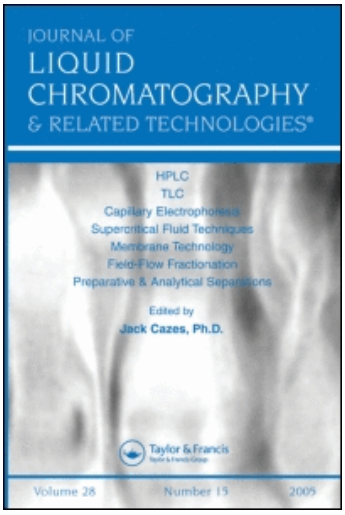

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In Vivo Monitoring of Brain Glutamate by Microdialysis Coupled to Capillary Electrophoresis and Laser Induced Fluorescence Detection

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IN VIVO MONITORING OF BRAIN GLUTAMATE BY MICRODIALYSIS COUPLED TO CAPILLARY ELECTROPHORESIS AND LASER INDUCED FLUORESCENCE DETECTION

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ABSTRACT

A method to measure glutamate in brain dialysates was developed. Nanomolar concentration of glutamate labeled with naphthalene-2,3-dicarboxaldehyde (NDA) was measured by using capillary electrophoresis and laser-induced fluorescence detection. The limit of mass detection was in the zeptomole concentration (10^{-21} M) range. The technique was linear between 10^{-5} M to 10^{-8} M.

It was possible to reduce the migration time of brain dialysate glutamate to 60 seconds when capillaries of 15 μm inside diameter were used. The potential of capillary electrophoresis coupled to laser-induced fluorescence detection for **in vivo** monitoring of neurotransmitters is discussed.

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INTRODUCTION

Glutamate is the most conspicuous and ubiquitous excitatory neurotransmitter in the brain (1). It is released and uptaken by specialized neurons and during these processes glutamate participates in fast behavioral actions such as learning and memory, and brain damage (2). Due to its widespread functions, **in vivo** monitoring techniques such as brain microdialysis have been developed, among other goals, to study glutamate actions (3-5). In brain microdialysis, a minute semipermeable membrane is inserted into the brain of an anesthetized or a freely moving rat. Extracellular glutamate diffuses into the probe and is collected for biochemical analysis.

The analytical methods available so far and, utilized in conjunction with brain microdialysis, are high-performance liquid chromatography (HPLC) coupled to electrochemical (EC) and HPLC coupled to fluorimetric detection (FD). With HPLC-EC or HPLC-FD, nanomolar concentrations of glutamate can be detected. Thus, with the availability of these methods, it has been possible to establish that the basal concentration of glutamate present in aliquots of microliter samples of brain dialysates, ranges on the micromolar level (5,6). It has also been shown that drastic manipulations such as occlusion of the brain blood vessels causes a massive release of glutamate (7,8). This excess of glutamate causes neuronal death.

In physiological conditions, however, glutamate is a fast acting neurotransmitter released in a fraction of a second and it is sensitive to Ca^{++} depletion and tetrodoxin (TTX), a fish poison that suppresses nervous activity. By contrast, the basal release of glutamate observed in brain dialysates is insensitive to Ca^{++} depletion or to TTX (Rada, P., personal communication) suggesting that basal glutamate might not be of neuronal origin. Despite these facts, most of the **in vivo** studies of glutamate have been carried out in dialysis samples collected for several minutes.

Several factors contribute to this incongruity. The technique for the chemical analysis of the dialysates is probably the most captious element. The mass sensitivity of HPLC-EC or HPLC-FD is at the low picomole range. This limit of mass detection proves to be inadequate for fast analysis. Assuming a micromolar concentration of glutamate in the dialysate, 1 µl of dialysate will contain 1 picomole of glutamate which is not enough to be detected. For this reason, when brain microdialysis is used for **in vivo** monitoring of glutamate, perfusion rates larger than 2 µl/min and collection times larger than five minutes, have to be used to collect enough analyte for the analytical technique.

One way to decrease the sampling time is to increase the perfusion flow-rate. This procedure increases the concentration gradient across the membrane and augments the absolute amount of collected glutamate. But even at high perfusion flow rate (e.g., 5 μl/min), between 1 and 2 min of collection time is required. These conditions turn out to be excessively large for a neurotransmitter that completes its release and uptake cycle in hundredths of a second. Considering that perfusion flow rates above 100 nl/min already deplete the neurotransmitter in the tissue creating non physiological conditions around the probe (9,10). This analysis leads us to the conclusion that faster and more sensitive analytical techniques are required for **in vivo** monitoring of glutamate.

Currently there are two options: biosensors and capillary electrophoresis with laser induced fluorescence detection system. Biosensors are made by adsorbing enzymes that degrade glutamate, on a solid support matrix. The products of the enzymatic reaction are then detected by either fluorescence or by oxidation (11-14). The main disadvantage of this technique is the low sensitivity, usually in the high micromolar range. In addition, for some biosensors, naturally occurring products such as ascorbic acid or carbon dioxide interfere with the enzymatic reactions. At the present time, and despite all the inconveniences, the use of biosensors is the only system that offers the possibility of continuous monitoring glutamate.

Capillary electrophoresis with laser-induced fluorescence detection (CE-LIFD), on the other hand, is a technique offering an excellent mass sensitivity and fast resolution. It has been possible to reach the molecular counting level (e.g., 1 zeptomole = 10^{-21} moles = 600 molecules, and 1 yoctomole = 10^{-24} moles = 0.6 molecules) (15,16). Moreover, when very narrow columns (less than 20 μm bore) are used for capillary electrophoresis the speed of separation is enhanced (17). Resolution of a mixture of aminoacids, including glutamate, can be achieved in less than 70 seconds. In addition, naphthalene-2,3-dicarboxaldehyde reacts with primary amines in the presence of cyanide to produce highly fluorescent 1-cyano-2-substituted-benz[f]isoindole derivatives. These derivatives are also chemiluminescent and electrochemically active, and they are very stable, exhibiting minimal degradation over a 10 hr period (18). The features of CE-LIFD, and the derivatization of analytes with NDA, are then very attractive for measuring glutamate in brain dialysates.

In the present report, we demonstrate that the measuring of zeptomole amounts of glutamate in striatal dialysates with analysis time shorter than 70 seconds by means of CE-LIFD is feasible. The potential of the technique is discussed.

EXPERIMENTAL

Reagents

All chemicals were obtained at the highest purity level available from the manufacturer and were used without additional purification. Sodium hydroxide, sodium cyanide, sodium chloride, potassium chloride, calcium chloride, boric acid, glutamic acid and methanol (HPLC grade) were purchased from Sigma Chemical Co (St. Louis, MO). Naphthalene-2,3-dicarboxaldehyde was obtained from Molecular Probes (Eugene, OR). Reagent solutions and buffers were prepared using triply distilled and deionized water and were routinely degassed and sonicated under vacuum after filtration. Millex disposable filter units (0.22 μm) were purchased from Millipore Corporation (Bedford, MA), and fused-silica capillary columns were obtained from Scientific Glass Engineering (Austin, TX), and Polymicro Technologies (Phoenix, AZ).

Instrumentation

A laboratory-made apparatus was used. This instrument consisted of a capillary electrophoresis section and a laser-induced fluorescence detection system using a collinear or confocal geometry previously described (19). Briefly, the detection system consisted of an epilumination fluorescence microscope in which the UV lamp had been replaced by a Liconix Helium Cadmiun laser of 442 nm wavelength with 10 mW power. This optical line matches the excitation peak of the NDA derivatized primary amines. Inside the epilumination condenser the laser beam (with a large wavelength range) was narrowed to a very small wavelength range by a band pass filter centered at 442 nm and a ± 10 nm bandwidth (Andover Corp., Salem, NH). The purified laser beam was then reflected by a dichroic mirror centered at 460 nm (Carl Zeiss, Oberkochen, Germany) and focused on the capillary column by a 40 X 0.85 NA objective (Carl Zeiss). The emitted radiation was collected by the same objective, refracted by the dichroic mirror, filtered through a notch filter centered at 442 nm with ± 10 nm bandwidth (Andover Corp.) and focused through an eye piece on a photomultiplier tube (PMT). This tube was a 928 multialkali, with a side quartz window and sensitive to radiation in the range of 180 nm to 960 nm (Hamamatsu Co., Bridgewater, NJ). The PMT was operated at 700 V by a model 2005 high-voltage power supply (Bertan, Hicksville, NY). The electrical current generated by the PMT was converted to voltage by a laboratory-made current-to-voltage converter which consisted of a 5013 operational amplifier with field effect transistor at the input. The circuit was offset by means of a 1 MΩ precision potentiometer. The signal was then fil-

tered and recorded on a strip-chart recorder (Linseis, Princeton Junction, NJ).

The CE instrument was equipped with a 30 cm (20 cm to the detector) x 15 μm I.D. capillary column. The grounded electrode (cathodic) terminal was equipped with a T-shaped glass tube which served as the cathodic buffer reservoir. In addition, the T tube allowed the rinsing of the capillary by means of a vacuum pump and the recycling of old buffer for new one. The change of buffer was carried out without disturbing the capillary. On the other hand, the anodic terminal consisted of a semicylinder made of a plastic syringe which supported the reservoir containing the buffer or the sample. The reservoir itself consisted of a 200 μl Eppendorf tip, a convenient system to replace the buffer (and the tip containing the buffer) between runs to prevent contamination. The power supply used for these experiments was a model 2030 Bertan power supply (Bertan, Hicksville, NY).

Methods

Experiment I. The dynamic range of the derivatization procedure was tested according to the following protocol: Derivatization of the standard solutions of glutamate was performed by mixing 1340 μl of reaction buffer mixture (electrophoresis buffer [20 mM KCl, 10 mM boric acid at pH 9.5] mixed with methanol in a 75:25 ratio respectively), 200 μl of 20 mM NDA in methanol, and 100 μl of one of the following glutamate solutions: 6.8×10^{-4} M, 6.8×10^{-5} M, 6.8×10^{-6} M, and 6.8×10^{-7} M (the final concentration of the amino acids were 3.69×10^{-5} M, 3.69×10^{-6} M, 3.69×10^{-7} M, and 3.69×10^{-8} M). After one hour of reaction in the dark, these solutions were filtered and injected electrokinetically (6 kV for 5 sec) into the capillary column filled with electrophoresis buffer. The analytes were separated by applying a voltage of 24 kV. Peak height of the separated analytes were measured and the log of the peak height was plotted against the log of the concentration to evaluate the linearity of the method.

Experiment II. In this experiment, the optimal molar excess to label low concentrations of glutamate was measured. For this purpose, 10 μl of a 3.5×10^{-5} M solution of glutamate was derivatized according to the protocol used in Experiment I except that the concentration of the NDA solution was decreased by a factor of two, starting with a 20×10^{-3} M solution until reaching a 1.9×10^{-5} M solution. As a consequence a total of 11 different concentrations in the indicated range were generated. The excess of moles of NDA was plotted against the peak height. Electrophoresis and detection procedures were the same as in Experiment I.

Experiment III. A guide shaft aimed to the striatum was implanted under Ketalar anesthesia in a rat. The guide shaft was a 10 mm long, 21 gauge stainless steel tube. Two anchors and the guide shaft were fixed on the skull with jeweler screws and dental cement. The coordinates to implant the guide shaft were 8.3 mm anterior to the interaural line, 3 mm lateral to the midsagittal suture and 4 mm ventral to the surface of the skull. After 7 days of recovery, microdialysis was performed. Microdialysis probes were made of concentric fused-silica tubing inside a 26 gauge stainless steel tube with a piece of cellulose tubing attached at the end. The cellulose tubing was 4 mm long, 200 μm diameter, and a molecular weight cut-off of 6000 (see reference 4). A microdialysis probe was connected to a syringe filled with a Ringer solution made of 146 mM NaCl, 3.7 mM KCl, and 1.2 mM $CaCl_2$. The syringe was set on a pump at a flow rate of 0.1 μl/min. The microdialysis probe was inserted into the guide shaft, and the dialysate collected every 10 min and derivatized.

The derivatization procedure was similar to the one used in experiments I and II but the proportion of sample and reactants varied as follows: 1 μl of Ringer solution or dialysate, 35 μl of 10 mM NDA, 35 μl of 10 mM NaCN, and 170 μl of reacting buffer. The mixture was allowed to react for 30 min prior to analysis. For this experiment, the blank and the samples were injected at the anodic end of the capillary by applying 6 kV for 5 sec and separated at 30 kV.

RESULTS

Experiment I. The results of this experiment are summarized in Figure 1. A linear relationship between the log of glutamic acid concentration and the log of the signal between 10^{-5} and 10^{-8} M concentration range was achieved. The best fitting equation was $y = 10.2 + x$. The regression coefficient was $r = 0.99$, and the regression analysis showed that this fitting was statistically significant [$F(1/2) = 256.8$, $p < 0.004$]. The glutamate solution of a concentration of 3.69×10^{-8} M produced a peak with a signal-to-noise ratio of 25:1. Even at the high nanomolar concentration the signal to noise ratio was still larger than 3:1.

Experiment II. A correlation between an increase in the concentration of NDA and an increase in the glutamate-NDA signal was achieved. Figure 2 shows the plotting of NDA excess in moles vs the peak height curve. An exponential curve was obtained reaching a plateau at the low mole excess. The insert represents an expanded

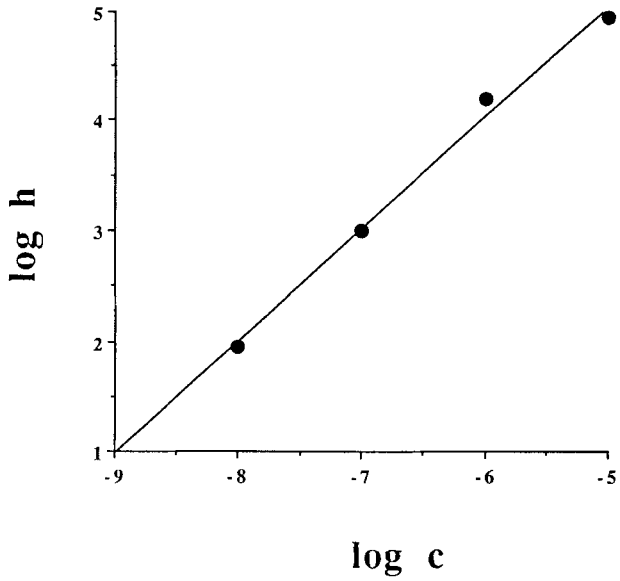

Figure 1. Linear Relationship Between the Logarithm of the Concentration of Glutamate and the Logarithm of the Peak Height of Glutamate: The linearity of the logarithm of the concentration (log c) of glutamate and the logarithm of peak height (log h) of glutamate was calculated and it was found that the correlation was statistically significant (see text for details).

view of the six points of the lowest portion of the curve. It clearly shows that at the fifth point, which represents 176 mole excess, the curve reaches an asymptotic level.

Experiment III. As shown in Figure 3, the derivatized samples showed 4 different peaks as compared with the blank. Peak number 3 corresponds to glutamate-NDA, having a migration time of 65 sec. Three more peaks were observed in the dialysates, two peaks still unidentified and a third one probably corresponding to aspartate.

DISCUSSION

The present report demonstrates that is feasible to derivatize glutamate at the nanomolar concentration level by means of NDA and

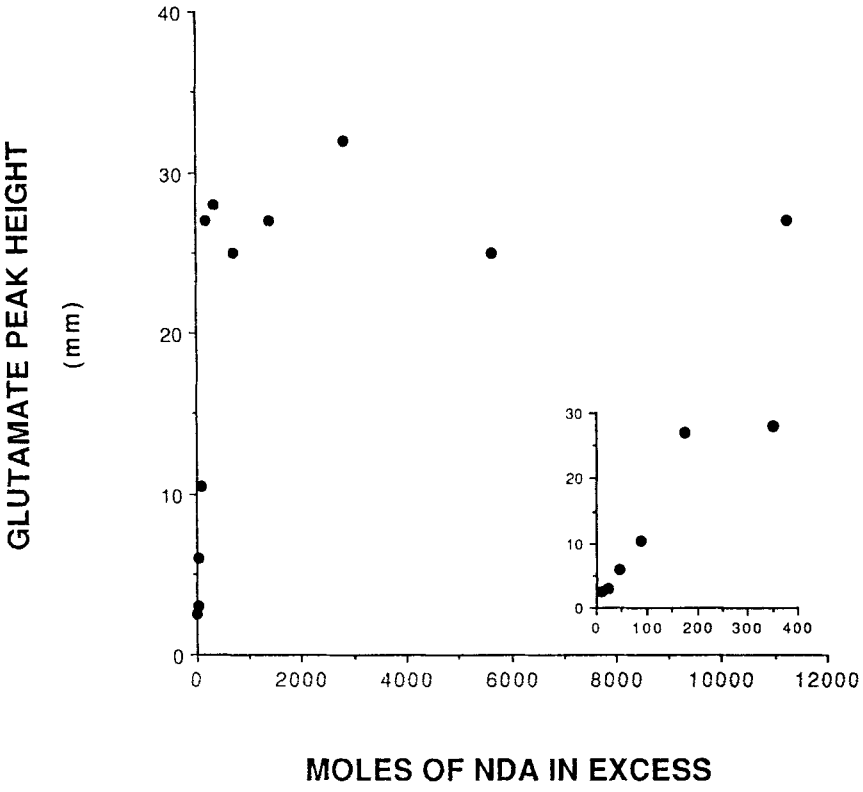

Figure 2. Relationship Between the Moles of NDA in Excess and the Glutamate Peak Height: An exponential curve was obtained reaching a plateau at the low mole excess. Approximately 200 moles of NDA in excess, with respect to glutamate, are needed to obtain maximal labeling of glutamate. As shown in the main graphic, at a concentration of NDA in excess corresponding between 200 and 12000 moles, no further labeling of glutamate was observed. The insert shows an expanded view of the first six points of the main graphic (the coordinates represent the same units). The maximum labeling was obtained at approximately 170 moles of NDA in excess.

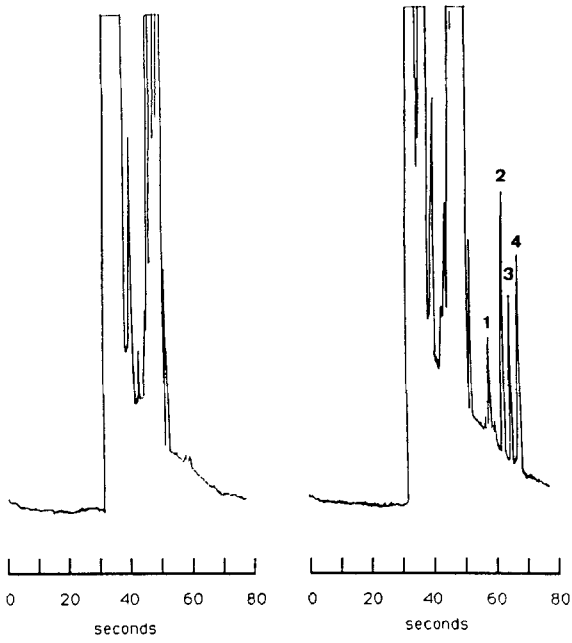

Figure 3. Capillary Electrophoresis Profile of Brain Dialysate Samples: Electropherograms of the NDA-derivatized samples of the control Ringer solution (left side) and of the dialysate of the caudate nucleus (right side) are shown. The electropherogram corresponding to the dialysate sample shows 4 new peaks when compared to to control sample. Based on experiments comparing migration time and spiking of the sample with a standard solution, it was determined that peak 3 correspond to that of glutamate.

sodium cyanide. The detection level achieved in this work compares well with the most sensitive technique for the determination of glutamate such as chemiluminescence (20). The present technique shows an adequate dynamic range since the expected concentration of glutamate in the extracellular fluid in the brain is at the micromolar level. The concentration of glutamate obtained here is within the linear part of the curve of log concentration vs log signal. Assuming an injection volume of 1 nanoliter, approximately 50 picoliters of glutamate solution were actually injected into the capillary column. Using a glutamate solution with a concentration of 3.69×10^{-8} M, a mass of 1.5 at-

tomole of glutamate was injected into the column. Since the signal-to-noise ratio was 25:1, the limit of mass detection was 180 zeptomoles. The mass detection limit achieved in this report, represents a substantial improvement when compared with previous techniques for the determination of glutamate.

The detection of glutamate in brain dialysates was accomplished with some simplicity. One factor that contributes to the success of this technique is the migration time of glutamate. At pH 9.5 glutamate is a negative molecule, therefore, glutamate migrates later than unreacted molecules of NDA, and later than NDA-derivatized components of the Ringer solution. The capillary electrophoresis migration time of glutamate-NDA was 65 seconds, reducing considerable the entire analysis time of the sample. There is some possibility, at least in principle, that by using a 10 μm I.D. capillary column the separation of the derivatized glutamate can be achieved even in a shorter period of time. Previous observations (16), demonstrate that the capillary internal diameter vs signal curve reaches a plateau at 10 μm I.D. Therefore, using a 10 μm I.D. capillary column should not reduce the sensitivity of the technique, and in fact present the opportunity of improving the resolution time of brain microdialysis substances. It seems that the main limiting factor for determining brain substances in dialysates is the need to collect enough analyte for analysis. Normally, the process of recollection of brain dialysates fluctuate in the range of 10 to 30 minutes, but occasionally may fluctuate from a minimum of 2 min to a maximum of 90 min lapses. At the present time, with the exception of microbore HPLC, most analytical techniques require large sample volumes of dialysates (in the microliter range) for analysis.

The results presented in this report were accomplished by utilizing the impressive minimum volume of one nanoliter, when CE-LIFD was used as the technique of analysis. A record of analysis volume never achieved before for determining substances present in brain dialysates. Since only 4 picoliters of dialysate (from the original mixture containing dialysate plus buffer and all reagents) were actually injected into the capillary column and this volume was in fact collected in 4 milliseconds, therefore, we are actually measuring the amount of glutamate collected in 4 milliseconds. Future work will require the necessity to create the appropriate tools to handle nanoliter samples in order to further exploit the technology of CE-LIFD. In the present work, the analysis of glutamate was carried out using microliter samples obtained in 10 minutes collection rather than nanoliters obtained in shorter period of times. The problem resides in the handling of nanoliter quantities of reactants rather than in the constraint of the analytical technique. We should concentrate then in the creation of automatic methods to derivatize nanoliter sample of material in order to

achieve subsecond measurement of glutamate in brain dialysates. Currently, we are identifying substances other than glutamate (containing primary amino groups) present in brain dialysates that were also derivatized by NDA. In addition, we are exploring possibilities of microderivatization at low concentration and ultralow volumes using on-column derivatization procedures. For this, nanoliter quantities of substances can be derivatized **in situ** using the analyte concentrator concept (21-23).

In conclusion, we have explored the use of CE-LIFD for the determination of glutamate in brain dialysates and we have found that a significant improvement was achieved (when compared with existing techniques) at the level of separation time, at the level of detection sensitivity, and also at the level of minutes amounts of volumes used for analysis.

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