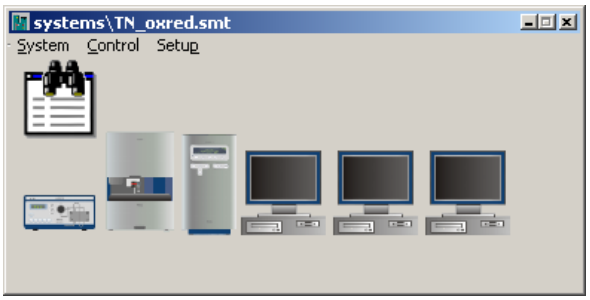


Fig. 15. System configuration with three recorders for data acquisition using DCC.

Because each recorder has its own method, including data file storage location, organisation of data becomes flexible.

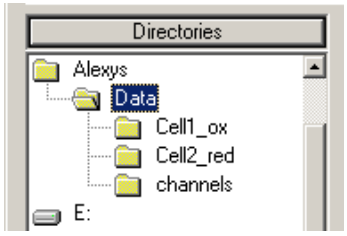


Fig. 16. Example of data tree using 3 recorders.

In the DECADE II driver the settings of the two cells can be programmed (Fig. 17).

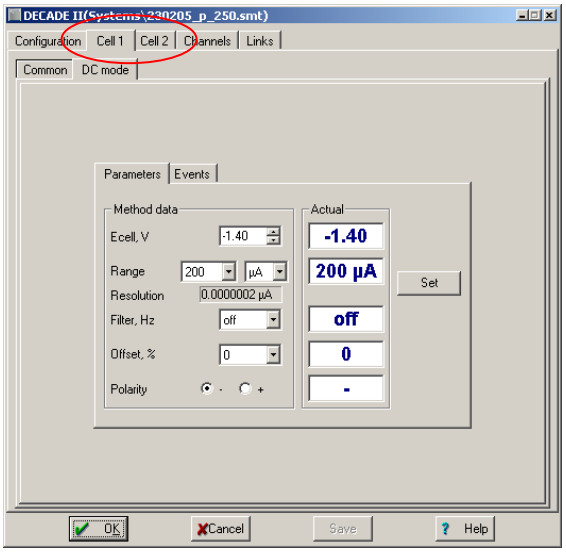


Fig. 17. DECADE II driver window with independent control of two cells.

If the data traces have to be modified relatively to each other, this can be done by changing the parameters in the channels setup (Fig. 18). For example, normalising one of the traces can be done by modifying the 'Coef' in the channels setup window, and a horizontal shift in one of the chromatograms can be applied after changing the value of 'shift'. Also the 'Name' field can be changed and is shown in the legend of an overlay.

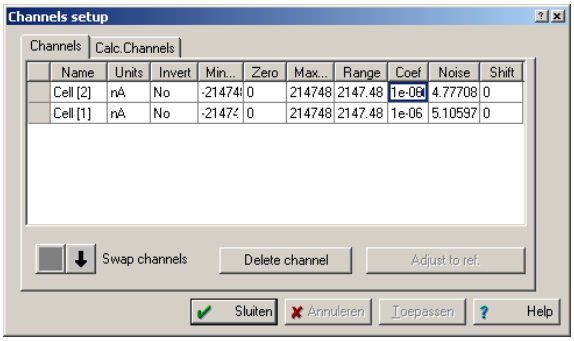


Fig. 18. Channels set-up window for data manipulation.

The queue editor is used to create a hydrodynamic voltammogram. First, a series of system files should be programmed with the correct working potential for cell 1 and cell2. Each system file has an incrementing working potential for one of the cells while keeping the other cell at a constant potential (Fig. 19). Note that when varying the working potential in the first cell, this will influence the signal in the second cell: the response of the second cell has to be acquired as well.

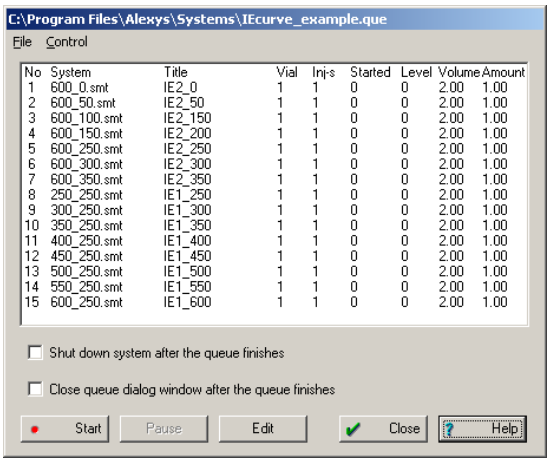


Fig. 19. Example of sample queue for the construction of a hydrodynamic voltammogram.

Stabilisation of cell current after increment of the potential is necessary. This can be done by programming a new potential in the event table of a system extending the run time with about 45 min. After the last peak of interest has been detected the potential is changed.
The last 15 minutes of a chromatogram can be used to measure the noise of the baseline.

CONCLUSION

Ox-red analysis has been applied for selectivity improvement in analysis of dialysates. Microbore HPLC is the method of choice because of the small microdialysis sample volume and higher conversion rate in a reactor cell. Detection limits down to 0.5 nmol/L have been found in the second (reductive) cell. Especially in the first 10 minutes of a chromatogram separation has improved considerably.

References

1. Application note 213-017: Serotonin, noradrenaline, dopamine and metabolites in microdialysates, Antec Leyden
2. Application note 220_001: Attomole detection limits in micro HPLC-ECD Detection, Antec Leyden
3. Application note 220-010: reactor cell, Antec Leyden
4. 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.