Determination of GABA in microdialysis samples by microbore HPLC and fluorescence detection

APPLICATION NOTE

▌ Why monitor GABA by microdialysis?

γ-aminobutyric acid (GABA) is one of the most thoroughly documented neurotransmitters in the CNS, being used by as many as 40% of all neurones. It is located predominantly in the cortex, basal ganglia, hippocampus, hypothalamus, amygdala, cerebellum, medulla and spinal cord.

Microdialysis permits monitoring of extracellular GABA levels as a reflection of GABA release and uptake under various pharmacological or physiological stimuli. It was reported previously that at least 50% of GABA overflow sampled by microdialysis is TTX and calcium sensitive (see Appl. Note 3 and refs. 1,2). This suggests that this portion of GABA is of vesicular origin.

## ▌ Why use microbore HPLC and fluorescence detection?

Determination of GABA in microdialysis samples requires an ultrasensitive analytical method, since the basal GABA levels in a typical microdialysis sample are often in the range of 0.1-0.5 pmol (5-50 nmol/l) . Previously, we developed an isocratic HPLC method based on electrochemical detection of GABA derivatized with OPA/t-butylthiol (3). The limit of detection was 50 fmol for GABA with a retention time of 3.5-4 minutes. However, several other peaks eluted after the GABA derivative. These corresponded to the excess of thiol from the derivatizing reagent, ammonia, ethanolamines and other unidentified compounds. Using an autosampler, the analysis time could be optimized by overlapping chromatograms leaving only the GABA peak clearly separated.

The main drawback of electrochemical detection is the shortened life-time of the electrode, most probably due to a high concentration of acetonitrile (50%) or electroactive impurities present in the mobile phase. However, the whole system is very sensitive and susceptible to errors, contamination and often unexplainable artifacts. In addition, the considerable odour of the t-butylthiol reagent can cause some practical problems with the location of HPLC apparatus, storage and handling of the reagent etc. Thus, success with this method requires skill and experience with liquid chromatography and electrochemistry. The result is that a substantial portion of "research time" is spent on system maintenance (Dr. W. O'Connor, Karolinska Institute, Stockholm, personal communication).

Using fluorescence detection of OPA/mercapto-ethanol (MCE) derivatized amino acids (see also Appl. Notes #16-18), the detector can be left unattended for at least 1500-2000 hours (half-life of the lamp). However, the limit of detection for GABA is only 0.5-1 pmol/sample for a typical chromatographic separation on a normal bore reversed-phase column with gradient elution (Fig. 1). To achieve GABA detection levels of 50-100 fmol, it is thus necessary to "scale down" the chromatographic system by using microbore columns and to reduce the retention time of the GABA peak by optimizing the mobile phase. The system can be further simplified by using isocratic separation with a step gradient, as described for the Asp/Glu method.

Fig. 1.
Chromatogram of a typical rat striatal microdialysis sample. OPA/MCE derivatized amino acids were separated on a 250 x 4 mm column packed with Nucleosil C18, 5 μm, using gradient elution at a flow rate 1 ml/min. Detection limit for GABA is about 1 pmol. Long retention of GABA causes large dilution by the mobile phase and gives enough time for partial degradation of GABA-OPA fluorophore.
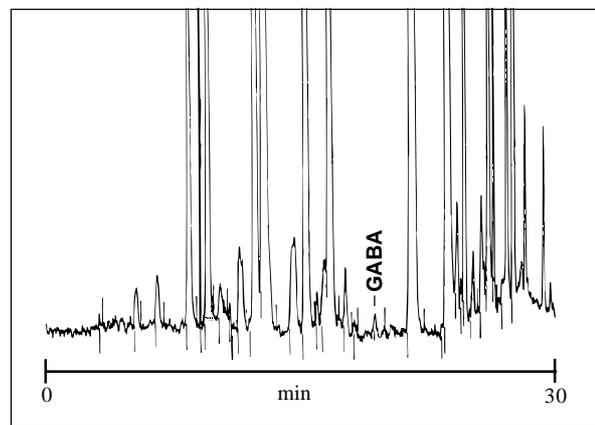
▌ How to separate GABA in standards and in microdialysis samples?

The following examples illustrate the separation of GABA in standards and in microdialysates from the rat hippocampus. Automated derivatizations were made using a CMA/200 Refrigerated Microsampler and OPA derivatives were detected using a CMA/280 Fluorescence Detector.

Chromatographic conditions: 100x1 mm microbore column packed with 3 μm ODS silica particles. Mobile phase: 0.1 M sodium acetate, pH 5.4 (adjusted with phosphoric acid), 20% acetonitrile, flow rate 70 μl/min. Normally, 10 μl sample is derivatized with 1 μl OPA/MCE reagent and 10 μl are injected on to the column.
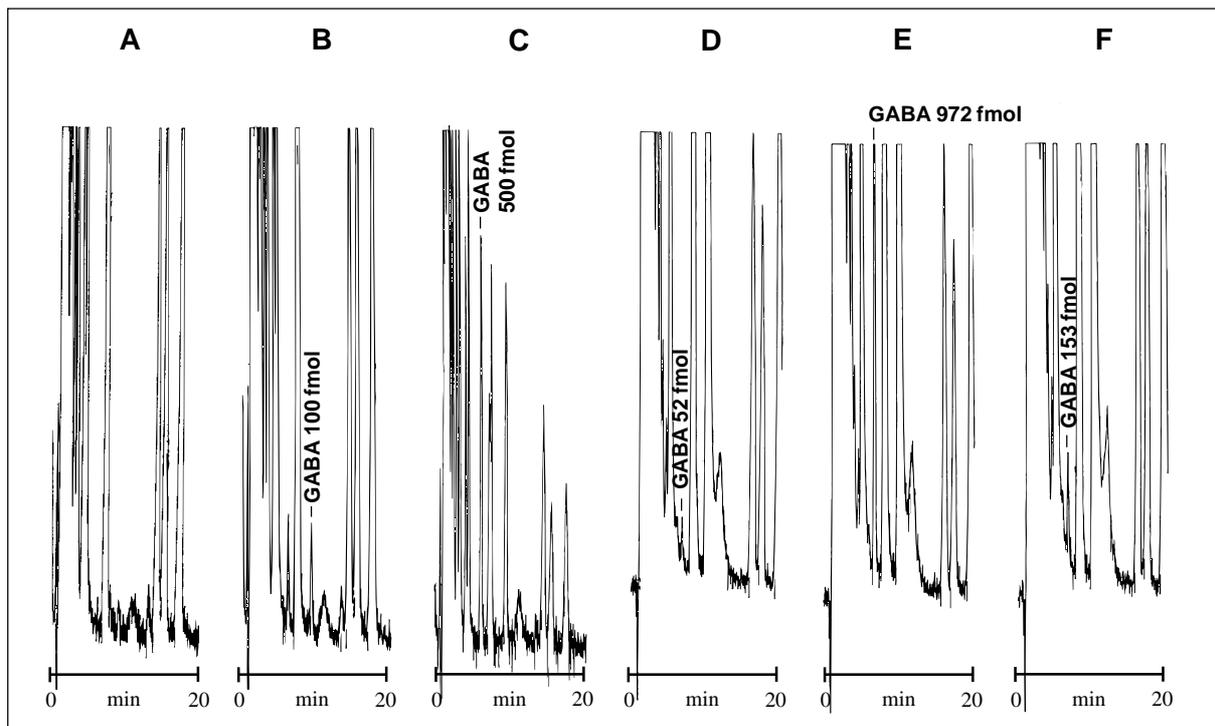

Fig. 2.
Chromatograms showing isocratic separation of GABA in standard mixtures and some typical microdialysis samples.

A) 10 μl standard mixture of physiological amino acids (Sigma AN) which does not contain GABA. Each sample diluted to 2.5 x 10^{-7} M.

B) 10 μl standard as above but spiked with GABA at a final concentration of 1 x 10^{-8} M.

C) 10 μl standard mixture of 32 physiological amino acids and related compounds (Sigma ANB) including GABA at a concentration of 5 x 10^{-8} M.

D) Microdialysis sample from rat hippocampus. Basal GABA level was 52 fmol/10 μl. CMA/12 Microdialysis Probe with 2 mm membrane was perfused with Ringer at 2 μl/min.

E) Potassium (100 mM K^+) stimulated release of GABA in the rat hippocampus. Estimated GABA concentration was 972 fmol/10 μl.

F) The effect of 100 mM K^+ stimulation in the presence of 12 mM Mg^{2+} and no Ca^{2+} on GABA release. GABA levels were suppressed by 90% compared to Fig.2 E. This may illustrate the calcium dependent portion of GABA release in this nucleus.

How can a step gradient speed up GABA analysis?

Previous chromatograms show GABA determination under isocratic conditions. The analysis time is rather long, 25-30 min, because of some late eluting peaks. The method can be further improved by installing an External Low Pressure switching Valve on the pump inlet. This allows faster flushing of unwanted peaks and reduces the analysis time down to about 16-18 min while still only using one isocratic pump.

Another possible complication could be a large matrix of other amino acids in some microdialysis samples. These amino acids are present at concentrations several times greater than that of GABA and thus cause column overload. To circumvent this problem, the mobile phase composition was changed in order to prolong retention of GABA and to facilitate proper separation from other peaks.

Chromatographic conditions were the same as above except:

Mobile phase A: 0.1 M sodium acetate, pH 5.4 (adjusted with phosphoric acid), 12% ACN, 2.5% 1-propanol.

Mobile phase B: 95% ACN, 2.5% 1-propanol, 2.5% water.

Flow rate: 70 µl/min.

The External Low Pressure Valve was programmed to switch to B after 11 min, and then switch back to A after 60 seconds. The column was then reequilibrated before the next injection (4-6 min).
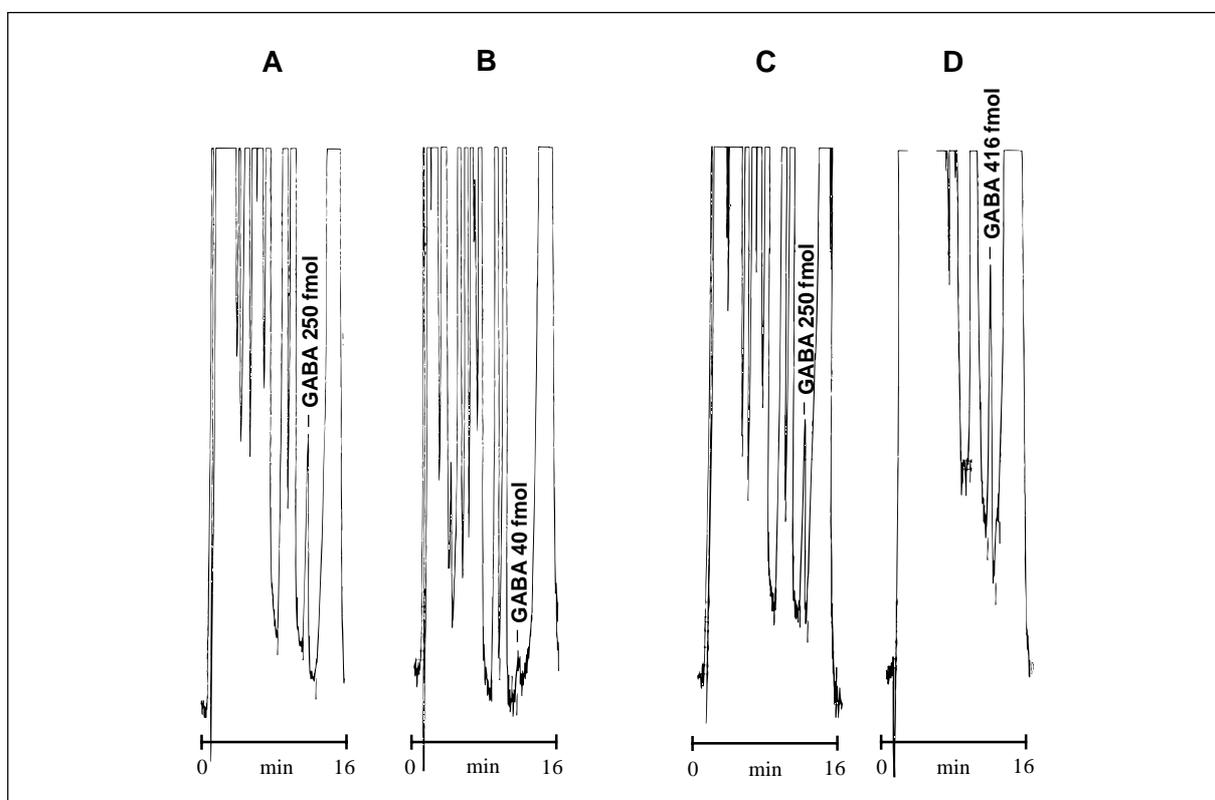

Fig. 3.

A) 20 µl amino acid standard mixture (Sigma AN) at concentrations of 5×10^{-7} M each spiked with GABA at final concentration of 25×10^{-9} M was derivatized with 2 µl OPA/MCE reagent (Sigma complete) for 1 min. Injected volume was 19 µl.

B) The same as (A) except that GABA concentration was reduced to 2×10^{-9} M.

C) 10 µl standard as (A) + 1 µl OPA/MCE. 10 µl injected.

D) 10 µl microdialysis sample from striatum of a conscious rat. A CMA/12 Microdialysis Probe with 2 mm membrane was perfused with Ringer solution at 2 µl/min.

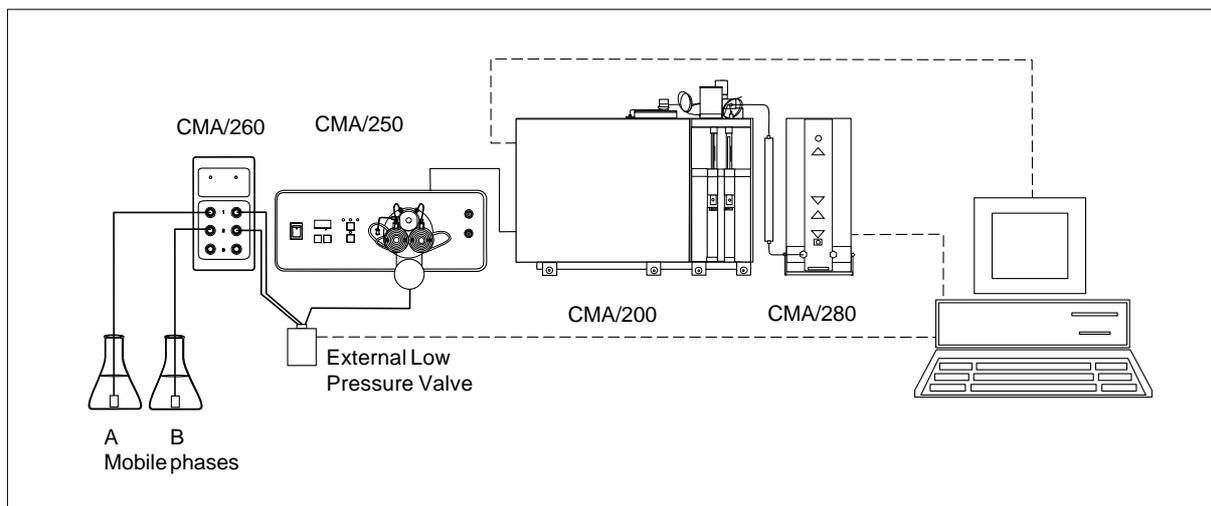## What are the requirements for a complete GABA system?

Fig. 4.
A complete system for the ultrasensitive detection of GABA in microdialysis or other biological samples consists of the following instruments:

1. CMA/260 Degasser
2. CMA/250 LC Pump
3. External Low Pressure Valve
4. CMA/200 Refrigerated Microsampler

5.CMA/280 Fluorescence Detector
6. Microbore column (recommended by CMA)
7. Integrator or data acquisition system (recommended by CMA).

What has been published on GABA determination by Microdialysis/HPLC?

1. Osborne P.G., O´Connor W.T., Drew K.L., and Ungerstedt U. (1990) An in vivo microdialysis characterisation of extracellular dopamine and GABA in dorsolateral striatum of awake freely moving and halothane anaesthetized rats. J. Neurosci. Meth. 34, 99-105.
2. Osborne P.G., O´Connor W.T., Kehr J., and Ungerstedt U. (1991) In vivo characterisation of extracellular dopamine, GABA and acetylcholine from the dorsolateral striatum of awake freely moving rats by chronic microdialysis. J. Neurosci. Meth. 37, 93-102.
3. Kehr J., and Ungerstedt U. (1988) Fast HPLC estimation of γ-aminobutyric acid in microdialysis perfusates: effect of nipecotic and 3-mercaptopropionic acids. J. Neurochem. 51, 1308-1310.

If you require further details on Microdialysis procedures, HPLC analysis, instrumentation or bibliography, please do not hesitate to contact:

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