

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)

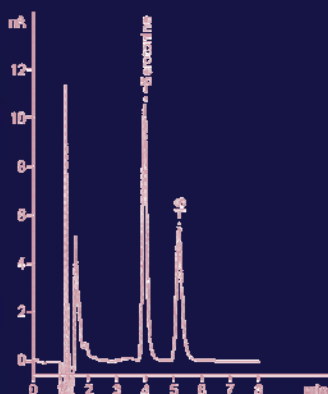
Choline and Acetylcholine

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, γ -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 and by anticonvulsant drugs. The GABA concentration in the brain is 200-1000 times greater than that of the monoamines or acetylcholine. Glutamate (Glu) is an excitatory neurotransmitter and a precursor for the synthesis of GABA in GABAergic neurons. Glutamate activates the N-methyl-D-aspartate (NMDA) receptors, which play a role in learning and memory and a number of other processes.

- One injection, two chromatograms
- Fully automated OPA derivatization
- Column switch: no late eluting system peaks
- Total analysis time < 25 min (including derivatization)

Summary

ALEXYS GABA and Glu Analyzer

A method is presented for analysis of GABA and Glu in microdialysates. Column switching is applied to resolve Glu from the front peaks and to reduce the retention time of GABA. It has a fully automated OPA derivatization taking place in the autosampler. Late eluting peaks from the OPA reagent that traditionally hamper routine analysis are conveniently removed by this column switch setup. The method is optimized for small sample volumes with a detection limit down to 15 and 35 fmol for Glu and GABA respectively.



Fig. 1. ALEXYS GABA and Glu Analyzer

Method

LC-EC conditions

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

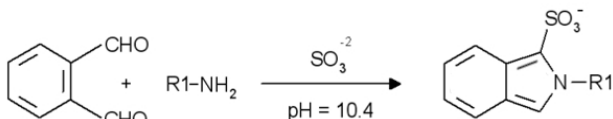


Fig. 2. Reaction scheme of the derivatization of primary alkyl amines with o-phthaldialdehyde and sulphite.

The formed N-alkyl-1-isindole sulphonate derivatives are separated and detected by HPLC with electrochemical detection.

Analysis of the two amino acid derivatives showed a large difference in retention times. Therefore, an ALEXYS[®] analyzer with a DECADE II[™] Dual Cell Control (DCC) and column switching has been applied (Fig. 3). This approach has three distinctive advantages:

- Automated OPA-sulphite derivatization in autosampler for better reproducibility.
- Glutamate, which is fast eluting, is well resolved from the chromatographic front using a 5 + 15 cm C18 column in series.
- The retention time of late eluting GABA is reduced considerably due to elution over the shorter 5 cm column only. Resulting in a total analysis time of 25 min per run, including the derivatization step.

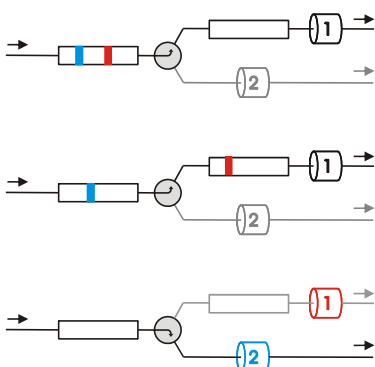


Fig. 3. Glutamate (red) is resolved from the front of the chromatogram and detected in cell 1. The run time of late eluting GABA (blue) is shortened by column switching followed by detection in cell 2.

The concept of column switching is depicted in Fig. 3. Glutamate is eluting over a 5 + 15 cm column in series. When glutamate is on the second column, a valve switches and a second pump takes

over column 2. The valve switches column 1 (with GABA still on it) to cell 2, thus bypassing the second column.

Table 1

Conditions	
HPLC	ALEXYS GABA, Glu analyzer
Oven temperature	35 °C (separation and detection)
Flow rate	200 µL/min
Flow cell	VT-03 with 2 mm GC WE and Ag/AgCl REF
ADF [™]	0.02 Hz

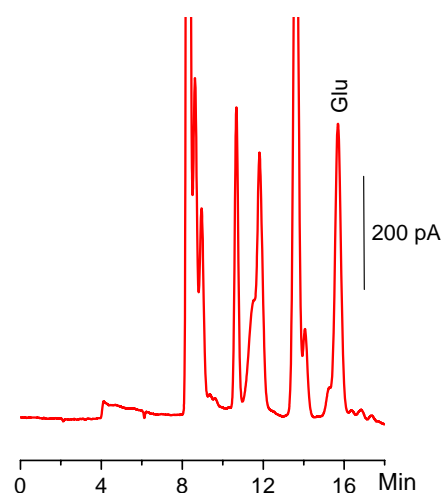
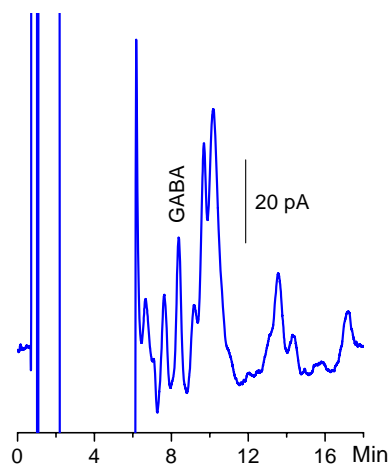


Fig.4. Analysis of GABA and Glutamate in pooled dialysate from Basal Lateral Amygdala.

Column switching

The system consists of 2 pumps, 2 columns, 2 cells and a switching valve (Fig. 5). To avoid large pressure drops after valve switching 2 restrictors are applied. In fact restrictor-205 balances the pressure drop over column-205, the same holds for column and restrictor 215. Both pumps have the same mobile phase and run at the same flow rate.

Balancing the pressure drop over the columns and restrictors is not only necessary to improve the column life time. It also improves baseline stability and reproducibility of the analysis. To balance the pressure of the different flow channels the following procedure is executed: Measurement of the back pressure of both LC channels (pump 1 and 2) with the CS valve in position A.

- Measurement of the back pressure of both LC channels with the CS valve in position B.
- Calculation of the length of tubing to be removed of restrictor 205 and 215 to balance the back pressure over the different LC channels.
- Subsequently, the calculated length of tubing is removed from the pressure restrictors using a tubing cutter. The resulting maximum pressure drop during column switching should not exceed 1 bar.

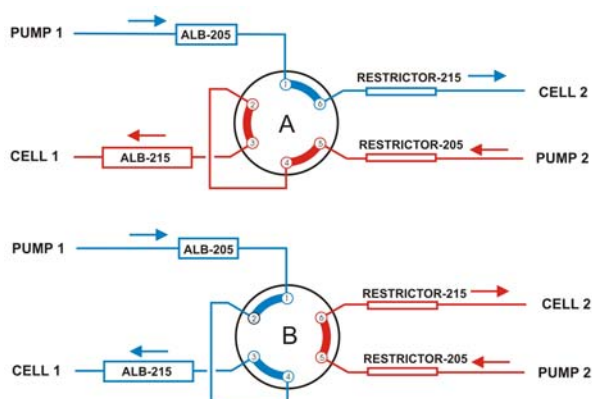


Fig. 5. Schematic representation of the two valve positions for column switching. Position A: 5 and 15 cm column in parallel, position B: 5 + 15 cm column in series. ($P_{red} = P_{blue}$).

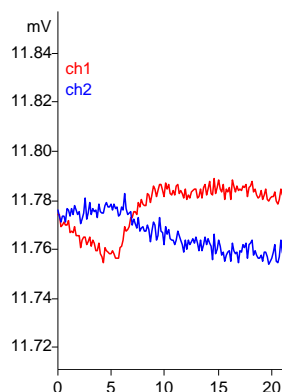


Fig. 6. Pressure change when switching valve at $t=6$ min is 0.3 bar. Output of 1 mV is 10 bar, system pressure is 118 bar.

A detailed description of the pressure balancing procedure can be found in the LC connection kit installation guide 180.7001A, appendix II [4].

OPA-sulphite derivatization

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

- 25 mg OPA is dissolved in 250 μ L methanol in a 10 mL glass autosampler vial.
- 250 μ L 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-sulphite reagent including the 1M sodium sulphite solution should be prepared fresh each day.

Derivatization procedure - the sample derivatization is completely automated by a user program in the autosampler. See document 213-019A, AS user program & settings for details [3]. The user program comprises of the following steps:

- Transfer of sample to a separate mixing vial.
- Addition of reagent to the mixing vial.
- Mixing of sample-reagent solution (Multiple steps).
- Injection of the derivatized sample on column.

Sample/reagent ratio - The sample/reagent ratio not only affects the sample dilution factor, but also the reproducibility of analysis. Reproducibility of the mixing ratio of 20 μ L sample with 2, 4 and 6 μ L reagent has been investigated using a user program [3]. The

optimum in dilution factor vs. reproducibility of the signal and peak area was found when adding 4 μL reagent.

It is not the transfer of small volumes that negatively affects reproducibility. In particular the final concentration in the derivatization mixture (OPA, sulphite as well as pH) must be sufficient, and is primarily affecting reproducibility. For example, if the pH of the mixture is not high enough it will result in poor reproducibility and decreased peak height.

Reproducibility

The reproducibility has been evaluated for standards of 10, 50 and 500 nmol/L GABA and Glu in water (Table 2) using 5 μL injection and column switching at 2 and 6 minutes. Standard deviation in peak height is about 1% at 500 nmol/L and 2-3% at 50 nmol/L. This is slightly better than the RSD in peak area.

Table 2				
Reproducibility (n=8)				
	Height, nA		Area, nA \cdot sec	
	Mean	%RSD	mean	%RSD
10 nM				
GABA	0.020	5	0.391	5
50 nM				
Glu	0.021	3.3	0.37	5.1
GABA	0.067	1.7	1.28	2.5
500 nM				
Glu	0.200	1.1	3.69	1.7
GABA	0.641	0.6	12.2	0.5

The biologically relevant concentration can typically range from 10nM - 50 nM for GABA [5, 6] to several μM for Glu [7, 8], depending on the brain region under investigation. At 10 nmol/L the standard deviation of GABA peak height is approximately 5%. Reproducibility of retention times is better than 0.2 % RSD in all our studies.

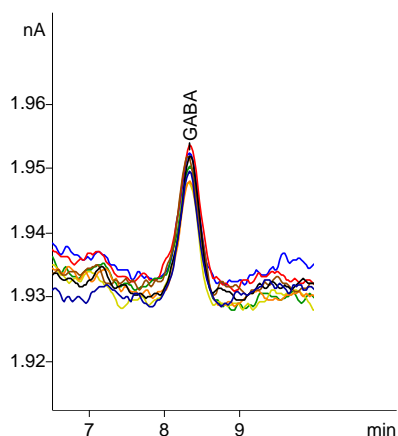


Fig. 7. Overlay of 8 chromatograms of 10 nM GABA. Injection volume 5 μL . Reproducibility parameters are listed in

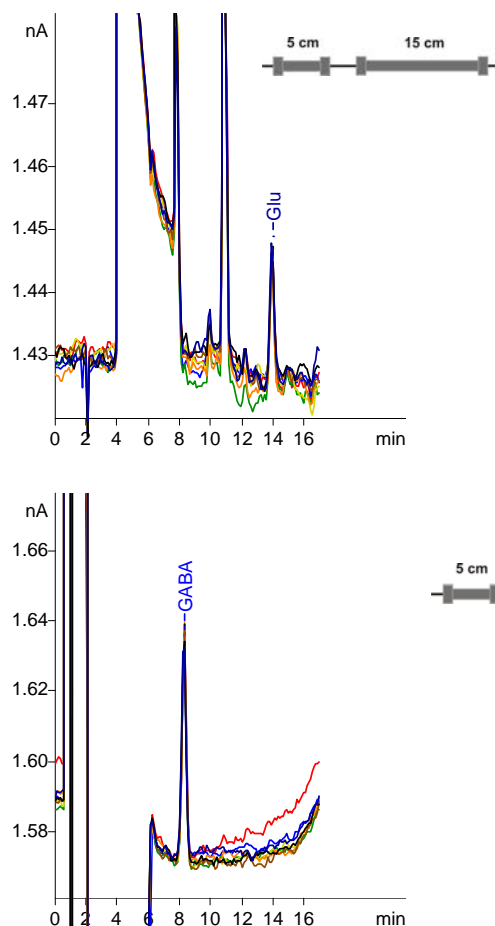


Fig. 8. Overlay of 8 chromatograms of 50 nM GABA (bottom) and Glu (top).

Linearity & Detection limits

The linearity of the method was determined in the biologically relevant concentration ranges for GABA and Glu. The method showed an excellent linear detector response with correlation coefficients of 0.999 or better for both GABA and Glu, for peak heights as well as peak areas.

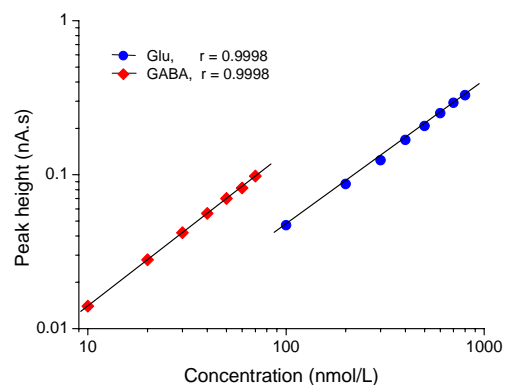


Fig. 9. Calibration plots of Glu and GABA in the concentration range 10-70 nM (GABA) and 10-800 nM (Glu). Top: peak height vs. concentration. Bottom: peak area vs. concentration.

Table 3

Linearity				
	Conc nM	Peak Height		
		a	b	r
GABA	10-70	0.00139	-0.00003	0.9998
Glu	100-800	0.00041	0.0051	0.9998

Linearity parameters of calibration curve ($Y = aX + b$) of GABA and Glu ($n=7$ and $n=8$, respectively).

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 7 nM for Glu and 3 nM for GABA could be reached under the specified conditions (5 μ L injections, flushed loop). This corresponds to an amount of 30 fmol Glu and 15 fmol GABA on column.

Analytical parameters

Several analytical parameters have been investigated during method optimization.

Chromatographic conditions

As a starting point the chromatographic conditions from reference [3] were applied. The conditions were modified to suite our specific needs. The modifications involved the addition of citric acid to the mobile phase to have optimal buffering capacity in the pH range 3 - 5, lowering both the MeOH concentration and pH for better separation and retention behaviour.

MeOH concentration - The percentage modifier typically affect retention of all sample components, increasing the percentage modifier will decrease retention times. In figure 10 the retention behaviour of GABA as function of modifier concentration is shown on the 15 cm ALB-215 column.

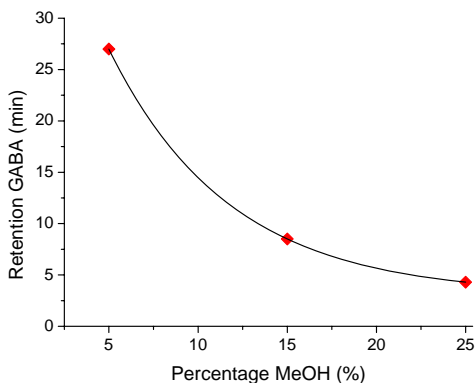


Fig. 10. Retention time of GABA derivative as a function of the percentage MeOH in the mobile phase on a 15 cm column (ALB-215) only. pH of the mobile phase was 4.5.

A modifier percentage of 5% MeOH was found to give the most optimum retention behaviour for the separation and detection of

GABA and Glu in our configuration. With 5% MeOH the retention time of GABA is around 8 minutes on the 5 cm ALB-205 column.

pH of mobile phase - To further optimize the separation of Glu the pH was varied between 4.5, 4.0 and 3.5.

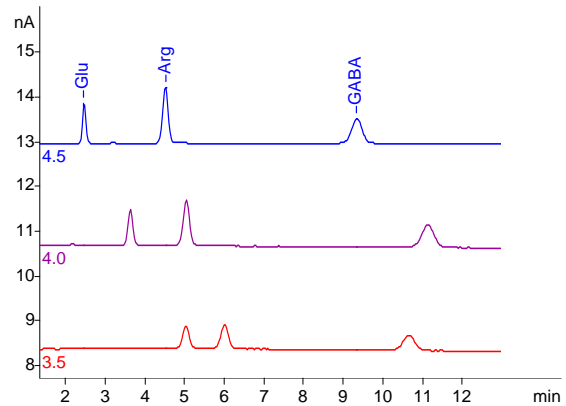


Fig. 11. Overlay of chromatograms of 100 nM Glu, Arg and GABA derivatives using a mobile phase with pH 4.5 (top), 4.0 (middle) and 3.5 (bottom) on a 5 cm ALB-205 column.

The retention of Glu increases with decreasing pH. A pH of 3.5 was necessary to resolve Glu from another co-eluting system peak. Glu has a retention time of 14 - 16 minutes when eluting over a 5 + 15 cm ALB column, and under such conditions is well resolved from the chromatographic front.

Table 4

Peak table				
	tR (min)	H (nA)	A (nA.s)	N TP/m
Glu	5.01	0.50	5.9	83256
Arg	5.99	0.54	7.3	92729
GABA	10.64	0.34	7.6	111791

Peak parameters for 100 nM Glu, Arg and GABA eluting over a 5 cm ALB-205 column at pH 3.5.

Both modifier concentration and pH of the mobile phase are important parameters for tuning of the chromatography in case baseline separation is not achieved for specific microdialysis samples.

Influence of column switching on plate number - The influence of the 6-port switching valve on peak shape has been determined. It can be concluded that the valve does not have a significant impact on peak shape. The plate number (plates/ meter) was 72500 for Glu and 82500 for Arg, with less than 1% difference in case a valve was connected between the two columns.

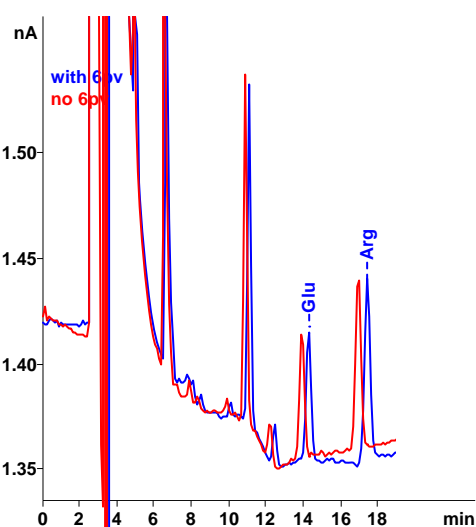


Fig. 12. Overlay of two chromatograms with (blue curve) and without (red curve) 6-port valve connected between the 5 and 15 cm column.

Sample preparation

The reaction time, sample pH and sample stability has been investigated during optimization of the sample preparation procedure.

Reaction time & sample pH - 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.

To determine the required reaction time for derivatization the delay time between mixing and injection of the sample-reagent solution was varied between 0 and 10 minutes. No difference in signal was found between injections with different delay time, indicating that all sample was already converted during the mixing step.

Microdialysates are often acidified with perchloric acid (PCA) immediately after sample collection to minimize sample degradation over time. It is advised not to acidify microdialysates prior to OPA derivation. 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.

Sample stability - It has been reported [1] that OPA-sulphite derivatives decompose at low pH (hydrolysis). This has been studied by means of the following experiment. 250 nM Glu was analyzed over a 5 cm column and 5 + 15 cm column. In the latter case Glu is exposed to pH 3.5 for a significant longer time period.

It is evident from Fig. 13 that the magnitude of the Glu signal (peak area) is dependent on the residence time on the analytical column. An increase in retention time from 3.7 to 15.5 minutes resulted in a loss of signal of 2,5 times.

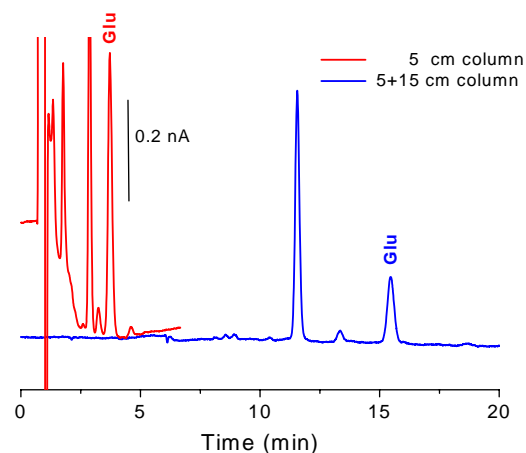


Fig. 13. Overlay of two chromatograms of a 250 nM Glu standard separated on a 5 (blue curve) and 5+15 cm column (red curve). The same flow cell was used for detection of Glu in both chromatograms.

Table 5

Peak table				
Column	Retention	Height	Area nA*sec	Plates
5 cm	3.72	0.56	6.7	47892
15 + 5	15.46	0.13	2.6	66928

Peak parameters for a 250 nM Glu standard eluting over a 5 cm and 5+15 cm ALB column at pH 3.5.

The stability of the OPA derivatives at low pH does not influence the robustness of this method. The automated derivatization and analysis procedure with fixed timing of each individual step assures good method reproducibility, as demonstrated by our results. Furthermore, the loss in sensitivity of a factor 2.5 for Glu is not a crucial factor, because the biologically relevant concentration range for Glu in microdialysates is much higher than the Limit of Quantitation (LOQ) of this method (LOQ is approximately 20 nM for Glu).

Analysis of microdialysates

The developed method has been applied for a number of dialysates. The occurrence of a number of interfering peaks in the first 15 minutes emphasized the importance of resolving Glu from the front peaks using the 2 columns in series. In Fig. 4 a pooled microdialysate from the basal lateral amygdala was analyzed and a concentration of 0.93 μ M Glu and 23 nM GABA was measured.



CONCLUSION

The ALEXYS GABA and Glu Analyzer is a dedicated LC- EC solution for the trace analysis of GABA and Glu in microdialysates. The system combines good chromatographic performance with ease of use. The method includes a fully automated derivatization and column switching step and shows good linearity, reproducibility and detection limits. The use of a column switching step results in a reduction of the total analysis time and an improvement of the separation of the fast-eluting Glu from the chromatographic front.

References

1. W.A. Jacobs, o-Phthalaldehyde-sulphite derivatization of primary amines for liquid chromatography-electro chemistry, *J. Chromatography* **392** (1987) 435-441
2. S. Smith, T. Sharp, Measurement of GABA in Rat Brain Microdialysates Using o-phthalaldehyde Sulphite Derivatization and High- Performance Liquid Chromatography with Electrochemical Detection, *J. Chromatography B* **652 (2)** (1994) 228-233
3. Antec Leyden, Gaba and Glutamate AS user program & settings, document 213-019A, (2005) 1-2
4. Antec Leyden, Installation guide LC connection kit DCC I-I CS, document 180.7018, (2006) 23 - 29
5. H. L. Rowley, K. F. Martin, C. A. Marsden, Determination of in vivo amino acid neurotransmitters by high-performance liquid chromatography with o-phthalaldehyde-sulphite derivatization, *J. of Neurosc. Meth.* **57 (1)** (1995) 93-99
6. S. Zhang, Y. Takeda, et. al., Measurement of GABA and Glutamate in vivo levels with high sensitivity and frequency, *Brain Research Protocols* **14** (2005) 61- 66
7. J. Kehr, Determination of glutamate and aspartate in microdialysis samples by reversed-phase column LC with fluorescence and electrochemical detection, *Journal of Chromatography B.* **708** (1998) 27 -38



Fig. 14. ALEXYS GABA and Glu Analyzer.

PART NUMBERS AND CONFIGURATIONS

180.0070B	ALEXYS GABA and Glu Analyzer
250.1095	ALB-205 column, 50x 2 mm, 3um C18
250.1096	ALB-215 column, 150x 2 mm, 3um C18
250.1700	In-line filter (aqueous)

GABA AND GLUTAMATE - SYSTEM SETTINGS

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)

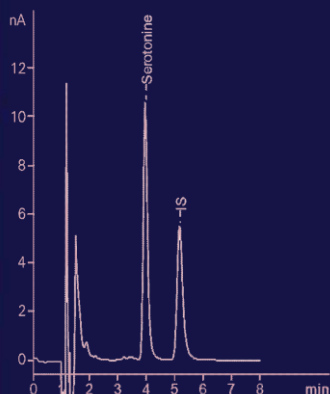
Acetylcholine (ACh)

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, γ -aminobutyrate, also called 4-aminobutyrate, (GABA) is a well-known inhibitor of pre-synaptic transmission in the Central Nervous System (CNS).

- One injection, two chromatograms
- Fully automated OPA derivatization
- Column switch: no late eluting system peaks
- Total analysis time < 25 min (including derivatization)

Summary

ALEXYS GABA and Glu Analyzer

A method is presented for analysis of GABA and Glu in microdialysates. Column switching is applied to resolve Glu from the front peaks and to reduce the retention time of GABA. It has a fully automated OPA derivatization taking place in the autosampler. Late eluting peaks from the OPA reagent that traditionally hamper routine analysis are conveniently removed by this column switch setup. The method is optimized for small sample volumes with a detection limit down to 15 and 35 fmol for Glu and GABA respectively.

Detailed system settings are given as used for application note '213_019 GABA and Glutamate'.



Fig. 1. ALEXYS GABA and Glu Analyzer

Automated derivatization and injection

In table 1 all steps of the AS 100 user program are listed for the analysis of GABA and Glutamate with the ALEXYS® 'GABA, Glu analyzer.

Table 1. AS 100 user program for derivatization & injection.

Step	Action	Speed	Volume (µL)
I Sample transfer			
001	Valve to Load / 6-1		
002	Wash		100
003	Aspirate from sample	1	0
004	Compressor ON		
005	Aspirate from sample		11
006	Compressor OFF		
007	Aspirate from sample	1	0
008	Dispense to destination	1	10
009	Dispense to waste	1	0
010	Syringe valve to wash		
011	Syringe Load		20
012	Syringe valve to needle		
013	Syringe Home		
014	Dispense to waste	2	0
II Addition of reagent			
015	Wash		100
016	Aspirate from reagent-A	1	10
017	Dispense to destination	1	1
018	Dispense to waste	1	0
019	Syringe valve to wash		
020	Syringe Load		20
021	Syringe valve to needle		
022	Syringe Home		
023	Dispense to waste	2	0
III Mixing			
024	Wash		100
025	Aspirate from reagent-B	1	20
026	Aspirate from destination	1	6
027	Dispense to destination	2	6
028	Aspirate from destination	1	6
029	Dispense to destination	2	6
030	Aspirate from destination	1	6
031	Dispense to destination	2	6
032	Aspirate from destination	1	6
033	Dispense to destination	2	6
IV Injection			
034	Aspirate from destination	1	12
035	Valve to inject / 1-2		
036	Marker inject		
037	Aspirate from destination	1	0
038	Dispense to waste	1	0
039	Syringe valve to wash		
040	Syringe load		20
041	Syringe valve to needle		
042	Syringe home		

043	Dispense to waste	1	0
044	Wash		300

Where:

- Speed: syringe speed used for aspiration or dispense steps (value: 1 – 9, see AS 100 user manual).
- Volume: aspirate, dispense or wash volume (in µL)
- Reagent A: OPA reagent in vial type C (Super LSV)
- Reagent B: Deionised water in Vial type C.

The steps in the AS 100 user program comprises the transfer of sample to a separate mix vial, the addition of reagent to sample, mixing steps, and injection of the derivatised sample.

In figure 1 a screen shot is shown of the User program interface in the method set-up of the Clarity HPLC software.

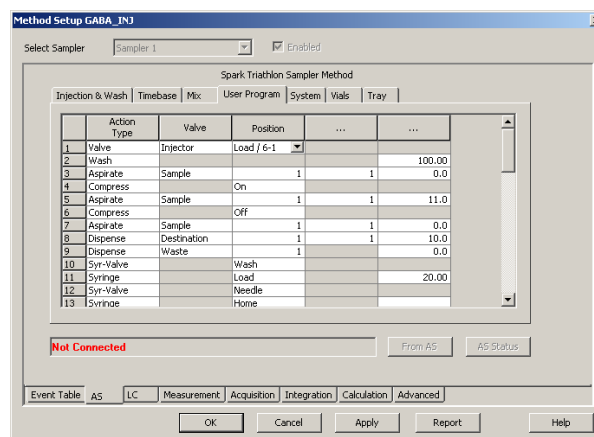


Figure 1. Screen shot of AS 100 user program in Clarity.

In the user program the destination vial is positioned relative to the sample vial location:

Destination Vial = Sample Vial + 40

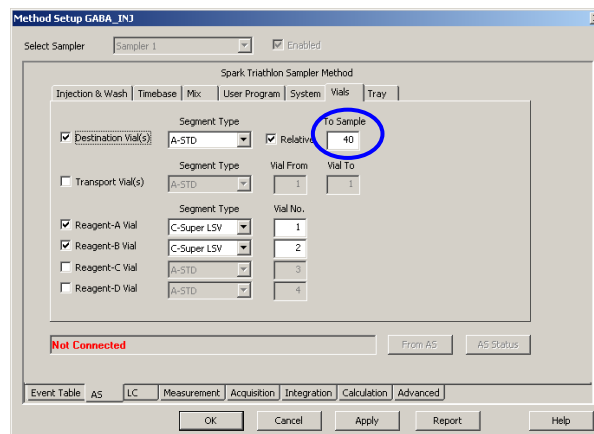


Figure 2. Screen shot of the vial menu in the AS 100 user program in Clarity.

So 40 is the shift in vial position relative to the position of the sample vial. For sample and destination vial type A is used.

Sample dilution

The AS 100 user program mode is a very versatile tool. It is possible to program automated sample dilution steps by means of a user program. Automated sample dilution can be useful to create calibration curves with standards, or in case of limited sample availability, to dilute the available microdialysate volume to match the minimal required sample volume for derivatization & injection. The latter is an option when the concentration levels of GABA & Glu in the microdialysates are sufficiently higher than the limit of quantitation.

In Table 2 an example of a modified user program is shown which results in a 1:2 dilution of the sample prior to derivatization. The user program steps 1-27 (sample transfer & dilution) in Table 2 replaces the steps 1-14 (sample transfer) in Table 1. Note that the total sample volume after dilution in the destination vial is the same as for the undiluted sample, 10 μ L. So the sample/reagent ratio in the derivatization step itself is not affected by the dilution step.

Table 2. Example of sample dilution with user program.

Step	Action	Speed	Volume (μ L)
I Sample transfer & dilution			
001	Valve to Load / 6-1		
002	Wash		100
003	Aspirate from sample	1	0
004	Compressor ON		
005	Aspirate from sample		6
006	Compressor OFF		
007	Aspirate from sample	1	0
008	Dispense to destination	1	5
009	Dispense to waste	1	0
010	Syringe valve to wash		
011	Syringe Load		20
012	Syringe valve to needle		
013	Syringe Home		
014	Dispense to waste	2	0
015	Wash		100
016	Aspirate from reagent-B	1	0
017	Compressor ON		
018	Aspirate from reagent-B		6
019	Compressor OFF		
020	Aspirate from reagent-B	1	0
021	Dispense to destination	1	5
022	Dispense to waste	1	0
023	Syringe valve to wash		
024	Syringe Load		20
025	Syringe valve to needle		
026	Syringe Home		
027	Dispense to waste	2	0

Multiple injections from one sample vial

For reproducibility studies it can be useful to inject replicates from a single sample vial. This can be achieved by preparing several Clarity method files with different relative positions of the destination vial. The most easy way to achieve this is to copy the original method file several times and change the destination vial position as shown in Table 3. The parameter can be found under the "Vials" tab in the AS 100 menu (see figure 2).

Table 3. Programming of Clarity method files in case of replicate injections from 1 sample vial.

Run	Method file	Vial	Rel. position of destination vial
1	GABA_INJ_V40.met	1	sample vial + 40
2	GABA_INJ_V41.met	1	sample vial + 41
3	GABA_INJ_V42.met	1	sample vial + 42
4	GABA_INJ_V43.met	1	sample vial + 43
5	GABA_INJ_V44.met	1	sample vial + 44

In case of the example in Table 3 five replicate injections from sample vial 1 will be processed using the destination vials 41 to 45 for mixing and dilution. It is good practice to name the method files in a systematic way for sake of clarity. In Table 3 the destination position 'Vxxx' is included in the system filename (for example GABA_INJ_V44.met).

Needle height adjustment

To minimise the occurrence of entrapped air bubbles in the mixing vials (Fig.) a *needle height* of 1 mm was programmed in the AS 100 system setting in the Clarity method (instead of the default 2 mm). Note: the actual needle height should be visually checked and adjusted if necessary (see AS 100 user manual), to prevent damage to the needle.

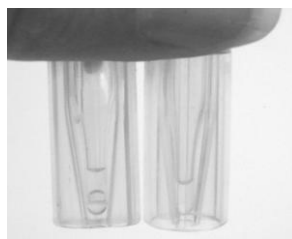


Fig. 3. On the bottom of the left destination vial an entrapped air bubble is visible. The destination vial on the right was correctly filled.

