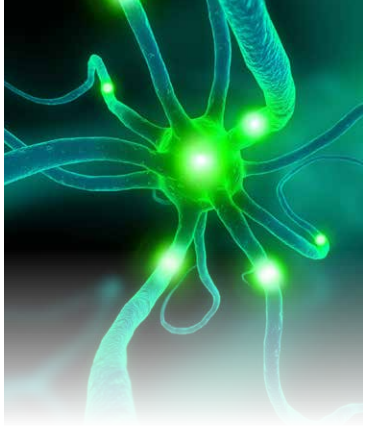
Application Note
Neuroscience



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].

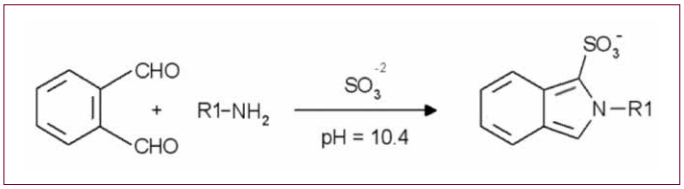


Figure 2: Reaction scheme of the derivatization of primary alkyl amines with OPA and sulphite.

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)

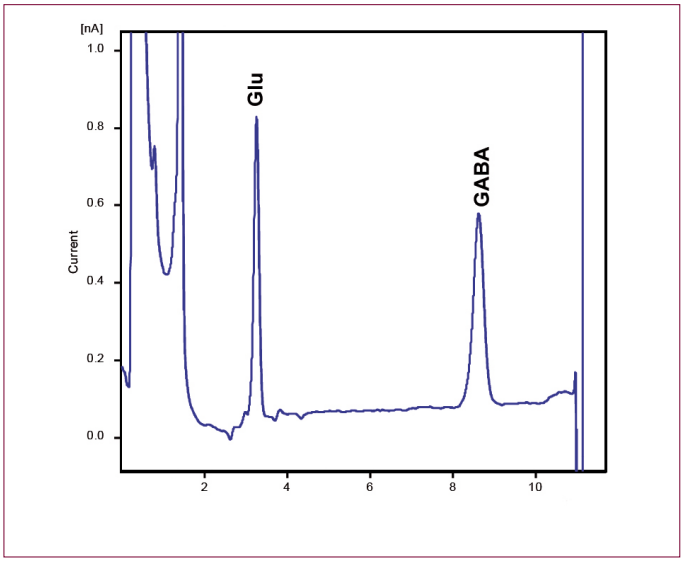


Figure 3: . Chromatogram of a 0.5 µmol/L GABA & Glu standard mixture in Ringer.

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).

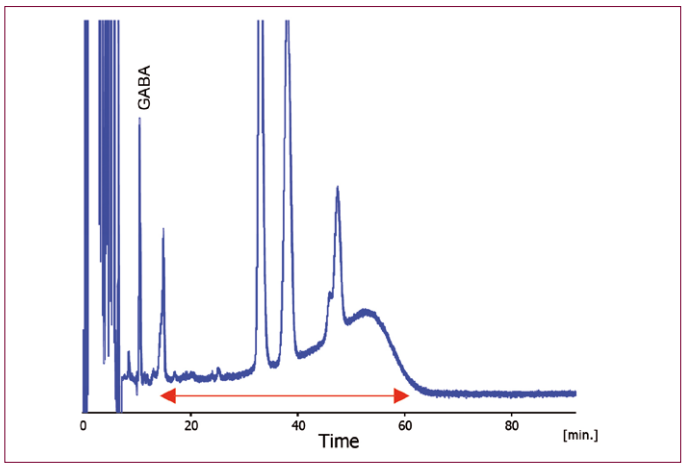


Figure 4: Chromatogram of a rat dialysate showing several late eluting peaks between 15 and 60 min (red arrow).

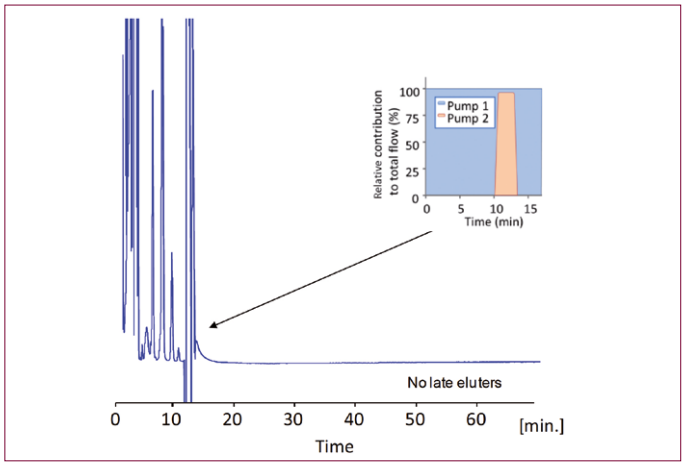


Figure 5: Chromatogram of a rat dialysate with a post-separation step-gradient. Late eluting peaks are absent from the baseline.

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 %	

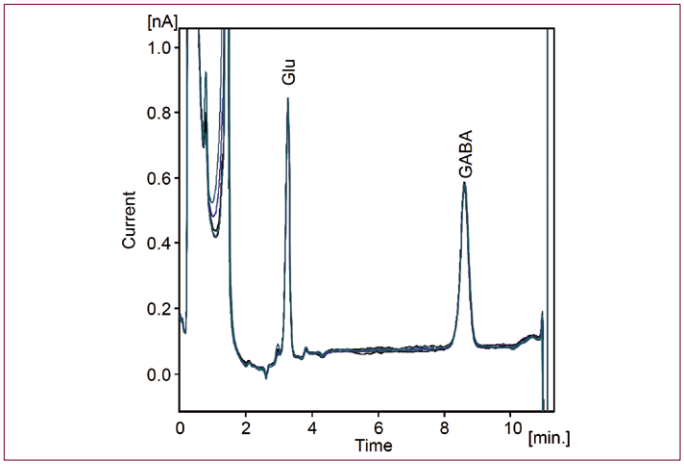


Figure 6: Overlay of 6 chromatograms of 500 nmol/L GABA and Glu in Ringer's solution. Step-gradient applied between 10-12 min. Total run-time per sample: 19 min (includes derivatisation, separation, column flush and stabilization time).

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.

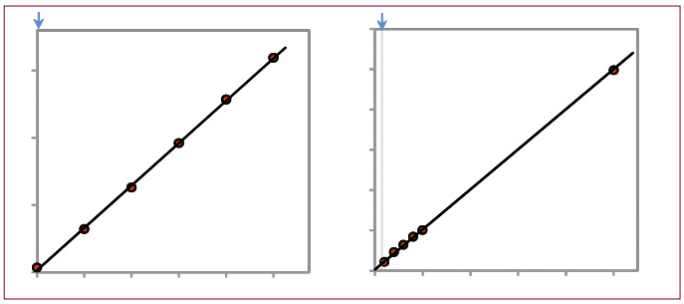


Figure 2: Calibration plots of Glu and GABA with linear regression line through the data points.

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.

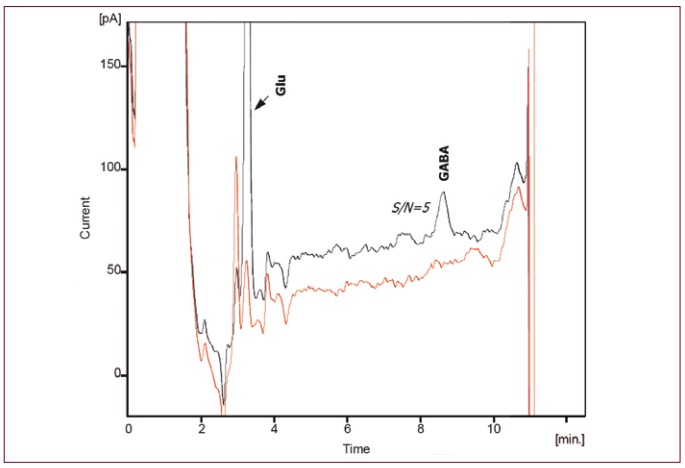


Figure 8: Overlay of chromatograms of a blank (ringer) and a mix of 200 nmolL Glu/20 nmol/L GABA in Ringer

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.

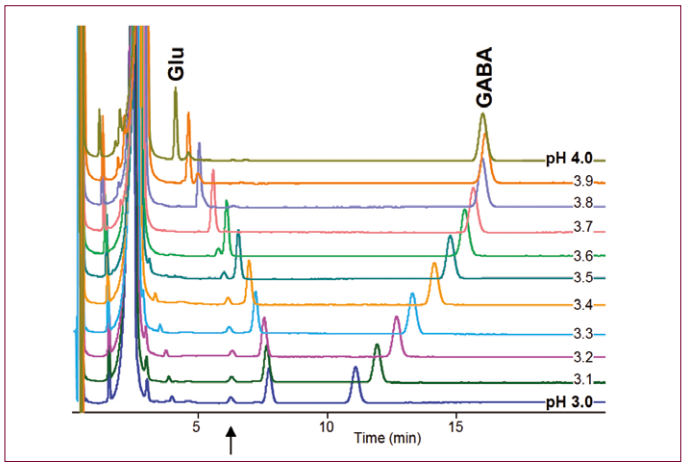


Figure 9: Effect of mobile phase pH on separation: overlay of GABA & Glu standard mixture chromatograms recorded in the range of pH 3- 4 (separation & detection performed at T=35°C).

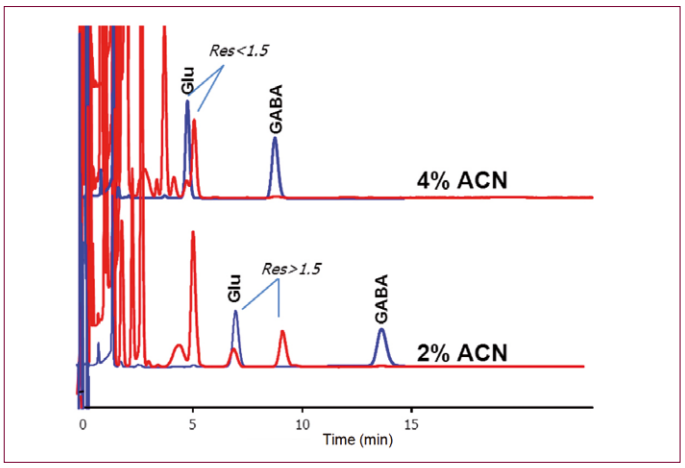


Figure 10: Overlay of two sets of chromatograms recorded with different modifier concentration (2 and 4% acetonitrile). Red trace: pooled rat dialysate from the Hippocampus. Blue trace: 5 µmol/L GABA & Glu standard mixture in Ringer. (T=35 °C, separation & detection).

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.

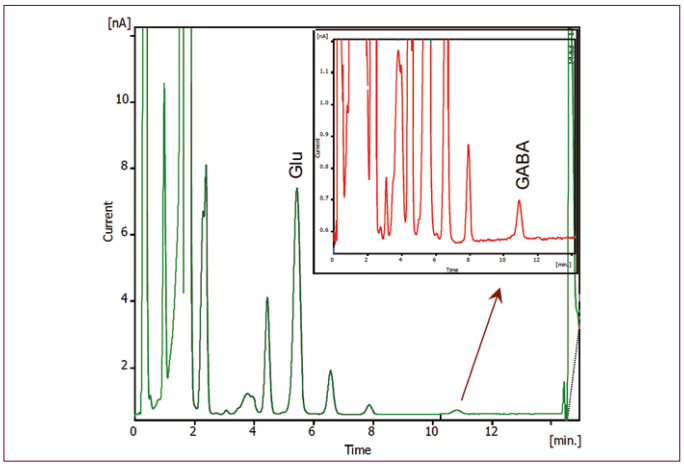


Figure 11: Example chromatogram of the analysis of GABA and Glu in pooled rat dialysate from the Nucleus Accumbens. Chromatogram recorded with a µVT-03 flowcell.

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.

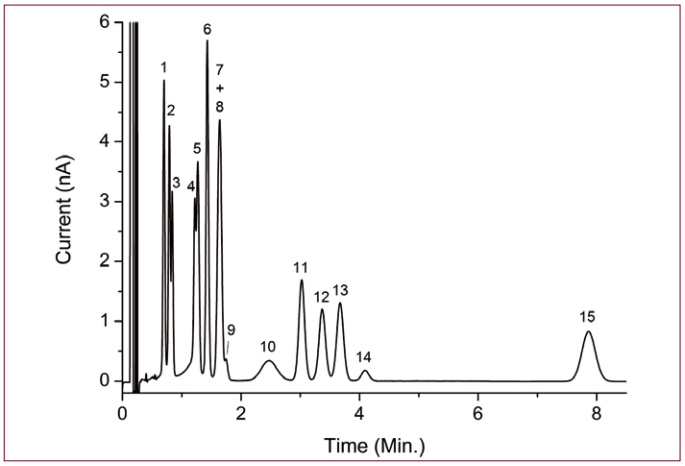


Figure 12: Analysis of 1.5 µL injection of a mixture of 14 amino acids and related substances in water at a concentration of 2.5 µmol/L. Peaks are OPA derivatives of (1) serine, (2) taurine, (3) asparagine, (5) glycine, (6) histidine, (7) aspartate, (8) glutamine, (9) cystine,(10) trans-4-hydroxy-L-proline,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

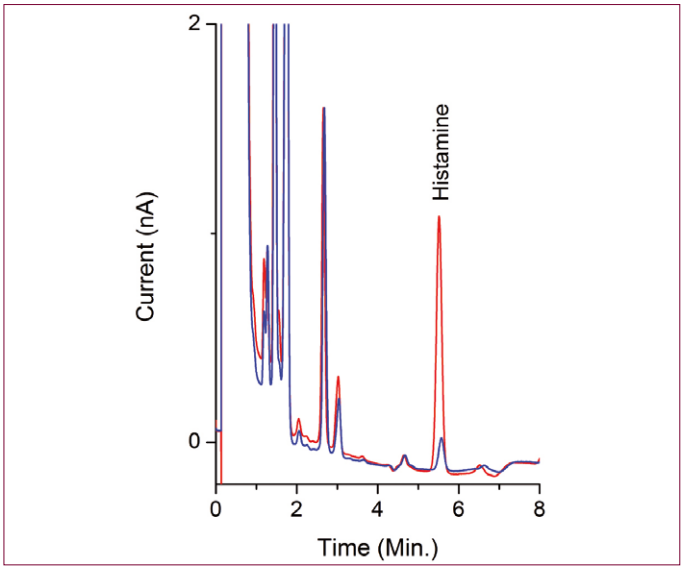


Figure 13: Analysis of the Histamine release in RBL-2H3 (mast cell model) after an allergen trigger. Chromatogram A (blue curve): Histamine level in blank (solution with RBL-2H3 cells before exposure to allergen. Chromatogram B (Red curve): Histamine level after exposure to DNP-BSA allergen. Chromatograms 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:

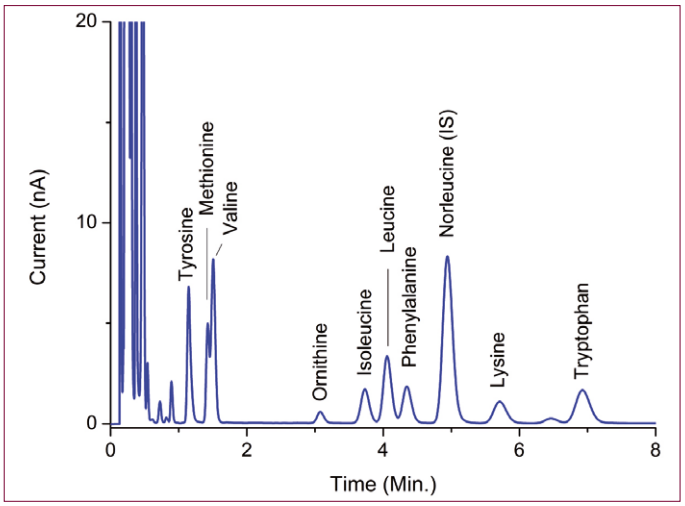


Figure 14: Analysis of extracted chicken plasma. Chromatogram courtesy of Mrs. Gerdien Korte-Bouws, Department of Pharmaceutical Sciences, division of Pharmacology, University of Utrecht, The Netherlands.

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

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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.

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

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

Antec (worldwide)
info@myantec.com
www.myAntec.com
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