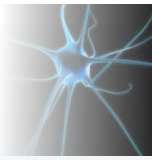
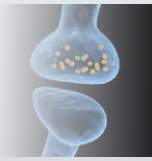
Antec® Scientific

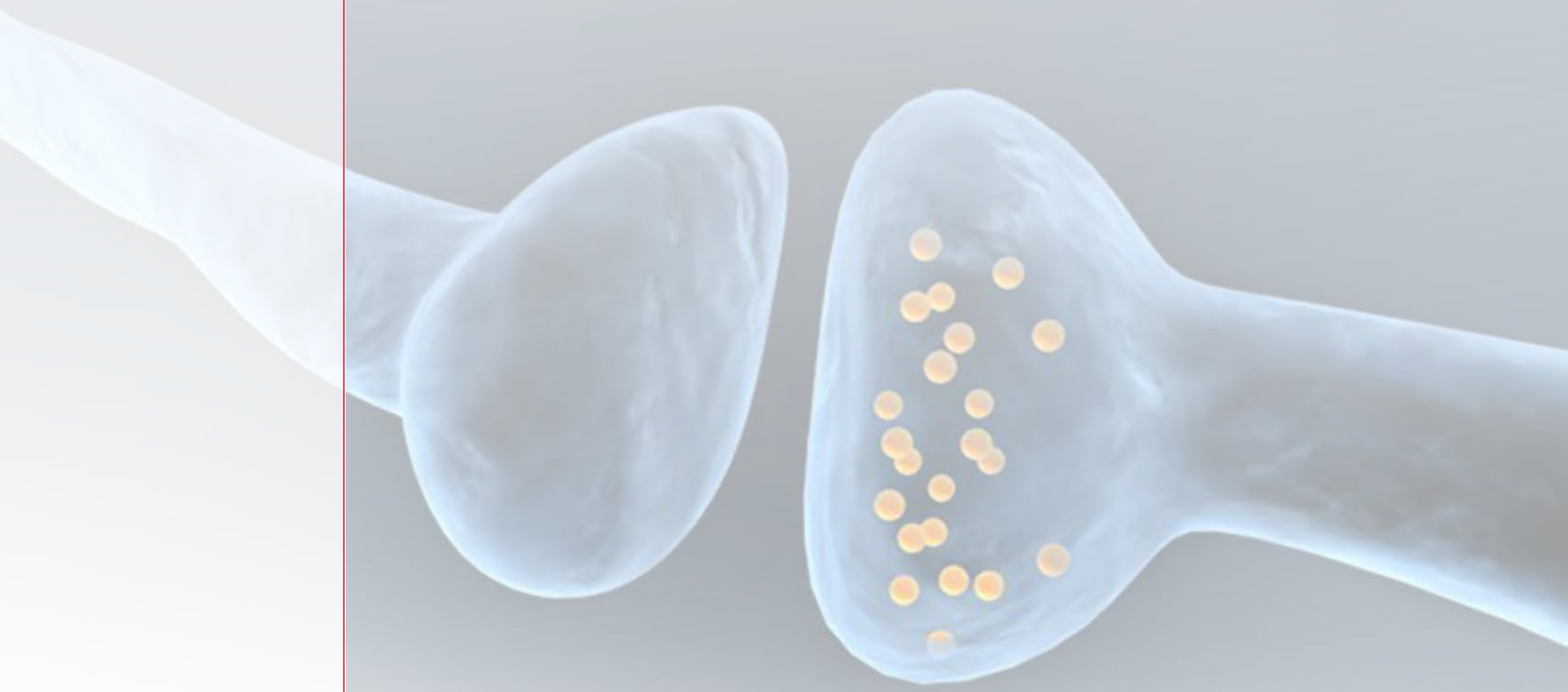




Applications Notebook

ALEXYS™ Analyzer
Unrivaled Neurotransmitter Analysis

Microdialysates, CSF, brain tissue homogenates



NEURO Notebook 213_070_06

Design: MGO-studio, Maarssen, NL
Illustrations: Antec Scientific

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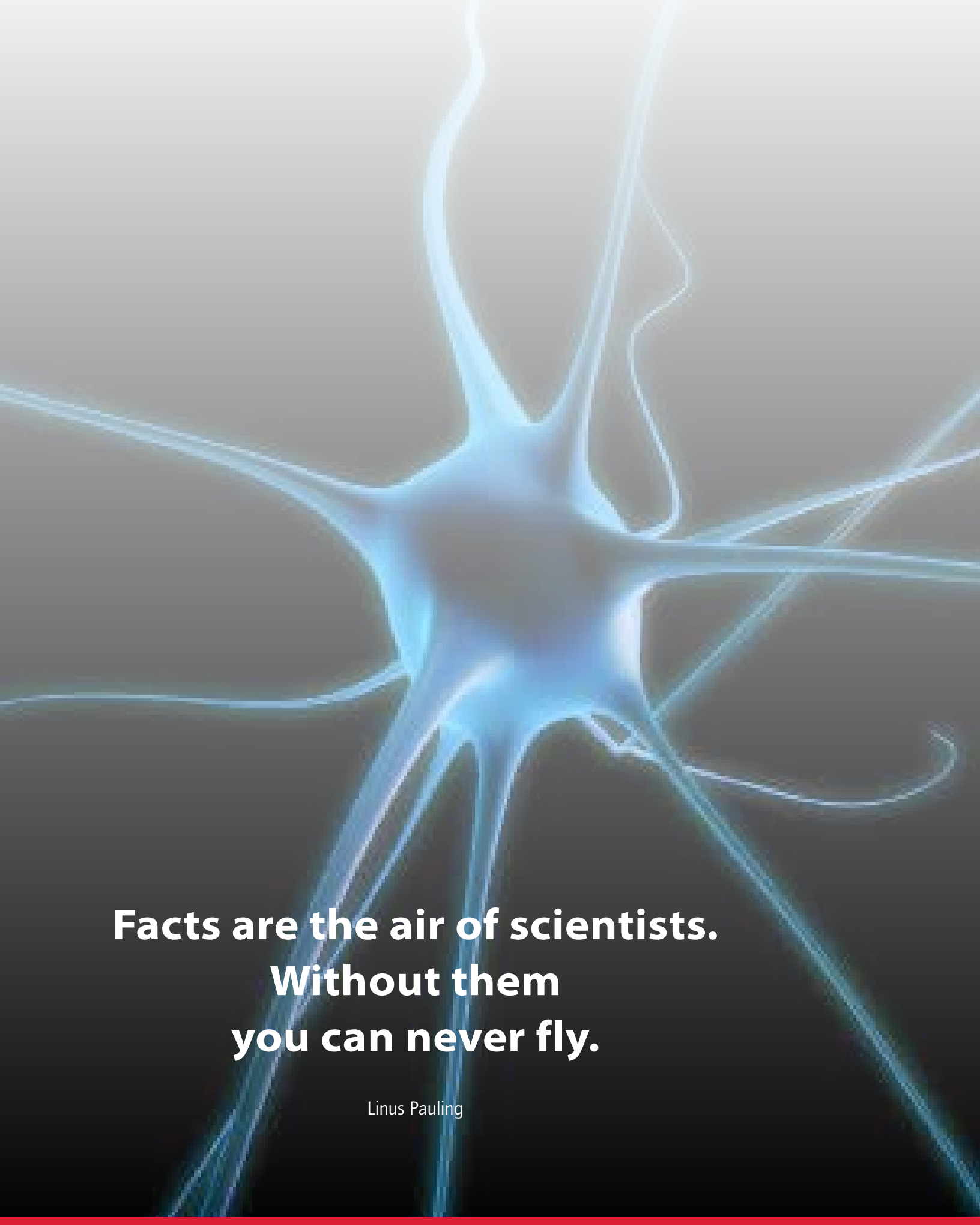
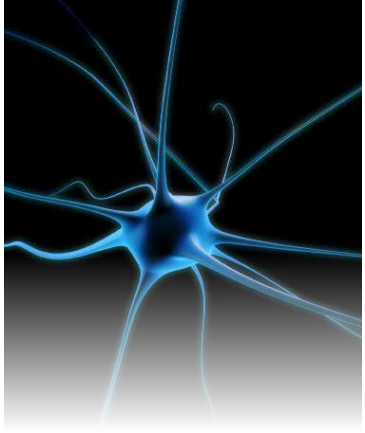
Contents



**Facts are the air of scientists.
Without them
you can never fly.**

Linus Pauling

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 Acetylcholine and Choline

- **Choline well separated from acetylcholine**
- **SenCell with programmable cleaning cycle**
- **Detection limit 0.5 nmol/L ACh (5 µL injection)**
- **Small total sample use of 10 µL**
- **Total analysis time < 10 min**

Summary

The ALEXYS Neurotransmitter Analyzer is a modular system for UHPLC with electrochemical detection (ECD) of neurotransmitters. This application note shows the use of the system to sensitively measure acetylcholine (ACh) in brain microdialysates. Such samples are challenging due to low levels of ACh and small available sample volumes (typically <20µL). Efficient separation of ACh is achieved on a reversed phase microbore column under ion-pairing conditions. Conversion of ACh to the electrochemically detectable hydrogen peroxide takes place in a post column IMER. A Sencell with platinum working electrode is used for detection. With this approach, the detection limit is about 0.5 nmol/L (2.5 fmol on column; 5 µL injection). Basal ACh levels are shown to be detectable in real microdialysis samples.

Introduction

Acetylcholine (ACh) is a neurotransmitter that activates muscles at the neuromuscular junction, and in brain it is a neuromodulator involved in processes like arousal, attention and motivation. In-vivo studies of the changes in ACh levels in the brain often use the well-established sampling technique of microdialysis [1]. This results in small samples (typically <20µL) with low nanomolar levels of ACh. A sensitive detection method is needed to quantify the ACh levels.

This note describes ACh measurements in brain microdialysates using the ALEXYS Neurotransmitter Analyzer (Figure 1). The ACh analysis is based on ion-pairing HPLC separation, followed by on-line enzymatic conversion of ACh to hydrogen peroxide, and detection on a Pt working electrode.

Figure 1: ALEXYS Neurotransmitter Analyzer for acetylcholine.

Method

ALEXYS Neurotransmitter Analyzer

The ALEXYS Neurotransmitter Analyzer consists of a OR 110 degasser unit, LC 110S pump(s), a DECADE Elite EC detector, an AS 110 autosampler and Clarity data acquisition software. The additional ACh kit contains the column and a SenCell with Pt working electrode. Other kits are available for neurotransmitters and amino acids such as dopamine, noradrenaline, serotonin, metabolites, GABA and glutamate [5].

Separation

Acetylcholine (Figure 2) is positively charged and is therefore separated on a C18 column with an ion-pairing agent in the mobile phase [2]. This new ion-pairing chromatography method is superior to the traditionally applied ion-exchange chromatography (see for example ref [2]). The new method shows a reversed elution order of Ch and ACh peaks with much better separation. A large late eluting peak at about 25-35 min observed in ion exchange LC, was not observed in the applied ion-pairing method.

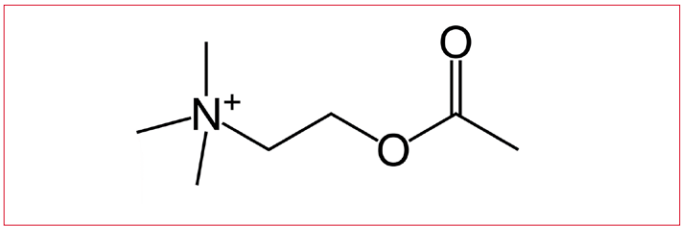


Figure 2: Structure of Acetylcholine.

Enzymatic conversion

After separation, ACh passes through an immobilized enzyme reactor (IMER) before entering the detector. The IMER contains covalently bound acetylcholine esterase (AChE) and choline oxidase (ChOx), which convert ACh to the electrochemically detectable hydrogen peroxide (Figure 3).

The AChE/ChOx IMER has some requirements for efficient enzymatic conversion:

- Mobile phase 100% aqueous, as enzymes denature in contact with organic solvents.
- Mobile phase with pH close to the enzyme's optimal pH of 8.2
- A low LC flow rate, as conversion efficiency and flow rate are inversely related.
- A thermostated oven at the optimal temperature of 35 °C.

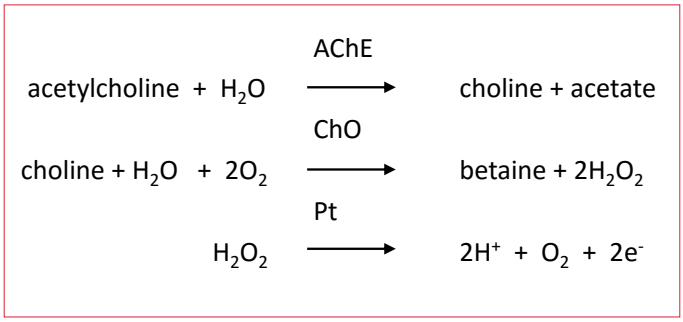


Figure 3: Enzymatic conversion of acetylcholine to electrochemically detectable hydrogen peroxide with the enzymes acetylcholine esterase (AChE) and choline oxidase (ChOx). These enzymes are covalently bound in an immobilized enzyme reactor (IMER) that is placed between the separation column and detector.

Detection

Hydrogen peroxide is electrochemically detected on a Pt working electrode. The working potential of the detector has to be set as low as possible to ensure selectivity, but high enough to generate a clear response. A potential of 0.2V is chosen as optimum (Figure 4). Applying a higher potential unnecessarily decreased the selectivity of the method by showing more and larger interfering peaks (Figure 5).

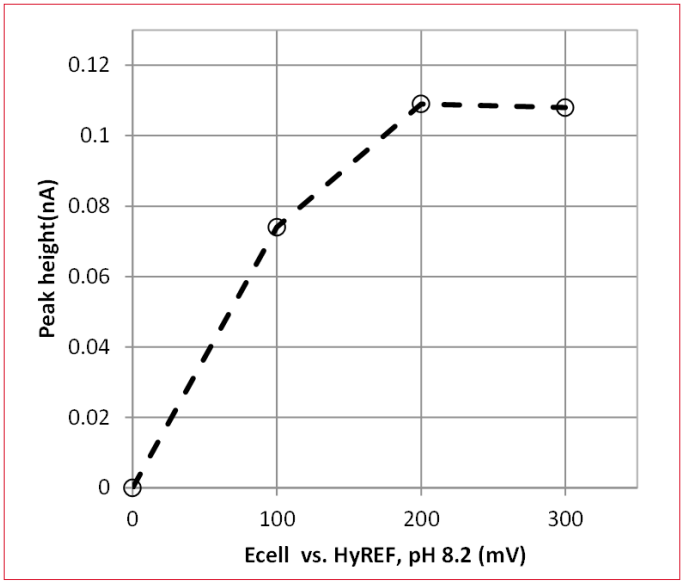


Figure 4: Plot of peak height vs. potential for the analysis of acetylcholine after conversion to hydrogen peroxide. The system was left to stabilize for 2.5 hours before each measurement.

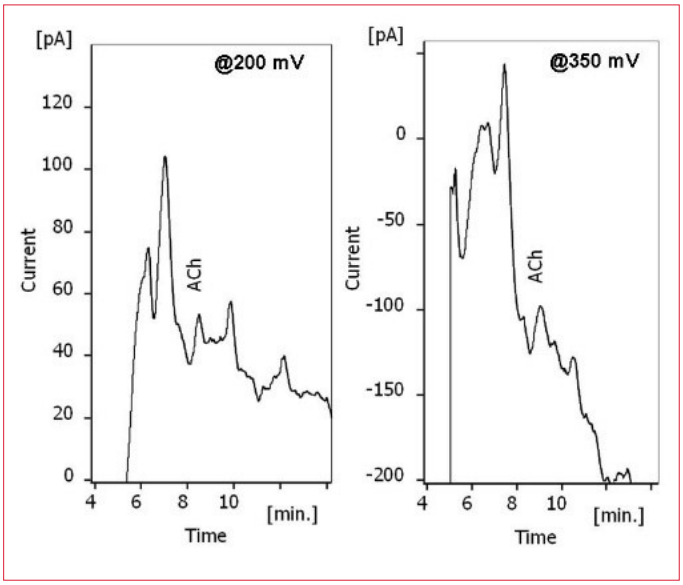


Figure 5: Chromatograms of brain microdialysate sample detected with potential settings of 200 mV and 350 mV. Notice the relatively higher response of non-relevant signals at the higher working potential.

Electrode (re)activation

A new flow cell with Pt electrode requires a one-time initialization step. After installation of the UHPLC/ECD system, it is necessary to apply an activation pulse. The detector is set to PAD mode (E1=+1.0V, E2=-1.0V, t1=1000ms, t2=1000ms, ts=20ms), and the pump is set to the standard flow rate and mobile phase. After 10 minutes [3] the signal is stabilized in DC mode at 0.2V for at least 30 min. The background current should drop below 25 nA in less than 30 min. This activation procedure can be programmed in the DECADE Elite detector and Clarity software. The pulse mode is not available in the SDC or Lite versions of the detector.

An inherent feature of the Pt electrode is a gradual decrease of responsiveness over time due to formation of Pt oxide (Figure 6). Noise level has a slight tendency to decrease as well, but overall sensitivity decreases over time as well. Regular 2-hourly recalibration of the response is advised, and this "calibration bracketing" is an automated feature in Clarity.

To bring back the response (Figure 7), a short reactivation pulse is advised every 2 days: E=-0.5V for 12 s followed by E=0.8V for 12s. The background current should drop below 25 nA in less than 10 min after this pulse.

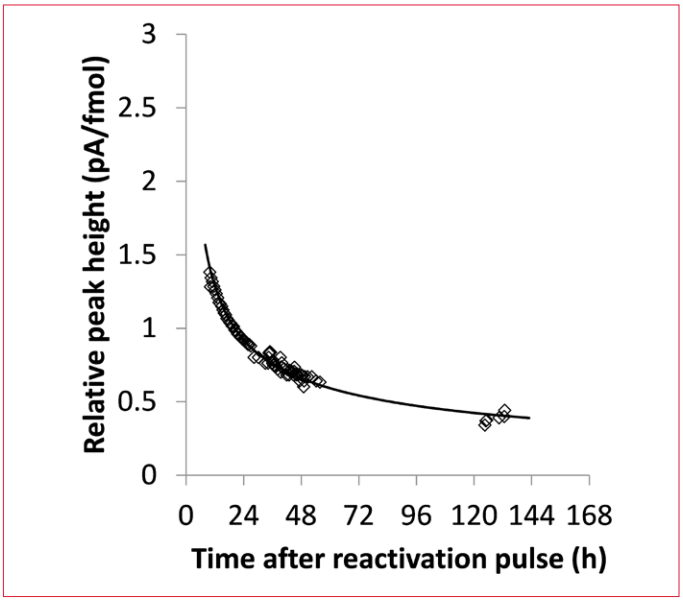


Figure 6: Response over time of ACh standard in Ringer solution, detected on a Pt working electrode at 0.2V vs HyREF. At t=0, a short reactivation pulse was applied (E=-0.5V for 12 s and E=0.8V for 12s).

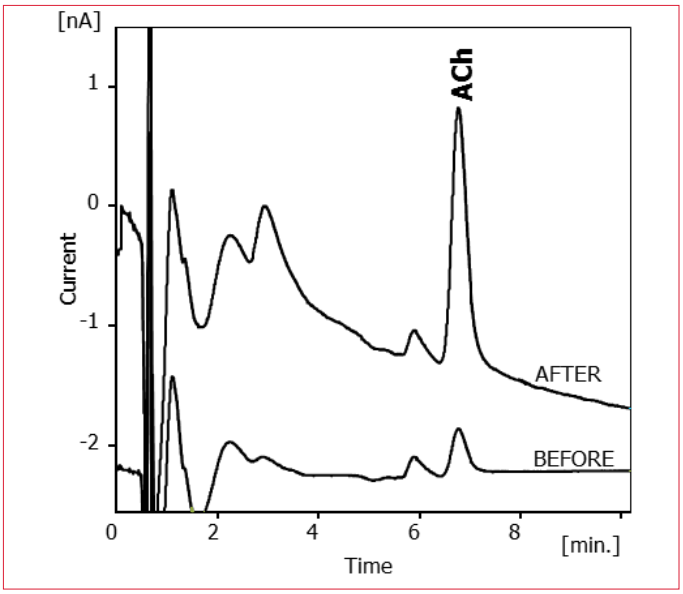


Figure 7: Overlay of LC-ECD chromatograms of an ACh standard in Ringer solution, recorded with a Pt working electrode before and 10 min after an electrochemical reactivation pulse of the electrode.

Sample use

The maximum volume that can be injected onto the column is 5 µL. The injection of larger volumes only results in the ACh peak becoming wider; not higher. A dedicated injection program was applied to inject the maximum volume of 5 µL while only using a total of 10 µL sample [4].

Table 1

Recommended conditions and settings for ACh analysis	
HPLC	ALEXYS Neurotransmitters Analyzer with AS110 autosampler and Acetylcholine kit
Column*	Acquity UPLC HSS T3 50 x 1 mm 1.8 µm Waters)
IMER	IMER, 1 mm ø x 4 mm
Mobile phase*	50 mmol/L sodium dihydrogen phosphate 0.5 mmol/L EDTA.Na2 pH 7.5 set with 50% NaOH 1.6 g/L octanesulfonic acid, sodium salt 0.5 mM tetramethylammonium chloride
Temperature	35 °C (separation and detection)
Flow rate	50 µL/min
Backpressure	100-150 bar
Pump piston wash	Water (HPLC grade; refresh at least once per week)
Needle wash	Water (HPLC grade; refresh at least once per week)
Flow cell	SenCell with 2mm Pt WE, AST position 1
Potential	200 mV vs. HyREF™ reference electrode
ADF	0.02Hz
Range	50 nA/V; change to 1 nA/V around ACh peak
Icell	5-15 nA
$V_{injection}$	5 µL max
Sample loop	5 µL
Injection method	Dedicated user program with 10 µL total sample use
Standards	Acetylcholine in Ringer solution (147 mM NaCl, 3 mM KCl, 1.2 mM MgCl2, 1.2 mM CaCl2)

* Revised conditions; chromatograms may differ slightly.

Results

Repeatability, linearity and detection limit

The repeatability was evaluated with a 100 nmol/L ACh standard in Ringer solution, analyzed in a sequence with a time frame of about 1 hour per 6 runs. A repeatability of peak area better than 3% RSD (n = 6) was found, 16 h after a reactivation pulse. The long term drop in response affects the inter-day RSD for peak area and height. Retention time of the ACh peak shows an RSD better than 0.2%.

Working with an application that shows a steady decrease of responsiveness makes it important to regularly calibrate the system with standards (calibration bracketing). A 5-point linear calibration in the range between 0-10 nmol/L ACh showed a correlation coefficient better than 0.998.

A detection limit down to 2.5 fmol ACh was measured using a well performing IMER and a noise level below 2 pA. Best detection limits are obtained within a few hours after the reacti-

vation pulse. Figure 8 shows the chromatogram of a near-LOD concentration of ACh.

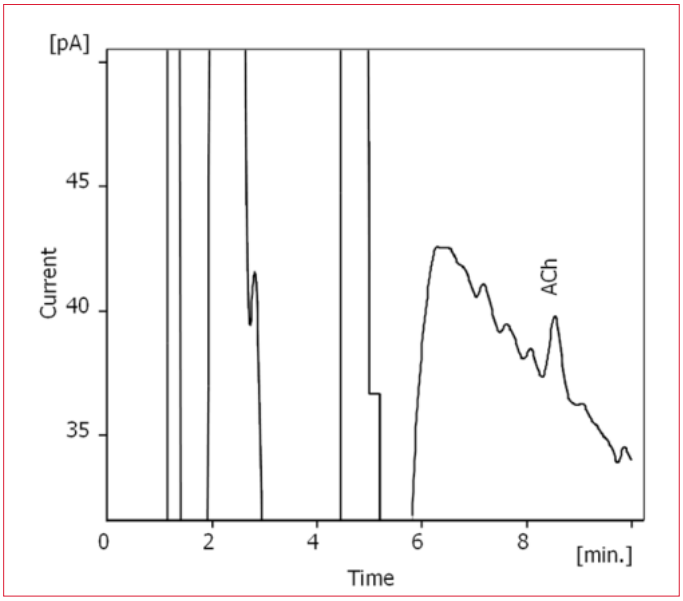


Figure 8: Chromatogram of 0.5 nM acetylcholine in Ringer solution. This concentration is near the LOD.

Brain microdialysate sample

Acetylcholine can be detected in basal brain microdialysate samples using the presented method (Figure 9). The large peak of choline (the metabolite of ACh) is also detectable in the chromatogram, but it does not interfere with the detection of ACh as both peaks are well separated from each other using this method (Figure 10).

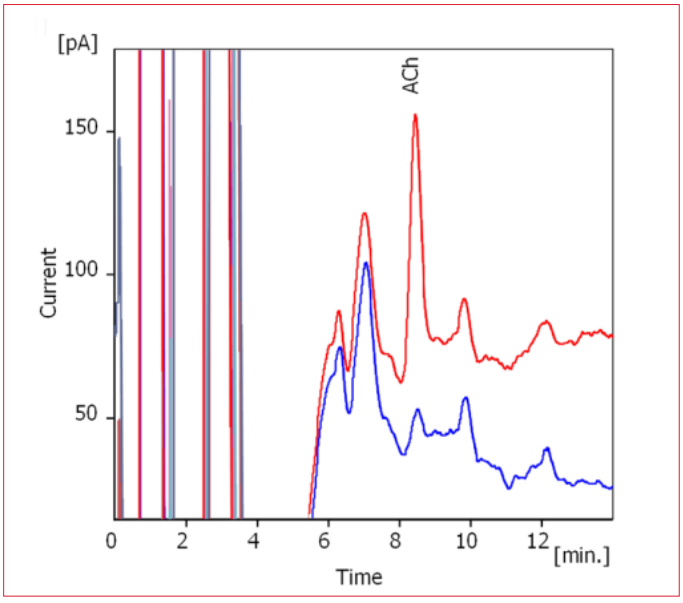


Figure 9: Overlay of chromatograms from pooled rat medial Prefrontal Cortex (mPFC) microdialysate sample, spiked with 10 nM acetylcholine (red trace) and not-spiked (blue trace).

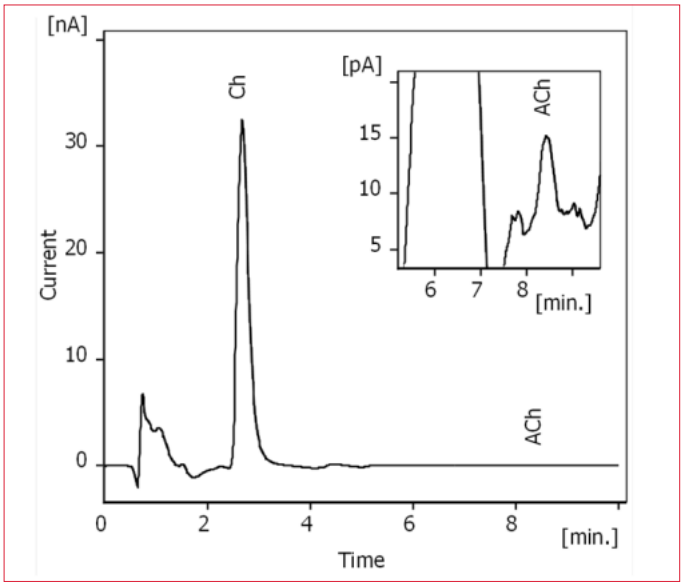


Figure 10: Chromatogram of basal level microdialysate sample from medial prefrontal cortex of a male Wistar rat. Inset is zoomed-in part of chromatogram. Concentrations were quantified as 1 nM acetylcholine and 40 µM choline. Sample origin: 30 µL samples were collected by dialysis at a flow rate of 1.25 µL/min using aCSF (166.5 mM NaCl, 27.5 mM NaHCO3, 2.4 mM KCl, 1.2 mM CaCl2, 0.5 mM KH2PO4 and 1.0 mM glucose (pH 7.1)). Samples were kept frozen at -20°C until analysis. Samples were kindly provided by Sarah Beggiato, Maryland Psychiatric Research Center, Baltimore (MD), USA

Conclusion

The ALEXYS Neurotransmitter Analyzer for Acetylcholine results in a fast and sensitive detection method of ACh. A detection limit of 2.5 fmol is obtained which facilitates basal level ACh detection without the need for Acetylcholinesterase inhibitors. The UHPLC ion-pair separation is superior to existing ion-exchange methods, by reversing the elution order of Ch and ACh, resulting in a better separation and no late eluting peaks.

References

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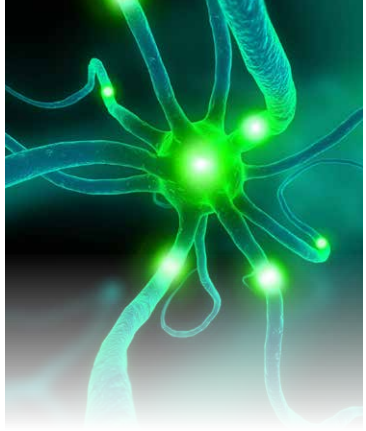
2. Sotoyama, H.; Zhu, Y.; Gitzen, J.; Xie, F.; Kissinger, P. Feasibility of ion-pair reversed-phase liquid chromatography/electrochemistry detection for determination of acetylcholine in microdialysates collected without acetylcholinesterase inhibitors. Cur. Separations 2002, 20, 11-16.
3. Sencell user manual, Antec document 116_0010.
4. Micro volume injections, Antec technical note 220_011.
5. Analysis of Glutamate, GABA, Noradrenaline, Dopamine, Serotonin and Metabolites using microbore UHPLC with Electrochemical Detection, Reinhoud NJ, Brouwer HJ, van Heerwaarden LM, Korte-Bouws GA.; ACS Chem Neurosci. 2013, 4:888–894

Ordering information

ALEXYS Neurotransmitter Analyzer for Acetylcholine	
180.0091E	ALEXYS Neurotransmitters
191.0035UL	AS 110 Autosampler UHPLC cool 6p
250.3532	AChE/ChOx post column-IMER
180.0505	Acetylcholine kit

Parts in Acetylcholine kit (180.0505)	
116.4322	SenCell 2 mm Pt HyREF
250.1160	Acquity UPLC HSS T3 50 x 1 mm column, 1.8 µm
180.0227A	IMER connection assembly

Application Note
Neuroscience



ALEXYS Neurotransmitter Analyzer for GABA & Glutamate, Histamine, LNAAs and other Amino Acids

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

- **Detection of Glu and GABA within 12 minutes**
- **Fully automated 'in-needle' OPA derivatization**
- **Post separation step-gradient to eliminate late eluters**
- **Small sample use of 5 µL**
- **Histamine and LNAAs analyses**

Summary

In this application note a fast and sensitive method is presented for the analysis of the amino acid neurotransmitters GABA and glutamate using the ALEXYS Neurotransmitter Analyzer [1].

Method features:

- Automated odorless in-needle OPA-sulphite derivatization.
- Sample use per analysis: 5 uL
- Fast and efficient separation using sub-2 µm particle column
- Post separation step-gradient eliminates late eluting peaks

With this approach, a high sample throughput and low detection limit of around 10 nmol/L GABA is achievable.

Introduction

The ALEXYS Neurotransmitter Analyzer is a modular UHPLC/ECD system with application kits for the analysis of various neurotransmitters including GABA and glutamate.
The amino acid derivative γ-aminobutyrate (GABA) is a well-known inhibitor of presynaptic transmission in the Central Nervous System (CNS). The activity of GABA is increased by Valium (Diazepam) and by anticonvulsant drugs.

Glutamate (Glu) is an excitatory neurotransmitter and a precursor for the synthesis of GABA in neurons. Glu activates the N-methyl-D-aspartate (NMDA) receptors, which play a role in learning and memory and a number of other processes.

Other Amino Acids e.g. Histmine, LNAAs (Tyr, Val, Met, Orn, Leu, Ile, Phe, Lys, Trp) can be analyzed too using the Neurotransmitter Analyzer.

ALEXYS Neurotransmitter Analyzer

The ALEXYS Neurotransmitter Analyzer is a modular system that can be customized for the analysis of specific neurotransmitters. The system consists of the OR 110 degasser unit, LC 110S pump(s), the AS 110S autosampler, the DECADE II electrochemical detector and Clarity data acquisition software. Different evaluated additional hardware kits are available for the analysis of for instance monoamines, metabolites, acetylcholine (ACh) and choline (Ch): one system for all neurotransmitters.

Figure 1: ALEXYS Neurotransmitter Analyzer with additional hardware kit for analysis of GABA and Glu

Method and results

GABA and Glu are not directly detectable with electrochemistry (EC), nor with UV. Therefore, a pre-column derivatization with OPA and sulphite must be applied [2-3].

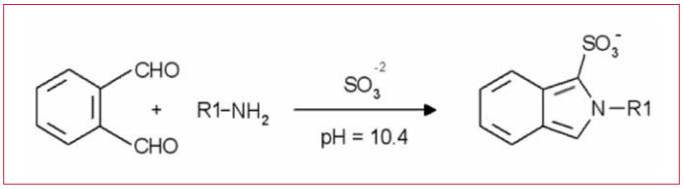


Figure 2: Reaction scheme of the derivatization of primary alkyl amines with OPA and sulphite.

The derivatization procedure and composition of the OPA reagent was modified from Smith and Sharp [3]. The rate of derivatization with OPA-sulphite reagent is strongly pH dependent. At high pH (> 9.5) the reaction occurs almost instantaneous [2]. Therefore, the OPA-sulphite reagent is buffered at pH 10.4 by means of a 0.1 mol/L borate buffer to assure fast conversion of the amino acids.

Reagent - The OPA reagent as well as the sodium sulphite solution should be prepared fresh each day for optimal performance.

Sample/reagent ratio - The sample/reagent ratio affects the sample dilution factor but also chromatographic performance (due to difference in pH between derivatised sample and mobile phase). A reagent:sample mix ratio between 1:10 and 1:20 was found to give optimal results. For derivatisation of 5 uL samples, the reagent should therefore be diluted 1:1 with water before use.

Sample constraints - Microdialysis samples are often acidified immediately after sample collection to minimize catecholamine degradation over time. GABA and Glu are more stabile in microdialysates and acidification is not necessary. However, if GABA and Glu analysis has to be performed in acidified samples, the derivatisation efficiency (thus sensitivity) will decrease if the buffering capacity of the reagent is not adjusted/increased.

'In-needle' derivatization procedure - The sample derivatization procedure is completely automated by an optimized pre-defined 'user program' for the autosampler. It comprises of the following steps:

- Aspiration of reagent
- Aspiration of sample
- Mixing of sample and reagent in the autosampler tubing
- Injection of the derivatized sample
- Extensive wash of autosampler flow path

In comparison with the method described in application note 213-019 [4], the procedure is now significantly faster and simpler. The derivatization procedure time is reduced by a factor of five (8 min versus 1 ½ min). Furthermore, with the 'in-needle' derivatisation procedure, separate mixing vials for every sample are not required any longer. Therefore almost all vial positions in the sample tray of the autosampler (2x 96 position plates) can be used for samples; only 4 positions are reserved for reagent.

UHPLC/ECD conditions

The EC detectable N-alkyl-1-isoindole sulphonate derivatives that are formed are separated using isocratic conditions on a 5 cm sub-2 micron C18 UHPLC column. Typically, the method results in a chromatogram as shown in Fig. 3, and using standards a column efficiency in the range of 100,000 -130,000 can be obtained (GABA peak).

Table 1

Conditions for GABA and Glu analysis	
HPLC	ALEXYS Neurotransmitter Analyzer (pn 180.0091E) with AS 110 UHPLC cool 6-p autosampler (pn 191.0035UL)
Column	Acquity UPLC HSS T3 1.0 x 50 mm column, 1.8 µm
Pre-column filter	Acquity in-line filter kit + 6 frits
Mobile phase A (separation)	50 mmol/L phosphoric acid, 50 mmol/L citric acid and 0,1 mmol/L EDTA, pH 3.5, 2% Acetonitrile
Mobile phase B (post-separation)	50 mmol/L phosphoric acid, 50 mmol/L citric acid and 0,1 mmol/L EDTA, pH 3.5, 50% Acetonitrile
Flow rate	200 µL/min
Temperature	40 °C (separation and detection)
AS wash solution	Water (refresh weekly)
Pump piston wash	15% isopropanol in water (refresh weekly)
Vinjection	1.5 µL full loop injection as part of automated in-needle derivatisation user defined program
Total sample use	5 uL
Flow cell	SenCell with 2 mm GC WE and saltbridge reference electrode, AST setting: 0.5
Ecell	850 mV vs. Ag/AgCl (salt bridge)
Range	50 nA/V for Glu; 5 nA/V for GABA
ADF™	0.1 Hz
Icell	2-5 nA
Noise	1-4 pA (@range 5 nA/V, ADF 0.1 Hz)

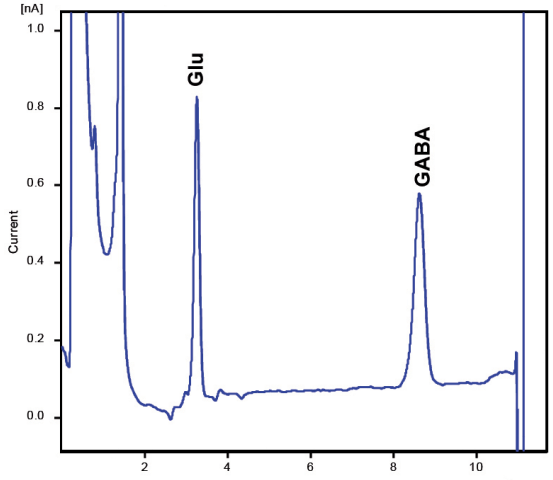


Figure 3: . Chromatogram of a 0.5 µmol/L GABA & Glu standard mixture in Ringer.

Table 2

Peak table for 0.5 µmol/L GABA & Glu standard in Ringer (Fig. 3)

Compound Name	Glu	GABA
Retention time [min]	3.3	8.6
Area [nA.s]	6.9	9.3
Height [nA]	0.77	0.50
Capacity [-]	16	45
Asymmetry [-]	0.9	0.9
Eff [t.p./m]	63500	105000

Post-separation step-gradient

After the elution of the last component of interest (GABA derivate), many other sample components elute off the column between t = 15 and 60 minutes under isocratic conditions (Fig. 4). Either analyses run times will be very long (60 min), or the late eluting peaks will disturb the baseline of the consecutive runs if no precautions are taken.

To combine short analysis times and a stabile baseline in consecutive analyses, a short step-gradient with a second strongly elut-ing mobile phase is applied. This mobile phase contains 50% acetonitrile and runs shortly through the system after elution of GABA (between 10 - 13 min). The later eluting components are thus quickly flushed off from the analytical column, and the baseline is stabile again within 5 min (Fig. 5).

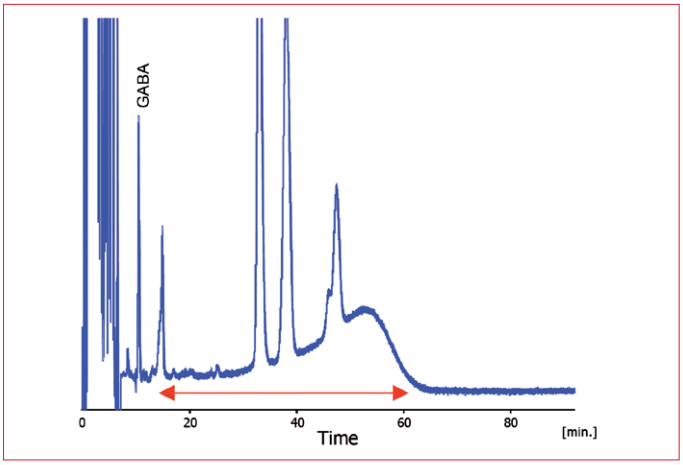


Figure 4: Chromatogram of a rat dialysate showing several late eluting peaks between 15 and 60 min (red arrow).

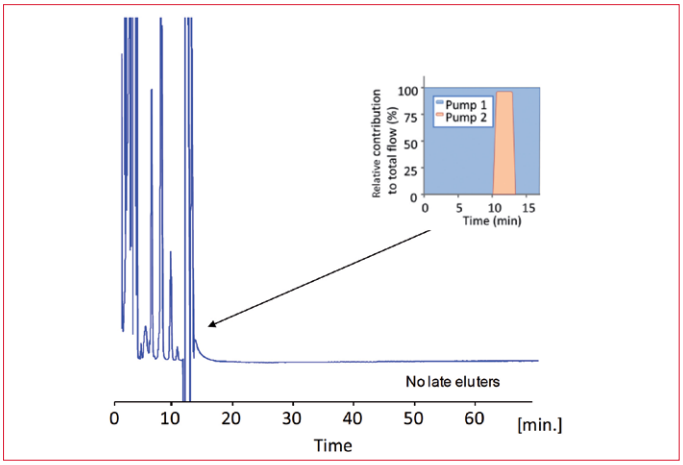


Figure 5: Chromatogram of a rat dialysate with a post-separation step-gradient. Late eluting peaks are absent from the baseline.

Repeatability

Depending on the brain region under investigation, basal concentrations typically range around 10 - 50 nmol/L GABA [6, 7] and several µmol/L Glu [8, 9]. in microdialysis samples. For the repeatability study, biologically relevant concentrations of GABA and Glu standards in Ringer's solution were analyzed after the in-needle derivatisation procedure (which contains a 1.5 µL flushed loop fill injection). Table 2 shows typical RSD values and Fig. 6 shows an overlay of chromatograms.

Table 3

Relative Standard Deviation (RSD) for peak area; n=6 (standards)		
	Glu	GABA
50 nmol/L	< 5 %	< 3 %
0.5 µmol/L	< 2 %	< 2 %
2.5 µmol/L	< 2 %	

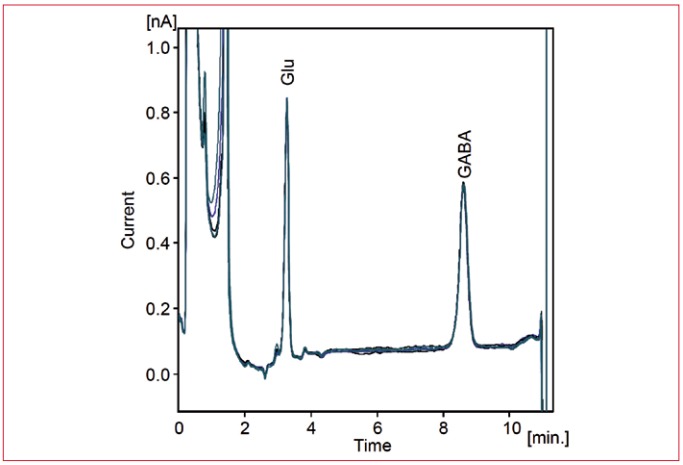


Figure 6: Overlay of 6 chromatograms of 500 nmol/L GABA and Glu in Ringer's solution. Step-gradient applied between 10-12 min. Total run-time per sample: 19 min (includes derivatisation, separation, column flush and stabilization time).

Linearity

The linearity of the method was determined in the concentration ranges of 0.2 -1 µmol/L Glu and 20 – 100/500 nmol/L GABA (Fig. 7). The method showed a good linear detector response with correlation coefficients of 0.998 or better for both GABA and Glu.

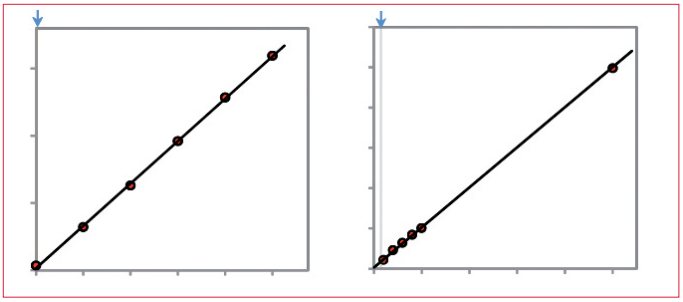


Figure 2: Calibration plots of Glu and GABA with linear regression line through the data points.

Limit of detection

Calculated detection limits (signal-to-noise ratio: 3) were about 12 nmol/L GABA and about 8 nmol/L Glu based on total sample use per analysis of only 5 uL. This corresponds to an amount of 6 pg GABA or Glu per sample of 5 uL and 12-18 fmol on column load.

A signal for 20 nM GABA is clearly visible as can be seen in Fig. 8. Note that the blank chromatogram shows a small peak with the retention time of Glu corresponding with a concentration of 17 nmol/L. In comparison to the basal concentration of Glu in microdialysates (in the range of several µmol/L) the intensity of the interference is relatively small.

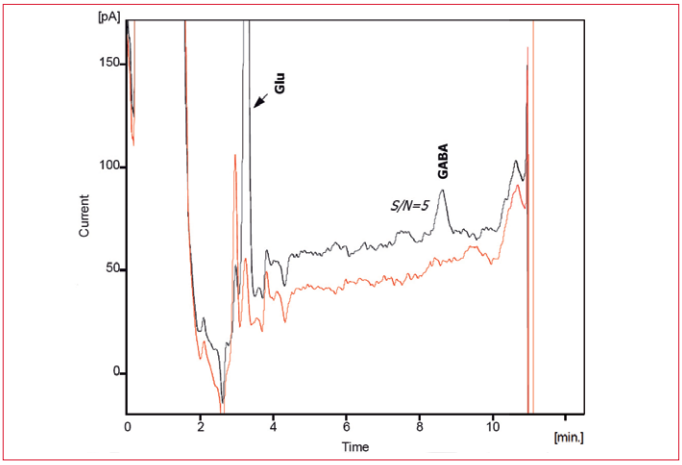


Figure 8: Overlay of chromatograms of a blank (ringer) and a mix of 200 nmolL Glu/20 nmol/L GABA in Ringer

Mobile phase optimization

During method development, a pH of 3.5 in combination with a modifier concentration of 2% acetonitrile was found to give good separation. However, the complexity of chromatograms from microdialysis samples can vary with brain region and by the experimental treatment. In case sufficient separation is not achieved for specific microdialysis samples, the mobile phase composition can be tuned in an attempt for improvement. Two parameters that can be used for tuning are mobile phase pH and modifier concentration.

Automated mobile phase optimization - As the ALEXYS neurotransmitter Analyzer with hardware kit for GABA-Glu analysis contains two pumps to run a gradient, mobile phase optimization can be automated. The overlay of chromatograms presented in Fig. 9 is an example of a set of data that was obtained by preprogrammed automated mixing of two compositions of mobile phase with the two pumps.

pH - The influence of pH on retention of GABA and Glu is shown in Fig. 9. Responses of GABA and Glu retention to a small change in mobile phase pH are opposite: lowering the pH results in more retention for Glu, whereas GABA will elute faster. The retention behavior of the other peaks in the chromatogram makes it also evident that the pH is a powerful tool to tune the separation.

Modifier – Acetonitrile is preferred as modifier above methanol as it will not increase the mobile phase viscosity [8] and system pressure as much as it would with methanol. The addition of acetonitrile as modifier speeds up the elution of all components. However, not all peaks respond to the same degree to changes in mobile phase acetonitrile concentration as can be seen in Fig. 10. Therefore acetonitrile concentration is also a useful parameter to tune elution patterns.

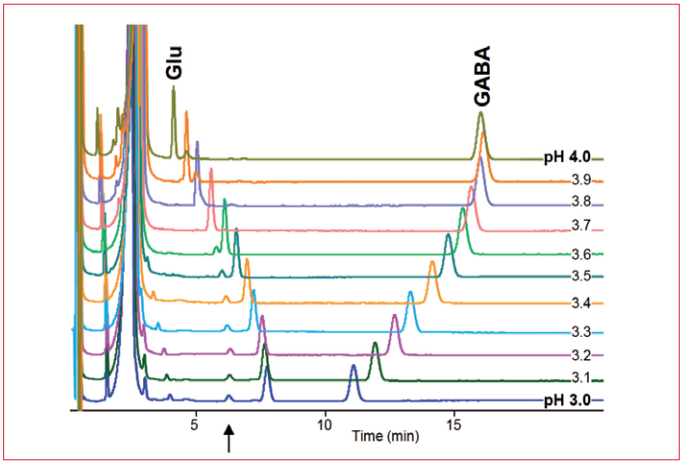


Figure 9: Effect of mobile phase pH on separation: overlay of GABA & Glu standard mixture chromatograms recorded in the range of pH 3- 4 (separation & detection performed at T=35°C).

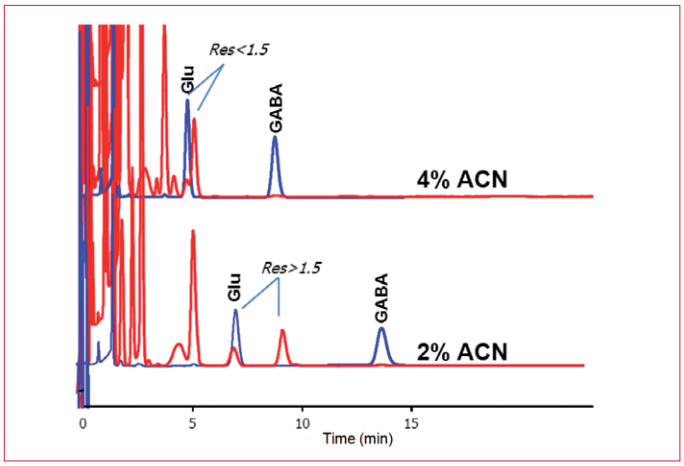


Figure 10: Overlay of two sets of chromatograms recorded with different modifier concentration (2 and 4% acetonitrile). Red trace: pooled rat dialysate from the Hippocampus. Blue trace: 5 µmol/L GABA & Glu standard mixture in Ringer. (T=35 °C, separation & detection).

Temperature

Another parameter to take into consideration with respect to optimizing of the separation is the temperature. At higher temperatures components will elute faster, thus decreases the analysis time. However it can also result in poorer separation. For this method a temperature of 40°C was chosen as the optimum with respect to separation versus analysis speed.

Analysis of microdialysates

During method development several microdialysate samples were analyzed to check the performance with real samples. Pooled basal-level rat microdialysates of different brain regions (Nucleus Accumbens and Hippocampus) were provided by Abbot Healthcare Products B.V., Weesp, the Netherlands. The samples were obtained by dialysis of 8 test animals for 16 hours at a flow rate of 2 µL/min using perfusion fluid consisting of 147 mmol/L NaCl, 4.0 mmol/L KCl, 1.2 mmol/L MgCl2 and 0.7 mmol/L CaCl2. After a sterility check, all samples (per brain region) were pooled and frozen at – 80°C until analysis.

An example chromatogram of the analysis of GABA and Glu in pooled rat dialysate from the Nucleus Accumbens is shown in Fig. 11. The insert in the top-right corner is a zoom in on the GABA peak. In Fig. 10 chromatograms are shown of pooled hippocampus rat dialysate (red curve). For the rat dialysate from the hippocampus a concentrations of 1.9 µmol/L Glu and 120 nmol/L GABA was measured.

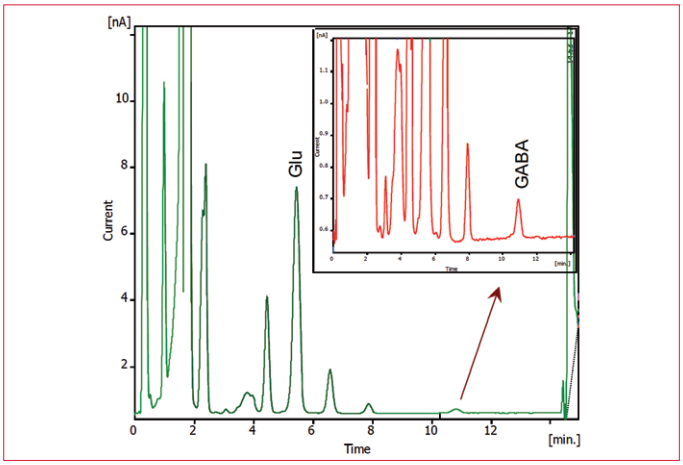


Figure 11: Example chromatogram of the analysis of GABA and Glu in pooled rat dialysate from the Nucleus Accumbens. Chromatogram recorded with a µVT-03 flowcell.

Analysis of other amino acids

In principle, the presented method in this application note is applicable to a wide range of other amino acids and related substances as well. As an example in figure 12 a chromatogram is shown of a mixture of 14 different amino acids and related substances in water (concentration 2.5 µM). It is evident that depending on the analytes of interest the chromatographic conditions should be optimized for optimal separation. See the mobile phase optimization section on the previous page for guidelines.

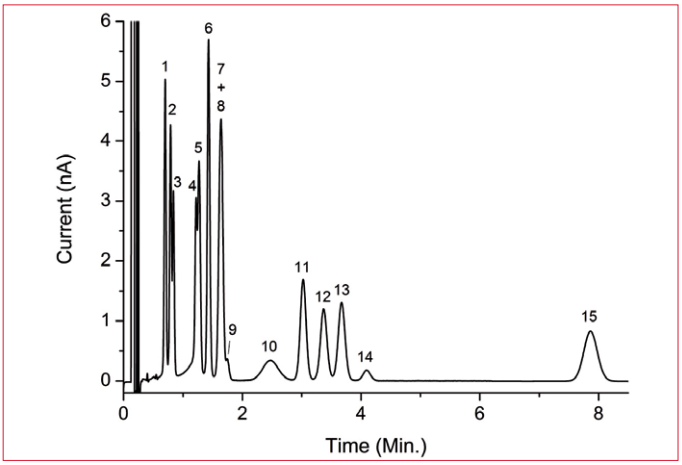


Figure 12: Analysis of 1.5 µL injection of a mixture of 14 amino acids and related substances in water at a concentration of 2.5 µmol/L. Peaks are OPA derivatives of (1) serine, (2) taurine, (3) asparagine, (5) glycine, (6) histidine, (7) aspartate, (8) glutamine, (9) cystine,(10) trans-4-hydroxy-L-proline, (11) alanine, (12) citrulline, (13) glutamate, (14) arginine, and (15) GABA; (4) is an OPA reagent peak.

Table 4

Conditions for GABA-Glu analysis*	
HPLC	ALEXYS Neurotransmitter Analyzer (pn 180.0091E) with AS 110 UHPLC cool 6-p autosampler (pn 191.0035UL)
Column	Acquity UPLC HSS T3 1.0 x 50 mm column, 1.8 µm
Pre-column filter	Acquity in-line filter kit + 6 frits
Mobile phase A (separation)	50 mM phosphoric acid, 50mM citric acid, 0.1 mM EDTA, pH3.28, 2% methanol, 1% Acetonitrile
Mobile phase B (post-separation)	40% Mobile phase A: 60% Acetonitrile
Flow rate	200 µL/min
Temperature	40 °C (separation and detection)
AS wash solution	Water/Methanol (80/20 v%)
Vinjection	1.5 µL full loop injection as part of automated in-needle derivatization user defined program
Total sample use	9 uL
Flow cell	µ-VT-03 flow cell with 0.7 mm GC WE and Salt-bridge reference electrode, spacer 25 µm
Ecell	V= 850 mV vs Ag/AgCl (SB)
Range	50 nA/V
ADF™	Off (Glu), 0.01 Hz (for GABA, set at t= 6.20 min)
Noise	1- 3 pA

*)Courtesy of Mrs. Gerdien Korte-Bouws, Department of Pharmaceutical Sciences, division of Pharmacology, University of Utrecht, The Netherlands

Table 5

Step-gradient programGABA-Glu (UU)		
Time (min)	**%A**	**%B**
Initial	100.0	0.0
12.00	100.0	0.0
12.50	5.0	95.0
14.50	5.0	95.0
15.00	100.0	0.0

Histamine

Another example is the analysis of the biogenic amine Histamine using the ALEXYS Neurotransmitter Analyzer. Histamine is considered as one of the most important mediators of allergic reactions and inflammations. Histamine is an amine, formed by decarboxylation of the amino acid histidine. It is involved in local immune responses as well as regulating physiological function in the gut and acting as a neurotransmitter. In peripheral tissues histamine is stored in mast cells, basophil granulocytes and enterochromaffin cells. Mast cell histamine plays an important role in the pathogenesis of various allergic conditions.

In figure 13 two example chromatograms are shown from a study (performed at the University of Utrecht) of the Histamine release from RBL-2H3 (mast cell model) after an allergen trigger.

Sample preparation: prior to analysis the samples were deproteinized using perchloric acid, centrifuged and the supernatant collected. The pH of the supernatant was subsequently adjusted to a pH > 8 using a sodium hydroxide solution to assure efficient derivatization with OPA. After filtering over a 4 mm diameter 0.2 µm syringe filter, 1.5 µL of the derivatized solution was injected.

To eliminate carry-over of histamine during the injection cycle a wash solution with > 20% methanol was used in the autosampler.

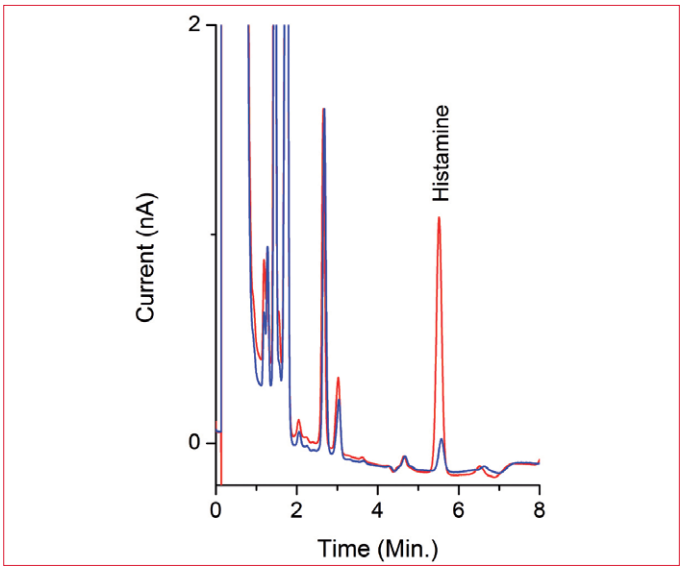


Figure 13: Analysis of the Histamine release in RBL-2H3 (mast cell model) after an allergen trigger. Chromatogram A (blue curve): Histamine level in blank (solution with RBL-2H3 cells before exposure to allergen. Chromatogram B (Red curve): Histamine level after exposure to DNP-BSA allergen. Chromatograms courtesy of Mrs. Gerdien Korte-Bouws, Department of Pharmaceutical Sciences, division of Pharmacology, University of Utrecht, The Netherlands.

Table 6

Conditions for Histamine analysis*	
HPLC	ALEXYS Neurotransmitter Analyzer (pn 180.0091E) with AS 110 UHPLC cool 6-p autosampler (pn 191.0035UL)
Column	Acquity UPLC HSS T3 1.0 x 50 mm column, 1.8 µm
Pre-column filter	Acquity in-line filter kit + 6 frits
Mobile phase A (separation)	50 mM phosphoric acid, 50mM citric acid, 0.1 mM EDTA and 8 mM KCl, pH6.0, 2% methanol, 1% Acetonitrile
Mobile phase B (post-separation)	40% Mobile phase A: 60% Acetonitrile
Flow rate	200 µL/min
Temperature	40 °C (separation and detection)
AS wash solution	Water
Vinjection	1.5 µL full loop injection as part of automated in-needle derivatization user defined program
Total sample use	9 uL
Flow cell	µ-VT-03 flow cell with 0.7 mm GC WE and ISAAC reference electrode, spacer 25 µm
Ecell	V= 0.70 V vs Ag/AgCl (ISAAC)
Range	50 nA/V
ADF™	advised between 0.5 - 0.01 Hz
Noise	1- 3 pA

*)Courtesy of Mrs. Gerdien Korte-Bouws, Department of Pharmaceutical Sciences, division of Pharmacology, University of Utrecht, The Netherlands.

Table 7

Step-gradient program Histamine (UU)		
Time (min)	**%A**	**%B**
Initial	100.0	0.0
8.00	100.0	0.0
8.50	5.0	95.0
10.50	5.0	95.0
11.00	100.0	0.0

Large neutral amino acids (LNAA's)

LNAA's (Tyr, Val, Met, Orn, Leu, Ile, Phe, Lys, Trp) can also be measured with the ALEXYS Neurotransmitter Analyzer using a mobile phase which contains a larger content of modifier. An example of an extracted chicken plasma sample is shown in the figure below:

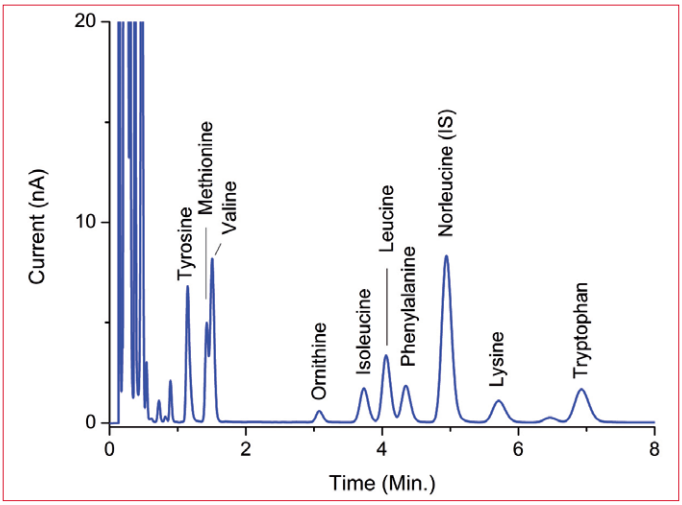


Figure 14: Analysis of extracted chicken plasma. Chromatogram courtesy of Mrs. Gerdien Korte-Bouws, Department of Pharmaceutical Sciences, division of Pharmacology, University of Utrecht, The Netherlands.

Table 8

Conditions for the analysis of LNAAs*	
HPLC	ALEXYS Neurotransmitter Analyzer (pn 180.0091E) with AS 110 UHPLC cool 6-p autosampler (pn 191.0035UL)
Column	Acquity UPLC HSS T3 1.0 x 50 mm column, 1.8 µm
Pre-column filter	Acquity in-line filter kit + 6 frits
Mobile phase A (separation)	50 mM phosphoric acid, 50mM citric acid and 0.1 mM EDTA, pH4.5, 10% methanol, 8% Acetonitrile
Mobile phase B (post-separation)	50% Mobile phase A: 50% Acetonitile
Flow rate	200 µL/min
Temperature	40 °C (separation and detection)
AS wash solution	Water
Vinjection	1.5 µL full loop injection as part of automated in-needle derivatization user defined program
Total sample use	9 uL
Flow cell	µ-VT-03 flow cell with 0.7 mm GC WE and Salt-bridge reference electrode, spacer 25 µm
Ecell	V= 850 mV vs Ag/AgCl (SB)
Range	50 nA/V
ADF™	advised between 0.1 – 0.01 Hz
Noise	1- 3 pA

*)Courtesy of Mrs. Gerdien Korte-Bouws, Department of Pharmaceutical Sciences, division of Pharmacology, University of Utrecht, The Netherlands

Table 9

Step-gradient program LNAAs		
Time (min)	**%A**	**%B**
Initial	100.0	0.0
8.00	100.0	0.0
8.50	5.0	95.0
10.50	5.0	95.0
11.00	100.0	0.0

References

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3. S. Smith, T. Sharp, Measurement of GABA in Rat Brain Microdialysates Using o-phthaldialdehyde Sulphite Derivatization and High- Performance Liquid Chromatography with Electrochemical Detection, J. Chromatography B 652 (2) (1994) 228-233
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8. J. Kehr, Determination of glutamate and aspartate in microdialysis samples by reversed-phase column LC with fluorescence and electrochemical detection, Journal of Chromatography B. 708 (1998) 27 -38
9. L.R. Snyder, J.J. Kirkland, J.W. Dolan, Introduction to Modern Liquid Chromatography, Wiley, 3rd ed. (2010)

Ordering information

ALEXYS Neurotransmitter Analyzer for GABA and Glutamate	
180.0091E	ALEXYS Neurotransmitters BP, 1 ch
191.0035UL	AS 110 Autosampler UHPLC cool 6p
180.0602	LC step-gradient upgrade
180.0504	ALEXYS GABA/Glu kit

Conclusion

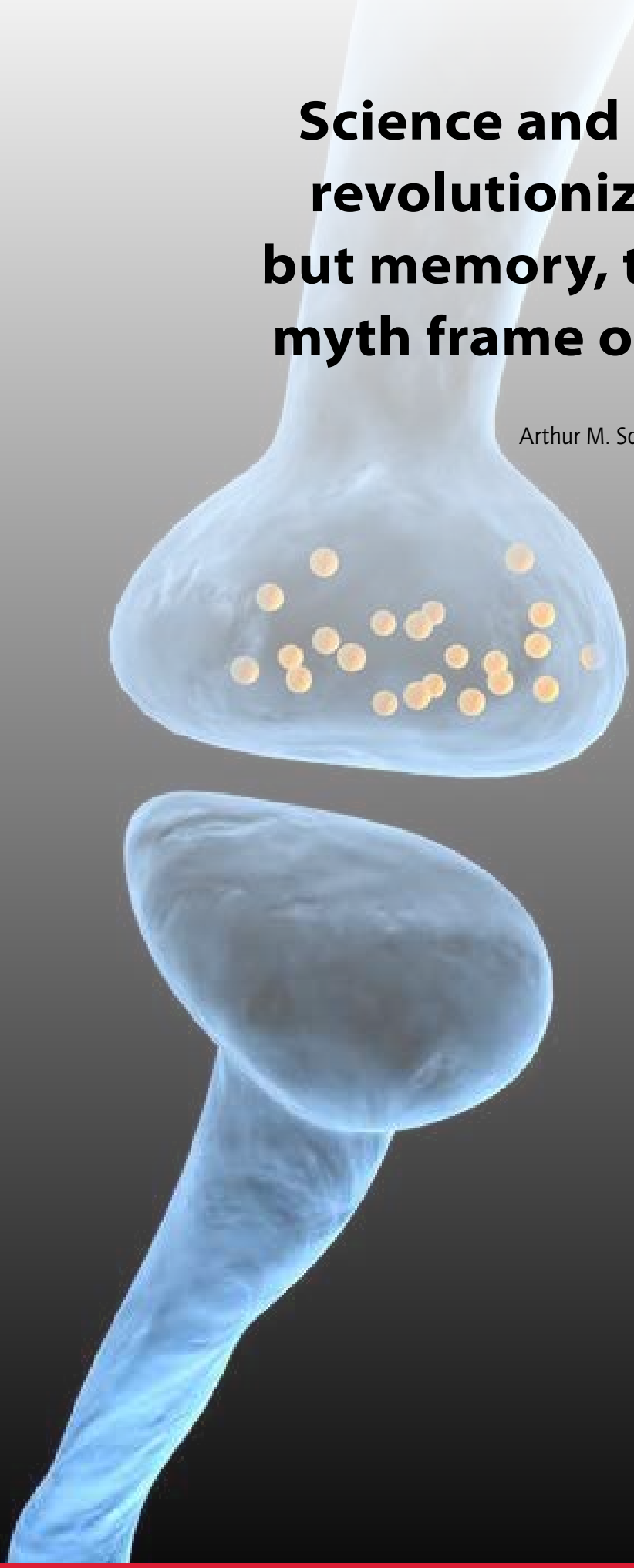
The ALEXYS Neurotransmitter Analyzer utilizes the extraordinary separation power of sub-2 µm packed columns. Plate numbers, retention times and detection sensitivity have been pushed to their limits.

The application for Amino Acids e.g. GABA/Glutamate, Histamine, LNAAs in microdialysates is a robust and suitable for routine based analysis. Optimized methods with fully automated in-the-needle derivatization, post separation step-gradient to eliminate late eluters and samples injection volumes of 5ul results in a total analysis time <12 minutes.

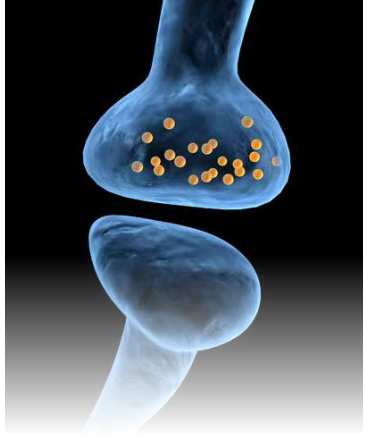
The ALEXYS Neurotransmitter Analyzer can be extended with several options and kits for any combination of other neurotransmitters, Acethylcholine and Choline as well as Monoamines and Metabolites.

Science and technology revolutionize our lives, but memory, tradition and myth frame our response.

Arthur M. Schlesinger



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 their Acidic Metabolites

- **ALEXYS Neurotransmitter Analyzer**
- **Analysis of NA, DA, 5-HT, HVA, 5-HIAA, DOPAC**
- **Insect neurotransmitters: tyramine, tyrosine, octopamine, and tryptophan**
- **LOD: 0.1 - 0.5 fmol on-column (below 100 pmol/L in less than 10 µL sample)**

Summary

The ALEXYS Neurotransmitter Analyzer using UHPLC and electrochemical detection (ECD) has been applied for the analysis of neurotransmitters in microdialysis samples, cerebrospinal fluid (CSF) and brain tissue homogenates. HPLC and ECD settings are optimized for different target compounds with respect to selectivity and detection sensitivity. The system applies a DECADE Elite ECD with a SenCell, a powerful combination for the best possible detection limits. The AS110 autosampler facilitates micro volume sample handling (few microliters), in a dedicated injection method. Detection limits are in the range of 0.1 - 0.5 fmol on column (below 100 pmol/L in less than 10 µL sample) and repeatability is better than 2% RSD for most components.

ALEXYS Neurotransmitter Analyzer for Monoamines and their Acidic Metabolites

Introduction

Microdialysis of neurotransmitters in vivo has become an invaluable tool to study neurotransmission in the living brain. Extracellular fluid of the brain is sampled via a microdialysis probe and fractions are collected for further analysis. HPLC in combination with electrochemical detection is often used to analyze neurotransmitters and metabolites [1-3]. The indolamines, monoamines (catecholamines), and metabolites are electrochemically active and are detectable with high sensitivity without the need for derivatization. The amino acid neurotransmitters and choline and acetylcholine can be detected using the same instrumentation (Figure 1).

Method requirements for analysis of neurotransmitters in microdialysis samples are challenging with respect to selectivity, sensitivity and available sample volume. There is a growing interest for collecting smaller fractions as this results in a better temporal resolution of the microdialysis experiment. Typical flow rates in microdialysis are 1 - 2 µL/min, decreasing the fraction size to a few microliters would enable a temporal resolution of a few minutes. The concentrations of NA, DA and 5-HT in microdialysis fractions can be below 100 pmol/L. In combination with a small sample volume of a few microliters, this requires an extremely low limit of detection down to the range of 0.1 – 0.5 fmol on column [1-8].

The concentrations of the metabolites DOPAC, HVA and 5-HIAA are usually considerably higher (about 100 – 1000 times), which places another challenge on the analytical method. The peak resolution should be sufficient to enable quantification of the minor peaks next to the major metabolite peaks. Finally, the analysis should be completed within an acceptable run time. This is challenging because of differences in polarity of the substances of interest.

Over the years, many papers have appeared on improving the speed of separations, or analyzing small sample volumes at low detection limits [1-8]. In this application note a scalable, fast UHPLC method for noradrenaline (NA), dopamine (DA) and serotonin (5-HT), and their metabolites homovanillic acid (HVA), 5-hydroxyindole acetic acid (5-HIAA), and 3,4-dihydroxyphenylacetic acid (DOPAC) is presented. In addition, separation and optimized detection settings for insect neurotransmitters tyramine, tyrosine, octopamine, and tryptophan are given as well.

Figure 1: ALEXYS Neurotransmitter Analyzer with AS110 autosampler and DECADE Elite.

Method

ALEXYS Neurotransmitter Analyzer

The ALEXYS Neurotransmitter Analyzer (Antec, Zoeterwoude, the Netherlands) for analysis of monoamines and metabolites consists of an OR 110 degasser unit with pulse damper(s), LC 110S pump(s), a DECADE Elite electrochemical detector, Clarity chromatography software of DataApex (Prague, The Czech Republic) and an AS 110 autosampler (other injector options are a manual injector and on-line coupling to a microdialysis experiment). A SenCell flow cell with a 2 mm glassy carbon working electrode and a sub-2µm particle 50 or 100 mm length 1.0 mm ID separation column are bundled in the additional application specific 'ALEXYS Monoamine kit' (see ordering info). Other kits are available as well, such as kits for acetylcholine, GABA and glutamate [3, 4].

Sample preparation

Before injection, sample preparation should be applied to produce a sample that is relatively free of interferences to prevent damage like clogging of the system or column. Another consideration to treat a sample is to prevent degradation of the components of interest if it will not be analyzed immediately after collection. These are the treatment advises for different samples:

- *Microdialysate samples*

  These samples are relatively clean and can be injected in the system without the need for filtering or other treatment. However, to prevent degradation of the monoamines, acidification with or without an anti-oxidant is most often applied to the sample [5, 6]

- *Brain homogenate samples*

  Preparation of a sample from brain tissue usually consists of homogenization in a dilution of perchloric acid, followed by a centrifugation step to remove debris [5, 7].

- *Cerebrospinal fluid*

  These sample are relatively more complex compared to microdialysis samples, and in literature it can be found that such samples either are not processed before injection, or they are acidified and centrifuged [8], or acid/anti-oxidant mix added before injection [5]. We highly recommend to apply at least a centrifugation or filtration step before injection to remove particles: sub-2 micron columns have a higher risk of clogging compared to the larger particle columns as used in older research.

- *Blood and urine (in clinical analysis)*

  For the analysis of catecholamines in blood or urine, complete SPE work-up kits are commercially available (e.g. at Chromsystems or BioRad). Such samples, however, have a clinical/diagnostics background, and the details are covered in another Antec application note [9].

Injection

A dedicated and reproducible injection program has been developed for the AS110 autosampler that efficiently handles small samples of only a few microliters. The details are described elsewhere [10], in short the injection program works without 'loop overfill' that is usually applied in full loop injections. It efficiently transports only 2 µL in addition to the injection volume between air bubbles to the loop without 'wasting' any additional sample.

Another mode of injection is the direct coupling of microdialysis to the ALEXYS using an electric valve. In principle, the continuous flow runs through an injection valve and at regular intervals a sample is injected. The analyses described in this application note can be applied to such on-line microdialysis set-up. Details about this set-up have been described elsewhere [11].

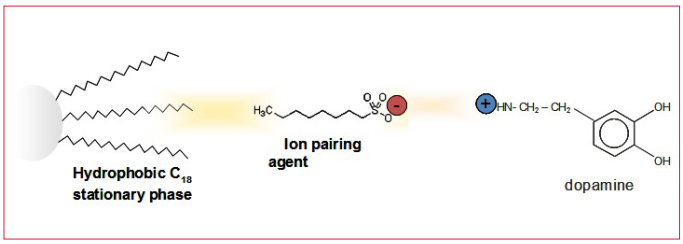


Figure 2: Schematic representation of ion-pairing principle for HPLC separation of monoamines on C18 particles

Separation

In the eighties of last century, a lot of research was done to develop and optimize the analysis of catecholamines, the precursors and metabolites, but this field is still progressing until today, see for example references [12 - 20]. Monoamines have a positive charge at pH<7, and they can gain retention on a (neutrally charged) reversed phase column when ion-pairing agent is added to the mobile phase (Figure 2). The monoamine retention times respond to the concentration of ion-pairing agent in the mobile phase (Figure 3).

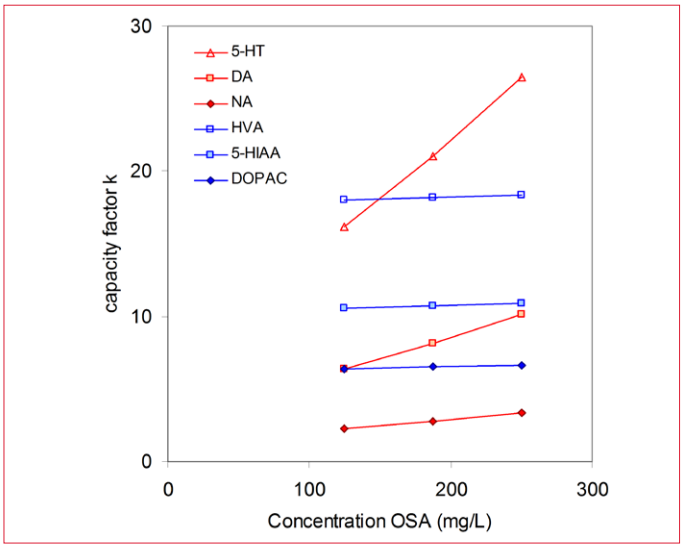


Figure 3: Effect of the ion-pairing agent octane sulfonic acid sodium salt (OSA) on retention behavior of monoamines (red) and acidic metabolites (blue).

The acidic metabolites have a carboxyl group with a pKa value of 4.7. They are best retained on reversed phase columns when applying a mobile phase with acidic pH. When applying a neutral pH, the negative charge of the carboxyl group makes them elute in the solvent front. The pH of the mobile phase therefore strongly affects the separation and retention times of acidic metabolites (fig 4.).

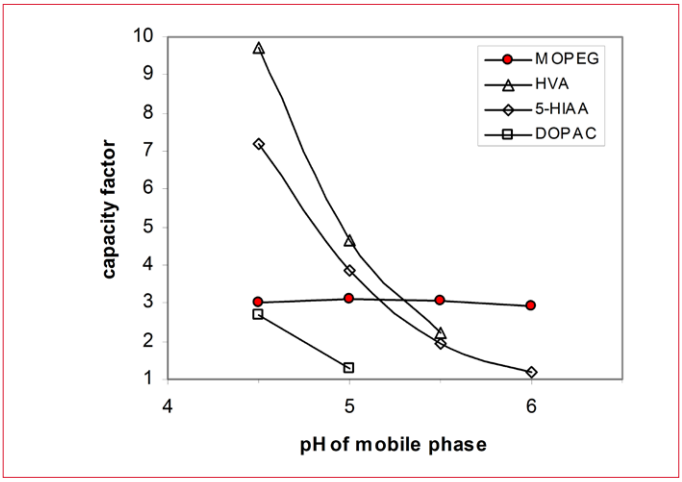


Figure 4: Effect of pH on retention behavior of molecules with a carboxyl group. For reference, the red dots indicate the retention of a molecule without a carboxyl group.

Detection

Monoamines and acidic metabolites are electrochemically detectable on a glassy carbon working electrode. A number of excellent papers are available reporting voltammetric behavior of relevant biogenic amines and metabolites [12 - 14]. Nagao and Tanimura [14] classified the biogenic amines in four groups depending on their electrochemical behavior in a mobile phase at pH 3.6 and flow cell with glassy carbon and Ag/AgCl electrodes. The four groups are: catechol compounds such as the catecholamines, DOPAC and DOPA ($E_{1/2}$ = 380-500 mV), indoles such as 5-HT and 5-HIAA ($E_{1/2}$ = 480-520 mV), vanillic compounds such as VMA, HVA and MHPG ($E_{1/2}$ = 640-680 mV) and monohydroxyphenols such as tryptophan and tyrosine ($E_{1/2}$ = 870 mV). It should be noted that these given values are affected by pH (shift of about 60 mV for every pH unit), mobile phase composition and differences in glassy carbon working electrode materials. It may be clear that the working potential has to be set as low as possible to ensure selectivity, but high enough to generate a clear response for the specific component(s) of interest. The working potential can also be used as a tool to enhance selectivity of the method:

- If there is only interest in the analysis of DA (and/or NA), but not 5-HT, then the working potential can be set to a lower value compared to the setting suggested in the settings table. In such case, 5-HT (and many other components) will not generate a signal.

- For detection of the monohydroxyphenols, a relatively high potential is necessary.

Electrode activation

It is important to realize that a new or freshly polished electrode can behave differently from an electrode that is in use for a longer time. A flow cell can build up a 'history' which can result in a chromatogram with different relative peak heights compared to a new cell. However, flow cells can often be 're-initialized' by applying an electrochemical pulse. The HPLC is not changed, the pump is on and the usual mobile phase is applied. The detector is set to PULSE mode for about 10 min with pulse settings E1=+1.0V, E2=-1.0V, t1=1000ms, t2=1000ms, t3=0 and ts=20ms. After 10 minutes the detector is set to DC mode at the detection potential [21]. The background current should drop below 25 nA in less than 30 min. This activation procedure can be programmed in the DECADE Elite detector and Clarity software for automated application. The pulse mode is not available in the SDC or Lite versions of the detector.

Results and discussion

Method optimization for analysis of neurotransmitters was carried out in two steps. Firstly, the HPLC separation was optimized with special attention to injection volume, selectivity and total analysis time. In a second step the working potential and detection settings were optimized for best detection sensitivity. It is not always required to measure all neurotransmitters and metabolites together, therefore several methods are described for different selections of target substances. Small differences in detection potential or mobile phase composition can have a considerable effect on assay validation parameters. All the presented applications show good performance with repeatability of signal (n=6) better than 2% RSD for peak area, and correlation coefficients better than 0.998.

Analysis of monoamines and acidic metabolites and some other related components

For the 'single shot' analysis of the monoamines NA, DA and 5-HT, and the acidic metabolites DOPAC, 5-HIAA and HVA, the settings in Table 1 were used to obtain a chromatogram as given in Figure 5. Ten other components of interest were added to the standard mix as well to demonstrate the separation performance. The column efficiency is better than 200.000 plates/m for most substances, resolution is >1.4 and total elution time is <12 min.

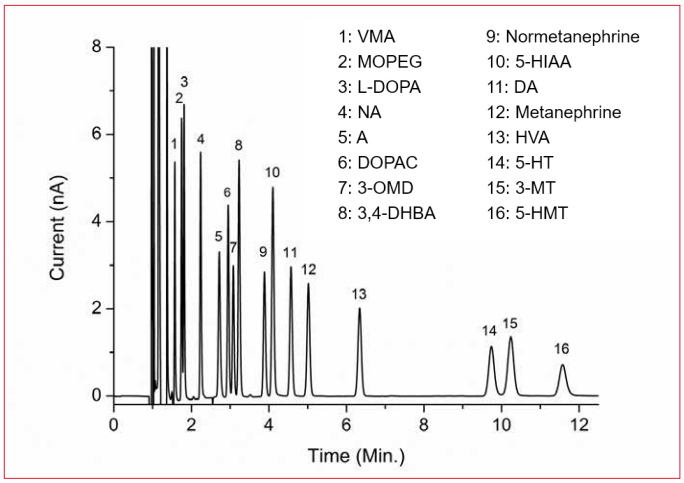


Figure 5: Chromatogram of 100 nmol/L standards in Ringer solution with 10 mmol/L acetic acid. Injection volume 2 µL. Conditions as in Table 1.

Table 1

LC-ECD settings for analysis of all monoamines and their acidic metabolites

HPLC	ALEXYS Neurotransmitters Analyzer with Monoamines kit
Column	Acquity UPLC BEH C18, 1.7µm, 1 x 100 mm (Waters)
Mobile phase	100 mM phosphoric acid, 100 mM citric acid, 0.1 mM EDTA.Na2 set to pH 3.0, 600 mg/L octanesulfonic acid sodium salt, 8% acetonitrile Refresh at least every 3 days.
Temperature	37 °C (separation and detection)
Flow rate	50 µL/min
Pump piston wash	15% isopropanol in water (HPLC grade; refresh at least once per week)
Flow cell	SenCell with 2 mm GC WE, AST position 1
Potential	0.8 V vs. salt bridge reference electrode
ADF	0.5 Hz
Range	1 nA/V for near-LOD signals 50 nA/V or higher for large signals
$V_{injection}$	2 µL (5 µL max)
Needle wash	Water (HPLC grade; refresh at least once per week)
Backpressure	about 250 bar
Icell	about 3 nA

The theoretical maximum loadability of a microbore column with about 200.000 plates/m is in the range of 0.5-3 µL for peaks between 2-12 min assuming no more than 5% contribution by injection dispersion to the total column band broadening [22]. The combination of C18 column material and a mobile phase with ion pairing agent and a few percent organic solvent seems to extend the loadability of the column: for the peaks between 3-12 min the plate numbers and asymmetry were not affected up to 5 µL injections, while the earlier peaks only showed peak broadening above 2.5 µL injections. To avoid any unwanted peak broadening, the advice for the application settings presented in Table 1 is to inject 2 µL on column. An injection volume of 5 µL, is a bit of a trade-off, it results in a decreased peak efficiency but peaks are higher and thus a better sensitivity (Figure 7). Sensitivity of the analysis of monoamines and acidic metabolites was checked with 5 µL full loop injections and showed a detection limit of 0.2-0.4 fmol on column (40-80 pmol/L for 5 µL injections).

A microdialysate sample was analyzed with the conditions from Table 1 to show the applicability of the method (Figure 6). Concentrations of the monoamines was calculated to be in the range of 0.1-0.9 nmol/L, which is near the detection limit. The selectivity and sensitivity of the method is sufficient to analyze these samples.

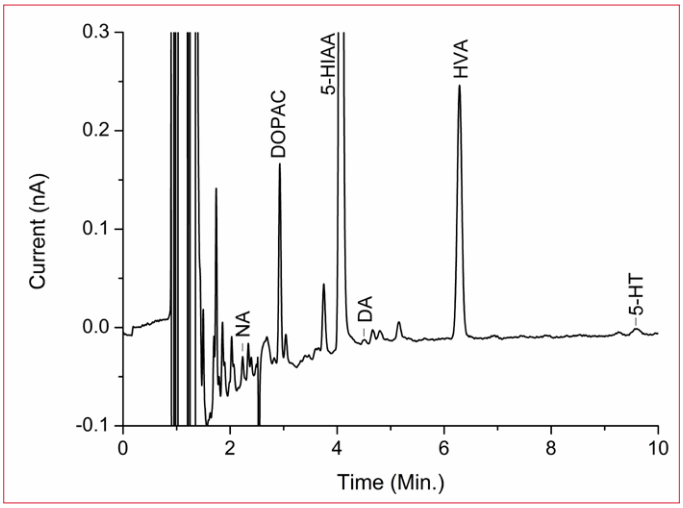


Figure 6: Chromatogram of rat prefrontal Cortex microdialysate (after administration NA re-uptake inhibitor). Sample kindly provided by Gerdien Korte-Bouws, Department of Phychopharmacology, University of Utrecht. Injection volume 2 µL. Conditions as in Table 1, but with µVT-03 flow cell and ISAAC reference electrode vs 8 mmol/L KCl (640 mV).

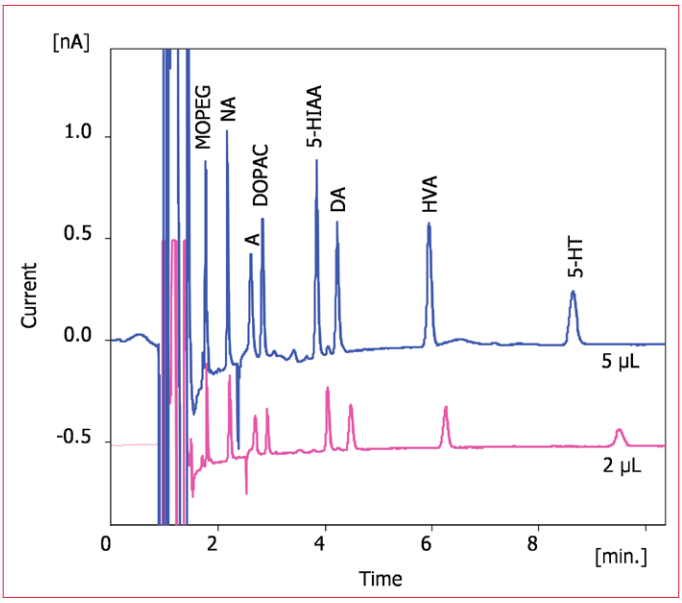


Figure 7: . Effect of injection volume on chromatograms of 10 nmol/L standards in Ringers solution with 10 mmol/L acetic acid. Conditions as in Table 1, but with µVT-03 flow cell and ISAAC reference electrode vs 8 mmol/L KCl (640 mV).

For (more) complex samples, or generally spoken in case the selectivity is not sufficient, a so called DCC (dual cell control) setup is advised. In such setup a dual channel HPLC system is used with different HPLC conditions for both channels. One sample is simultaneously injected with a dual loop valve and analyzed under different conditions as described elsewhere [23].

Analysis of acidic metabolites

For the analysis of the acidic metabolites DOPAC, 5-HIAA and HVA, the settings in Table 2 can generate a chromatogram as given in Figure 8. As the mobile phase does not contain ion-pairing agent, the monoamines will not appear in the chromatogram as they will elute as part of the solvent front.
Sensitivity of the analysis of acidic metabolites was checked with 2 µL full loop injections and showed a detection limit of about 0.2 nmol/L.

Two different microdialysis samples were analyzed to show the applicability of the method (Figure 9). Concentrations of the acidic metabolites were calculated to be in the range of 4-240 nmol/L, which is well above the detection limit of the application. It should be noted that there are brain region specific peaks eluting after the last peak of interest. Analysis time of 1.5 min instead of 1 min may have to be applied to make sure that such peaks do not show up in the following chromatogram. In case the sample shows a need for more separation, the acetonitrile concentration in the mobile phase can be lowered from 10% to 5% (which would double the analysis time to 2 min).

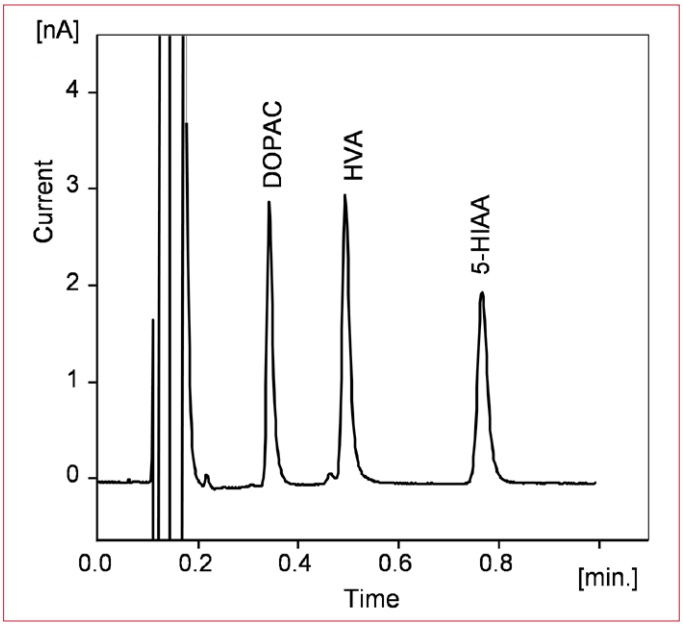


Figure 8: . Chromatogram of 100 nmol/L DOPAC, HVA and 5-HIAA in Ringer solution with 10 mmol/L acetic acid. Injection volume 1 µL. Conditions as in Table 2

Table 2

LC-ECD settings for analysis of the acidic metabolites DOPAC, HVA and 5-HIAA	
HPLC	ALEXYS Neurotransmitters Analyzer with ALEXYS Monoamines kit, 50 mm
Column	Acquity UPLC BEH C18, 1.7µm, 1 x 50 mm (Waters)
Mobile phase	100 mM phosphoric acid, 100 mM citric acid, 0.1 mM EDTA.Na2 set to pH 3.0, 10% acetonitrile Refresh at least every 3 days.
Temperature	37 °C (separation and detection)
Flow rate	175 µL/min
Pump piston wash	15% isopropanol in water (HPLC grade; refresh at least once per week)
Flow cell	SenCell with 2 mm GC WE, AST position 1
Potential	0.8 V vs. salt bridge reference electrode
ADF	off
Range	1 nA/V for near-LOD signals 50 nA/V or higher for large signals
$V_{injection}$	1 µL
Needle wash	Water (HPLC grade; refresh at least once per week)
Backpressure	about 450 bar
Icell	about 3 nA

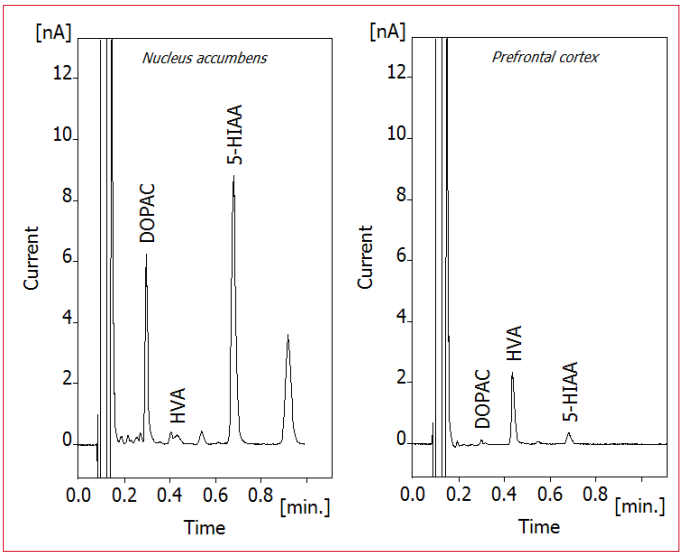


Figure 9: Chromatograms of rat brain microdialysate from prefrontal cortex and nucleus accumbens. Samples kindly provided by Gerdien Korte-Bouws, Department of Phychopharmacology, University of Utrecht. Injection volume 2 µL. Conditions as in Table 2, but with flow rate of 0.2 mL/min.

Analysis of NA, DA and 5-HT

For the selective analysis of monoamines, the acidic metabolites can be moved out of the chromatogram by increasing the pH of the mobile phase. Applying the mobile phase composition as given in Table 3, resulted in chromatograms as in Figure 10 and Figure 11. Detection limit is 0.1 nmol/L for NA and DA, and 0.3 nmol/L for 5-HT using an injection volume of 5 µL. Better detection limits for 5-HT are feasible if more selective settings would be applied (see below)

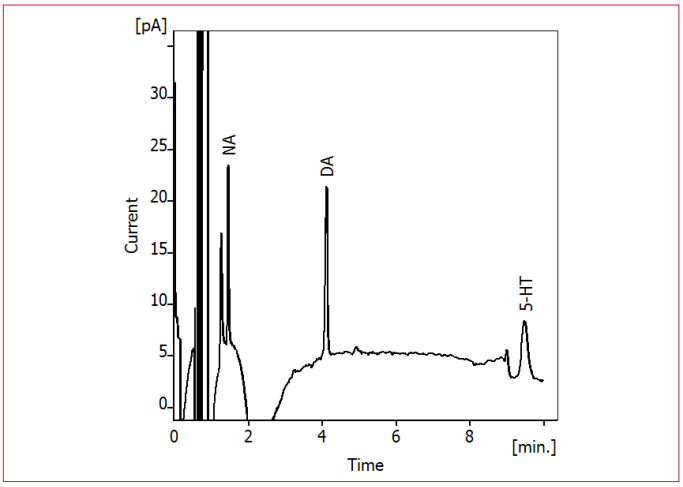


Figure 10: Chromatogram of 2 nmol/L standard of NA, DA and 5-HT in Ringer solution with 10 mmol/L acetic acid. Injection volume 5 µL. Conditions as in Table 3, but with the use of a µVT-03 flow cell.

A microdialysate sample was analyzed to show the applicability of the method (Figure 11). In this chromatogram, concentrations of the monoamines were calculated to be 0.3 nmol/L NA, 1.6 nmol/L DA and 0.8 nmol/L 5-HT.

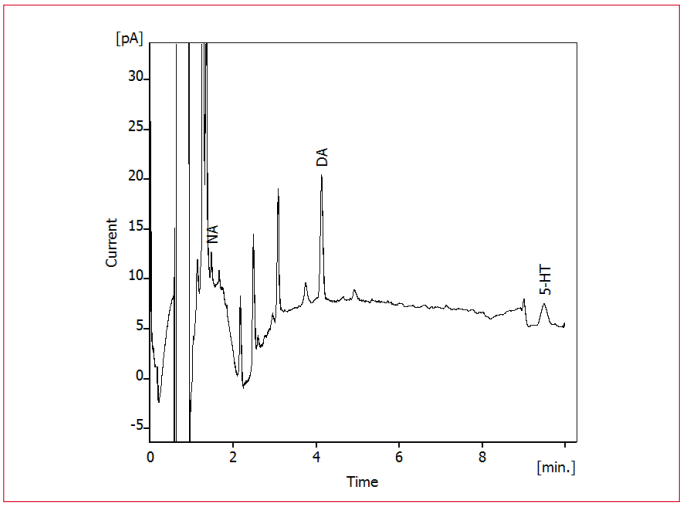


Figure 11: Chromatogram of rat brain Nuccleus accumbens dialysate, acidified during collection with acetic acid (10 mmol/L final concentration). Injection volume 5 µL. Conditions as in Table 3, but with the use of a µVT-03 flow cell.

Table 3

LC-ECD settings for analysis of NA, DA and 5-HT	
HPLC	ALEXYS Neurotransmitters Analyzer with ALEXYS Monoamines kit
Column	Acquity UPLC BEH C18, 1.7µm, 1 x 100 mm (Waters)
Mobile phase	100 mM citric acid, 100 mM phosphoric acid, pH 6.0, 0.1 mM EDTA, 950 mg/L OSA, 5% acetonitrile Refresh at least every 3 days.
Temperature	42 °C (separation and detection)
Flow rate	75 µL/min
Pump piston wash	15% isopropanol in water (HPLC grade; refresh at least once per week)
Flow cell	SenCell with 2 mm GC WE, AST position 1
Potential	0.46 V vs. salt bridge reference electrode
ADF	off
Range	1 nA/V
$V_{injection}$	2 µL
Needle wash	Water (HPLC grade; refresh at least once per week)
Backpressure	about 370 bar
Icell	about 0.5 nA

Target analysis of NA/DA or DA/5-HT

Analysis of NA and DA is accomplished by applying the conditions as given in in Table 3, but lowering the working potential to about 300 mV. At such low potential a number of peaks (incl. 5-HT) will not be visible anymore resulting in a rather 'clean' chromatogram. This clearly shows how the working potential can improve the selectivity for this analysis.

For analysis of DA and 5-HT, the acidic metabolites are selectively moved to the unretained front in the chromatogram by increasing the pH of the mobile phase. DA and 5-HT are both sufficiently retained and easily separated even when a much shorter column is used. A shorter column results in elution of NA in the solvent front, but more important it has less on-column dilution, which results in smaller peak volumes and therefore better sensitivity compared to using the longer column.

Sensitivity of the analysis of DA and 5-HT was checked with 1.5 µL injections (and 3 µL total sample use) and showed a detection limit of 100 pmol/L (0.15 fmol on column).

A microdialysate sample was analyzed with the conditions from Table 4 to show the applicability of the method (Figure 13). Concentrations of the monoamines were calculated of be 0.7 nmol/L DA and 0.2 nmol/L 5-HT.

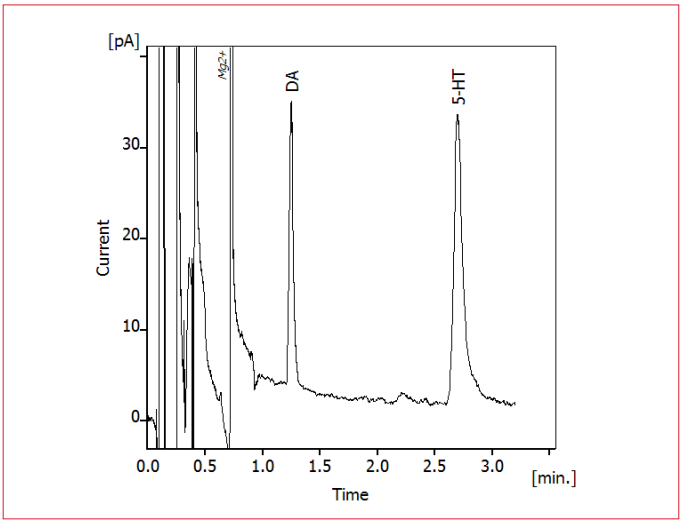


Figure 12: Chromatogram of 1 nmol/L DA and 5-HT in Ringer solution (which contains among others 1.2 mmol/L Mg2+) with 10 mmol/L acetic acid. Injection volume 1.5 µL. Conditions as in Table 4

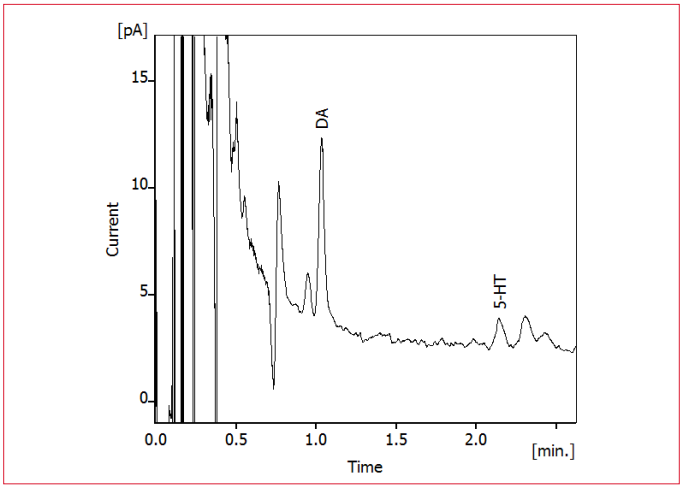


Figure 13: Chromatogram of rat brain basal level microdialysate sample with preservative mix (containing citric acid, EDTA and acetic acid). Sample kindly provided by Jolien van Schoors, Department of Pharmaceutical Chemistry and Drug Analysis, Vrije Universiteit Brussel. Injection volume 1.5 µL. Conditions as in Table 4.

Table 4

LC-ECD settings for analysis of DA and 5-HT

HPLC	ALEXYS Neurotransmitters Analyzer with ALEXYS Monoamines kit, 50 mm
Column	Acquity UPLC BEH C18, 1.7µm, 1 x 50 mm (Waters)
Mobile phase	200 mM acetic acid, 0.1 mM EDTA.Na2, pH 5.8, 250 mg/L sodium 1-decanesulfonate, 7.5 % acetonitrile Refresh at least every 3 days.
Temperature	35 °C (separation and detection)
Flow rate	175 µL/min
Pump piston wash	15% isopropanol in water (HPLC grade; refresh at least once per week)
Flow cell	SenCell with 2 mm GC WE, AST position 1
Potential	0.46 V vs. salt bridge reference electrode
ADF	off
Range	1 nA/V
$V_{injection}$	2 µL
Needle wash	Water (HPLC grade; refresh at least once per week)
Backpressure	about 470 bar
Icell	about 0.2 nA

Target analysis of DA or 5-HT only

For selective analysis of DA or 5-HT only, the working potential is an important parameter as can be seen in Figure 14. In case of DA analysis, lowering the working potential makes other peaks less/not visible. As a result fast run times are feasible with excellent detection sensitivity. A very short total analysis time of only 1 min is feasible in case there is only the need to measure 5-HT (Figure 15). The mobile phase composition and short column make all the other components elute in the solvent front. Sensitivity of the analysis of DA and 5-HT was checked with 1.5 µL injections (and 3 µL total sample use) and showed a detection limit of 100 pmol/L (0.15 fmol on column). A microdialysis sample was analyzed with the conditions from Table 5 to show the applicability of the method (Figure 16). Concentrations of the monoamines were calculated of be 0.7 nmol/L DA and 0.2 nmol/L 5-HT.

Table 5

LC-ECD settings for analysis of 5-HT	
HPLC	ALEXYS Neurotransmitters Analyzer with ALEXYS Monoamines kit, 50 mm
Column	Acquity UPLC BEH C18, 1.7µm, 1 x 50 mm (Waters)
Mobile phase	100 mM citric acid, 100 mM phosphoric acid, 0.1 mM EDTA.Na2, pH 6.0, 5 % acetonitrile, 25 mg/L octane sulfonic acid, sodium salt Refresh at least every 3 days.
Temperature	37 °C (separation and detection)
Flow rate	100 µL/min
Pump piston wash	15% isopropanol in water (HPLC grade; refresh at least once per week)
Flow cell	SenCell with 2 mm GC WE, AST position 1
Potential	0.46 V vs. salt bridge reference electrode
ADF	off
Range	1 nA/V
$V_{injection}$	2 µL
Needle wash	Water (HPLC grade; refresh at least once per week)
Backpressure	about 270 bar
Icell	about 0.7 nA

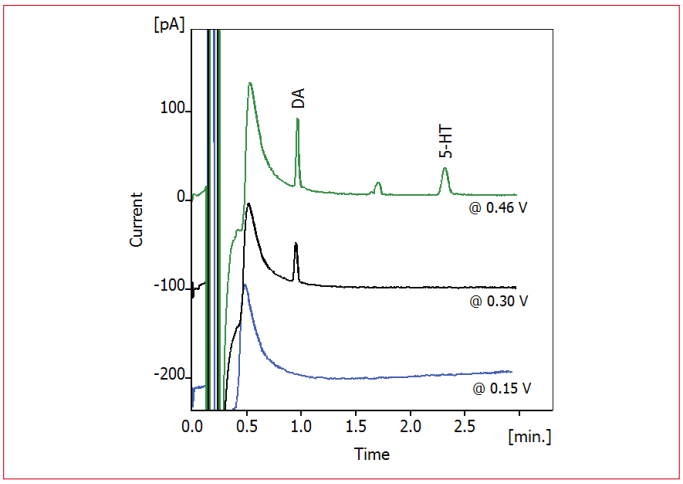


Figure 14: Chromatogram of 1 nmol/L DA and 5-HT in Ringer solution with 10 mmol/L acetic acid, analyzed with different working potentials . Injection volume 1.5 µL. Conditions as in Table 5, but with flow rate of 150 µL / min (pressure about 400 bar) and 250 mg/L octane sulfonic acid sodium salt in the mobile phase.

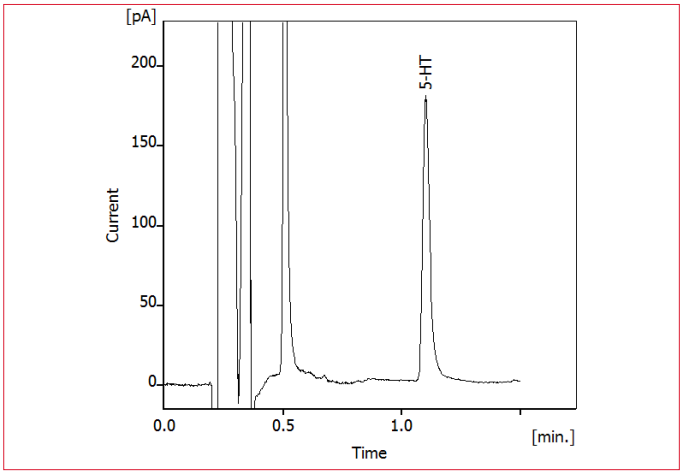


Figure 15: Chromatogram of 10 nmol/L 5-HT in Ringer solution with 10 mmol/L acetic acid. Injection volume 1.5 µL. Conditions as in Table 5.

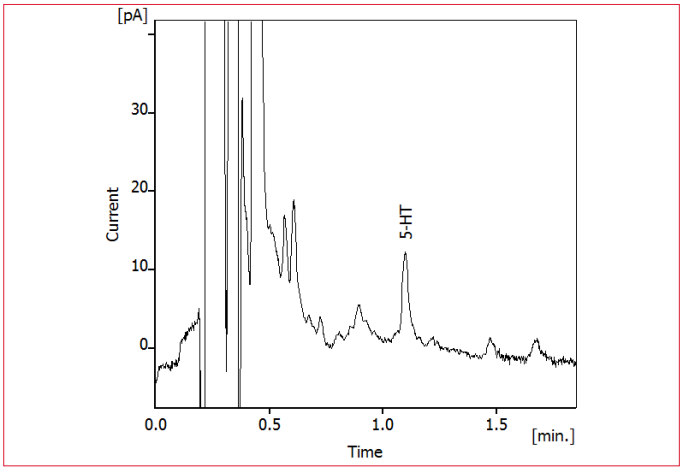


Figure 16: Chromatograms of rat brain microdialysate from prefrontal cortex. Samples kindly provided by Gerdien Korte-Bouws, Department of Phychopharmacology, University of Utrecht. Injection volume 1.5 µL. Conditions as in Table 5.

Analysis of tyramine, tyrosine, octopamine, tryptophan

The analysis of the monohydroxyphenols require a higher working potential for their detection compared to the previously described components. Using the conditions as given in Table 2, they are not detectable. Only after increasing the potential with about 0.2 V did these components show a signal in the chromatogram (Figure 17).

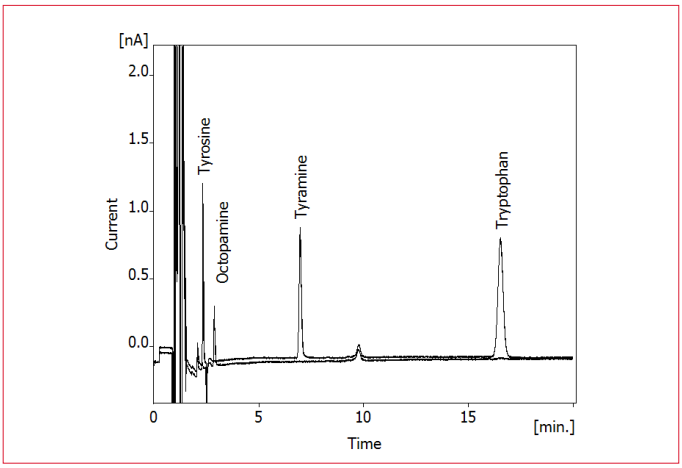


Figure 17: . Chromatograms of 100 nmol/L standards in Ringers solution with 10 mmol/L acetic acid. Conditions as in Table 1, but with µVT-03 flow cell and ISAAC reference electrode vs 8 mmol/L KCl (850 mV).

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Ordering information

ALEXYS Neurotransmitter Analyzer for Monoamines and metabolites	
180.0091E	ALEXYS Neurotransmitters Analyzer
191.0035UL	AS 110 autosampler UHPLC cool 6p

Application specific hardware kits

Parts in ALEXYS Monoamines kit (180.0502)	
116.4120	SenCell with 2 mm GC WE and sb REF
250.1163	Acquity UPLC BEH C18, 1.7 µm, 1 x 100 mm

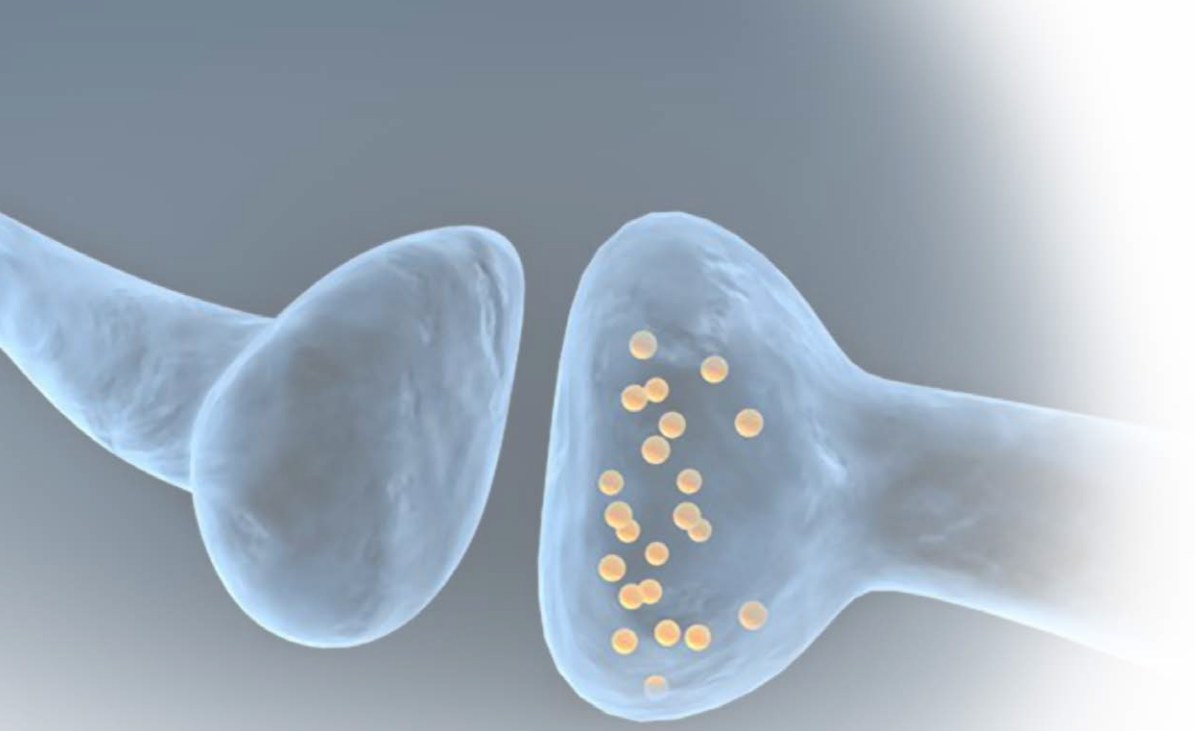
Parts in ALEXYS Monoamines kit, 50 mm (180.0503)	
116.4120	SenCell with 2 mm GC WE and sb REF
250.1160	Acquity UPLC BEH C18, 1.7µm, 1 x 50mm

Conclusion

The ALEXYS Neurotransmitter Analyzer is a dedicated platform for fast analysis of monoamines and their metabolites in small samples with excellent detection limits. The system applies UHPLC with a DECADE Elite detector, an amperometric SenCell, and a dedicated autosampler for handling micro volumes. Settings for different sets of target compounds are presented with excellent sensitivity and repeatability, as well as applicability to real samples. Detection limits are in the range of 0.1 - 0.5 fmol on column (below 100 pmol/L in less than 10 µL sample) and repeatability is better than 2% RSD for most components.

Men love to wonder, and that is the seed of science.

Ralph Waldo Emerson



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