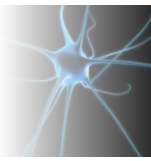
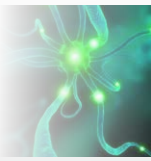
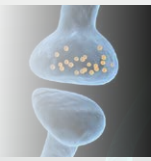






Applications Notebook

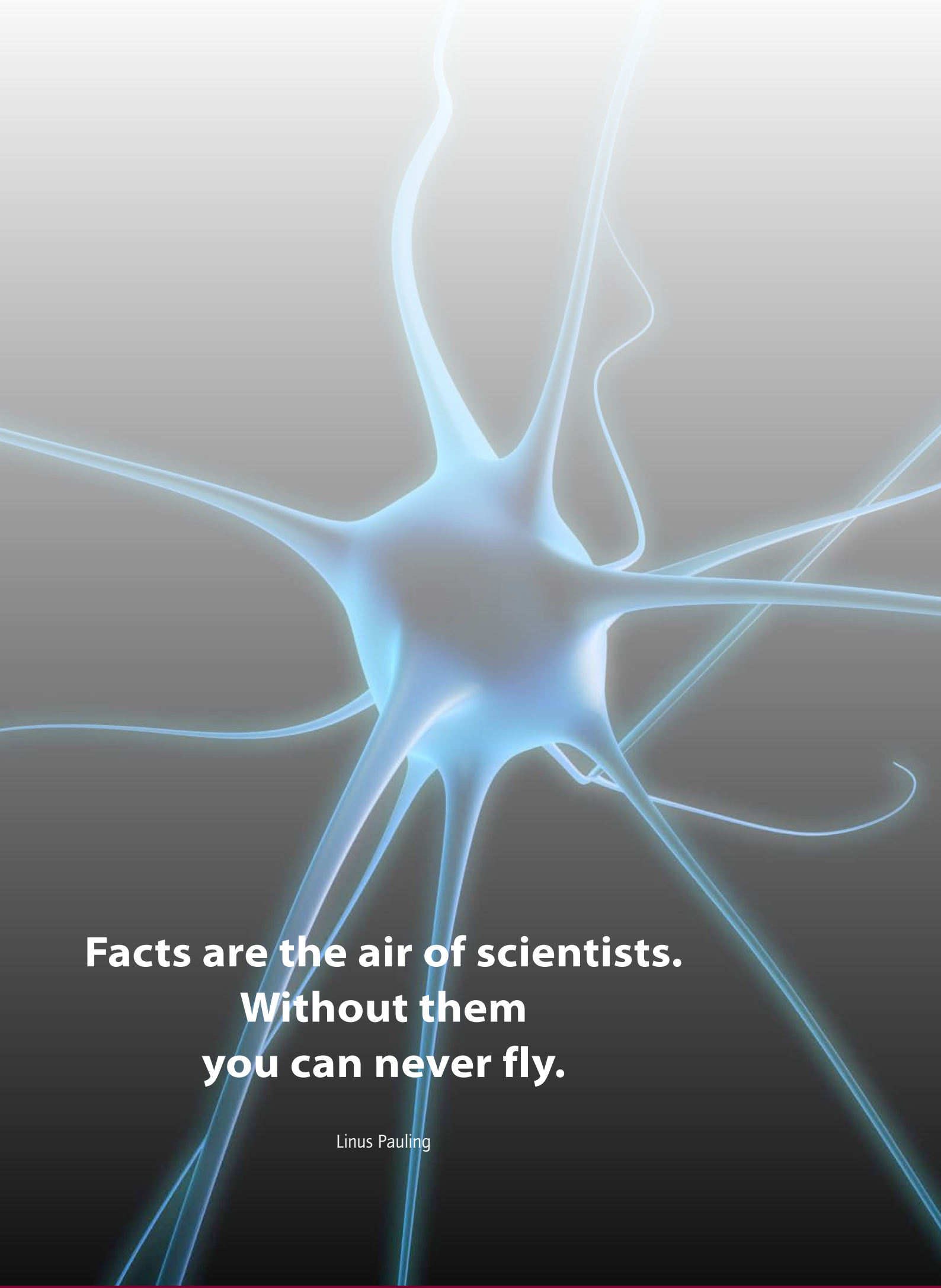
Neurotransmitter Analysis using the ALEXYS™ Analyzer for Highest Sensitivity

Design: MGO-studio, Maarssen, NL
Illustrations: Antec
Printing: EPS, Amsterdam, NL

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the ALEXYS system. The actual performance may be affected by factors beyond Antec's control. Specifications mentioned in this notebook are subject to change without further notice.

The author and publisher acknowledge their duty to provide as accurate a publication as possible. Nevertheless, they cannot be held liable for any possible inaccuracies in this publication.

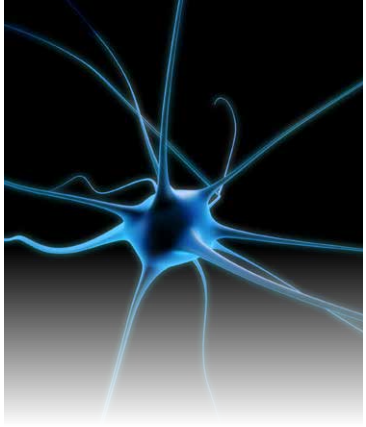
Contents



**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

- **Fast separation on a sub-2µm UHPLC column**
- **Flexcell with easily exchangeable electrodes**
- **Detection limit down to 0.3 nmole/L ACh**
- **Total analysis time < 6 min**

Introduction

The ALEXYS® Acetylcholine analyzer featuring a FLEXCELL™ with easily exchangeable working electrode disks can be used in combination with a peroxidase kit for fast and sensitive detection of basal ACh levels in microdialysis samples.

Analysis of acetylcholine (ACh) and Choline (Ch) by HPLC-ECD is based on an ion-pairing separation, followed by post-column enzymatic conversion to hydrogen peroxide with acetylcholinesterase (AChE) and choline oxidase (ChO) [1]. Both enzymes are covalently bound to a stationary phase in an immobilized enzyme reactor (IMER). After conversion, the hydrogen peroxide can be detected electrochemically on a glassy carbon electrode coated with horseradish peroxidase (HRP).

Summary

The ALEXYS Neurotransmitter Analyzer is a modular system that can be customized for specific neurotransmitters. In this application note a fast and sensitive method is presented for the analysis of acetylcholine. Fast and efficient separation is achieved using a sub-2 µm particle UHPLC column. A post column IMER is applied to convert ACh in hydrogen peroxide which is detected. With this approach a detection limit down to 0.3 nmol/L is obtained using a total sample of 10 µL (3 fmol).

Figure 1: ALEXYS Neurotransmitter Analyzer for acetylcholine.

The ALEXYS Neurotransmitter Analyzer consists of the OR 110 degasser unit, LC 110S pump(s), the AS 110S autosampler, the DECADE II EC detector and Clarity data acquisition software. Complementary kits have been developed for common neurotransmitters such as dopamine (DA), noradrenaline (NA), serotonin (5HT) and metabolites, or acetylcholine (ACh) and choline (Ch).

Method and results

ACh and Ch are very polar molecules (Fig. 2), and are positively charged at neutral pH. They are separated using a C18 column with an ion-pairing agent in the mobile phase [2].

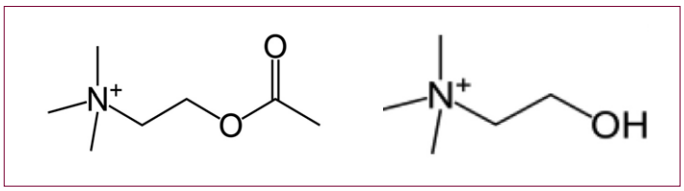


Figure 2: Structure of Acetylcholine (left) and Choline.

To convert ACh and Ch to the electrochemically detectable hydrogen peroxide (Fig. 3), an immobilized enzyme reactor (IMER) containing acetylcholine esterase and choline oxidase is connected directly behind the analytical column.

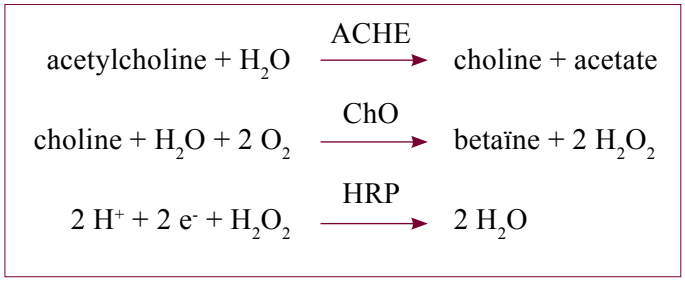


Figure 3: Enzymatic conversion of acetylcholine and choline to electrochemically detectable hydrogen peroxide in post-column immobilized enzyme reactor (IMER).

The reduction of hydrogen peroxide involves a two electron transfer per molecule. Reduction results in negative peaks in a chromatogram; this can be inverted by setting the detector polarity to 'negative'.

Electrode coating procedure

Coating a glassy carbon working electrode is done by drying a drop of 'surfactant solution' followed by a drop of 'peroxidase/polymer coating solution' from the peroxidase electrode refill kit, and letting it dry overnight [4]. It is recommended to coat two electrodes at the same time, the second electrode can serve a as a back-up or replacement electrode (shelf life: approximately 1 week in the refrigerator).

Repeatability, linearity and detection limit

The repeatability (n = 6) using a 5 nmol/L ACh in Ringer solution was found to be better than 0.20% RSD for retention time and better than 3% for peak area and height. However a significant drop in response was observed over time affecting the interday RSD's for peak area and height. This is caused by a loss in activity of the HRP enzyme coating on the working electrode which should be replaced regularly (after a few days). This highlights the importance of having short runtimes per sample and regular calibration with standards.

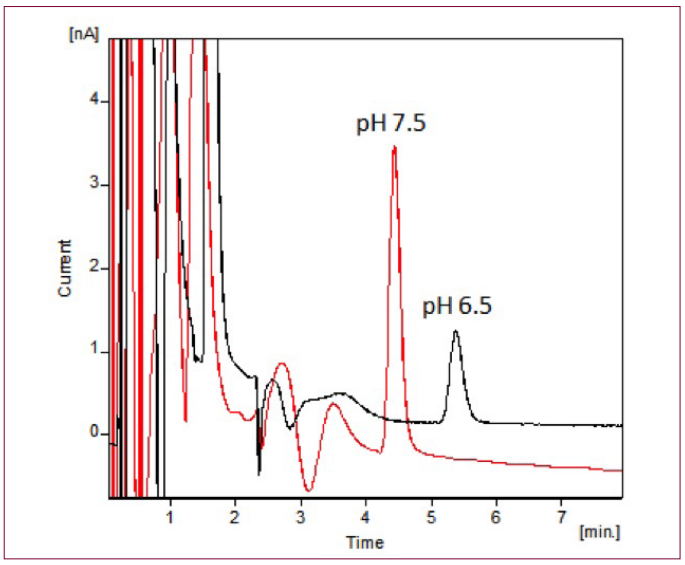


Figure 4: Mobile phase pH is affecting the IMER performance and peak height, pH 7.5 is used in this method.

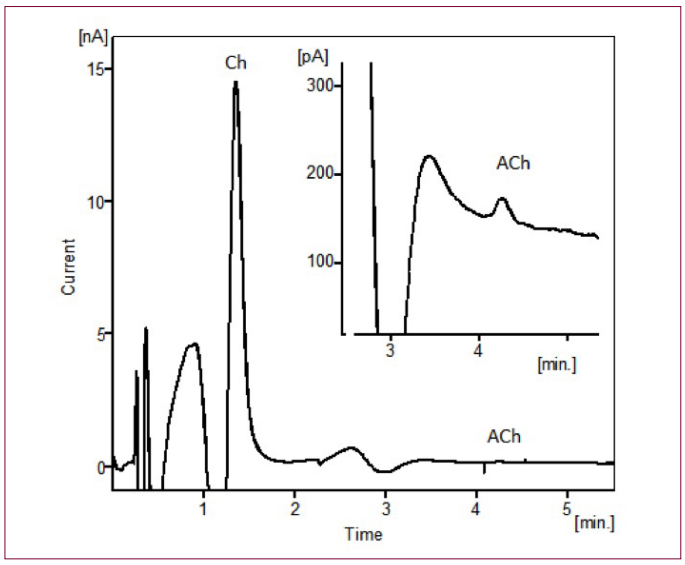


Figure 6: Chromatogram of a basal level rat microdialysate sample. The acetylcholine concentration was calculated to be 1 nmole/L.

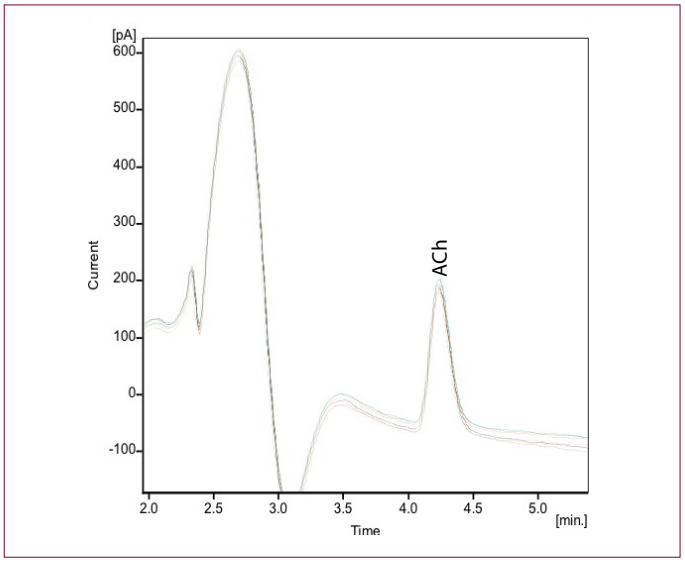


Figure 5: Overlay of 6 chromatograms of 5 nmol/L ACh in Ringer's solution. Injection with method UP101 that uses 10 µL of sample in total.

A detection limit down to 3 fmol has been obtained for ACh using a well performing IMER, extensively stabilized system with a noise level below 5 pA, making this system sensitive enough to measure basal levels of ACh in microdialysate samples.

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.

Due to an optimized and dedicated method using an extremely selective enzyme reactor which is also ion-pair LC compatible, an improved selectivity is obtained in combination with very short run times of < 6min. The detection limit at basal level down to 0.3 nmol/L is obtained using a total sample of 10 µL.

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

Ion-pair vs. ion-exchange chromatography

Instead of ion-pairing chromatography sometimes ion-exchange chromatography is used (see f.e. ref [8]) for analysis of Ach and Ch. With ion exchange the elution order of the peaks is reversed. In such case often the small ACh peak and large Ch peak are not very well separated, and a large late eluting peak is present in the chromatogram (at about 25-35 min) resulting in long runtimes. Therefore, the method presented in this application note is preferred in that respect.

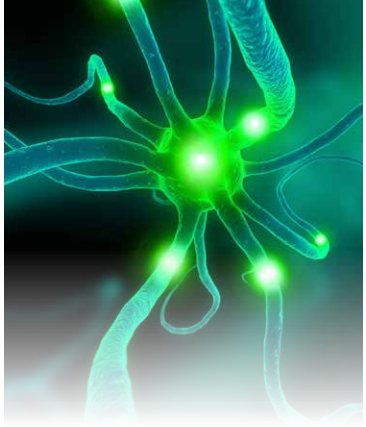
References

1. *Liquid chromatographic methods used for microdialysis: an overview*, Sarre, S., Michotte, Y; Reference: from Westerink, B.H.C. and Cremers, T.I.F.H. (Eds.) Handbook of Microdialysis, vol. 16, 2007 Elsevier B.V.
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 acetylcholin-esterase inhibitors.* Cur. Separations 2002, 20, 11-16.
3. De Bundel, D.; Sarre, S., Van Eeckhaut, A; Smolders, I.; Michotte, Y. *Critical evaluation of acetylcholine determination in rat brain microdialysates using ion-pair liquid chromatography with amperometric detection.* Sensors 2008, 8, 5171-5185.
4. Peroxidase Electrode Kit. BASi instruction manual (MF-9079).
5. *Flexcell user manual.* Antec (102.0010a)
6. Ichikawa, J., Dai, J., Meltzer, H.Y. *Acetylcholinesterase inhibitors are neither necessary nor desirable for microdialysis studies of brain acetylcholine.* Cur. Separations 2000, 19, 37-43.
7. Kehr, J, Dechent, P., Kato, T., Ögren, S. *Simultaneous determination of acetylcholine, choline and physostigmine in microdialysis samples from rat hippocampus by microbore liquid chromatography/electrochemistry on peroxidase redox polymer coated electrodes.* Journal of Neuroscience Methods 83 (1998) 143–150
8. *Acetylcholine and Choline.* Application note, Antec (213-022)

Ordering number

ALEXYS Neurotransmitter Analyzer for Acetylcholine	
180.0091U	ALEXYS neurotransmitters
191.0035U	AS 110 autosampler UHPLC cool 6p
180.0506	Acetylcholine SCC kit
250.3531	AChE/ChOx post column-IMER

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 GABA & Glutamate, Histamine, LNAAs and other Amino Acids

- **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**

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.

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.

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].

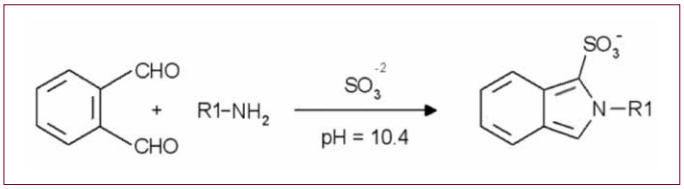


Figure 2: Reaction scheme of the derivatization of primary alkyl amines with OPA and sulphite.

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 solu-tion should be prepared fresh each day for optimal performance.

Sample/reagent ratio - The sample/reagent ratio affects the sam-ple 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 rea-gent 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 microdi-alysates and acidification is not necessary. However, if GABA and Glu analysis has to be performed in acidified samples, the derivat-isation 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 col-umn 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 180.0091U) with AS 110 UHPLC cool 6-pv autosampler (pn 191.0035U)
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)
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)

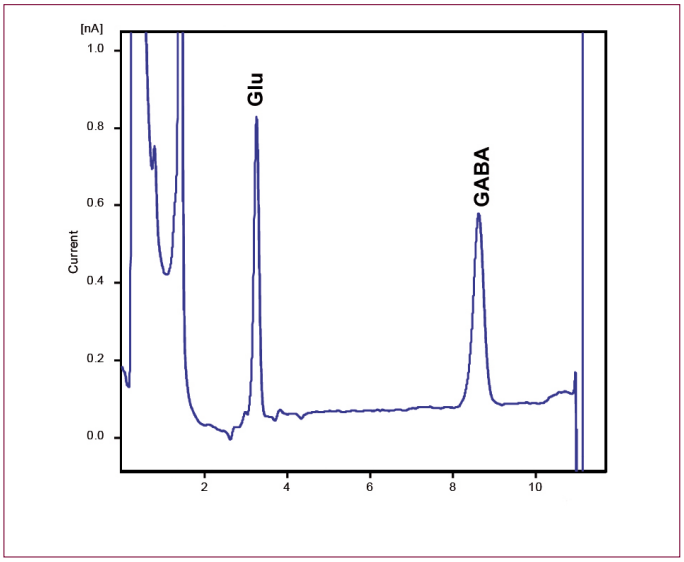


Figure 3: . Chromatogram of a 0.5 µmol/L GABA & Glu standard mixture in Ringer.

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 de-rivate), 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 pre-cautions are taken.

To combine short analysis times and a stabile baseline in consec-utive 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 base-line is stabile again within 5 min (Fig. 5).

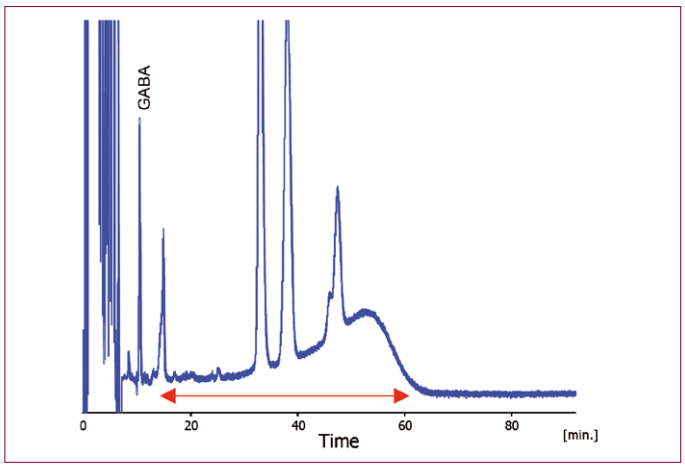


Figure 4: Chromatogram of a rat dialysate showing several late eluting peaks between 15 and 60 min (red arrow).

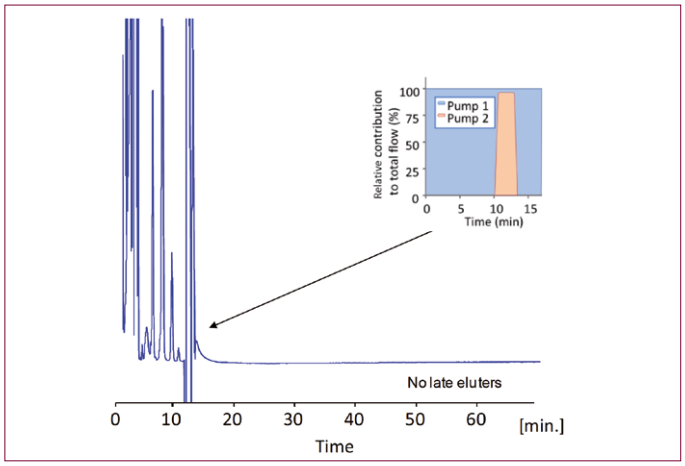


Figure 5: Chromatogram of a rat dialysate with a post-separation step-gradient. Late eluting peaks are absent from the baseline.

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 %	

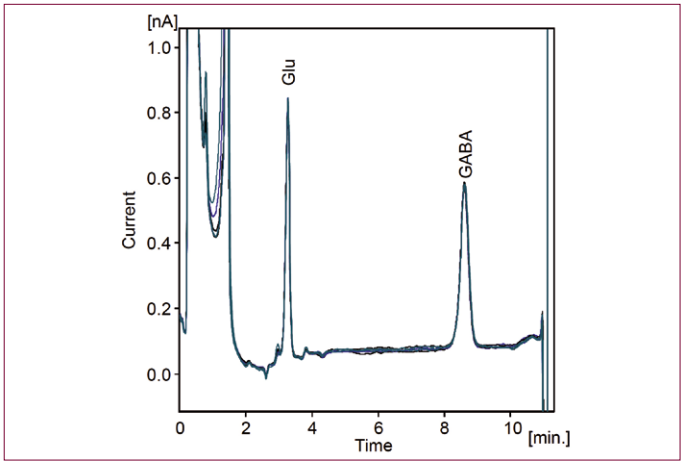


Figure 6: Overlay of 6 chromatograms of 500 nmol/L GABA and Glu in Ringer's solution. Step-gradient applied between 10-12 min. Total run-time per sample: 19 min (includes derivatisation, separation, column flush and stabilization time).

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.

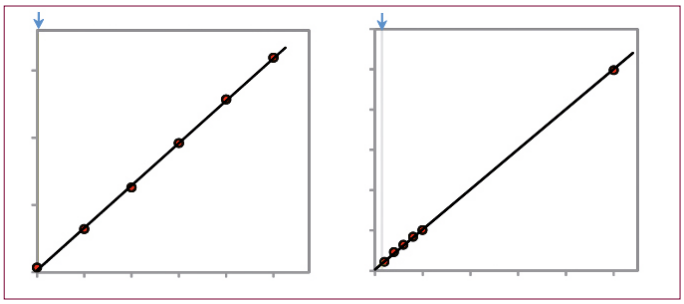


Figure 2: Calibration plots of Glu and GABA with linear regression line through the data points.

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 micro-dialysates (in the range of several µmol/L) the intensity of the interference is relatively small.

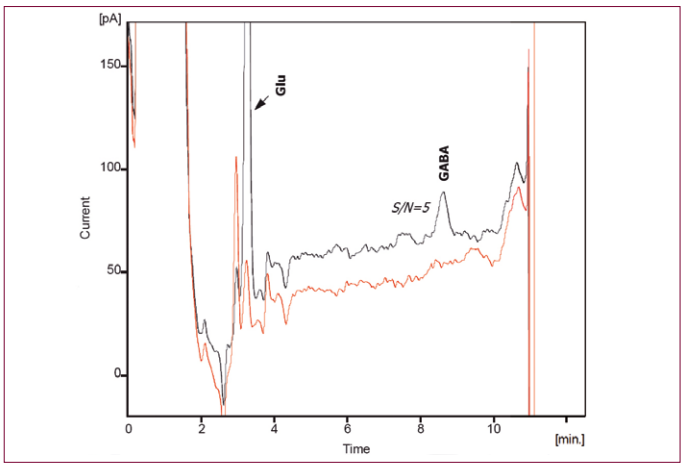


Figure 8: Overlay of chromatograms of a blank (ringer) and a mix of 200 nmolL Glu/20 nmol/L GABA in Ringer

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.

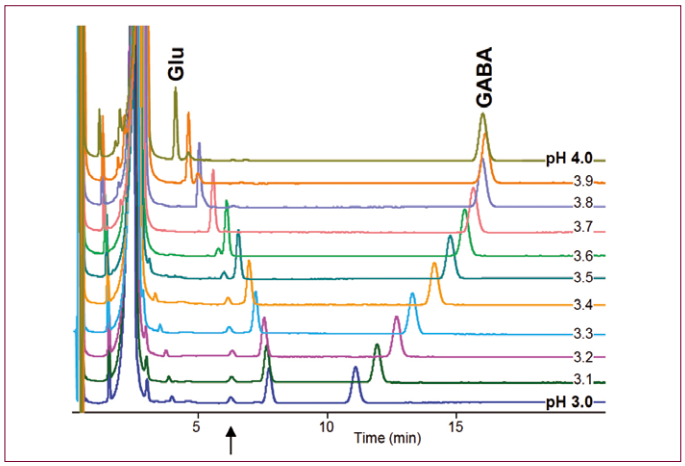


Figure 9: Effect of mobile phase pH on separation: overlay of GABA & Glu standard mixture chromatograms recorded in the range of pH 3- 4 (separation & detection performed at T=35°C).

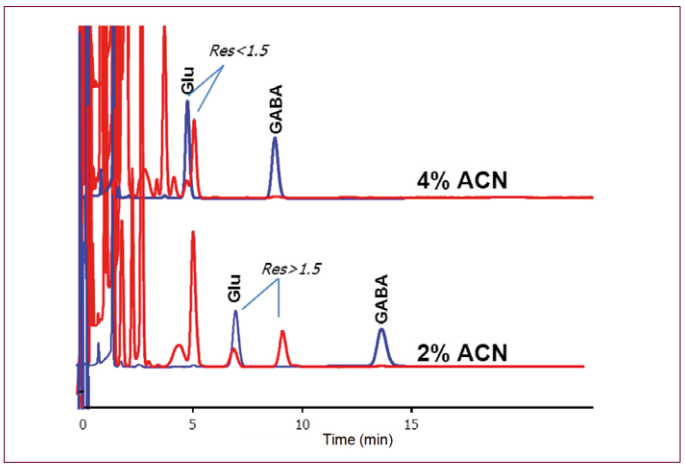


Figure 10: Overlay of two sets of chromatograms recorded with different modifier concentration (2 and 4% acetonitrile). Red trace: pooled rat dialysate from the Hippocampus. Blue trace: 5 µmol/L GABA & Glu standard mixture in Ringer. (T=35 °C, separation & detection).

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 hippocam-pus rat dialysate (red curve). For the rat dialysate from the hippo-campus a concentrations of 1.9 µmol/L Glu and 120 nmol/L GABA was measured.

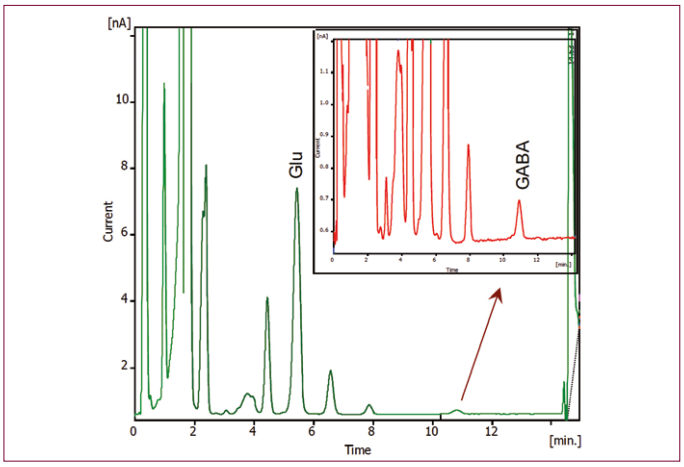


Figure 11: Example chromatogram of the analysis of GABA and Glu in pooled rat dialysate from the Nucleus Accumbens. Chromatogram recorded with a µVT-03 flowcell.

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.

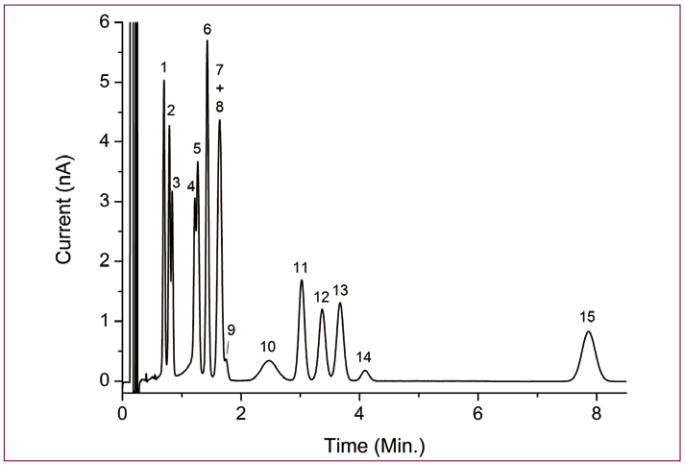


Figure 12: Analysis of 1.5 µL injection of a mixture of 14 amino acids and related substances in water at a concentration of 2.5 µmol/L. Peaks are OPA derivatives of (1) serine, (2) taurine, (3) asparagine, (5) glycine, (6) histidine, (7) aspartate, (8) glutamine, (9) cystine,(10) trans-4-hydroxy-L-proline, (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 180.0091U) with AS 110 micro cool 6-PV, UHPLC auto sampler
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 auto sampler.

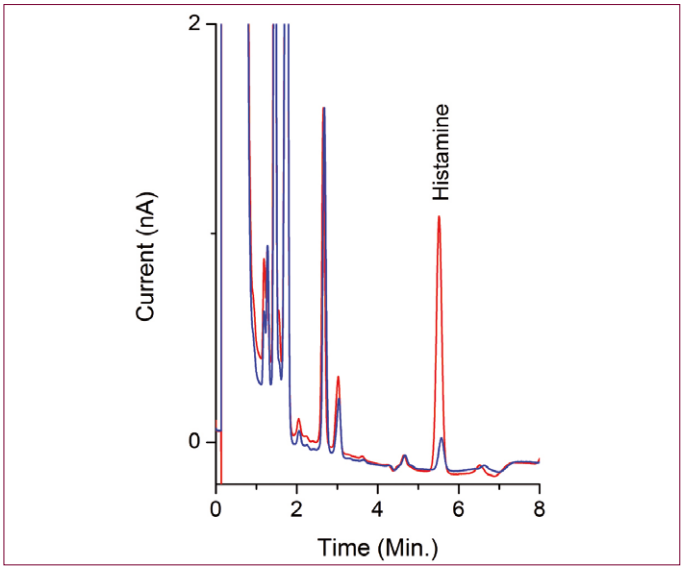


Figure 13: Analysis of the Histamine release in RBL-2H3 (mast cell model) after an allergen trigger. Chromatogram A (blue curve): Histamine level in blank (solution with RBL-2H3 cells before exposure to allergen. Chromatogram B (Red curve): Histamine level after exposure to DNP-BSA allergen. Chromatograms 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 180.0091U) with AS 110 UHPLC cool micro 6-PV autosampler (pn 191.0037U)
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/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 ISAAC reference electrode, spacer 25 µm
Ecell	V= 0.70 V vs Ag/AgCl (ISAAC)
Range	50 nA/V
ADF™	Off
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:

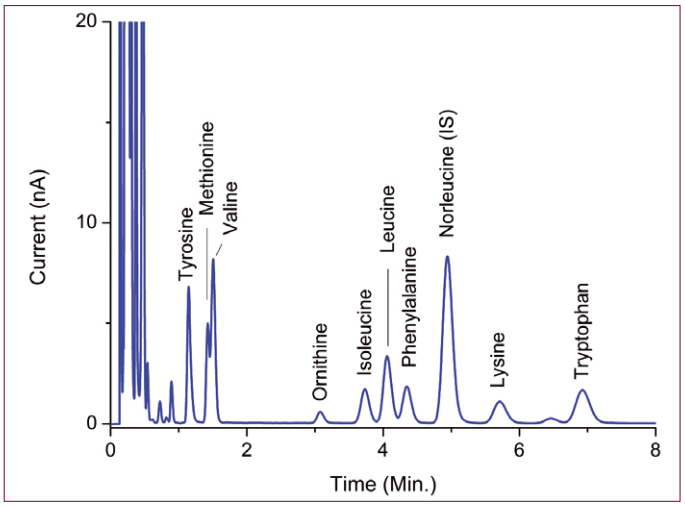


Figure 14: Analysis of extracted chicken plasma. Chromatogram courtesy of Mrs. Gerdien Korte-Bouws, Department of Pharmaceutical Sciences, division of Pharmacology, University of Utrecht, The Netherlands.

Table 8

Conditions for the analysis of LNAAs*	
HPLC	ALEXYS Neurotransmitter Analyzer (pn 180.0091U) with AS 110 micro cool 6-PV, UHPLC auto sampler
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/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 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

1. N.J. Reinhoud, H.J. Brouwer, L.M. van Heerwaarden, G.A.H. Korte-Bouws, Analysis of Glutamate, GABA, Noradrenaline, Dopamine, Serotonin, and Metabolites Using Microbore UHPLC with Electro-chemical Detection, ACS Chem. Neurosci., 4 (2013), 888–894
2. W.A. Jacobs, o-Phthalaldehyde-sulphite derivatization of primary amines for liquid chromatography-electro chemistry, J. Chromatog-raphy 392 (1987) 435-441
3. S. Smith, T. Sharp, Measurement of GABA in Rat Brain Microdialy-sates Using o-phthaldialdehyde Sulphite Derivatization and High- Per-formance Liquid Chromatography with Electrochemical Detection, J. Chromatography B 652 (2) (1994) 228-233
4. Antec Leyden, Application note Gaba and Glutamate; Appendix: AS user program & settings, document 213-019 & 213-019A
5. Antec Leyden, Installation guide LC connection kit DCC I-I CS, docu-ment 180.7018, (2006) 23 - 29
6. H. L. Rowley, K. F. Martin, C. A. Marsden, Determination of in vivo amino acid neurotransmitters by high-performance liquid chromatog-raphy with o-phthalaldehyde-sulphite derivatization, J. of Neurosc. Meth. 57 (1) (1995) 93-99
7. S. Zhang, Y. Takeda, et. al., Measurement of GABA and Glutamate in vivo levels with high sensitivity and frequency, Brain Research Proto-cols 14 (2005) 61- 66
8. J. Kehr, Determination of glutamate and aspartate in microdialysis samples by reversed-phase column LC with fluorescence and elec-trochemical 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 number

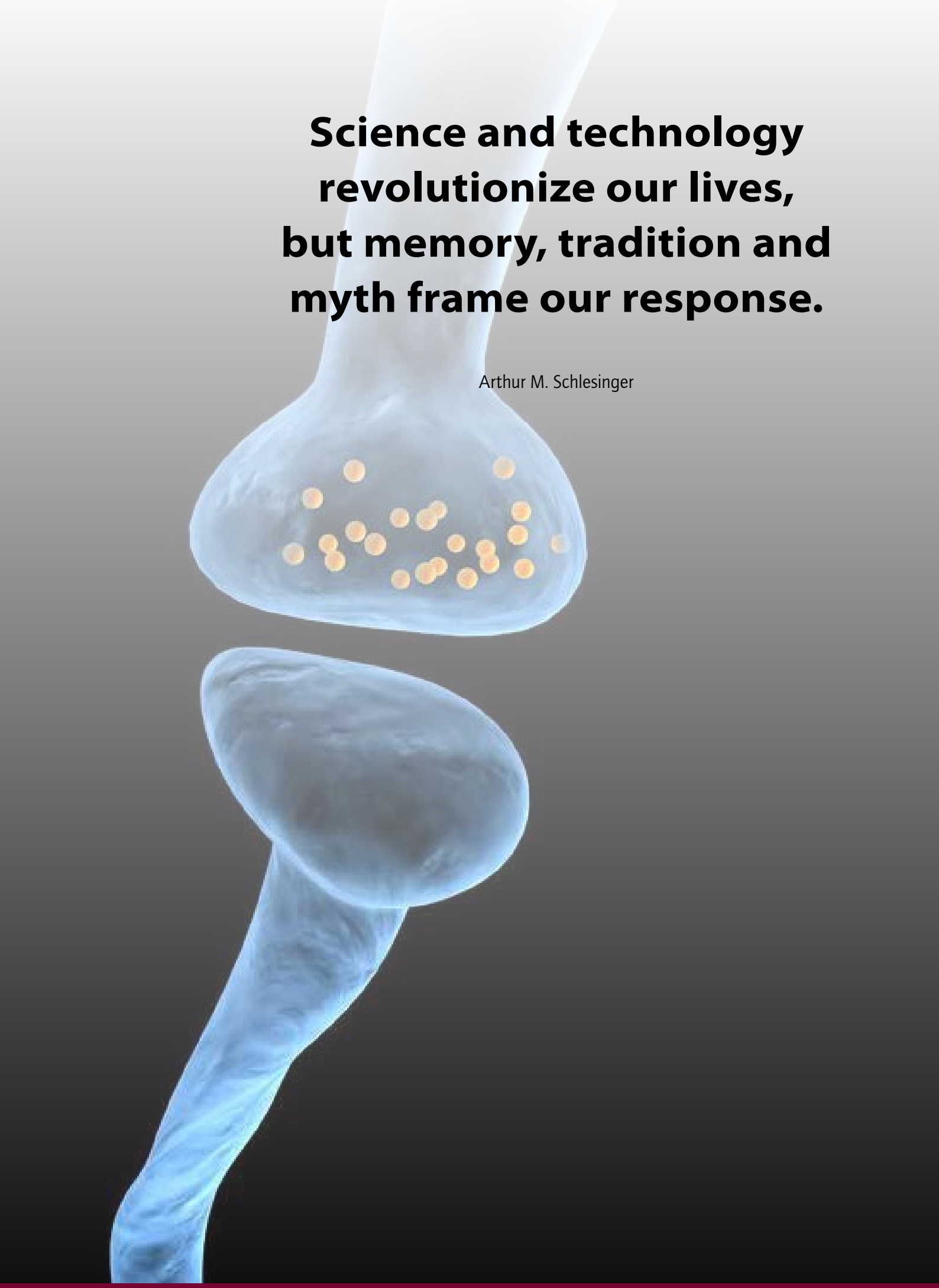
ALEXYS Neurotransmitter Analyzer for GABA and glutamate	
180.0091U	ALEXYS neurotransmitters BP, 1 ch
191.0035U	AS 110 autosampler UHPLC cool 6p
180.0602	LC step-gradient upgrade
180.0504	ALEXYS GABA/Glu SCC kit

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 analyzes. Optimized methods e.g. fully automated in-the-needle derivatization, post separation step-gradient to eliminate late eluters and samples injection volumes of 5ul results in a total analyzes time <12 minutes.

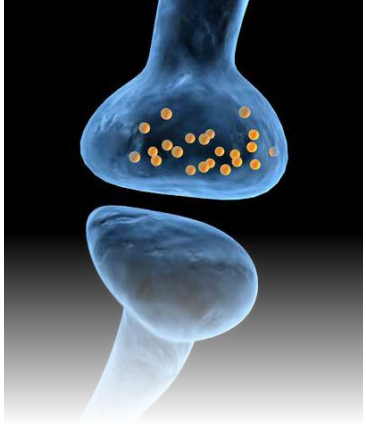
The ALEXYS Neurotransmitter Analyzer can be extended with several options and kits for any combination of other neurotransmitters, Acethylcholine and Choline as well as Monoamines and Metabolites.



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 Metabolites

- **Fast separation on a sub-2µm UHPLC column**
- **Wall-jet flow cell for best detection limit**
- **Small injection volume, for better time resolution**
- **Optimized separation for multi-component analysis**
- **Dual channel option for parallel separations**

Introduction

Microdialysis of neurotransmitters *in vivo* has become an invaluable tool to study neurotransmission in living brain. Cerebrospinal fluid of the brain is sampled trough a microdialysis device and analyzed by HPLC with electrochemical detection [1-7].

A neurotransmitter analyzer has been developed with features that meet the most demanding requirements. The required injection volume is small, as a smaller volume means a better time resolution in microdialysis.

The method has a low limit of detection, as some neurotransmitters have a concentration below 100 picomole/L. Separation and selectivity are optimized for multicomponent analysis, to get as much information as possible from a drop of dialysate. Where possible the analysis time has been shortened by any means such as by using UHPLC.

Summary

In this application note a fast and sensitive method is presented for the analysis of monoamines and metabolites. A microbore UHPLC column is applied in combination with electrochemical detection using a high sensitivity wall jet flow cell. Detection limits down to 50 pmol/L have been achieved for dopamine. Analysis times vary between 1-15 minutes depending on the complexity of the sample matrix and the number of substances of interest. The system can be equipped with an additional channel for simultaneous analysis of (for example) the metabolites.

Figure 1: ALEXYS Neurotransmitters Analyzer.

The ALEXYS Neurotransmitter Analyzer consists of a DECADE II electrochemical detector, an OR 110 degasser unit and LC 110S pump(s), an AS 110S autosampler and Clarity data acquisition software. Complementary kits for analysis of noradrenaline, dopamine, serotonin and metabolites, GABA and glutamate or acetylcholine and choline are available.

Method and results

In method development for the analysis of monoamines and metabolites a number of parameters are optimized to meet the requirements for detection limits, the use of small samples, and short analysis times of multiple components.

Small sample volume and low detection limits

In a previous communication the optimization for best possible detection limit using small samples has been described [8]. Briefly, a wall-jet micro flow cell has been used which is fully compatible with microbore HPLC. It is well known that small samples are best analyzed using microbore LC with less peak dilution, resulting in more signal. Miniaturization using micro electrodes results in smaller noise. With this combination an improved signal-to-noise ratio with detection limits down to 50 pmol/L (dopamine) have been achieved for the analysis of standards (Fig. 2).

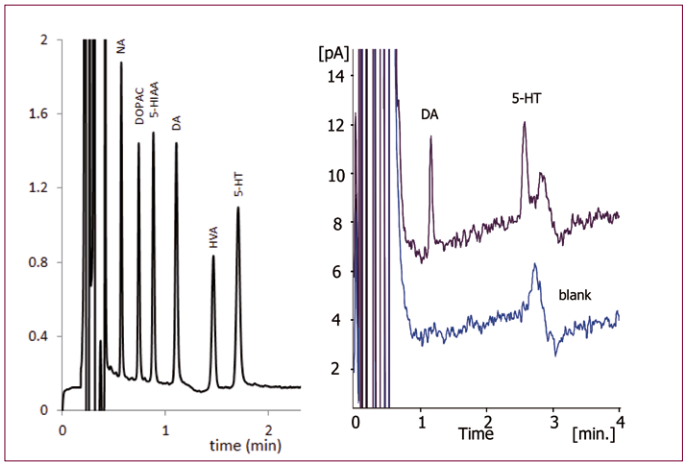


Figure 2: Analysis of monoamines and metabolites (standards) showing a separation within 2 (left) and a 5 µL injection of 100 pmol/L DA and 5HT in Ringer with 10 mmol/L HAc (right). A LOD of 50 pmol/L for DA and 65 pmol/L for 5HT is obtained. There is a trade-off in speed of analysis vs. detection limit and resolution in biological samples.

Separation and speed of analysis

To meet the requirement for fast analysis of multiple components is a matter of selecting a suitable column and optimizing the mobile phase. An analysis time less than 2 minutes is feasible for standards using UHPLC (Fig. 2), however we found there is a trade-off in analysis time vs. detection limit and resolution when analyzing biological samples.

Speeding up a standard HPLC analysis with a factor 2 – 4 using UHPLC columns is feasible, depending on sample matrix and injection volume. At higher velocities a few things are limiting the performance. One of the most critical was the analysis of NA which is close to the solvent front (see figure 4). At high flow rates the peaks overlapped or disappeared in the front. Assumingly this is due to non-ideal behavior of high concentration matrix components eluting in the front peak.

The method and results presented describe an approach, not a fixed set of conditions. It is very well possible or even required to tune the method for a different matrix composition (f.e. measuring in a different brain area) or a different set of neurotransmitters or metabolites of interest. In other words, the ALEXYS system is a flexible system which is not limited to a few applications. There is range of columns available to assist you in optimizing the chromatography to your specific application needs.

Secondary HPLC channel for metabolites

To extend the possibilities a secondary channel can be added to the ALEXYS Neurotransmitter system (Fig. 1). The dual channel system contains one additional pump, a column, and uses an autosampler with a 10 port valve and a dual channel detector.

Both channels share the same autosampler and electrochemical detector (Fig. 3). Detection parameters and HPLC conditions are optimized for each channel depending on the substances of interest. Channel 1 is optimized for NA, DA and 5-HT. Channel 2 is for the acidic metabolites 5-hydroxyindole acetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA).

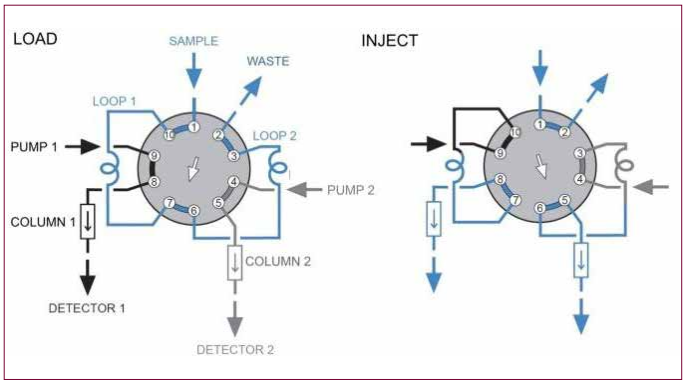


Figure 3: Schematic configuration of sampling 2 parallel systems with a 10-port valve.

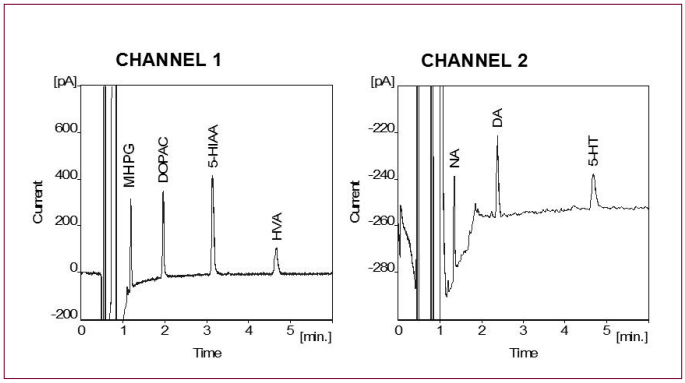
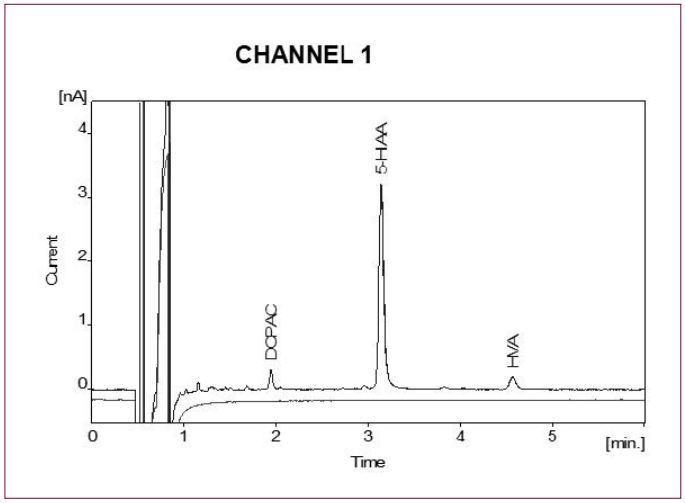


Figure 4: Analysis of standards 5 nmol/L acidic metabolites and 0.5 nmol/L monoamines in Ringer solution with 10 mmol/L acetic acid.

Linearity and repeatability

Using a dual channel configuration the acidic metabolites and the monoamines are measured (Fig. 4). They are loaded simultane-ously on both HPLC's in a single injection and analyzed under completely different and optimized conditions.

The relative standard deviation (RSD) has been investigated for 8 injections of a mix of metabolites (100 nmol/L) and monoamines (10 nmol/L). The RSD in retention times is better than 0.5%. The RSD of peak areas and heights is better than 2% for the metabo-lites and better than 1.5 % for the monoamines. Linearity shows a correlation coefficient better than 0.998 in the range of 2 to 100 nmol/L.



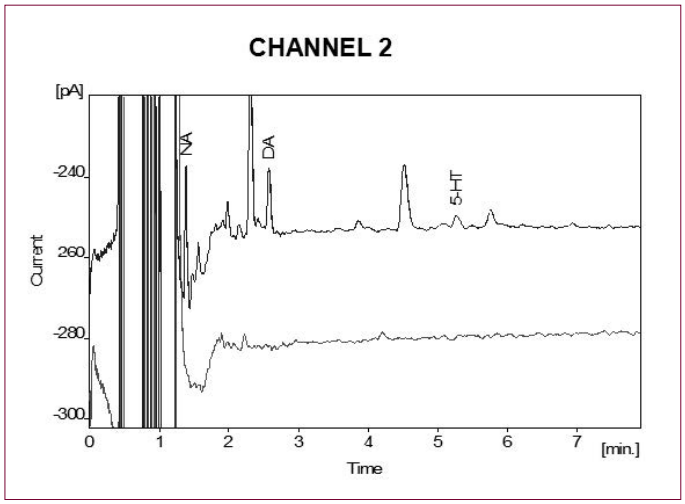


Figure 5: Analysis from a single injection of basal level rat Prefrontal Cortex (PFC) microdialysate. In both chromatograms the bottom trace is an injection of blank (Ringer solution). Samples kindly provided by Mrs. Gerdien Korte-Bouws, Department of Psychopharmacology, University of Utrecht, The Netherlands.

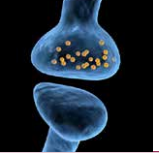


Table 1

Conditions for analysis of monoamines (NA,DA, 5-HT) and metabolites	
HPLC	ALEXYS Neurotransmitter Analyzer
Oven temperature	38 °C (separation and detection)
Injection method	5 µL per column
ASSAY 1 (MPHG DOPAC, 5-HIAA, HVA)	
Flow rate	100 µL/min, pressure about 420 bar
Flow cell	Sencell 2 mm GC, sb, spacing 0.5
Column	Acquity HSS T3 1 x100 mm, 1.8 um + pre-filter
ADF™	off
Range	10 nA/V
Ecell	800 mV
Icell	0.9 nA
Mobile phase	50 mM phosphoric acid, 0.1 mM EDTA, pH 3.0, 10 % ACN
ASSAY 2 (NA, DA and 5-HT)	
Flow rate	100 µL/min, pressure about 420 bar
Flow cell	Sencell 2 mm GC, sb, spacing 0.5
Column	Acquity HSS T3 1.0x100 mm, 1.8 um
ADF™	Off
Range	1 nA/V
Ecell	460 mV
Icell	0.6 nA
Mobile phase	200 mM Acetic Acid, 0.1 mM EDTA, 300 mg/L DSA, pH 5.5, 14 % ACN

LOD: 80 pM NA (0.40 fmol), 70 pM DA (0.35 fmol), 100 pM 5-HT (0.50 fmol), 169 pM DOPAC (0.85 fmol), 69 pM 5-HIAA (0.34 fmol), 208 pM HVA (1.04 fmol)

Analysis of microdialysis samples

The matrix of microdialysate samples typically consist of the Ringer solution or artificial cerebrospinal fluid (aCSF) that is used for dialysis. The main constituent of these solutions is NaCl in a concentration of almost 0.15 mol/L.

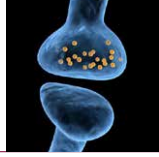
Monoamines and some metabolites are not stabile in these solu-tions and break down rapidly. Adding a few microliters of concen-trated preservative mix or acid to each collected fraction can prevent this. Care must be taken that the added preservative is not interfering with the chromatographic analysis later on.

Good results have been obtained using 1:4 addition of 0.1 mol/L acetic acid to samples in Ringer. Adding a high concentration of perchloric acid (PCA) sometimes interferes with chromatography and causes deformation of peaks.

The results shown in this note are based on the analysis of stand-ards in Ringer solution, acidified with acetic acid (final concentra-tion 10 mmol/L HAc) unless mentioned otherwise. In case of UHPLC additional care must be taken that the sample does not contain small particles. Centrifugation or filtration is required in such case to avoid clogging of the column. To illustrate the applicability of the method a microdialysis fraction of basal level rat Prefrontal Cortex (PFC) has been analyzed (Fig. 5). Depending on the sample composition a little tuning might be required to get a bit more retention for NA. In case only DA and 5HT are of interest, the time of analysis can be decreased further.

The concentrations of monoamines in the microdialysis fraction are 0.24 (NA), 0.21 (DA), and 0.12 nmol/L (5HT). The concentra-tions of metabolites are 2.6 (DOPAC), 36.1 (5-HIAA), and 7.8 nmol/L (HVA).

Under these conditions, the calculated detection limits in pmol/L are (brackets: amounts in fmol) for monoamines NA 80 (0.40), DA 70 (0.35), 5-HT 100 (0.50) and for the metabolites DOPAC 169 (0.85), 5-HIAA 69 (0.34), HVA 208 (1.04).



References

1. Y. Ikarashi en Y. Maruyama, Determination of catecholamines, indoleamines, and related metabolites in rat brain with LC with ECD, Biogenic amines 2 (1985) 101-110
2. J. P. Crombeen, J. C. Kraak en H. Poppe, Reversed-phase systems for the analysis of catecholamines and related compounds by HPLC, J. Chromatogr., 167 (1978) 219-230
3. Nagao T, Tanimura T., Simultaneous determination of biogenic amines, their precursors and metabolites in a single brain of the cricket using high-performance liquid chromatography with am-perometric detection, J Chromatogr. 496(1) (1989) 39-53
4. Joseph MH, Kadam BV, Risby D., Simple high-performance liquid chromatographic method for the concurrent determination of the amine metabolites vanillylmandelic acid, 3-methoxy-4-hydroxyphenylglycol, 5-hydroxyindoleacetic acid, dihydroxy-phenylacetic acid and homovanillic acid in urine using electro-chemical detection, J Chromatogr., 226 (2) (1981) 361-368
5. Krstulovic AM, Dziedzic SW, Bertani-Dziedzic L, DiRico DE., Plasma catecholamines in hypertension and pheochromocytoma determined using ion-pair RP chromatography with amperometric detection: investigation of the separation mechanism and clinical methodology. J Chromatogr., 217 (1981) 523-537
6. Katrien Thorré, Miloslav Pravda, Sophie Sarre, Guy Ebinger and Yvette Michotte, New antioxidant mixture for long term stabil-ity of serotonin, dopamine and their metabolites in automated microbore liquid chromatography with dual electrochemical detec-tion, Journal of Chromatography B: Biomedical Sciences and Applications, Volume 694, Issue 2, 4 (1997), 297-303
7. A.T. Nguyen, T. Aerts, D. Van Dam and P.P. De Deyn, Biogenic amines and their metabolites in mouse brain tissue: Development, optimization and validation of an analytical HPLC method, Journal of Chromatography B Volume 878, Issue 29, 1 (2010) 3003-3014
8. Antec application note: 220_001 - Attomole detection limits

Ordering number

ALEXYS Neurotransmitter Analyzer for Monoamines single channel	
180.0091U	ALEXYS neurotransmitters, 1 ch
191.0035U	AS 110 autosampler UHPLC cool 6p
180.0502	ALEXYS Monoamines SSC kit
dual channel	
180.0092U	ALEXYS neurotransmitters, 2 ch
191.0041U	AS 110 autosampler UHPLC cool 10p
180.0502	ALEXYS Monoamines SSC kit (2x)

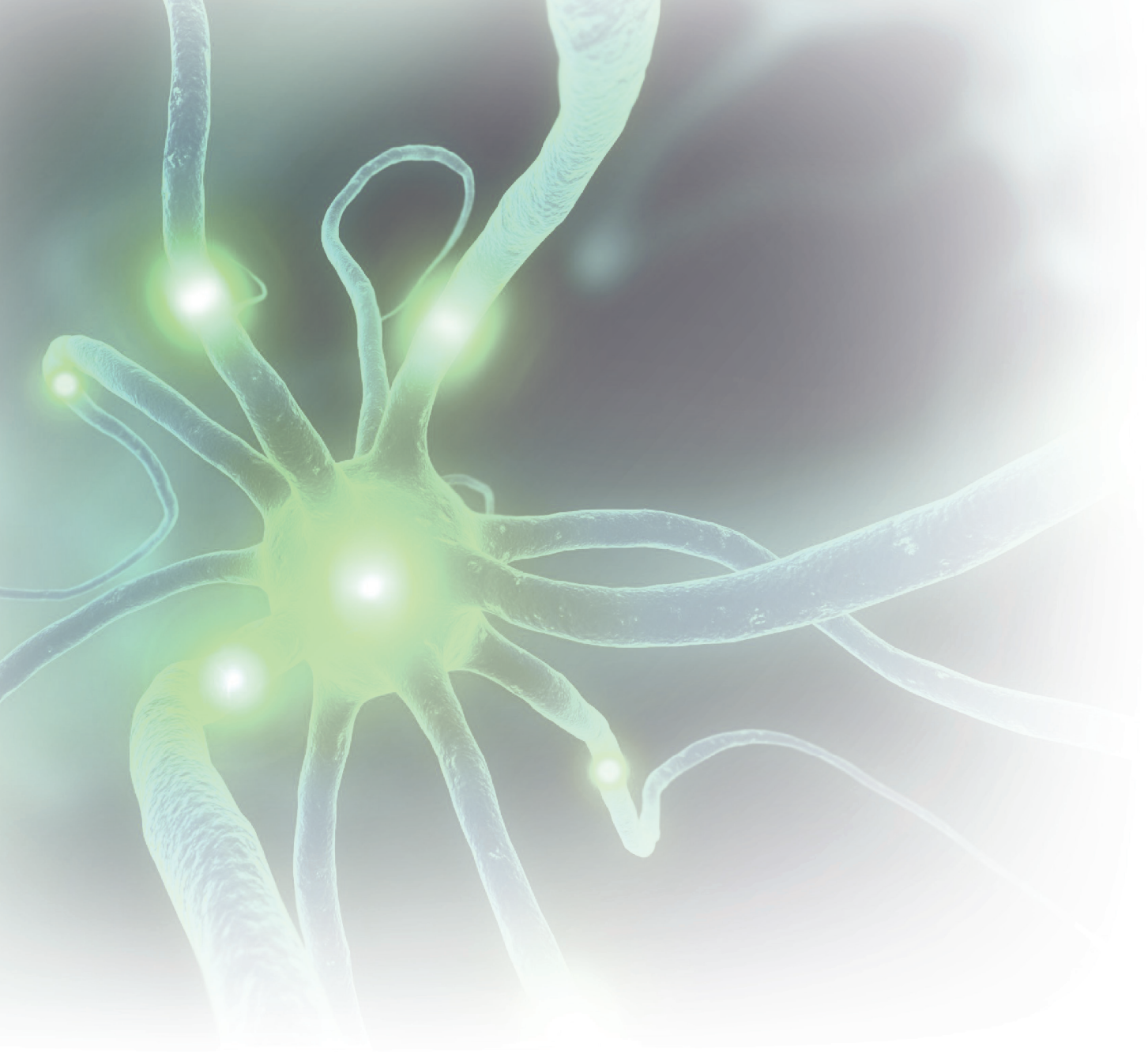
Conclusion

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 Monoamines and their Metabolites in microdialysates is robust and suitable for routine based analyzes. Optimized method files are developed e.g. to prevent sample loss, to minimize retention times without compromises on the sensitivity and to inject samples volumes of 2-10 uL. Typical detection limits of 50 pmol/L are feasible.

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


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



Antec (USA)
info@myantec.com
www.myAntec.com
phone (888) 572-0012 (toll free)

Antec (worldwide)
info@myantec.com
www.myAntec.com
phone +31 71 5813333

Antec®
Proven Performance!