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Monitoring γ-aminobutyric acid in human brain and plasma microdialysates using micellar electrokinetic chromatography and laser-induced fluorescence detection

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Abstract

Due to its low electrophoretic mobility, few studies have been able to measure gamma aminobutyric acid (GABA) in biological samples by means of capillary zone electrophoresis. Nevertheless, in micellar electrokinetic chromatography (MEKC) by adding a surfactant to the mobile phase separation can be carried out on the basis of the partition coefficient of the molecules rather than their electrophoretic mobility. In the present study microdialysis coupled to MEKC with laser induced fluorescence detection was used to successfully monitor GABA from cerebrospinal fluid and plasma dialysates. Moreover, we monitored changes in extracellular GABA from a human brain. Microdialysis samples were collected from a Parkinson's disease patient undergoing a thallamotomy as part of her treatment. Significant decreases in extracellular GABA were detected during high frequency electrical stimulation and following a thermolesion of the thalamus. These results demonstrate the feasibility of MEKC coupled to laser-induced fluorescence detection in resolving neutral amino acids, specifically GABA, from different human body fluids.

Keywords: γ-Aminobutyric acid

1. Introduction

Capillary zone electrophoresis (CZE) is an analytical technique which offers high resolution (usually in the hundred-thousand theoretical plates) and requires very small sample volumes (picoliter to nanoliter volumes) [1,2]. By applying a high voltage through a narrow bore capillary, substances are separated by their electrophoretic mobility. However, neutral molecules are not separated at all. Terabe and collaborators overcame this limitation by adding a surfactant to the mobile phase to effectively separate neutral compounds on the basis of their partition coefficient [3,4]. This method is called micellar electrokinetic chromatography (MEKC).

Most of the reports to this date, using cerebral spinal fluid (CSF) or microdialysates combined with CZE, have measured charged amino acids like glutamate and aspartate [2,5–11]. Few have monitored neutral amino acids, in part because of the

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above mentioned difficulties of CZE in separating them. One of the amino acids which coelutes with neutral ones is gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the mammalian brain. GABA, a zwitterion, comigrates with other neutral amino acids found in the central nervous system such as: threonine [12], valine [8,13], glutamine [9] and tyrosine [13]. Separation of these five amino acids is necessary to monitor them in a complex biological sample.

A previous methodological report demonstrates the detection of glutamine, valine and GABA from the periaqueductal gray matter using brain microdialysis and CZE detection [8]. In the present report we used MEKC and laser-induced fluorescence detection (LIFD) to achieve a better separation of different neutral amino acids including GABA. Then we showed the feasibility of this technique by measuring GABA in cerebrospinal fluid or plasma dialysate of human patients. Finally, a GABA neural circuit was studied by brain microdialysis-MEKC-LIFD in a human patient undergoing thallamotomy as part of her treatment for Parkinson's disease.

2. Experimental

2.1. Capillary electrophoresis with a laser-induced fluorescence detection instrument

A Meridialysis® CZE system (Model R202, Mérida, Venezuela) was used in these experiments. It is equipped with an argon laser (Ion Laser Technology, Salt Lake City, UT, USA) tuned to 488 nm. A fiber optic conducted the laser beam onto a dichroic mirror centered at 510 nm (Omega optics). The laser is focused on the window of a capillary (Polymicro Technologies, Phoenix, AZ, USA) by means of a 0.75 NA objective. The window was made by burning out the polyimide coating at 38 cm from the anodic end of a 50 cm long, 27 μm ID×346 μm OD, fused-silica capillary. The fluorescence was collected through the objective and focused on a R120-05 multialkali photomultiplier tube (PMT) (Hamamatsu, Bridgewater, NJ, USA). A high pass filter and a notch filter centered at 520 and 488 nm (Andover, Salem, NH,USA) respectively, attenuated stray radiation.

2.2. Capillary electrophoresis

A borate buffer (23 m*M*) with sodium dodecyl sulfate (SDS, 120 m*M*) and methanol (1%) was found to be the best running buffer. The sample was loaded at the anodic end by applying a negative pressure (19 p.s.i. for 1 s) at the cathodic end of the capillary. Electrophoretic separation was obtained by applying for 12 min 26 kV between the anode and the cathode. Between runs the capillary was rinsed with 0.1 *M* NaOH for 1 min followed by water for 1 min and running buffer for 4 min.

2.3. Sample and standard derivatization

Optimal FITC/GABA relation for maximal derivatization of GABA was calculated by mixing 1 ml of a 10^{-5} *M* GABA dissolved in artificial cerebrospinal fluid with increasing volumes of a mixture of 6.4×10^{-3} *M* solution of FITC in acetone and 20 m*M* carbonate buffer at pH 9.4 in a 1:1 (v/v) ratio (FITC–carbonate mixture). The final solutions had 1, 5, 25, 50, 75, 125, 250, 500, 750 and 1000 molecules of FITC in excess, respectively for each GABA molecule.

A calibration curve for GABA analysis was also prepared by derivatizing increasing concentrations of the standard (5×10^{-9} to 5×10^{-7} *M*) while keeping the FITC concentration constant (10^{-3} *M*).

Cerebrospinal fluid (CSF) and dialysates (500 nl) from human patients, and standards were derivatized by adding 500 nl of the FITC–carbonate buffer mixture. A syringe loaded with a FITC–carbonate mixture was placed on a precision pump and the flow-rate was set at 1 μl/min. By turning the pump on for 30 s we delivered 500 nl of the reaction mixture into the tube containing the sample. The samples reacted overnight (14 h) at room temperature in a water-saturated chamber to minimize evaporation [6].

2.4. Subjects and samples

2.4.1. Plasma microdialysates

In patients a removable dialysis probe, a flexible version of the brain microdialysis probe, was used. The details of the probe and the procedure have been

previously described [14–16]. Briefly, the active area of the removable probe was made of a 20 mm long cellulose hollow fiber, 220 μm o.d. and 13,000 molecular weight cut-off (Spectrum-Medical Industries, Los Angeles, CA, USA). The probes and connectors were sterilized with ethylene oxide at least 24 h before use. During the dialysis session the probe was perfused with sterile 0.9% NaCl solution at 1 μl/min. A guiding catheter was introduced into an arm's vein and the dialysis probe was inserted through the catheter. The probe was fixed in place and 90 min after probe insertion samples were collected every 30 min (these samples were also used to monitor serotonin by means of HPLC).

2.4.2. Cerebrospinal fluid

CSF was obtained, at the local hospital, from children who underwent a spinal tap as a diagnostic procedure to discard meningitis. A parental consent form was signed before performing the procedure.

2.4.3. Patient material and surgery

Brain dialysates were obtained from a female patient, age 54, undergoing a thalamotomy. This is a routine neurosurgical procedure used in the treatment of Parkinson's disease, and is intended to relieve tremor in those patients with little or no relieve with pharmacological treatment. In this procedure an electrical stimulating electrode is stereotaxically driven into the thalamus to localize and lesion the ventral intermediate nucleus (Vim).

A Leksell stereotaxic system was fixed to the skull of the patient under local anesthesia (lidocaine 5 mg/ml). The position of the electrode was checked by CAT scan. The patient remained awake throughout the surgery.

2.4.4. Brain microdialysis procedure

The microdialysis procedure used in this protocol was approved by the University's ethical committee prior experimentation and with the signed consent of the patient.

A thorough description of the microdialysis probe has been previously reported [17,18]. Briefly, the probe consists of fused-silica capillary and 26 g stainless steel tube with a tip of exposed cellulose membrane 1.5 cm long.

The microdialysis probe was placed beside the stimulation electrode and stereotaxically implanted into the thalamus 20 min before sampling started. Samples were taken every fifteen minutes for a 45 min control period followed by two samples during intermittent high frequency (>50 Hz) electrical stimulation. Another sample was collected immedi-

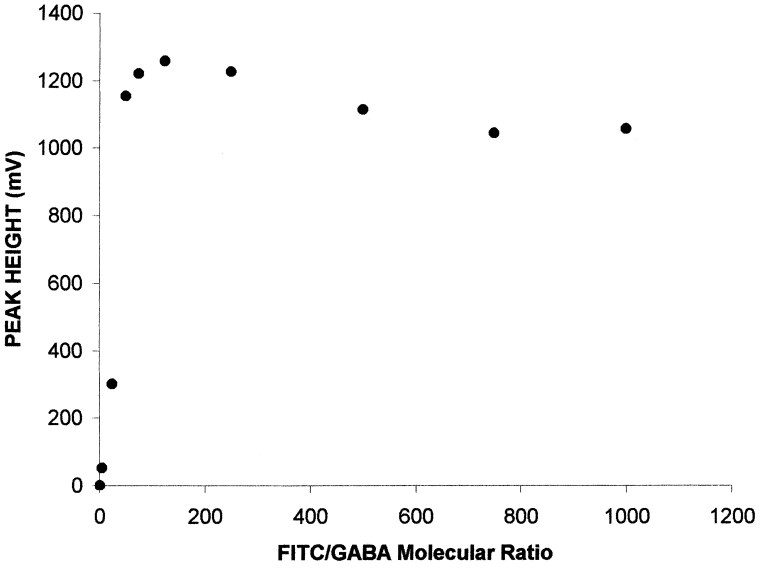

Fig. 1. A 75-fold excess of FITC was sufficient to maximally label a 10^{-5} *M* GABA standard.

ately after stimulation and four samples after the thermolesion of the thalamus. The probe was perfused with a sterile artificial cerebrospinal fluid (135 m*M* NaCl, 3.7 m*M* KCl, 1.0 m*M* Mg Cl_2, 1.2 m*M* $CaCl_2$ and 10 m*M* $NaHCO_3$, pH 7.4) and the perfusion flow-rate was set at 1 μl/min.

3. Results

Different concentrations of borate, SDS and methanol were tested and it was found that 23 m*M* Borate, 120 m*M* SDS and 1% methanol offered the best resolution (data not shown).

3.1. Optimal FITC/GABA relation for derivatization

Fig. 1 shows that a 75-fold excess of FITC was sufficient to maximally label a 10^{-5} *M* GABA standard. At the fifth point, which represents a 75-fold excess of FITC, the curve reaches an asymptote. Assuming that the concentration of GABA in the sample is nanomolar, then the same amount of FITC used in the standards corresponds to 500 000 fold excess in the sample. This suggests that the amount of FITC that was used in the present experiments will label GABA in plasma or brain dialysates despite the presence of other primary amine containing compounds, as previously shown [9,19].

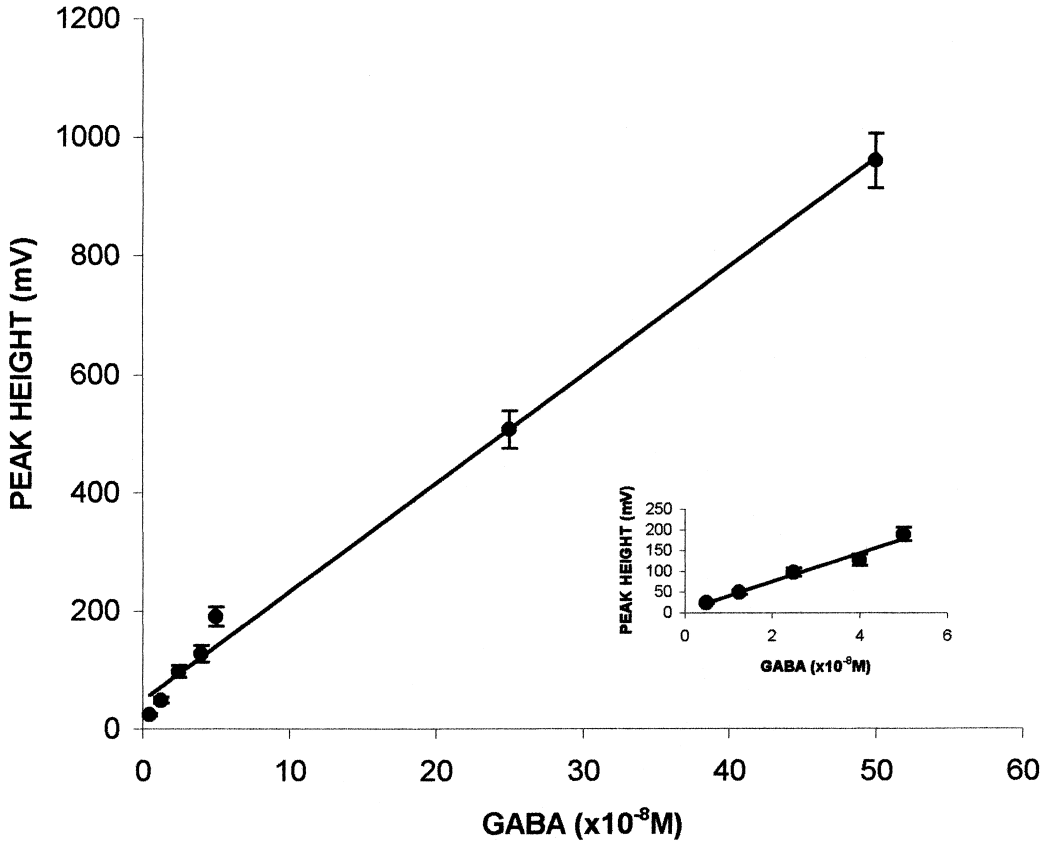

Fig. 2. Detection of GABA standards was linear in the range between 5×10^{-7} *M* to 5×10^{-9} *M*. The inset shows the linearity of the method in the 5×10^{-8} *M* to 5×10^{-9} *M* range.

3.2. Calibration curve

Detection of different concentrations of GABA standards was linear in the range between 5×10^{-7} to 5×10^{-9} *M* (y=18.36x+48.42; r=0.997; Fig. 2). However, two different slopes were observed in the calibration curve. One for higher concentration (5×10^{-8} to 5×10^{-7} *M*) and another one for low concentrations (5×10^{-9} to 5×10^{-8} *M*) as shown in the inset of Fig. 2. Each data point corresponds to the mean of three replicates ± the standard error of the mean. Higher concentrations than 10^{-7} *M* were not tested because previous works have found, using brain microdialysis, extracellular GABA levels in the 10^{-8} *M* range [20]. Fig. 3 shows the electropherogram of a GABA standard (2.5×10^{-6} *M*) and a blank prepared with the same characteristics as the standard.

3.3. Separation of GABA from four other neutral amino acids

Fig. 4 shows that the method efficiently resolves all five neutral amino acids (including GABA) in a 10^{-6} *M* standard mixture. Each amino acid is identified by adding an excess of FITC-amino acid to

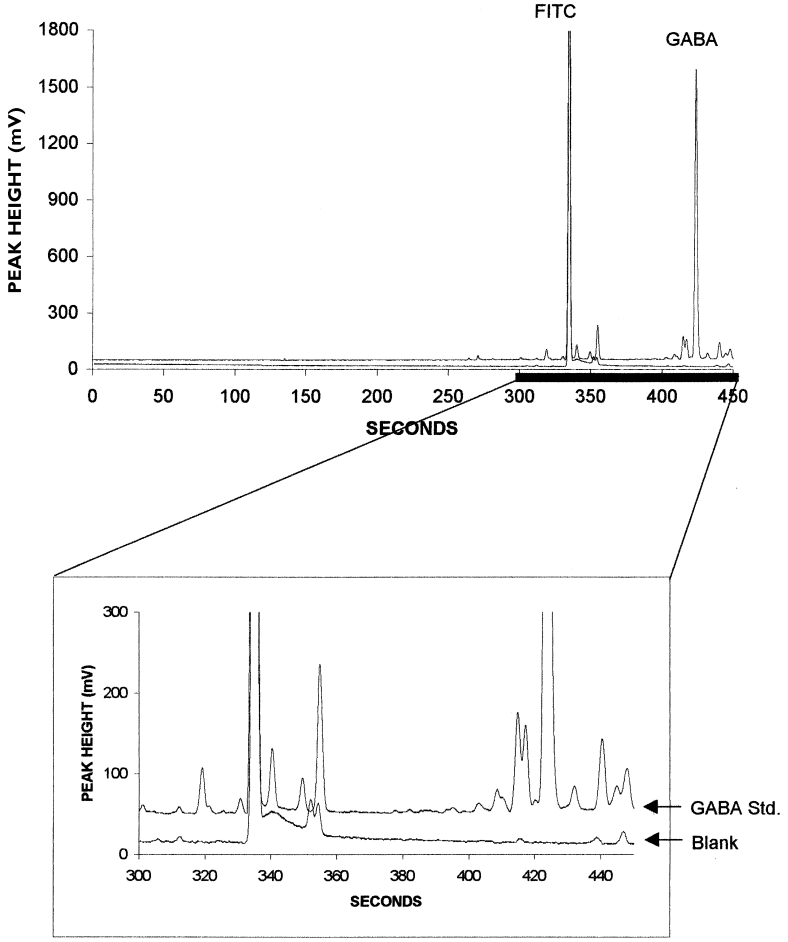

Fig. 3. Electropherograms of a GABA standard (2.5×10^{-6} *M*) and a blank prepared with the same solutions and concentrations of FITC.

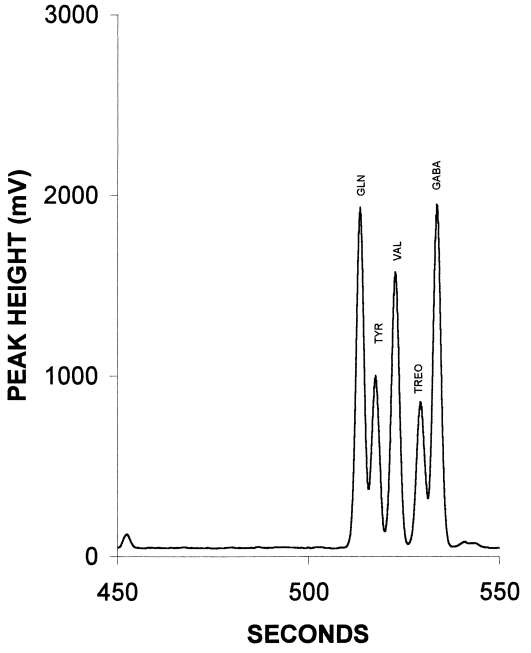

Fig. 4. This electropherogram shows a successful separation of five neutral amino acids (10^{-6} *M*): glutamine (GLN), tyrosine (TYR), valine (VAL), threonine (TREO) and GABA.

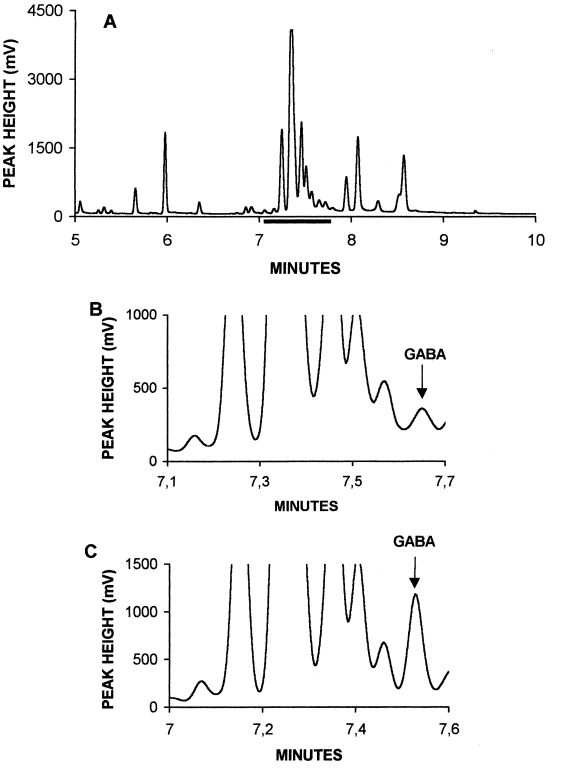

Fig. 5. (A). This portion of the figure shows a complete electropherogram of a plasma dialysate. (B). Magnification of the area between 450 and 550 s of the original electropherogram. (C). Spiking of the plasma sample with FTC-GABA. Notice the enlargement of the FTC-GABA peak.

the standard mixture (spiking of the standard mixture).

3.4. Analysis of GABA from plasma dialysates

GABA was well resolved from other neutral amino acids in a plasma dialysate (Fig. 5A and B). The GABA peak was identified by spiking the sample with an excess of FTC-GABA (Fig. 5C). The concentration of GABA corresponded to 1.2×10^{-8} *M*.

3.5. Analysis of GABA in human CSF

Fig. 6A shows that this method was capable of separating GABA from other neutral amino acids in human CSF. The concentration of GABA in the sample corresponded to 7×10^{-9} *M*. A spiking of the sample with FTC-GABA and FTC-threonine standards permitted the identification of the peaks (Fig. 6B).

3.6. Extracellular GABA levels from human brain dialysates

GABA was well separated from other neutral amino acids in dialysates from human thalamus. Fig. 7(A and B) is an electropherogram showing basal levels of GABA. A spiked sample confirms that the peak does correspond to GABA (Fig. 7C). Extracellular concentrations of GABA decreased during the intermittent high-frequency electrical stimulation and also after the thalamic lesion (Fig. 8). Mean GABA concentrations of basal samples was $5.7\pm0.2\times10^{-8}$ *M*.

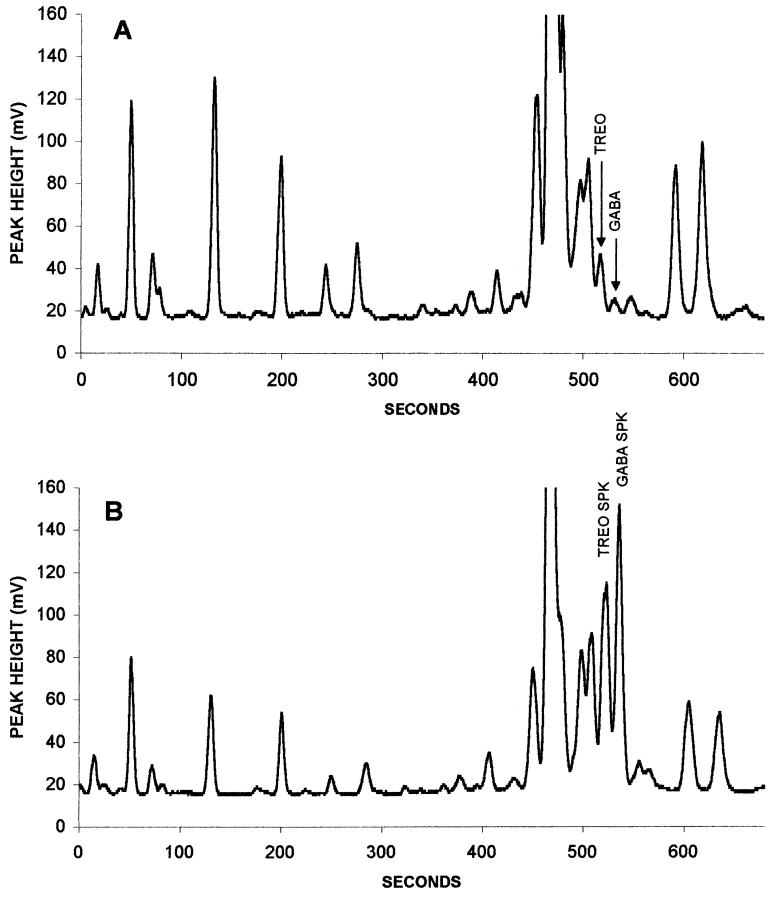

Fig. 6. Electropherogram of a CSF sample (A) and spiking of the same sample with FTC-GABA and FTC-treonine (B).

4. Discussion

The present report shows the feasibility of measuring GABA in human brain and plasma dialysates as well as in cerebrospinal fluid by means of MEKC-LIFD. This was accomplished by using a high concentration of sodium dodecyl sulfate (SDS) and a 27 μm bore capillaries. In a recent report Berquist et al. used 30 m*M* SDS to measure GABA in periaqueductal gray matter dialysates in rats and found good resolution for glutamine, valine and GABA [8]. However, in our hands, when the same procedure was used to monitor GABA in the human thalamus, threonine as well as tyrosine comigrated with GABA. This discrepancy could be due, in part, to different derivitizing agents used, to species difference and to different brain sites studied. For example, the extracellular concentrations of threonine and tyrosine could have been too low to alter GABA, glutamine and valine in rats. In the present report, by increasing the SDS concentration to 120 m*M*, a better resolution of GABA was achieved in human dialysates and CSF samples.

Detection of analytes using CZE or MEKC-LIFD requires a prior derivatization process. In small volume samples (CSF or dialysates) the actual amount of the analytes is also small, so extra-care has to be taken to assure that all the molecules are derivitized. In our experiments we have used a 500 000-fold excess of FITC in the sample to make

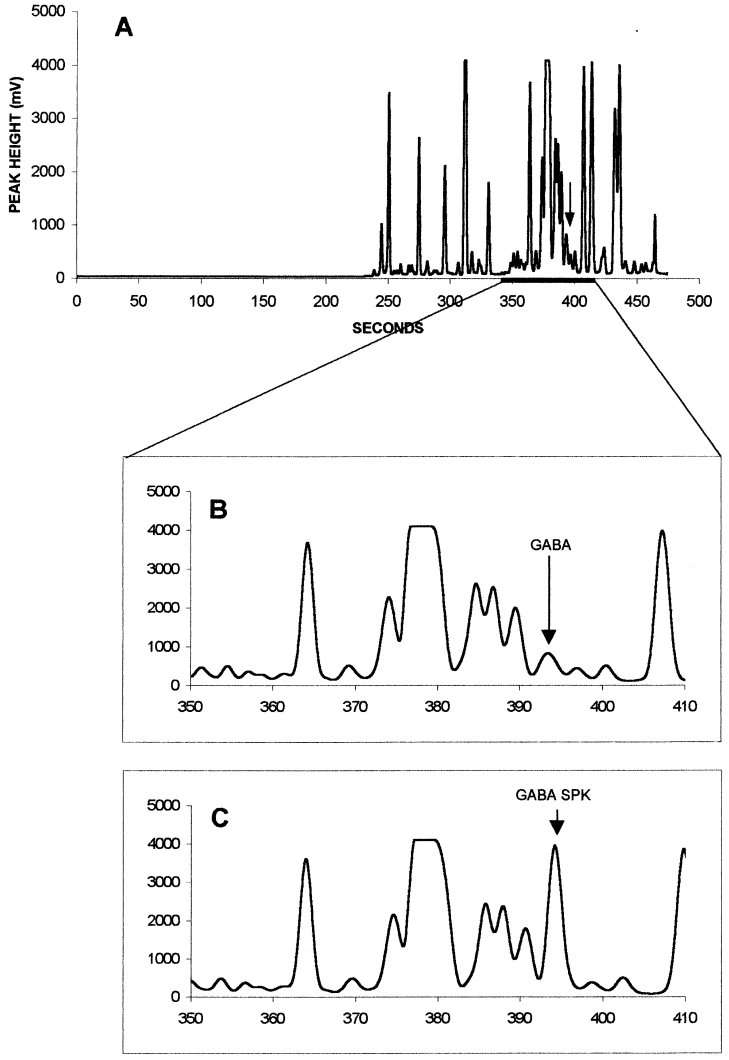

Fig. 7. (A). Electropherogram of a thalamic microdialysate from a human patient. The arrow shows the FTC-GABA peak. Portions (B) and (C) of the graph show a magnification of the electropherogram. In (C) a spiking of the sample with FTC-GABA was done.

certain that most, if not all, of the analytes were derivitized. By using this excess of FITC we obtained a limit of concentration of 5×10^{-9} *M* for GABA, which is similar to the detection limits that have been found for other amino acids when FITC was used as derivitizing agent [1,19,21,22].

Most MEKC studies to this date have used 50 μm I.D. or larger diameter capillaries [4]. In the present report a 27 μm I.D. capillary was reliably used. A small diameter capillary diminishes band broadening and improves resolution because the low Joule heating effect. In addition smaller internal diameter capillaries dissipate heat better due to their larger surface/volume ratio also diminishing band broadening.

GABA, the main inhibitory neurotransmitter, is an amino acid also found in plasma and CSF. Low levels of GABA have been recently measured in the CSF, using HPLC–EC, in patients with Alzheimer's disease [23]. Similarly, other researchers have shown that low levels of GABA in plasma seem to be correlated with mood disorders [24,25]. Few reports,

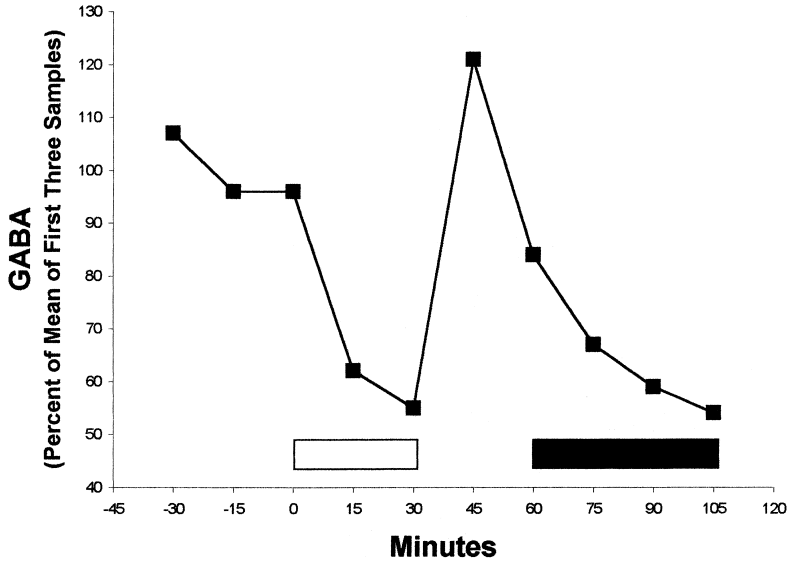

Fig. 8. The relative concentrations of GABA, in a human thalamic microdialysate, significantly decreased during intermittent electrical stimulation (open bar) and following the thermolesion (closed bar).

however, have measured GABA in CSF or plasma using MEKC-LIFD [26]. Our results confirm previous work showing that MEKC-LIFD is capable of detecting this amino acid from complex biological samples.

Most microdialysis studies of the human brain have focused on measuring neurotransmitters and metabolites in traumatic brain injuries, subarachnoid hemorrhage, epilepsy and brain ischemia [27–30]. There is only one report on the use of brain microdialysis in Parkinson's disease in which basal levels of GABA were monitored from the thalamus, specifically the Vim, of four parkinsonian patients using HPLC–EC [31]. In the present report we found a significant decrease in extracellular GABA in the thalamus both during high-frequency electrical stimulation and following a thermolesion of the thalamus.

In conclusion, these experiments show a different approach, using MECK coupled to LIF detection, to monitor neutral amino acids from human dialysates (brain and plasma) and CSF. A better peak and time resolution was achieved by increasing the concentration of SDS and by decreasing the diameter of the capillary. It was also shown that this method is capable of determining changes in extracellular levels of GABA during intermittent electrical stimulation and after a thermolesion of the thalamus in a human patient.

Acknowledgements

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