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Capillary electrophoresis–laser-induced fluorescence detection of amphetamine in the brain

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Abstract

Prefrontal cortex microdialysis was done in rats that had received intraperitoneal amphetamine (AMPH). Samples were derivatized with 10^{-4} *M* fluorescein isothiocyanate and incubated for 18 h. AMPH was separated by capillary electrophoresis (CE) and detected by laser-induced fluorescence detection (LIFD) from 30 to 150 min after injection. The limit of mass detection was 3 amol, which is three orders of magnitude lower than that in gas chromatography–mass spectrometry, and the limit of concentration detection was $3 \cdot 10^{-9}$ *M*. The results showed that CE–LIFD is a good method for detecting AMPH in brain dialysates of rats.

Keywords: Capillary electrophoresis; Laser-induced fluorescence detector; Derivatization, electrophoresis; Amphetamine; Amines

1. Introduction

The assessment of drugs in living organisms requires in vivo extraction of the drug and trace analysis in aqueous solutions. This puts strong demands on the sensitivity of the analytical technique. Recent developments in in vivo monitoring techniques such as microdialysis and capillary electrophoresis (CE) have created interesting alternatives for drug body distribution evaluation. CE with laser-induced fluorescence detection (LIFD) has high sensitivity; zeptomolar and even yoctomolar masses have been detected with this technique [1–3]. However, most of the compounds of interest do not exhibit native fluorescence and have to be derivatized with fluorescent tags. Fortunately, there is a large variety of fluorochromes with high affinity for primary amine or carboxylic groups which are abundant in biologically active compounds. Although these fluorochromes have different excitation spectra, it is now easy to obtain a laser line very close to almost any absorption peak of any fluorochrome [4]. However, the derivatization procedure itself poses interesting problems. At low concentration, the target analyte does not react with the fluorescent probe on a molar to molar basis. A high molar excess of the fluorescent probe is required to label successfully trace amounts of analyte. This in turn introduces spurious peaks that sometimes mask the peak of the target analyte. Alternatively, analyte preconcentration enhances the yield of tagged products.

The extraction of the drug from the body can be performed with different techniques to sample in vivo biological fluids. Microdialysis is a technique successfully used over the last 10 years

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[5–7]. In this technique a semipermeable bag is perfused with artificial cerebrospinal fluid (ACSF) and inserted in the brain. According to their molecular masses, the compounds diffuse into the bag and are dragged out by the ACSF current.

In the present experiments, we tested the off-line combination of brain microdialysis with CE–LIFD for the assessment of amphetamine (AMPH) given intraperioneally. AMPH is a drug of abuse of medical and forensic value [8–10] and several techniques have been developed for the determination of this drug and its derivatives. These techniques include HPLC with UV, fluorescence, chemiluminescence and electrochemical detection [11–16], GC–MS [17–22], immunoassays (I) [23–25] and CE, which is an attractive alternative to all the other techniques [26]. Except for CE, the limit of mass detection (LOMD) of these techniques is in the picomole to femtomole range and the limits of concentration detection (LOCD) are in the micromolar to nanomolar range. Sensitivity levels as good as those reported for other techniques have been obtained with CE. However, most of the CE work has been done with UV detection [26]. To our knowledge, no attempt to apply CE–LIFD to AMPH detection in biological fluids have been made.

AMPH is a phenethylamine with a primary amine group which can be derivatized with different flurochromes. In a previous study we showed that CE with fluorescence detection allows the detection of fluorescein isothiocyanante (FITC)- or fluorescamine-labelled AMPH [27]. In this work, we used FITC because it has an excellent quantum efficiency and its absorption peak matches very well the blue line of argon ion lasers. The brain area that we chose to dialyse was the prefrontal cortex because some of the biomedically relevant actions of AMPH are mediated by the prefrontal cortex.

2. Experimental

2.1. Subjects and surgery

Three male Wistar rats weighing between 300 and 350 g were individually housed with food and water ad libitum and 12/12 h light/dark cycles. Under ketamine chlorohydrate anaesthesia [45 mg/kg intraperitoneal (i.p.)], a 10-mm long, 21-gauge stainless-steel guide tube (the guide shaft) targeted above the prefrontal cortex was stereotaxically implanted in each rat. The coordinates (with level skull) were 2.5 mm anterior to bregma, 0.5 mm lateral to the sagittal sinus and 1.5 mm ventral to the skull surface according to the the Paxinos and Watson atlas [28]. The guide shaft was fixed to the skull with screws and acrylic cement. An obturator was placed inside the guide shaft to keep patency. The removable dialysis probe construction has been described elsewhere [29]. The dialysis section is made of a 3 mm long cellulose hollow fibre (Spectrum Medical Industries), molecular mass cut-off 6000 and 200 mm O.D.

2.2. Dialysis session

The sessions began 3–4 days after surgery between 7.00 a.m. and 13.00 p.m. The general procedure of dialysis has been described elsewhere [29]. Briefly, the rat was placed into a cage with a swivel joint to prevent tangling of the connecting tubing. The collection of samples began 60–90 min after insertion of the probe that protruded 5 mm off the guide shaft. The probe was perfused with a filtered and degassed ACSF (135 m*M* NaCl–3.7 m*M* KCl–1.0 m*M* $MgCl_2$–1.2 m*M* $CaCl_2$–10.0 m*M* $NaHCO_3$, pH 7.4) at 1 ml/min using a sp210iw syringe pump (World Precision Instruments, Sarasota, FL, USA). Dailysates were collected before and after the i.p. injection of 3 mg/kg of *d*-AMPH sulfate. The sessions ended with the collection of the fifth postreatment sample. The dialysates were immediately frozen until derivatization.

2.3. Capillary electrophoresis and laser-induced fluorescence detection instrument

The instrument has been described previously [30]. The laser radiation used for excitation was the 488 nm line of a Model 5425 air-cooled argon ion laser (Ion Laser Technology, Salt Lake City, UT, USA). The laser beam was reflected by a 510-nm centred dichroic mirror at a 45° angle

and focused on the capillary window through a 0.75 NA objective (Zeiss, Caracas, Venezuela). The fluroescence was collected through the same objective. A high-pass filter centred at 520 nm and a notch filter centred at 488 nm attenuated the stray radiation (Andover, Salem, NH, USA). The light was detected with a Model R928 multialkali photomultiplier tube (PMT) from Hamamatsu (Bridgewater, NJ, USA), operated at 700 V, and connected to a laboratory-made current-to-voltage converter. The signal was fed to a 386 computer and acquired and processed with MAXIMA software from Waters.

The CE separations were performed in fused-silica capillaries obtained from Polymicro Technologies (Phoenix, AZ, USA) and filled with 20 m*M* carbonate buffer. The length of the capillaries was 30 cm, the effective length was 20 cm and the I.D. was 20–25 mm. The samples were injected by applying a −19 p.s.i., 0.3 s vacuum pulse at the cathodic end of the capillary while the anodic end was immersed in the sample reservoir. Separation was carried out by applying 20 kV with a Model 30R high-voltage power supply (Bertan, Hicksville, NY, USA). The electrodes were platinum–iridium wires.

2.4. Derivatization procedure

A standard solution of AMPH was prepared by dissolving 1 mg of AMPH in 1 ml of ACSF to obtain a $7 \cdot 10^{-3}$ *M* solution, then successive dilutions of 10^{-6}, 10^{-7} and 10^{-8} *M* were prepared in ACSF. A $2.6 \cdot 10^{-3}$ *M* FITC solution was prepared by dissolving 1 mg of FITC in 1 ml of acetone. This solution was diluted with acetone to obtain 10^{-4} and 10^{-5} *M* FITC solutions. The derivatization procedure consisted in mixing 30 μl of either blank, standard or sample with 75 μl of carbonate buffer and 15 μl of FITC solution. The mixture was incubated in the dark and analysed as described above.

2.5. Reagents

d-Amphetamine sulfate was obtained from California (Sunnyvale, CA, USA) and fluorescein isothiocyanate isomer I (FITC), sodium carbonate, sodium bicarbonate, sodium chloride, potassium chloride, calcium chloride and magnesium chloride from Sigma (St. Louis, MO, USA). Doubly distilled, deionized (18 MΩ) pure water was used in the preparation of solutions.

2.6. Experiments

Two experiments were carried out. In the first experiment, the conditions for the derivatization reaction were tested as follows. (1) Concentration of FITC: 10^{-4} and 10^{-5} *M* FITC solutions were used to react with 10^{-7} *M* AMPH standard solution for 8 h and analysed. (2) Reaction time: reaction times of 1, 8 and 24 h were allowed after mixing 10^{-6} *M* AMPH standard solution with 10^{-5} *M* FITC. (3) Linearity of the AMPH calibration graph: 10^{-6}, 10^{-7} and 10^{-8} *M* AMPH solutions were derivatized with 10^{-5} *M* FITC for 8 h. The logarithm of the magnitude of the signal and the logarithm of the concentration were compared by regression analysis. (4) Optimization of AMPH peak height in brain dialysates samples: for this experiment, the derivatization procedure consisted in mixing 15 μl of dialysate collected as described before and 10 μl of $3.3 \cdot 10^{-5}$ *M* AMPH with 25 μl of carbonate buffer and 50 μl of one of thirteen concentrations of FITC increasing from $1.1 \cdot 10^{-6}$ to $6.6 \cdot 10^{-3}$ *M*. The mixtures were incubated in the dark for 18 h. The logarithm of the magnitude of the signal of FITC–AMPH in the dialysate spiked with AMPH and the FITC in excess were compared by regression analysis.

In the second experiment, the detection of AMPH in the rat brain was performed after the i.p. injection of 3 mg/kg of *d*-AMPH sulfate. In the cortical dialysates AMPH was detected using 10^{-4} *M* FITC and the reaction time was 18 h.

2.7. Histology

At the end of the experiments the animals were killed with an overdose of chloroform and then perfused intracardially with 10% formalin. The brains were removed and sectioned with a freezing microtome to locate the tracks of the guides and probes.

3. Results and discussion

3.1. Optimum derivatization conditions

The derivatization of standard solutions with 10^{-4} *M* FITC gave higher peaks of AMPH than derivatization with 10^{-5} *M* FITC. More "ghost peaks" were observed with 10^{-4} *M* FITC, however. These peaks correspond to different chemical species of fluorescein. A cation, three neutral species, two anions and a dianion form have been reported, the relative concentrations of which vary depending on the surrounding environment [31]. By contrast, derivatization with 10^{-5} *M* FITC gave a cleaner electropherogram but smaller signals (Fig. 1). A 100 molar excess of FITC seems to be the best for AMPH derivatization in standard solutions, but exceeding the optimum excess of FITC would enlarge the base of the FITC peak and hinder AMPH detection. Derivatization of AMPH in brain samples needed a larger FITC excess than derivatization of AMPH in standard solutions. Fig. 2 shows the correlation between the increase in moles of FITC and the increase in the FITC–AMPH signal ($y = 1.336 \log x + 0.676$; $r = 0.941$) in brain dialysates spiked with AMPH. At the twelfth point, which represents a 512 molar excess of FITC, the curve reached an asymptotic level. This means that a ca. 500 molar excess of FITC is needed to obtain maximum labelling of AMPH in dialysates. This excess is larger than that needed for maximum labelling of the AMPH in standard solutions owing to the presence of other primary amines in the brain dialysate. In AMPH standard solutions, compared with a 1-h reaction time the 24-h reaction time increased the signal, reduced the FITC peak and eliminated several unknown peaks (Fig. 3). After 8 and 24 h the heights of the AMPH peaks were virtually the same but at 24 h the other peaks were drastically reduced. Similar results were obtained with 10^{-7} *M* AMPH.

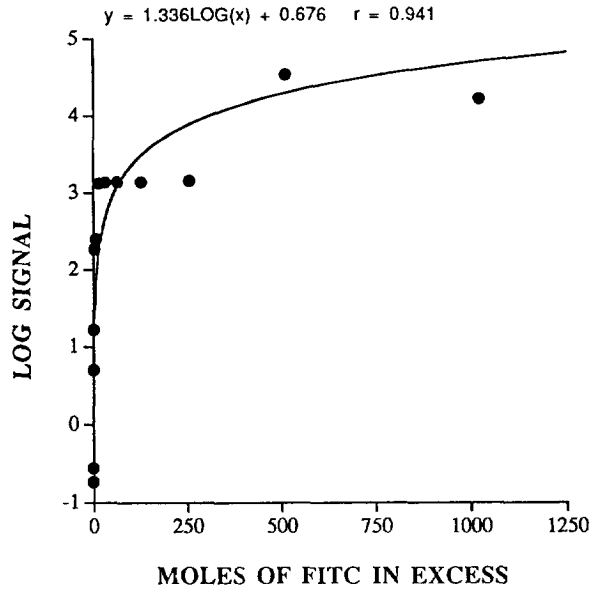

Fig. 2. Optimization of FITC–AMPH peak height in brain dialysates spiked with 10^{-5} *M* AMPH. The logarithmic curve of AMPH signal height in volts versus FITC excess in moles shows that a ca. 500 molar excess of FITC with respect to AMPH is needed to obtain maximum labelling of AMPH.

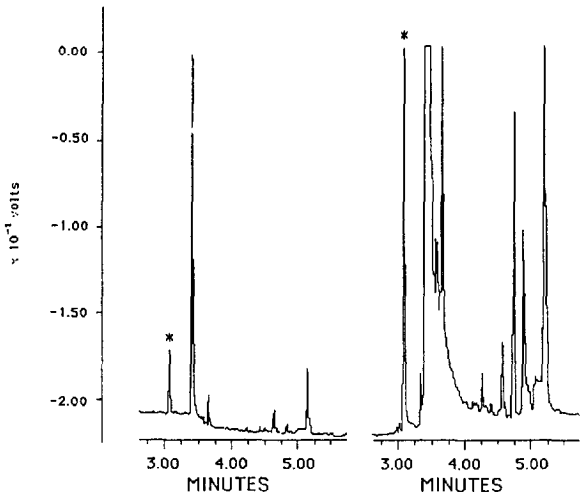

Fig. 1. FITC concentration and FITC–AMPH peak height. Left, derivatization of 10^{-7} *M* AMPH standard solution for 8 h with 10^{-5} *M* FITC; right, derivatization with 10^{-4} *M* FITC. The asterisk marks the FITC–AMPH peak.

Fig. 4 shows a linear relationship in the 10^{-6}–10^{-8} *M* range of AMPH standards derivatized with 10^{-5} *M* FITC for 8 h. There was a 31:1 signal-to-noise ratio for 10^{-8} *M* AMPH derivatized with 10^{-4} *M* FITC and reacted for 8

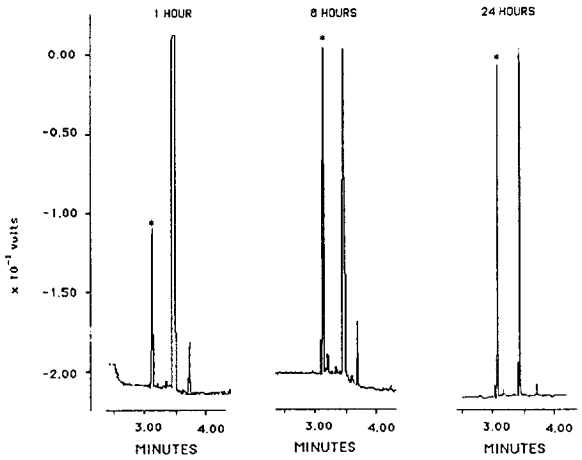

Fig. 3. Reaction time and FITC–AMPH peak height. Derivatization of 10^{-6} *M* AMPH standard solution with 10^{-5} *M* FITC solution for 1, 8 and 24 h. The asterisk marks the FITC–AMPH peak.

h. This indicates that it is possible to have an acceptable signal-to-noise ratio for detecting AMPH in the nanomolar range.

As the injection volume was calculated as 0.3 nl, the limit of mass detection was 3 amol and the limit of concentration detection was $3 \cdot 10^{-9}$ *M*. This method offers a three orders of magnitude lower limit of mass detection than GC–MS, which is the most sensitive method for AMPH determination. The limit of concentration detection is as good as for GC–MS. With the combination of microdialysis and CE, the determination of AMPH in the samples was simpler and quicker than with GC–MS. There was no need for extraction procedures, the volume of sample was minimum and the separation and measurement of the drug with CE–LIFD took only a few minutes. On the other hand, the CE equipment is not as complex and expensive as a GC–MS system. The CE–LIFD technique does not require a high level of skill either for operating the system or for interpreting the results.

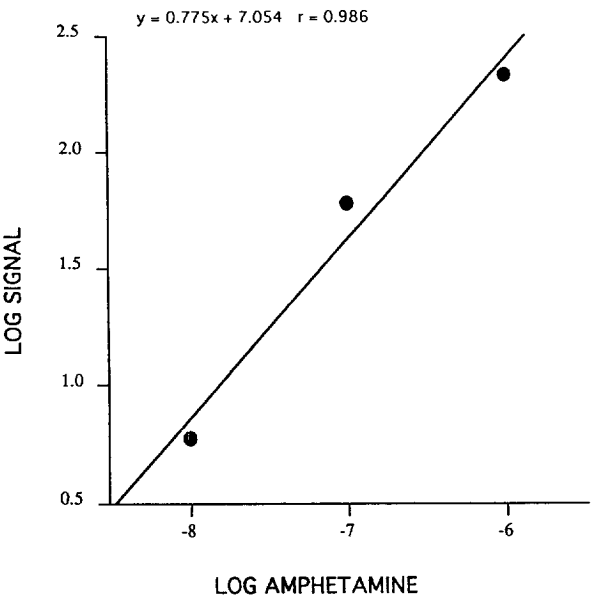

Fig. 4. AMPH calibration graph: linear regression of the logarithm of AMPH concentration with the logarithm of the signal size. Derivatization of AMPH standard solutions with 10^{-5} *M* FITC solution for 8 h.

3.2. Derivatization of brain dialysates of AMPH-treated rats

The chosen conditions for this experiment included 10^{-4} *M* FITC, which gave larger peaks, and a 24-h reaction time, which reduced the size and number of extra peaks. In the cortical dialysates the AMPH peak was absent in pretreatment samples and present from 30 to 150 min after administration of AMPH. The identification of the peak was verified by spiking the sample with 10^{-5} *M* AMPH standard solution (Figs. 5 and 6).

4. Conclusion

The present experiments show that CE–LIFD is a good method for detecting AMPH in brain dialysates from rats. The drug was detected at low concentrations in the brain without the need for sample preconcentration. Since the derivatized sample has a volume of 120 ml and only 1 nl was injected, the collection time could be reduced to 1 min or less. A time resolution improvement for brain dialysates has been demonstrated in glutamate measurements [32]. The dynamic range of CE–LIFD is good enough for

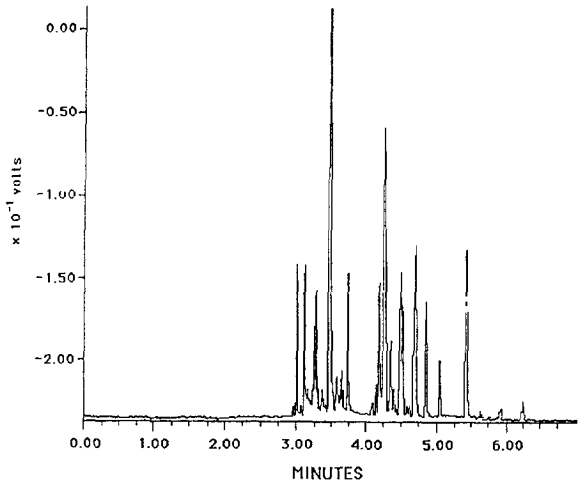

Fig. 5. Electropherogram of a dialysate from rat prefrontal cortex. The dialysate was derivatized with 10^{-4} *M* FITC solution for 18 h.

the concentration range found in brain dialysates. Sample preconcentration should enhance the limit of concentration detection in biological samples. The limit of mass detection for AMPH is 3 amol. However, this limit can be improved by using cylindrical mirrors and digital processing of the electropherograms. In any event, the mass sensitivity of the present method is good enough for forensic and medical applications. The limit of concentration detection is $3 \cdot 10^{-9}$ *M*.

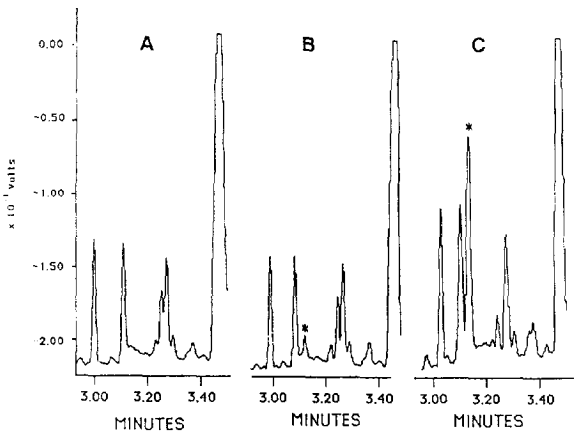

Fig. 6. Amplified electropherograms of rat prefrontal cortex dialysates before and after an i.p. injection of *d*-AMPH sulfate. (A) Preinjection baseline; (B) 30 min after AMPH injection; (C) 10^{-5} *M* AMPH standard solution added to the preinjection sample. Derivatization conditions as in Fig. 4. The asterisks indicate the FITC–AMPH peak.

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