CAPSULES
preliminary notes and applications from Bioanalytical Systems, Inc.

GABA in Rat Brain Tissue

Purpose
Isocratic determination of GABA in rat brain tissue.

γ-Aminobutyric acid (GABA) has been recognized as a major inhibitory neurotransmitter in the central nervous system of all vertebrates. It is found in mammalian cerebellum, striatum, cerebral cortex, and spinal cord. Its role in various neurological and mental disorders is being examined.

Existing Methods
Direct detection of GABA has been accomplished by an enzymatic cycling method, a radioreceptor assay, an isotachophoretic analyzer method, and GCMS.

Precolumn derivatization with o-phthalaldehyde (OPA) or dansylchloride and LCF has been used. However, the OPA-derivative is not stable and the dansylation procedure requires a relatively long reaction period. Post-column derivatization with OPA has been employed to circumvent the instability problem.

LCEC methods employing precolumn derivatization with trinitrobenzene sulfonic acid have been described. The procedures require that the trinitrophenyl derivatives be extracted from the electroactive derivatizing reagents. Both methods are based on the electrochemical reduction of the aromatic nitro groups in the trinitrophenyl derivative.

Reference
The procedure outlined below, from the BAS research laboratories, describes the determination of GABA by LCEC following its derivatization with OPA and tertiary-butylthiol (TBT). The reaction is depicted in Figure 1. The derivative is a thio-substituted isoindole which is easily oxidized on a glassy carbon electrode. Rather than β-mercaptoethanol, which is often used in this type of reaction (LCF method), TBT is used because it forms a more stable derivative (1 - 3).

OPA DERIVATIZATION

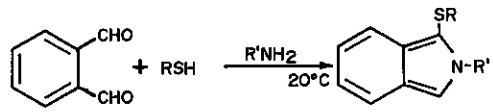

Figure 1. The reaction of OPA with primary amines in the presence of a thiol.

Conditions
System: LC-304 (BAS), BAS 400, BAS 460, or BAS 200
Electrode: Glassy Carbon (BAS, P/N MF1000)
Potential: +0.70 V vs. Ag/AgCl
Column: Biophase ODS, 5µm, C_{18}, 250 x 4.6mm (BAS P/N MF6017)
Mobile Phase: 55% 0.18 M sodium acetate (pH 5.0), 45% acetonitrile (v/v). Flow rate was 2.5 mL/min.
Sample Volume: 50 µl of derivatized sample

Reagents
0.1 M $HClO_4$: Dilute 8.5 mL of concentrated $HClO_4$ (70%) to 1L with deionized water.

GABA Stock: Dissolve 0.0258 g of γ-amino-n-butyric acid (GABA) in 100 mL of 0.1 M $HClO_4$. This yields a 2.5 mM GABA stock. Make serial dilutions from this stock into 0.1 M $HClO_4$, to give GABA concentrations of 1.2, 0.625, 0.313, and 0.156 mM respectively. Refrigerate all solutions when not in use.

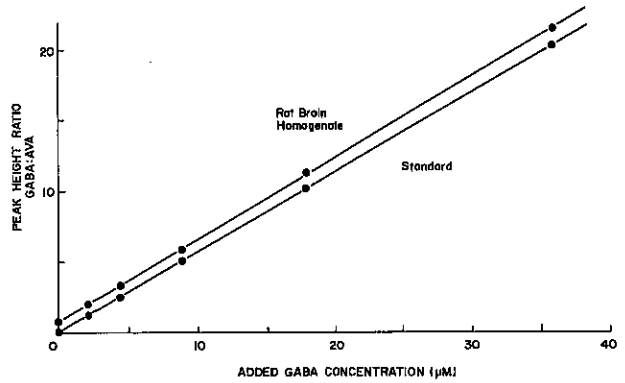

Figure 2. Linearity of GABA standard and spiked rat brain homogenate. Concentration (μM) is calculated as the final 1 mL reaction mixture, containing 200 μL of the spiked homogenate (or standard) and 800 μL of working reagent.

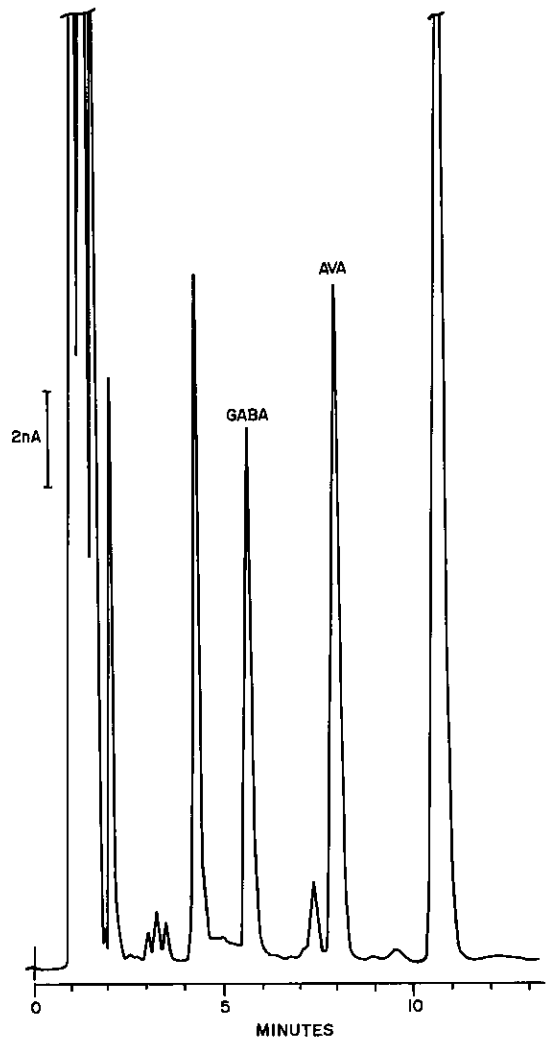

Figure 3. Chromatogram of OPA/TBT derivative of an aliquot in unspiked brain homogenate. AVA was added as an internal standard.

5-Aminovaleric acid (AVA) Stock: Dissolve 7 mg of AVA in 100 mL of 0.1 M $HClO_4$. This yields a 0.598 mM solution. Refrigerate when not in use.

1.0 M Carbonate Buffer: Add 84.01 g of sodium bicarbonate to 950 mL of deionized water and bring to pH 9.6 with NaOH (addition of NaOH enhances the carbonate going into solution). Make to 1L with deionized water.

Working Reagent: Dissolve 0.0671 g of phthalic dicarboxaldehyde (OPA) (Aldrich Chemical Company, Milwaukee, WI, is a good source) in 50 mL of methyl alcohol, add 56 μL of t-butylthiol (Aldrich), and 30 mL of deionized water. Water must be added at this stage to prevent a precipitate from forming when the carbonate buffer is added. Add 15 mL of carbonate buffer, mix well and dilute to 100 mL with deionized water. A closed container of working reagent should be usable for about one week.

Sample Preparation

In the present study 1g of rat brain was homogenized in 0.1 M $HClO_4$, and particulate matter removed by centrifugation and filtration. The clarified supernatant was diluted with 0.1 M $HClO_4$ to give a total dilution factor of 500 for the original brain sample. The homogenates were kept frozen until used.

Procedure

1. To 500 μL of the diluted brain homogenate add 20 μL of the AVA stock (internal standard), and 40 μL of a GABA stock (this will give a "spiked" brain sample, 40 μL of 0.1 M$HClO_4$ will give sample only response). Mix thoroughly.

2. Place 200 μL of the spiked or unspiked sample with internal standard (from #1 above) into a 1.5 mL capped microcentrifuge tube.

3. Add 800 μL of Working Reagent to the sample and mix thoroughly (keep capped to contain the thiol odor).

4. Let stand at R.T. for 6 minutes to allow for the reaction to go to completion. Inject 50 μL of the

derivatized sample at the 6 min. mark. Timing should be consistent in order to ensure precision.

Comments

Both rat brain homogenate and GABA standard responses were linear over the range defined by the GABA standards added (0.0 to 35.71 µM added/0.0 to 1.786 nanomoles injected). The corresponding slopes (F2) of the peak height ratio (GABA:AVA) vs. the concentration of GABA added are close enough to allow comparison between rat brain and GABA standards (spiked brain homogenate, m = 0.5846; standards, m = 0.5696; ± 1.8%). Using peak height ratio (of the sample) and the known concentration of the GABA standards, we may determine the rat brain concentration from:

$$\text{rat brain GABA conc.} = \frac{\text{pk. ht. ratio(unk)}}{\text{pk. ht. ratio(known)}} \times \text{(GABA) known}$$

The precision of the assay was determined by comparing five replicate samples of rat brain homogenate. The relative error was: 1.4% for the GABA derivative peak, 2.2% for the AVA derivative peak, and 0.9% for the peak height ratios.

A chromatogram of an aliquot of unspiked rat brain homogenate, derivatized as described above, is presented in Figure 3. Using the peak height ratio, the concentration of GABA in the original brain tissue was calculated to be 3.36 µmoles/g wet weight.

Appendix

Reverse-phase columns containing 3 µm packing materials exhibit a broad range of applicability to the separation and determination of organic analytes. The utilization of this packing requires some adaptation of the above methodology. The following outlines some changes that were required in switching from a 5 µm packing material to a 3 µm packing material for the determination of GABA in brain tissue.

Conditions

System: BAS 400 Electrode: Glassy carbon
Potential: +750 mV vs Ag/AgCl
Column: Phase II (3 µm, C_{18}, 100 x 3.2 mm cartridge,, BAS P/N MF- 6213) with guard column (7 µm, C_{18}, 15 x 3.2 mm cartridge, BAS P/N, MF-6206)
Temperature: 50°C
Mobile Phase: 36% 0.1 M potassium phosphate (pH 7.0) with 200 mg/L Na_2EDTA, 22% 0.1 M sodium acetate (pH 4.0) with 200 mg/L Na_2EDTA, 42% acetonitrile (v/v). Flow rate was 1.6 mL/min.
Sample volume: 20 µL of derivatized sample

Experimental

In this study 1 g of rat brain was homogenized in 5 mL of 0.1 M $HClO_4$ and clarified by centrifugation and filtration. This supernatant was diluted 1:10 with 0.1 M $HClO_4$ prior to derivatization.

Reagent mix (Working Reagent, see above) was prepared 24 hours before use (let stand at R.T): 5 mM OPA/5 mM t-butylthiol in 10% 1 M carbonate buffer (pH 9.6), 50% methanol. Derivatization of GABA was performed by mixing sample and reagent (1:2) at room temperature for 10 min. A 20 µL aliquot of the derivatized sample was injected into the chromatography system (F4). Using an external standard, the concentration of GABA in the original brain tissue was calculated to be 2.9 µmoles/g wet weight.

Related References

1. L.A. Allison, G.S. Mayer, and R.E. Shoup, Anal. Chem., 56(1984) 1089-1096.

2. W. Jacobs, Current Separations, 7(1986) No. 2, 39 (BAS Press).

3. D. Pallister, Current Separations, 8(1987) No. 3/4, 53 (BAS Press).

(Figure 4 on back side)

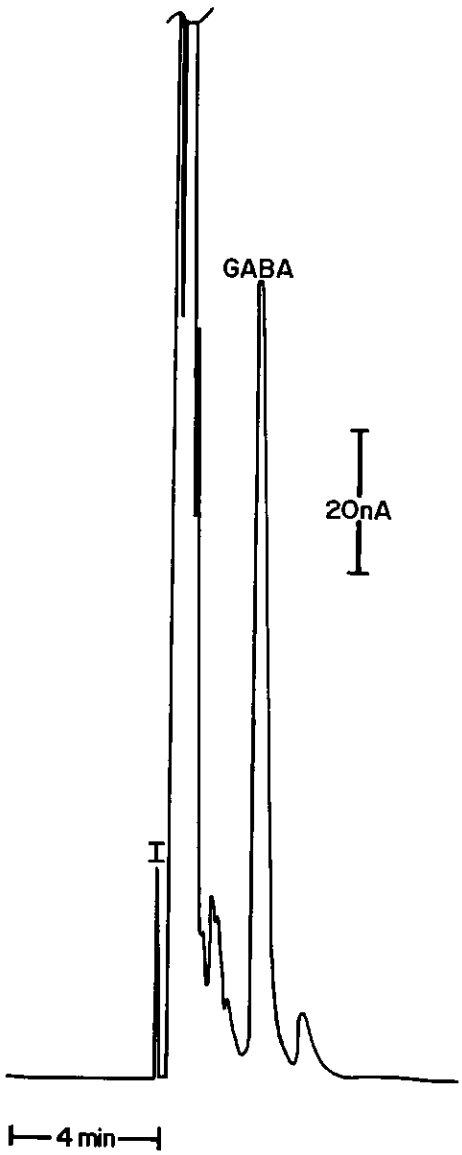

Figure 4. Chromatogram of OPA/TBT derivative of an aliquot of brain homogenate.