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In vivo monitoring of glutamate in the brain by microdialysis and capillary electrophoresis with laser-induced fluorescence detection

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ABSTRACT

Glutamic acid, an excitatory neurotransmitter, was monitored *in vivo* in the corpus striatum of freely moving rats by brain microdialysis and capillary electrophoresis with laser-induced fluorescence detection. A procedure to derivatize glutamate in complex matrices was developed. Capillary electrophoresis in 12 μm I.D. capillaries was performed to determine glutamate with a migration time of 195 s. Laser-induced fluorescence detection with 488-nm radiation from an argon ion laser and with collinear geometry was used. An injection of haloperidol decreased the concentration of glutamic acid in the dialysates. These experiments support the hypothesis that dopamine receptor blockade decreases glutamate release. The potential of these techniques for the study of chemicals in biomedical experiments is discussed.

INTRODUCTION

In vivo monitoring techniques such as brain microdialysis have been developed for collecting samples of extracellular fluid from different tissues in freely moving animals [1–5]. In this technique a semipermeable hollow fibre is inserted in an organ or a blood vessel. The wall of the fibre allows diffusion of chemicals from the extracellular fluid into the fibre. A concentric tube injects artificial cerebral spinal fluid into the hollow fibre and this current of liquid drags the chemicals from the living tissues into a vial. Once extracted, the chemicals have to be analysed.

An analytical technique suited to microdialysis has to deal with small volumes. In general, microdialysate volumes range from 5 to 30 μl. These volumes are appropriate for high-performance liquid chromatography (HPLC). This technique currently works with 10–50-μl loops in most applications to brain microdialysis.

Because of these volumes, perfusion flow-rates of 1–5 μl/min are most often used in microdialysis with collection times between 5 and 30 min. These perfusion flow-rates are far from the ideal for microdialysis. They produce 80–95% depletion of the chemicals in the extracellular space around the microdialysis probe [6,7]. Such depletion perturbs the extracellular environment and affects the function of the cells around the microdialysis probe. Perfusion flow-rates from microdialysis should be less than 100 nl/min [7]. However, if a conventional analytical technique such as HPLC is used together with a perfusion flow-rate of 100 nl/min, the perfusion time must be increased from 50 to 300 min to collect 5–30 μl. These collection times are generally unacceptable. The question is how to perfuse the probe at a low flow-rate, *i.e.*, 100 nl/min or less, and to maintain a perfusion time of 20 min or less.

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Interestingly, a flow-rate of 100 nl/min gives enough sample volume for capillary electrophoresis (CE). Indeed, 50 pl–10 nl are the sample volume requirements for CE. These volumes can be collected in 0.003–8 s. Therefore, from the sample volume point of view, CE is one of the best analytical techniques for microdialysis. We have already coupled microdialysis and capillary electrophoresis with UV detection [8]. With these techniques, transfer of phenobarbital from blood to brain was studied. However, endogenous compounds, such as neurotransmitters, cannot be studied with CE–UV because of the low concentration sensitivity of UV detection, the limit of concentration detection of CE–UV being about 10^{-6} *M*. The concentrations of many biologically active substances are often less than the micromolar level.

The most sensitive detection method for CE is laser-induced fluorescence detection (LIF), but it requires previous derivatization of the analytes. Most of the biologically interesting compounds exhibit poor native fluorescence at visible excitation wavelengths and some of them do not exhibit native fluorescence at all. Monoamines and amino acids with aromatic rings fluoresce at 260 nm but do not fluoresce at higher wavelengths. Therefore, most of the biologically active compounds have to be derivatized, *i.e.*, tagged with fluorescent molecules, and then analysed. With this procedure several molecules have been analysed by CE–LIF. However, it is technically difficult to derivatize nanolitre volumes. For this reason, some researchers have tried to develop nano-scale techniques for carrying out precolumn derivatization [9].

Another technical drawback of derivatization is that derivatizing agents do not react with their target compounds when the latter are not well concentrated. For instance, when the concentration of a compound is lower than 10^{-6} *M*, the derivatizing reagent does not react with the compound on a mole to mole ratio basis. In order to overcome this problem, excess of reagent can be used. In general, 100 mol in excess with respect to the estimated concentration of the compound will label at concentrations as low as 10^{-9} *M*. However, the excess of reagent will be detected by the detection system and can mask the labelled compound. This last problem can be addressed in different ways. One is to improve the separation efficiency of the technique and another is to measure compounds that migrate before or after the spurious peaks due to the excess of fluorescent agent. We chose the latter strategy to measure glutamic acid. At alkaline pH this compound is negatively charged and migrates later than the reagent peaks, as we have shown in earlier analyses [10].

In a previous study, we showed that it is possible to label glutamate at low concentrations with naphthalene dicarboxaldehyde [10]. In this work, glutamate from brain dialysates was labelled with fluorescein isothiocyanate (FITC), which has a higher quantum efficiency than naphthalenedicarboxaldehyde, and fluorescein thiocarbamyl amino acid (FTC)–glutamate was detected by CE–LIF. With this method the effect of haloperidol injections on glutamic acid levels in the striatum was studied. We describe here the linearity and reproducibility of the technique and then show that a single haloperidol injection lowers glutamic acid levels.

EXPERIMENTAL

Reagents

Fluorescein isothiocyanate isomer I, sodium chloride, potassium chloride and calcium chloride were obtained from Sigma (St. Louis, MO, USA), acetone and sodium hydroxide of HPLC grade from J.T. Baker (Phillipsburg, NJ, USA) and sodium carbonate and sodium hydrogencarbonate from Merck (Darmstadt, Germany). Water was doubly distilled and deionized in a Milli-Q system (Millipore, Bedford, MA, USA) until its conductivity reached 18 MΩ. Haloperidol (Haldol) (Janssen Pharmaceutica, Beerse, Belgium) was obtained from a pharmacy. The buffer for the derivatization and the separation was 20 m*M* carbonate (pH 9.5). Ringer's solution for microdialysis perfusion was 146 m*M* NaCl–3.7 m*M* $CaCl_2$–1.2 m*M* KCl (pH 6.0).

Instrumentation

The laser-induced fluorescence detector and the CE system are described elsewhere [11,12]. Briefly, the samples and standards were injected in a 30 cm × 12 μm I.D. × 150 μm O.D. fused-

silica capillary (Polymicro Technologies, Phoenix, AZ, USA). At 20 cm from the anodic end, a 2-mm wide window was opened by removing the polyimide cover of the capillary with a scalpel under a dissecting microscope. This method is less prone to leave fluorescent residues of charred polyimide, which are common with the conventional burning procedure to make the detection window. The anodic end of the buffer-filled capillary was placed in a buffer reservoir made of a 200-μl Eppendorf pipette tip. This is a very convenient disposable reservoir that prevents contamination with fluorescent material from one sample to another. The cathodic end of the capillary was immersed in a laboratory-made T-shaped buffer reservoir that allowed flushing of the cathode compartment with fresh buffer. This reservoir also allowed a vacuum to be applied at the cathodic end of the capillary for hydrodynamic injection of the sample. The capillary was set on the *xyz* displacer of a microscope (Zeiss, Oberkochen, Germany). An epillumination condenser conducted 488-nm radiation from an argon ion laser (Ion Laser Technology, Salt Lake City, UT, USA) through a 40×0.85 NA objective on to the capillary. For this purpose the excitation radiation was reflected by an FT 510 dichroic mirror than reflected radiation of wavelength shorter than 510 nm (Zeiss) and focused through the objective. The fluorescence (usually emitted at 532 nm) was filtered by a high-pass filter centred at 520 nm (LP520; Zeiss). Stray light was attenuated by a notch filter centred at 488 nm (Andover, Salem, NH, USA). The fluorescence was detected by a Model 928 multi-alkali photomultiplier (Hamamatsu, Bridgewater, NJ, USA) operated at 700 V by a Model 2203 high-voltage power supply (Bertan, Hicksville, NY, USA). The signal from the photomultiplier tube was passed through a laboratory-made current-to-voltage converter equipped with a precision potentiometer to offset the background current (due to dark current plus stray radiation) and registered on a strip-chart recorder (Linseis, Princeton, NJ, USA).

Surgical procedure

Thirteen Wistar male rats weighing between 300 and 350 g were anaesthetized with Ketalar (100 mg/kg) and placed on a stereotaxic instrument. The surgical procedure is described elsewhere [2]. Briefly, a guide shaft 10 mm long, made of 21-gauge stainless-steel tubing, was inserted into the brain at the coordinates lateral to the midsaggital suture 3 mm, posterior to bregma 1.2 mm and ventral to the surface of the skull 4 mm. The guide shaft was attached to the skull by jewellers' screws and cemented with acrylic. After 7 days of recovery the rats were ready for microdialysis.

Microdialysis procedure

A microdialysis probe was made of a stainless-steel tube with a cellulose hollow fibre attached to its end and a 10 cm $\times$ 76 μm I.D. $\times$ 150 μm O.D. fused-silica capillary inserted into the stainless-steel and cellulose tube [13]. The inlet of the probe was connected to a syringe pump loaded with Ringer's solution set at a flow-rate of 100 nl/min. The outlet tube was inserted in a 400-μl microcentrifugue tube. The probe was inserted in the brain of an awake rate. Each sample of dialysate was collected throughout the course of 20 min (final collection volume 2 μl). After two baseline samples had been collected, six rats received an intraperitoneal injection of haloperidol (5 mg/kg) and seven rats received saline, then ten more samples were collected.

Derivatization procedure

The samples were mixed with 5 μl of buffer and 1 μl of $6 \cdot 10^{-4}$ *M* FITC in acetone solution. A blank solution of 2 μl of Ringer's solution or 2 μl of standard solutions of $5 \cdot 10^{-4}$, $5 \cdot 10^{-5}$ and $5 \cdot 10^{-6}$ *M* glutamate were derivatized with the same protocol. These mixtures were allowed to react in the dark for 18 h, then 192 μl of buffer were added to each vial.

Fluorescence measurements

The samples, blank and standard were hydrodynamically injected [a negative pressure of 19 p.s.i. (1 p.s.i. = 6894.76 Pa) applied during 2 s], then 21 kV were applied between the two ends of the capillary. This voltage generated a 4-μA current. After each run the capillary was flushed with 0.1 *M* NaOH followed by water. The peaks for the sample were identified by the migration

times and measured by comparing their heights with those of peaks given by standard solutions.

Three experiments were conducted. In the first a $1 \cdot 10^{-8}$ *M* solution of derivatized glutamate was injected eight times to determine the reproducibility of the migration time and the peak height. In the second experiment four glutamate solutions of $1 \cdot 10^{-5}$, $1 \cdot 10^{-6}$, $1 \cdot 10^{-7}$ and $1 \cdot 10^{-8}$ *M* concentrations were derivatized to yield $1 \cdot 10^{-7}$, $1 \cdot 10^{-8}$, $1 \cdot 10^{-9}$ and $1 \cdot 10^{-10}$ *M* FTC–glutamate solutions. They were then injected to determine the linearity of the derivatization procedure. In the third experiment, the actual concentrations of glutamate in brain dialysates before and after haloperidol or saline injections were measured.

Either regression analysis or one-factor analysis of variance (ANOVA) with repeated samples were used for statistical analysis.

RESULTS

The electropherograms of the blank and $5 \cdot 10^{-9}$, $5 \cdot 10^{-8}$ and $5 \cdot 10^{-7}$ *M* standard solutions are shown in Fig. 1. The blank shows several peaks due to decomposition of FITC and reaction with the aqueous solvent and its impurities. The same peaks are observed for the standard solutions. However, a new peak is observed towards the end of the electropherogram. This peak corresponded to FTC–glutamate. Note that it is absent in the blank. The migration time of this peak was 3 min 15 s.

The results of the reproducibility tests were as follows: mean migration time 187 s (mean standard error ±1.3 s) and mean peak height 102 mm (mean standard error ±2.0 mm).

The migration time was reproducible with an error of ±0.69% and the peak height with an error of ±2%. The points of the log–log plot of concentration *vs.* peak height fitted a straight line with the equation $y = 9.1 + 0.9x$ and the goodness of fit was highly significant [$r = 0.99$, $F(1/2) = 1425$, $p < 0.001$].

Analysis of the dialysate showed a peak that corresponded to glutamate. In addition, several other peaks different to the glutamate peak and to the ghost peaks of the dye were seen (see Fig. 2). These peaks probably corresponded to other substances present in the dialysate and taken up from the brain. The average concentration of glutamate was $3.5 \cdot 10^{-5}$ *M*. This concentration was kept very similar for each rat. For instance, the electropherograms in Fig. 2 correspond to one rat. In six consecutive measurements the height of the glutamate peak remained very constant.

When the data were plotted it was found that haloperidol decreased the glutamate concentration significantly by the second and third samples after haloperidol injection [$F(1/11) = 6.0$, $p <$

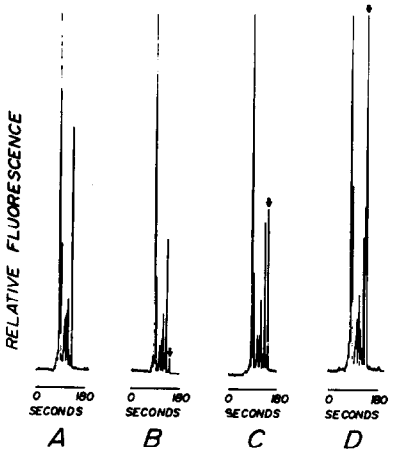

Fig. 1. Electropherograms of (A) a blank and (B) $5 \cdot 10^{-9}$, (C) $5 \cdot 10^{-8}$ and (D) $5 \cdot 10^{-7}$ *M* standard solutions of glutamate. Numerous peaks of FITC products are observed. The glutamate peak is indicated by an arrow.

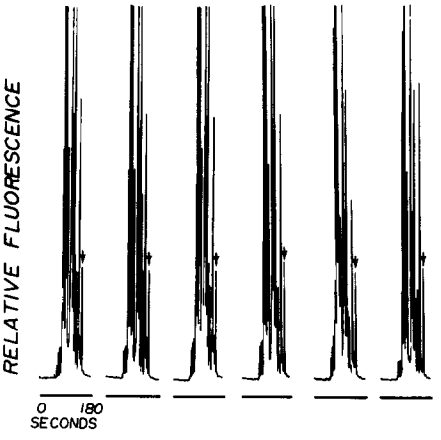

Fig. 2. Six electropherograms of dialysates showing FTC–glutamate signals (indicated by arrows) of consistent amplitude. The horizontal bars represent 180 s.

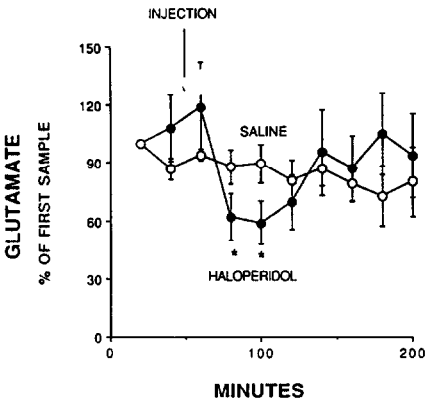

Fig. 3. Decrease of glutamate level in striatal dialysates in the presence of halopendol. Asterisks indicate statistically significant differences.

0.03]. This decrease lasted for about 60 min and then the glutamate level increased towards the base level (see Fig. 3).

DISCUSSION

The results show that it is possible to label nanomolar solutions of glutamate with FITC and detect, by laser-induced fluorescence, FTC–glutamate when a 100-fold molar excess of FITC is used. The linearity of the method is excellent in the range 10^{-7}–10^{-10} *M*, *i.e.*, three orders of magnitude. The reproducibility of the technique is reasonably good. This suggests that the technique might be very useful for the precise determination of glutamate in biological fluids.

The analysis takes 3 min or less, which is acceptable when compared with techniques such as HPLC. It is possible to shorten this time by using potassium-based buffers.

It is possible to measure glutamate in complex matrices such as brain dialysates, because glutamate migrates later than the peaks of FITC owing to the negative charge of FTC–glutamate. In such matrices several peaks corresponding to primary amines are observed. As primary amines are present in monoamines, amino acids and peptides, these unidentified peaks could be biologically interesting compounds.

The measurement of glutamate in brain dialysates requires very small volumes. In the present experiments we used a total sample volume of 2 μl for the measurements. This volume came from a perfusion flow-rate of 100 nl/min, which is very low when compared with the flow-rates currently used in brain microdialysis, which are between 1 and 5 μl/min, *i.e.*, from ten to fifty times greater.

The concentration of glutamate was between one and two orders of magnitude larger than that reported in previous experiments by other workers [3,4,14] using perfusion flow-rates greater than 1 μl/min. This discrepancy might be due to depletion of glutamate caused by the high-flow rates. In some experiments microdialysis probes have been inserted in the brain and microelectrodes for *in vivo* voltammetry have been placed in the neighbourhood of the microdialysis probe [6,7]. With this approach it is possible to determine the concentrations of the biological compounds at different distances from the microdialysis probe. It has been shown that at high flow-rates (2 μl/min or more) the concentration of Ca^{2+}, DOPAC or dopamine in the extracellular compartments is 80% lower than the normal concentration. This is particularly important because the depletion reaches 100 μm away from the microdialysis probe. However, when flow-rates of 100 nl/min or less are used, then the concentration of biological compounds is about normal around the probe.

An acute injection of haloperidol decreased glutamate in the striatum. There is considerable evidence that dopamine and glutamate interact in the striatum. Glutamate increases dopamine release and dopamine increases glutamate release. In this work we found that the blockade of dopamine receptors by haloperidol decreases the glutamate level. This finding supports the proposal that glutamic acid and dopamine regulate each other's release.

The final volume of the derivatized sample was 200 μl and 120 pl were injected for analysis. This means that the analysis of each sample can be repeated many times.

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