

## FAST ANALYSIS OF GABA AND GLUTAMATE

THE SMARTEST LC-EC APPLICATIONS FOR  
NEUROSCIENCE ANALYSIS  
EVER MASTERMINDED

## Monoamines and the metabolites

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

## OPA derivatized amines and amino acids

GABA and Glutamate

*4-aminobutyrate (GABA)**Glutamate (Glu)*

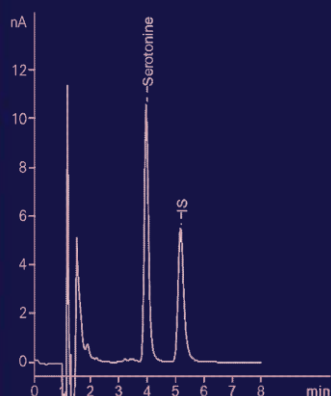
## Choline and Acetylcholine

*Choline (Ch)**Acetylcholine (ACh)*

## Markers for oxidative stress

*3-nitro-L-Tyrosine**8-OH-DPAT*

## Glutathione and other thiols



## INTRODUCTION

Certain amino acids not only serve as building blocks for proteins, but can also serve as neurotransmitters in the brain.

The amino acid derivative,  $\gamma$ -aminobutyrate, also called 4-aminobutyrate, (GABA) is a well-known inhibitor of pre-synaptic transmission in the Central Nervous System (CNS). The activity of GABA is increased by Valium (Diazepam) and by anticonvulsant drugs. The GABA concentration in the brain is 200-1000 times higher than that of the monoamines or acetylcholine. Glutamate (Glu) is an excitatory neurotransmitter and a precursor for the synthesis of GABA in GABAergic neurons. Glu activates the N-methyl-D-aspartate (NMDA) receptors, which play a role in learning and memory and a number of other processes.

- Fast separation on a sub-2 $\mu$ m UHPLC column
- Fully automated 'in-needle' OPA derivatization
- Post separation step-gradient to eliminate late eluters
- Separation of Glu and GABA within 12 minutes

## Summary

## ALEXYS GABA and Glu Analyzer

A fast and sensitive method is presented for the analysis of the amino acid neurotransmitters GABA and Glu in micro dialysates based on the ALEXYS<sup>®</sup> Neurotransmitter analyzer. Highly efficient separation is achieved using a sub-2  $\mu$ m particle UHPLC column. An automated pre-column derivatization with o-phthalaldehyde (OPA) is applied in the needle without the need of a mixing vial. A quick post separation step-gradient is used to eliminate late eluting substances. With this approach excellent detection sensitivity can be achieved with minimal sample consumption. Ultra-High Performance Liquid Chromatography (UHPLC) offers advantages in chromatographic resolution, analysis speed, and sensitivity over conventional HPLC systems. The combination of Electrochemical Detection (ECD) with UHPLC is a powerful solution to increase the sample throughput and sensitivity of neurotransmitter analysis in microdialysates and brain homogenates.



Fig. 1. ALEXYS Neurotransmitter analyzer for GABA and Glu.

## Method

### LC-ECD conditions

For the analysis of GABA and Glu in brain dialysates a pre-column derivatization with OPA and sulphite has been used [1-2].

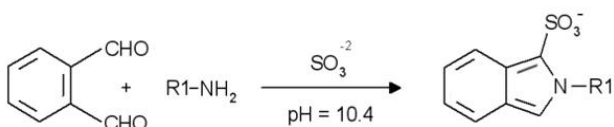


Fig. 2. Reaction scheme of the derivatization of primary alkyl amines with OPA and sulphite.

The N-alkyl-1-isoindole sulphonate derivatives that are formed are analyzed using UHPLC with ECD. The two amino acid derivatives of GABA and Glu show a large difference in retention times. Therefore, a new method was developed for GABA and Glu using the ALEXYS neurotransmitter analyzer. A 5 cm sub-2 micron C18 UHPLC column was used in combination with a post-separation step-gradient. The new ALEXYS system can operate at back pressures up to 650 bar (maximum rating 700 bar).

Table 1

| Conditions         |  |
|--------------------|--|
| <b>HPLC</b>        | ALEXYS Neurotransmitters analyzer with GABA-Glu application kit & LC step-gradient upgrade |
| <b>Flow cell</b>   | μVT-03 with 0.7 mm GC WE and Ag/AgCl REF   |
| <b>Temperature</b> | 40 °C (separation and detection)   |
| <b>Column</b>      | Acquity UPLC HSS T3 1.0 x 50 mm column, 1.8 μm   |
| <b>Flow rate</b>   | 200 μL/min   |
| <b>Pressure</b>    | about 400 bar  |

This new approach has several advantages:

- Automated in-needle OPA-sulphite derivatization.
- Fast and efficient separation using UHPLC
- Post separation step-gradient to eliminate late eluters

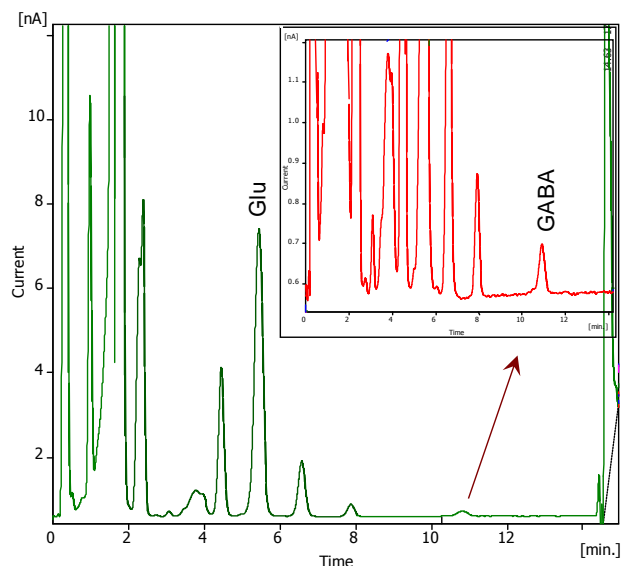


Fig. 3. Example chromatogram of the analysis of GABA and Glu in pooled rat dialysate from the Nucleus Accumbens.

### OPA-sulphite derivatization

**Reagent** - The derivatization procedure and composition of the OPA reagent was modified from Smith and Sharp [2]. The reagent is prepared in the following way:

- 25 mg OPA is dissolved in 250 μL methanol in a 10 mL glass autosampler vial.
- 250 μL 1M sodium sulphite solution is added.
- Followed by addition of 4.5 mL 0.1 M borate buffer (adjusted to pH 10.4 with 14 M sodium hydroxide).

The OPA reagent including the 1M sodium sulphite solution should be prepared fresh each day for optimal performance.

**'In-needle' derivatization procedure** - The sample derivatization is completely automated by a user program in the autosampler. The procedure is significantly improved in comparison to the method described in application note 213-019 [3]. The time required to perform the derivatization is reduced by a factor of two (duration < 4 min instead of 8 min). Furthermore, sample and reagent are mixed in the auto sampler flow path (buffer tubing) and mixing vials are not required. This has as advantage that almost all vial positions (2x 96 vial plates) can be used for samples, resulting in a total sample capacity in the auto sampler of 192 samples (4 vials are reserved for reagent).

The user program comprises of the following steps:

- Aspiration of 0.5 μL reagent
- Aspiration of 9.0 μL sample
- Mixing of sample-reagent solution
- Injection of the derivatized sample on column.

The procedure is depicted in Fig. 4 in more detail.

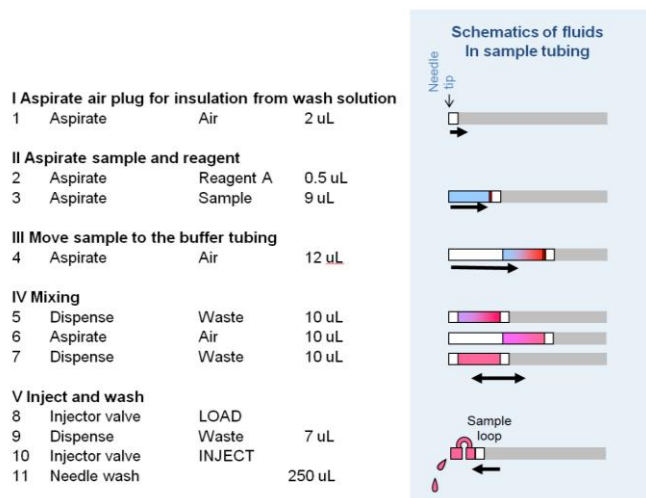


Fig. 4. Automated 'in needle' derivatization procedure (derivatization time < 4 min).

**Sample/reagent ratio** - The sample/reagent ratio affects both the sample dilution factor and the reproducibility. A mixing ratio of 0.5:9 uL reagent:sample was found to give the most optimum result in combination with the above-mentioned derivatization procedure. The transfer of small volumes are handled reproducibly. In particular the final concentration in the derivatization mixture (OPA, sulphite as well as pH) must be sufficient and is primarily affecting reproducibility.

The rate of derivatization with OPA-sulphite reagent is strongly pH dependent. At high pH (> 9.5) the reaction occurs almost instantaneous [1]. Therefore, the OPA-sulphite reagent is buffered at pH 10.4 by means of a 0.1 M borate buffer to assure fast conversion of the amino acids. While microdialysates are often acidified with perchloric acid (PCA) immediately after sample collection to minimize sample degradation over time, it is suggested that microdialysates are not acidified prior to OPA derivatization. The acidic sample pH will decrease the pH of the sample-reagent solution, which in turn will lead to a lower conversion of sample and subsequent loss of signal/sensitivity. For acidified samples, it might be necessary to adjust the composition/buffering capacity of the OPA/sulphite reagent to be able to reach a sufficiently high pH of the mixture to be able to derivatise the amino acids neurotransmitters in the sample.

#### Post-separation step-gradient

The analysis of microdialysate samples is hampered by late eluting substances. In Fig. 5 a typical chromatogram of a 90 minutes isocratic run of a pooled rat dialysate of the Nucleus Accumbens is shown.

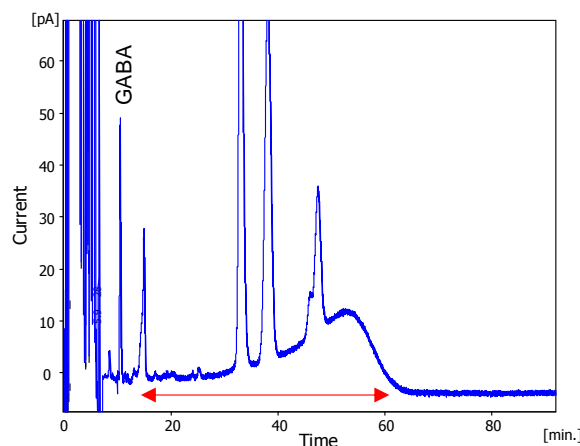


Fig. 5. Chromatogram of a rat dialysate showing several 'late eluters' between 15 and 60 min (red arrow).

It is evident from Fig. 5 that a series of components are eluting between  $t = 15$  and 60 minutes. These peaks will interfere in consecutive runs if no precautions are taken. To remove the strong retained interferences from the column a post-separation step-gradient is applied using a mobile phase with 60% acetonitrile after elution of GABA (from  $t = 12 - 14$  min) as shown in Fig. 6.

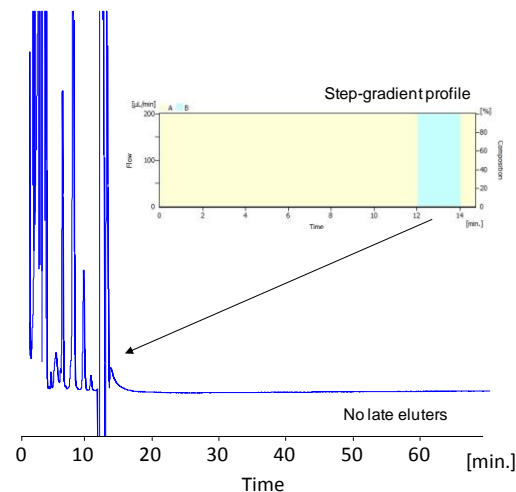


Fig. 6. Chromatogram of a rat dialysate with a post-separation step-gradient. No late eluting peaks are found.

### Repeatability, linearity & LOD

The biologically relevant concentration can typically range from 10nM - 50 nM for GABA [5, 6] to several  $\mu$ M for Glu [7, 8], depending on the brain region under investigation.

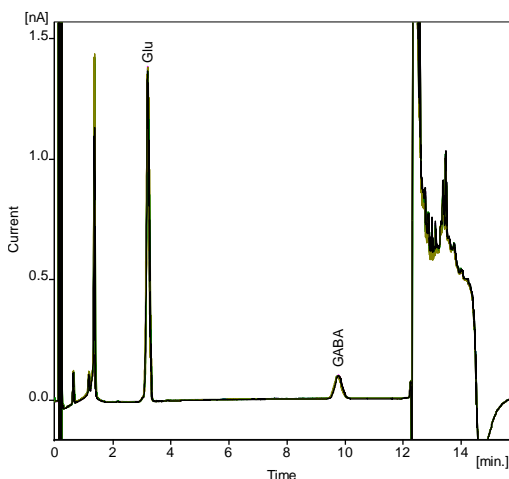


Fig. 7. Overlay of 8 chromatograms of 500 nM GABA and 2.5  $\mu$ M Glu in Ringer.

The repeatability has been studied for 50 and 500 nM GABA standards and 50 nM, 500 nM and 2.5  $\mu$ M Glu standards in Ringer's solution using 1.5  $\mu$ L flushed loop injections. See table 2 for typical RSD values obtained.

|             | Glu, RSD | GABA, RSD |
|-------------|----------|-----------|
| 50 nM       | < 5 %    | < 3 %     |
| 500 nM      | < 2 %    | < 2 %     |
| 2.5 $\mu$ M | < 2 %    |           |

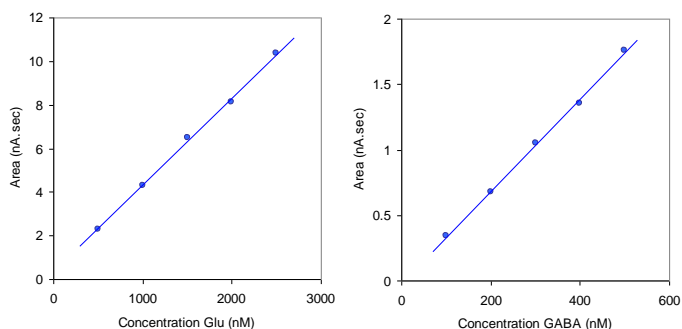


Fig. 8. Calibration plots (Peak Area) of Glu and GABA in the concentration range 0.5 – 2.5  $\mu$ M (Glu) and 100-500 nM (GABA).

The linearity of the method was determined in the concentration ranges of 0.5 -2.5  $\mu$ M and 100 – 500 nM for Glu and GABA respectively. The method showed a good linear detector response with correlation coefficients of 0.998 or better for both GABA and Glu.

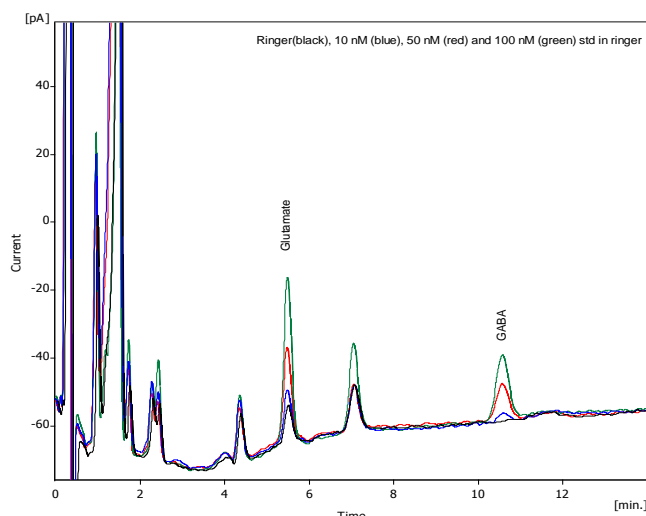


Fig. 9. Overlay of chromatograms of a blank (ringer), 10, 50 and 100 nM GABA and Glu standard in Ringer.

Detection limits were calculated as the concentration resulting in a signal that is 3 times the peak-to-peak noise of the baseline. With the ALEXYS system, a Limit of Detection of 10 nM for both GABA and Glu could be reached under the specified conditions (1.5  $\mu$ L injections, flushed loop). This corresponds to an amount of 15 fmol on column (2 – 3 pg). Total sample consumption in derivatization/injection procedure is 9  $\mu$ L. Note that under the Glu peak in the blank (ringer) an unidentified interference is present. In comparison to the expected biologically relevant concentration of Glu in dialysates ( $\mu$ M range) the intensity of the interference is relatively small.

## Analytical parameters

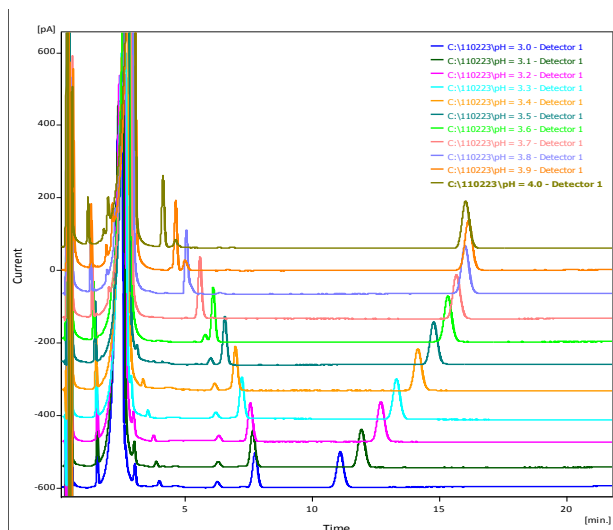


Fig. 10. Optimizing the separation using pH: overlay of chromatograms recorded in the pH range 3 – 4. of a GABA & Glu standard mixture ( $T=35^{\circ}\text{C}$ , separation & detection).

Several analytical parameters have been investigated during method optimization. Both modifier concentration and pH of the mobile phase are the most important parameters for tuning of the chromatography in case baseline separation is not achieved for specific microdialysis samples.

### pH

In Fig. 10 the influence of pH on the separation of GABA and Glu is shown. The pH was varied in the range 3 to 4 with steps of 0.1 pH units. At higher pH GABA is more strongly retained while Glu behaves opposite, it is eluting faster. It is evident that the pH is a powerful tool to tune the chromatography. With the ALEXYS GABA-Glu analyzer, which can operate as a high-pressure gradient system, such optimization can be performed in an automated procedure, using two mobile phases (A and B) with identical contents but different pH. By changing the mixing ratio of the two mobile phases in the gradient method one can control the pH used for the separation.

### Modifier concentration

The percentage modifier affects retention of all sample components, increasing the percentage modifier will decrease the retention times. Acetonitrile (ACN) is preferred as modifier above methanol as it will not increase the mobile phase viscosity [8] and system pressure.

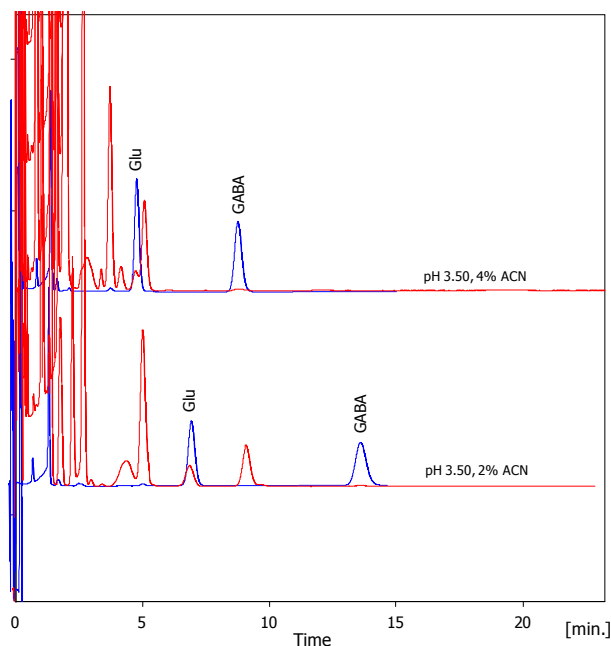


Fig. 11. Overlay of two sets of chromatograms recorded with different modifier concentration (2 and 4% ACN). Red: pooled rat dialysate from the Hippocampus. Blue:  $5\ \mu\text{M}$  GABA & Glu standard mixture in Ringer. ( $T=35^{\circ}\text{C}$ , separation & detection).

Fig. 11 demonstrates that both the pH the modifier concentration are useful parameters for optimizing of the chromatography in case of co-elution of interferences in microdialysates. Also optimization of the modifier concentration can be performed in an automated way with the ALEXYS GABA-Glu analyzer. A pH of 3.5 in combination with a modifier concentration of 2% ACN was found to be the optimum.

### Temperature

Another parameter to take into consideration with respect to optimizing of the separation is the temperature. At higher temperatures components will elute faster, increasing the analysis speed. However it can also result in poorer separation. The oven temperature of the ALEXYS analyzer can be varied between  $7^{\circ}\text{C}$  above room temperature and  $45^{\circ}\text{C}$  and can be used as a parameter for optimisation of the separation if necessary. For this method a temperature of  $40^{\circ}\text{C}$  was chosen as the optimum with respect to separation versus analysis speed.

### Performance

In Fig. 12 a chromatogram and peak table of a  $500\ \text{nM}$  GABA and Glu standard in Ringer's solution is shown to illustrate the performance of the system with standards under optimized LC conditions.

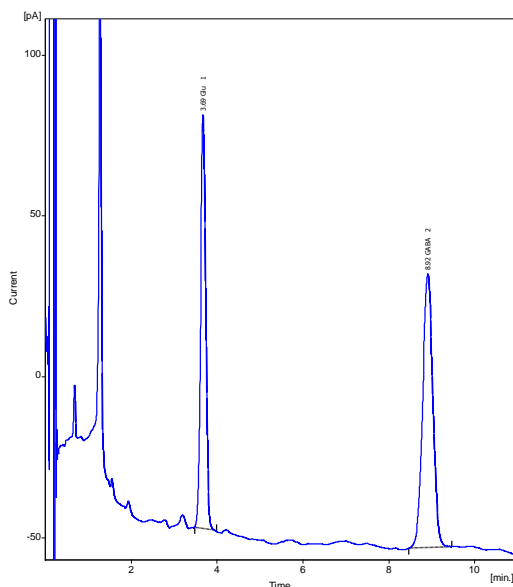


Fig. 12. Chromatogram of a 0.5 µM GABA & Glu standard mixture in Ringer (1.5 µL injection). Separation at oven temperature of 40 °C.

Table 3

| Peak table, 0.5 µM GABA & Glu standard in Ringer |       |        |
|--|-------|--------|
| Compound Name                                    | Glu   | GABA   |
| Reten. Time [min]                                | 3.69  | 8.92   |
| Area [nA.s]                                      | 1.059 | 1.412  |
| Height [nA]                                      | 0.129 | 0.085  |
| Capacity [-]                                     | 29.75 | 73.33  |
| Asymmetry [-]                                    | 1.043 | 1.014  |
| Eff/I [t.p./m]                                   | 91604 | 132102 |

Typically for standards, efficiencies in the range of 70,000 – 90,000 t.p./m for Glu and 100,000 – 130,000 for GABA are found.

### Analysis of microdialysates

During method development several microdialysate samples were analysed to check the performance with real samples. Pooled basal-level rat microdialysates of different brain regions (Nucleus Accumbens and Hippocampus) were provided by Mr. Niels Leguit, 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 mM NaCl, 4.0 mM KCl, 1.2 mM MgCl<sub>2</sub> and 0.7 mM CaCl<sub>2</sub>. 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. 3. The insert in the top-right corner is a zoom in on the GABA peak. In Fig. 11 chromatograms are shown of pooled hippocampus rat dialysate (red curve). For the rat dialysate from the hippocampus a concentrations of 1.9 µM Glu and 120 nM GABA was measured.

### CONCLUSION

A fast and sensitive method is presented for the analysis of the amino acid neurotransmitters GABA and Glu in microdialysates based on the new ALEXYS neurotransmitter analyzer. The system combines good chromatographic performance with ease of use. The method includes an improved fully automated derivatization and a post-separation step-gradient to eliminate late eluters.

### References

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Fig. 13. ALEXYS Neurotransmitter analyzer for GABA & Glu based on the modular ALEXYS neurotransmitters base system combined with dedicated GABA-Glu application kit and LC step-gradient upgrade.

| PART NUMBERS AND CONFIGURATIONS |   |
|---------------------------------|---|
| 180.0091U                       | ALEXYS Neurotransmitter Analyzer                    |
| 180.0502                        | ALEXYS GABA-Glu kit                                 |
| 180.0602                        | LC step-gradient upgrade                            |
| 250.1160                        | Acquity UPLC HSS T3 1.0 x 50 mm column, 1.8 $\mu$ m |
| 250.1700                        | In-line filter (aqueous)                            |

