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

GABA, Glutamate and Other Amino Acids

- **Detection of GABA and Glu within 10 min**
- **Fully automated OPA derivatization**
- **Post-separation step-gradient to eliminate late eluting peaks**
- **Small sample use down to 5 µL**
- **Detection limit down to 1.5 nmol/L**

Summary

In this application note a fully automated, fast and sensitive method is presented for the analysis of GABA and glutamate using the ALEXYS ® Neurotransmitter Analyzer in samples as small as 5 µL, and a detection limit down to 1.5 nmol/L GABA (with 10 µL sample use). The method is based on automated in-needle OPA-sulphite derivatization, fast and efficient separation using a sub-2 um particle column, and the elimination of late eluting peaks from previous injections using a post-separation step-gradient.

Introduction

The amino acid glutamate (Glu) and g-aminobutyrate (GABA) have been shown to act as neurotransmitters within the Central Nervous System (CNS). The principal role of GABA is reducing neuronal excitability throughout the CNS, and Glu has an excitatory role and is a precursor for the synthesis of GABA in neurons. Changes in the levels of glutamate and GABA are known to be associated with neurological disorders: measuring and tracking the changes in the level of GABA and Glu in the brain is important for various neuroscientific researches.

Analysis of these compounds in brain homogenates or microdialysate sample needs a method that is sensitive enough to detect the low-level presence of GABA in combination with good separation from the other more abundant components. GABA and Glu do not show a chromophore or molecular structure suitable for electrochemical detector (ECD). This can be overcome by making them EC active through a derivatization step.

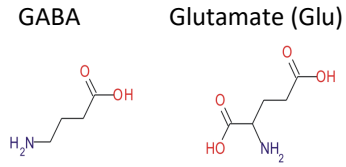


Figure 1: Structural formulas of GABA and glutamate

This note highlights the fully automated GABA and Glu analysis method that was optimized for the ALEXYS Neurotransmitter Analyzer (Fig. 2).

Method

ALEXYS Neurotransmitter Analyzer

The ALEXYS system is a versatile UHPLC-ECD platform that is dedicated to analyses that require sensitivity and small sample use [1]. This system can run different methods, like the analysis of monoamines, acidic metabolites, acetylcholine (ACh) and choline (Ch). Those methods are described in other dedicated application notes [2, 3].

The ALEXYS Neurotransmitter Analyzer consists of the DECADE Elite electrochemical detector (ECD) with integrated column oven to accommodate the flow cell and column, the AS110 autosampler with sample cooling option and fully programmable injection procedures, two P6.1L (U)HPLC pumps with an integrated degasser, pulse dampers, and Clarity data acquisition software for full automated control over the system

Figure 2: ALEXYS Neurotransmitter Analyzer

and easy data processing. The recommended flow cell for low-level detection of derivatized amino acids is the SenCell wall-jet electrochemical flow cell [4] with glassy carbon working electrode.

OPA-sulphite derivatization

GABA and Glu are not directly detectable with electrochemistry, but this can be changed with a pre-column derivatization with OPA and sulphite [5, 6]. This reagent (Fig. 3) derivatizes all primary amines (like amino acids) and this means there will be many peaks that show up in a chromatogram from a biological sample. Good separation will be key.

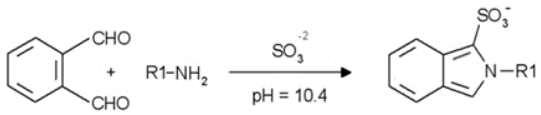
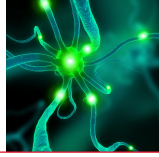


Figure 3: Reaction scheme of the derivatization of primary alkyl amines with OPA and sulphite.

The derivatization procedure and composition of the OPA reagent was modified from Smith and Sharp [6] and the preparation instructions are given in reference [7].

- *Reagent* - The rate of derivatization with OPA-sulphite reagent is strongly pH dependent. At high pH (> 9.5) the derivatization reaction occurs almost instantaneous [5]. 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. The derivates are instantly formed and fairly stabile. The OPA reagent as well as the sodium sulphite solution should be prepared fresh each day for optimal performance.

- *Sample pH* - Microdialysis samples are often acidified immediately after sample collection to minimize catecholamine degradation over time. Brain homogenates



are also acidic as they are typically prepared with 0.1 M perchloric acid. Acidification is not necessary for stabilization of GABA and Glu as they are also stabile under pH neutral conditions. Therefore, the OPA reagent composition was developed for pH neutral samples. Acidified samples need to be neutralized for the reagent to work, and we recommend to check and optimize a neutralization protocol specific for the samples under investigation, if necessary. Without neutralization, the reagent doesn't work in acidified samples and the peaks will not show up in the chromatograms. As an example, the chromatogram in Fig. 15 was recorded based on an acidic sample extract after neutralization.

- *Sample/reagent ratio* - The volume of reagent affects the sample dilution factor and response. The optimal reagent to sample volume ratio was between 1:10 and 1:20. In practice this means that 10 µL sample is mixed with 1 µL reagent. For samples as small as 5 µL, the original reagent has to be 2x diluted to maintain the correct mix ratio (the minimum step-size of the autosampler is 1 µL).

- *'In-needle' derivatization procedure* - The benefits of an automated derivatization procedure are a standardized timing between preparation of the sample and detection, which improves reproducibility over manual derivatization, and the user needs to perform less sample preparation. The sample derivatization procedure is completely automated and runs as part of the injection procedure through an optimized predefined 'user program' for the autosampler. The user only needs to load the sample vials in the sample tray and place one vial with reagent on a dedicated position.

The derivatization procedure is part of the injection method and consists of the following steps:

1. Aspiration of reagent
2. Aspiration of sample
3. Mixing of sample and reagent in the autosampler tubing
4. Loading of the sample loop
5. Injection of the derivatized sample
6. Extensive wash of autosampler flow path

The autosampler is then ready for the next automated injection. Since the reagent is going to get in contact with the sampled vial and react with the left-over sample, vials should not be sampled repeatedly: each analysis should take place from a separate unsampled vial for best reproducibility.

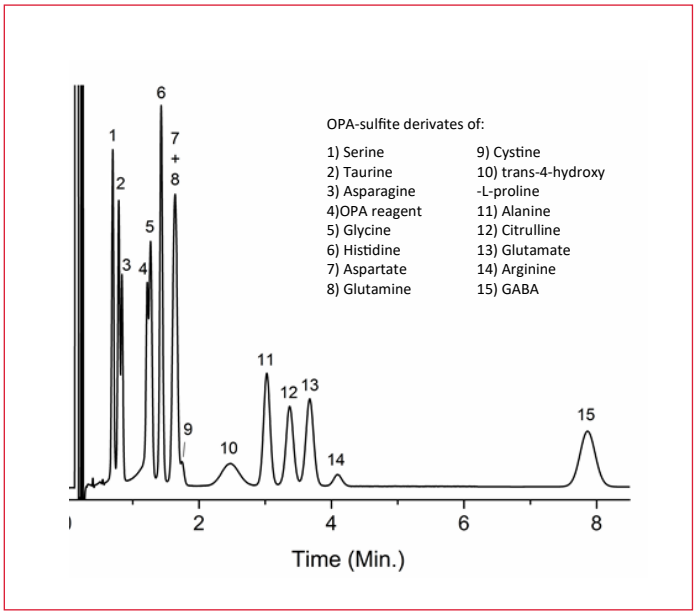


Figure 4: Chromatographic profile of OPA-sulfite derivatized standards mix of amino acids and related substances in water. Chromatographic conditions according to Table 1 (but with a mobile phase with pH set to 3.28 and 1% acetonitrile and 2 % methanol). With courtesy to Mrs. Gerdien Korte-Bouws, Department of Pharmaceutical Sciences, division of Pharmacology, University of Utrecht, The Netherlands.

UHPLC separation column

The derivatization products that are formed are separated using isocratic conditions on a 5 cm sub-2 micron C_{18} 1 mm ID UPLC column (see Table 1). This column will typically show an efficiency in the range of 100,000 -130,000 plates per meter (standard GABA peak), and the fact that we chose to apply a 1 mm ID column reduces the amount of mobile phase and waste with almost a factor 20 when comparing with the use of a traditional 4.6 mm ID column.

For the analysis of GABA and Glu, we recommend the conditions in Table 1 as a good starting point. As there may be other primary amines or amino acids present in the sample, Fig. 4 is shown to highlight their potential presence. This figure shows the chromatographic profile of 14 amino acids and related substances using the conditions of Table 1 (but slightly different mobile phase). This picture also shows that other analytes of interest (containing a primary amine) can be detected with this method.

The next paragraph highlights the mobile phase composition and how adjustments affect retention times.

Mobile phase

The mobile phase consist of an acidic phosphate/citrate buffer, EDTA to capture stray metal ions, a low level of acetonitrile as organic modifier and 'Type I' water with high resistivity (>18 MΩ-cm) and low TOC (<10 ppb), which has the least amount of electrochemically active contaminants possible. During method development, a pH of 3.5 in combination with 2% acetonitrile was found to give good separation for GABA and Glu from the neighboring peaks in some microdialysate samples. However, the complexity of chromatograms from microdialysis samples can vary with brain region and by the experimental treatment, so it may still be necessary to make small adjustments before running a whole set of samples.

Two mobile phase parameters that can be used to tune retention times are pH and modifier levels:

- *Organic modifier* – The addition of acetonitrile as modifier speeds up the elution of all components (Fig. 5). However, not all peaks respond to the same degree to changes in acetonitrile concentration in mobile phase. Therefore, the acetonitrile concentration is a useful parameter to change elution patterns. Another option is to use a mix of methanol and acetonitrile as modifier.

- *pH* - The influence of pH on retention of GABA and Glu is shown in Fig. 6. Small changes in mobile phase pH result in opposing changes in retention of GABA and Glu. Notice also the retention behavior of the (small) peak close to the Glu peak. This figure makes it evident that the pH is a powerful tool to tune the separation.

Note: the ALEXYS Neurotransmitter Analyzer (equipped with two pumps) can be fully programmed to run the mobile phase optimization. The overlay of chromatograms presented in Fig. 6 is an example of a set of data that was obtained from an automated overnight sequence, with methods that mixed two different mobile phases in different ratios.

Temperature

A fixed temperature is important for stabile retention times and baselines. An increase in temperature will speed up the elution and decrease the system back-pressure, but increase the baseline noise level. A temperature of 40°C was chosen as a good starting point for the analysis of GABA and glutamate. It was observed that a small change in temperature will not alter the retention of all components to the same extent. Therefore, a small decrease in temperature can be tried and tested when there is a need for a slightly different chromatographic separation when dealing with complex samples.

Post-separation step-gradient

A number of late eluting peaks have been observed long after the last component of interest eluted off the column when analyzing biological samples. It may take at least 60 minutes

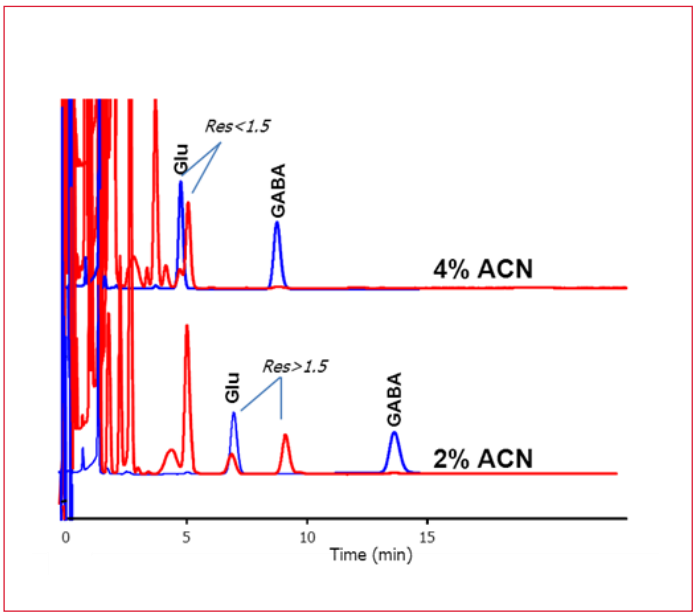


Figure 5: Overlay of a standard and sample chromatogram recorded with 2% or 4% acetonitrile in the mobile phase. Red trace: pooled rat dialysate from the Hippocampus. Blue trace: 5 µmol/L GABA and Glu standard mixture in Ringer solution (separation and detection at 35 °C). With courtesy to Niels Leguit, Abbot Healthcare Products B.V., Weesp, the Netherlands for providing the samples.

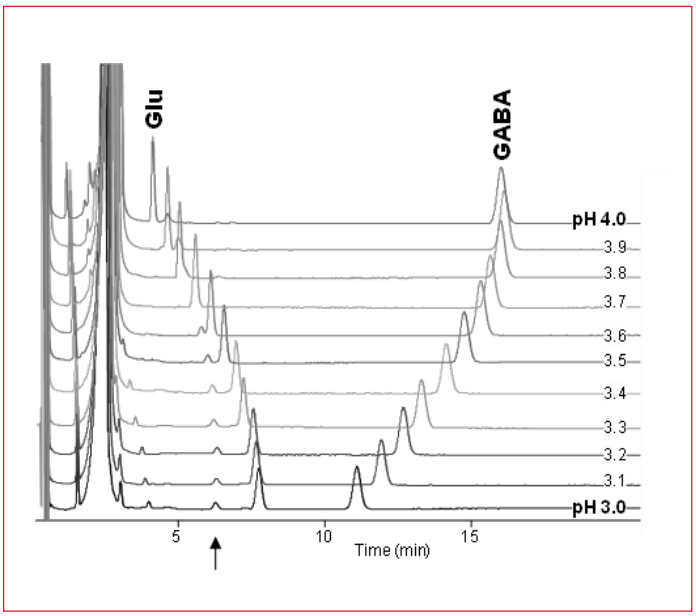
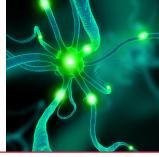


Figure 6: Effect of mobile phase pH on separation: overlay of chromatograms from OPA-sulfite derivatized GABA and Glu standards mixture (separation and detection performed at T=35°C).



before the baseline is stabile and ready for the next injection if no precautions are taken (Fig. 7). In such case, analysis run times will be very long (>60 min), or the late eluting peaks will show up on the baseline of the next analysis if shorter run times are chosen.

Therefore, a short post-separation washing step is applied with a mobile phase containing a high percentage of organic modifier (Fig. 7). This will quickly flush out all the components that were still progressing towards the end of the column.

We explored two ways to implement a washing step: using the standard Solvent Switch Valve (SSV) on the P6.1L pump, and the use of a second pump in High Pressure Gradient (HPG) mode. The HPG method is preferred as is has twice the sample throughput compared to the SSV method.

Flow cell installation

The SenCell glassy carbon working electrode must be in good condition, and installed according the instructions in the manual [8]. After running mobile phase through the cell for at least 10 min, a working potential (Ecell) of 850 mV is set and the cell turned on. The background current (Icell) should stabilize quickly and smoothly down and past 15 nA (Fig. 8), otherwise open and inspect the cell and make sure to refill it air free. Eventually Icell will drop into a range of 2-5 nA, but test injections to check the chromatography can already be started before reaching a completely stabile baseline.

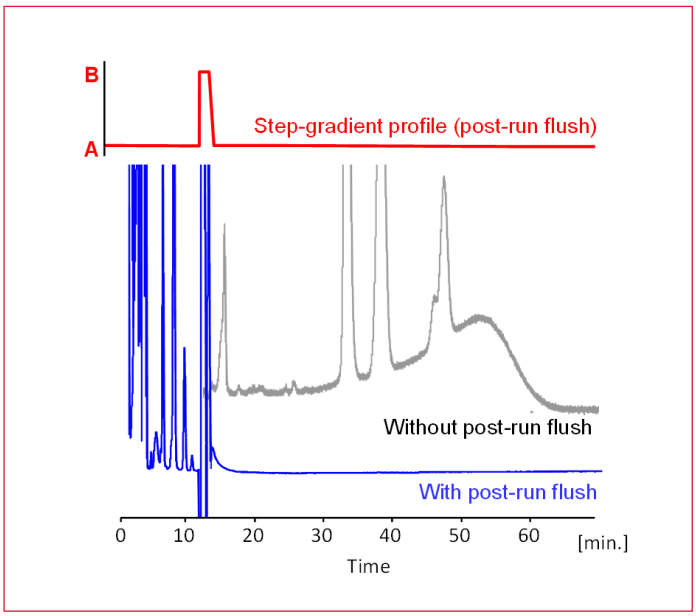


Figure 7: Chromatograms of OPA-sulfite derivatized microdialysate samples from nucleus accumbens recorded with and without a post-run flush as part of the method.

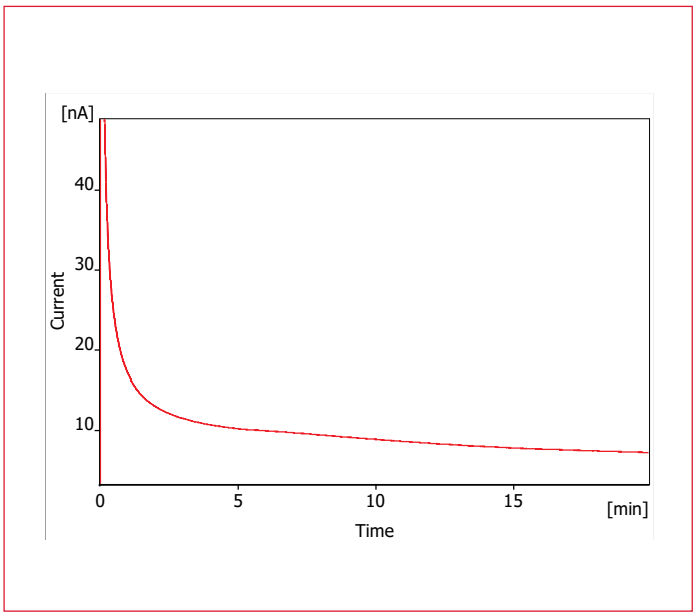


Figure 8: Typical stabilization pattern of a SenCell 2 mm GC flow cell. The baseline quickly stabilizes after turning on the flow cell.

Method evaluation

A fast and sensitive method (Table 1) was optimized and evaluated for GABA and Glu analysis in neutral microdialysates.

Initial condition evaluation

We advise to start with a few repeated initial test injections of 10 µmol/L GABA and Glu to check the general local conditions before proceeding. Under the standard conditions given in Table 1, the expected retention time is around 3 min for Glu , and around 8 min for GABA. Expected peak heights are around 15-20 nA for the Glu peak, and 10-15 nA with >100.000 plates per meter for the GABA peak .The baseline noise level can be expected to be around 10 pA at a 5nA range setting.

Linearity

Depending on the brain region under investigation, basal concentrations in microdialysate samples are typically in the range of 10 - 50 nmol/L GABA [9, 10] and several µmol/L Glu [10, 11]. We prepared mixed standards in a biologically relevant concentration range of 0.2 -1 µmol/L Glu and 20 – 500 nmol/L GABA in a background of Ringer's solution for the linearity evaluation of the method (Fig. 9). The correlation coefficient showed a value of 0.998 or better for both GABA and Glu.

Table 1

LC-ECD conditions for analysis of GABA and Glu

UHPLC	ALEXYS Neurotransmitter Analyzer
Column	Acquity UPLC HSS T3 1.0 x 50 mm, 1.8 µm (Waters)
Pre-column filter	Acquity in-line filter kit (Waters)
Mobile phase A (separation)	50 mmol/L phosphoric acid, 50 mmol/L citric acid and 0.1 mmol/L EDTA.Na_2, set to pH 3.5 with NaOH solution, 2% acetonitrile
Mobile phase B (post-separation flush)	50 mmol/L phosphoric acid, 50 mmol/L citric acid and 0.1 mmol/L EDTA.Na_2, set to pH 3.5 with NaOH solution, 50% acetonitrile
Flow rate	200 µL/mL 100% A during separation, 12% A 88% B during post-separation flush in HPG mode
Temperature	40 °C for separation and detection
Backpressure	About 400 bar (during separation)
$V_{injection}$	1.5 µL, full loop injection as part of automated in-needle derivatization program using 5 µL sample
Needle wash	Water (refresh weekly)
Pump piston wash	15% isopropanol in water (refresh weekly)
Flow cell	SenCell™ with 2 mm GC working electrode and saltbridge reference electrode, AST setting 1
Ecell	850 mV
Range	50 nA/V for Glu; 5 nA/V for GABA (event table)
ADF	0.1 Hz
I-cell	2 - 5 nA

Table 2

Typical peak area RSD's (n=6) for different concentations of GABA and Glu using the method from Table 1.

	Glu	*GABA*
50 nmol/L	< 5%	< 3%
500 nmol/L	< 2%	< 2%
2.5 µmol/L	< 2%	

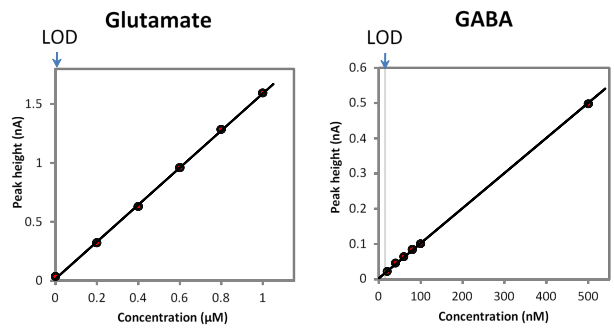


Figure 9: Calibration plots of Glu and GABA with linear regression line through the data points. Conditions according to Table 1.

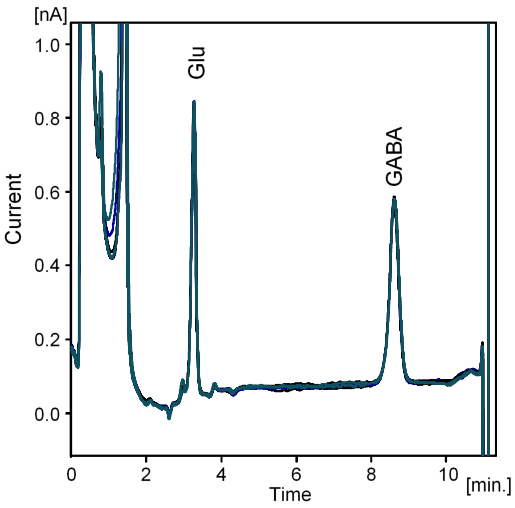


Figure 10: Overlay of 6 chromatograms of 500 nmol/L GABA and Glu in Ringer's solution. Conditions according to Table 1.

Reproducibility

For the repeatability study, different biologically relevant concentrations of GABA and Glu standards in Ringer's solution were analyzed. Table 2 shows the typical area RSD values for this method. Retention time RSD's are typically under 0.3 % RSD. Figure 10 shows an overlay of chromatograms from 500 nmol/L GABA and Glu in Ringer's solution.

Detection limit

The calculated detection limit (signal-to-noise ratio: 3) was about 12 nmol/L GABA and about 8 nmol/L Glu based on a 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 concentration of 20 nM GABA (near the detection limit) is clearly showing a peak (Fig. 11). Note that the presented blank chromatogram showed a small peak with the retention time of Glu, corresponding with a concentration of 17 nmol/L. This has been frequently observed in various laboratories and it is typical for Ringer's solution, and the size appears to becomes more prominent with the age of the solution. In comparison with the basal concentration of Glu in microdialysates (in the range of several µmol/L) the intensity of the interference is relatively small. Based on our observations we can recommend to use fresh Ringer's solution and run a blank to check for interfering peaks.

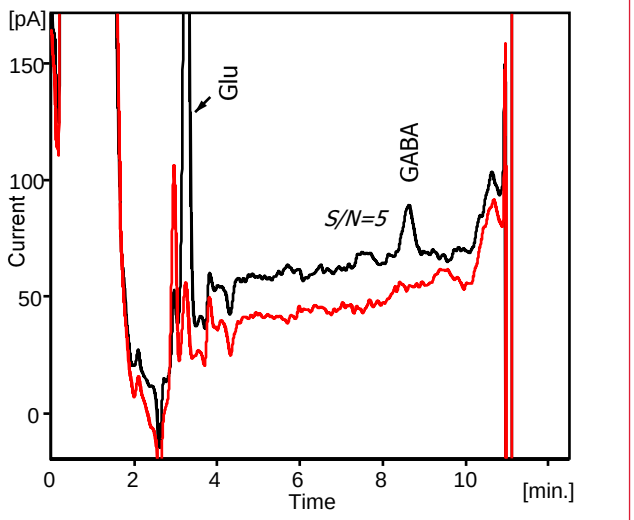


Figure 11: Chromatogram overlay of a blank and a standard of 200 nmol/L Glu and 20 nmol/L GABA in Ringer's solution. Conditions according to Table 1.

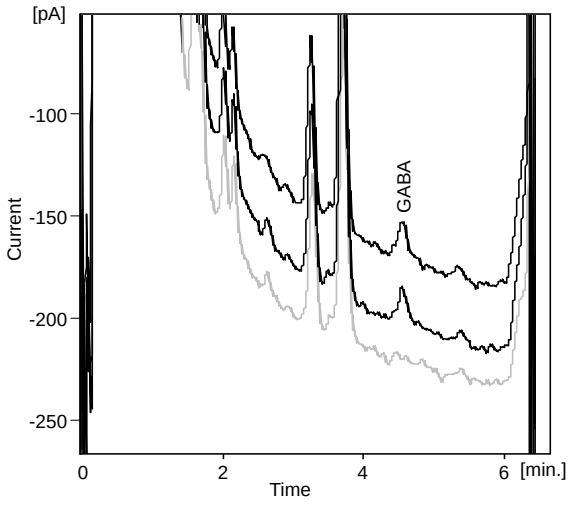


Figure 12: Analysis of a 2 nmol/L GABA standard prepared in Ringer solution (duplicate) in overlay with a blank (grey trace). Conditions according to Table 3. Chromatogram courtesy of Mrs. Lucy Pinder, RenaSci, Nottingham, UK.

Improved detection limit

The detection limits mentioned in the previous paragraph is based on the conditions in Table 1, with a focus on using small samples (5 µL). The detection limit drops to 1.5 nmol/L GABA (Fig. 12) with a few changes (Table 3).

The 1.5 µL sample loop is replaced for a 5 µL sample loop, and the total sample use is doubled to 10 µL. A stronger Advanced Digital Filter (ADF) setting is chosen for the DECADE Elite, to decrease the noise level and increase the signal-to-noise-ratio. The separation conditions are optimized for GABA in real microdialysate samples (not shown), with a slightly higher organic content in the mobile phase and a lower temperature.

Table 3

LC-ECD conditions (changes to Table 1) for improved detection limit of GABA

Mobile phase A (separation)	50 mmol/L phosphoric acid, 50 mmol/L citric acid and 0.1 mmol/L EDTA.Na_2, set to pH 3.5 with NaOH solution, 3,5% acetonitrile
ADF	0.01 Hz
Noise level	about 3 pA
Temperature	37 °C for separation and detection
Backpressure	about 450 bar
$V_{injection}$	5 µL, full loop injection as part of automated in-needle derivatization program that uses 10 µL sample

Analysis of microdialysate samples

During method development several microdialysate samples were analyzed to check the performance with real samples. Chromatograms from Hippocampus are shown in Fig. 5 (red

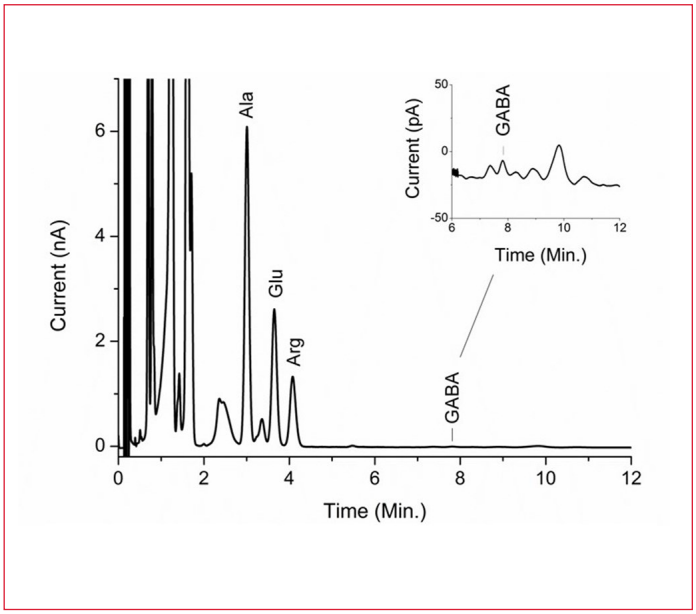


Figure 13: Chromatogram of microdialysate sample from a rat prefrontal cortex. The insert in the top-right corner is a zoom in on the GABA peak. Conditions according to Table 1, except the use of a flow cell with a 0.7 mm GC electrode and mobile phase with pH set to 3.28 and 1% acetonitrile and 2 % methanol. With courtesy to Mrs. Gerdien Korte-Bouws, Department of Pharmaceutical Sciences, division of Pharmacology, University of Utrecht, The Netherlands.

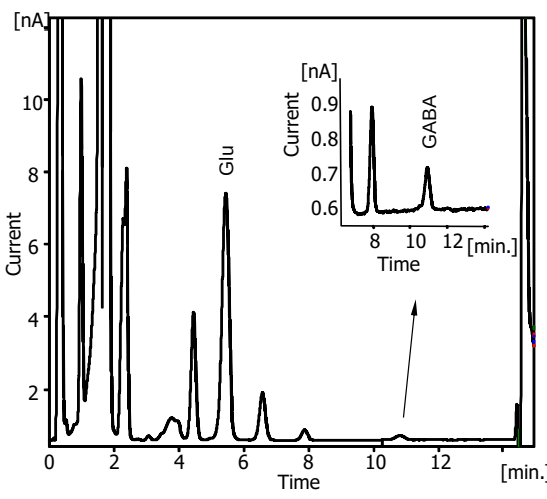


Figure 14: Chromatogram of microdialysate sample from a rat nucleus accumbens. The insert in the top-right corner is a zoom in on the GABA peak. Conditions according to Table 1, except the use of a flow cell with a 0.7 mm GC electrode. With courtesy to Niels Leguit, Abbot Healthcare Products B.V., Weesp, the Netherlands for providing the samples.

traces). The sample levels were quantified to be 1.9 µmol/L Glu and 120 nmol/L GABA. An example of a rat prefrontal cortex microdialysate is shown in Fig. 13. A chromatogram from a sample of the nucleus accumbens is shown in Fig. 14.

Analysis of other primary amines

As already visible in Fig. 4 and Fig. 13, this method will not only detect specifically GABA and glutamate, but also other amino acids and molecules with a primary amine. Here we want to highlight the fact that with a few modifications to the mobile phase, other combinations of amino acids and related substances can be measured as well using the ALEXYS system. See next page for examples.

Analysis of histamine

In Fig. 15, two example chromatograms from histamine 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. For further details we refer to the dedicated application note [12].

Analysis of Large Neutral Amino Acids (LNAA's)

LNAA's (Tyr, Val, Met, Orn, Leu, Ile, Phe, Lys, Trp) are a class of components that can also be measured with the ALEXYS system as shown with a sample of extracted chicken plasma in Fig. 16 (analysis performed at the University of Utrecht). For further details we refer to the dedicated application note [13].

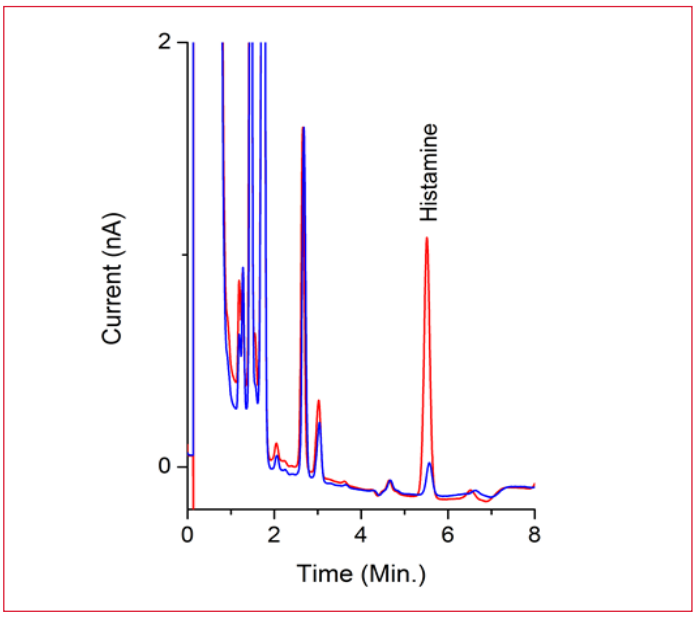


Figure 15: Analysis of the histamine release in RBL-2H3 (mast cell model) after an allergen trigger. Chromatogram overlay from a solution with RBL-2H3 cells before exposure to allergen (blue trace) and after exposure to DNP-BSA allergen (red trace). Chromatogram courtesy of Mrs. Gerdien Korte-Bouws, Department of Pharmaceutical Sciences, division of Pharmacology, University of Utrecht, The Netherlands.

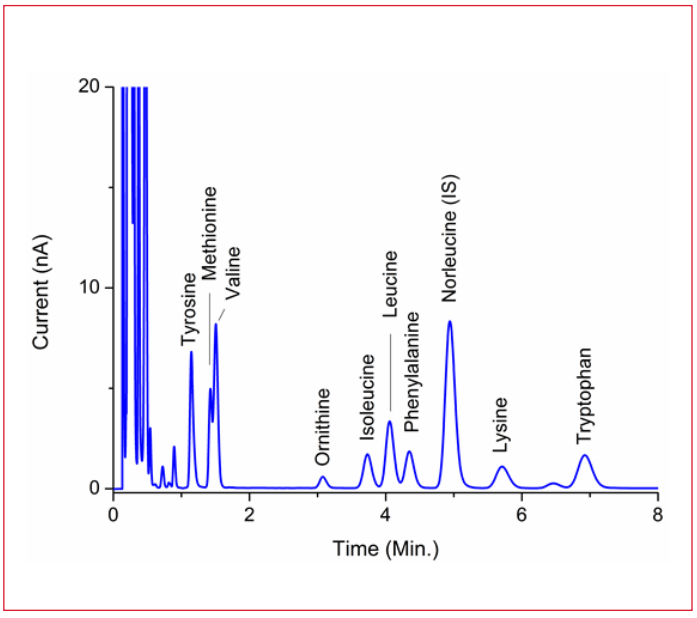
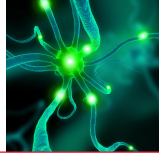


Figure 16: Analysis of extracted chicken plasma. Conditions according to Table 1, except the use of a flow cell with a 0.7 mm GC electrode and a mobile phase according to Table 5. Chromatogram courtesy of Mrs. Gerdien Korte-Bouws, Department of Pharmaceutical Sciences, division of Pharmacology, University of Utrecht, The Netherlands.



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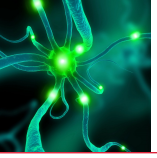
Conclusion

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 analysis of GABA and glutamate is robust and suitable for routine based analysis of various sample types. The method is also applicable for analysis of other primary amines like amino acids. The method utilizes fully automated in-needle derivatization, a post-separation step-gradient to eliminate late-eluting peaks, sample injection volumes down to 5 µL and a separation time <10 minutes.

The ALEXYS Neurotransmitter Analyzer is a versatile platform that can run methods for other neurotranmsitters as well, like the analysis of acetylcholine, choline, monoamines and metabolites.



Ordering information

ALEXYS Neurotransmitter Analyzer for analysis of GABA-Glu	
180.0091UW	ALEXYS Neurotransmitters SCC base
180.0504W	Add-on parts for GABA-Glu analysis (2-pump HPG option)
116.4120	SenCell with 2 mm GC WE and sb REF
250.1165*	Acquity UPLC in-line filter kit + 6 frits (205000343)
250.1160*	Acquity UPLC C18 HSS T3, 1x50 mm 1.8 µm (186003535)

*) Columns are products of Waters Corporation (Milford, USA). The Waters part numbers are given between parenthesis for reordering purposes.

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