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Journal of Chromatography A, 781 (1997) 81–89

JOURNAL OF CHROMATOGRAPHY A

Determination of kynurenic acid by capillary electrophoresis with laser-induced fluorescence detection

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Abstract

Kynurenic acid (KA) is an excitatory amino acid receptor antagonist that is believed to play an important role in a host of diseases of the neuropsychiatric and central nervous system. A method for the determination of KA in microdialysate samples using capillary electrophoresis (CE) separation with laser-induced fluorescence (LIF) detection is described. CE is advantageous for the analysis of microdialysis samples due to its short analysis times and small sample volume requirements. Three complexation approaches were evaluated in an attempt to achieve the best limit of detection. The best approach was found to be pre-column complexation with inclusion of Zn(II) in the background electrolyte. After optimization of the zinc acetate concentration and pH, a limit of detection of 1 n*M* KA was achieved. However, when KA was present in the dialysate, the limit of detection increased 50-fold. Even though the endogenous levels of KA in rat brain are below this limit of detection, this methodology could be used to monitor the increase of KA levels in rat brain following dosing with its precursors, tryptophan and kynurenine. © 1997 Elsevier Science B.V.

Keywords: Buffer composition; Microdialysis; Kynurenic acid; Metal complexes; Zinc

1. Introduction

Kynurenic acid (KA), a metabolite of tryptophan, is the only endogenous N-methyl-D-aspartic acid (NMDA) receptor antagonist currently identified in mammalian brain [1]. Because of the role of KA as a broad spectrum antagonist of excitatory amino acid receptors and the fact that it is capable of preventing neurodegeneration induced by excitatory amino acid receptor agonists [2], it has been the subject of several recent reports [3–5]. In vivo, KA is an especially potent antagonist of quinolinic acid-induced neurotoxicity [6], and has a high affinity for the glycine coagonist site of the NMDA receptor [7].

Endogenous levels of KA in rat brain extracellular fluid (ECF) have been reported to be in the low nanomolar range, and were elevated during seizure activity [3]. In humans, KA in ECF is increased in HIV-1 [8] and decreased in Parkinson's disease [9]. The cerebrospinal fluid (CSF) levels of KA are increased in patients with Down's syndrome [4] and decreased in patients with Huntington's disease [10].

Microdialysis, a technique used for sampling extracellular fluids, has been employed for the determination of many different neurotransmitters in the brain [11–15] as well as for studies of drug metabolism [16] and pharmacokinetics at various sites within the body [17–21]. This technique offers the distinct advantage of providing aqueous, protein- and enzyme-free samples that can be directly in-

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0021-9673/97/$17.00
PII S0021-9673(97)00534-7

jected onto an analytical system without further sample preparation. An additional advantage is that data for an entire study can be collected from one animal, thus reducing the number of experimental animals used and eliminating inter-animal variations.

One challenge associated with microdialysis sampling is analysis of the small sample volumes obtained. Temporal resolution in microdialysis is dependent on the sensitivity of the analytical technique, the sample volume requirements of the analytical system, and the recovery of the microdialysis probe. In some cases, the volume collected may not be large enough for reproducible injections, and longer sampling intervals result in decreased temporal resolution.

The low concentration of analyte present in the dialysate, which is partially dependent on the recovery of the probe, presents an additional challenge. Because probe recovery increases with decreasing flow-rate, low dialysis flow-rates are essential, as is a sensitive analytical system that is not limited by injection volume requirements.

Several reversed-phase liquid chromatograpy (RP-LC) methods have been reported for the determination of KA. LC with UV detection (LC–UV) has been used to detect KA extracted from brain tissue [22], but is not sensitive enough to detect endogenous levels of KA in ECF. LC with electrochemical detection (LC–ED) [9,10,23] has also been employed for the determination of KA in brain tissue extracts, but the selectivity is poor due to the high oxidation potential required. The most commonly utilized method for determination of KA is post-column Zn(II) complexation followed by fluorescence detection (FD) [1,3,23–25]. With this methodology, it is possible to detect the nanomolar concentrations of KA in rat brain microdialysates [1,3,24]. However, relatively large sample volumes (30–50 μl) are required for the analysis.

In this paper, a method for the determination of KA based on capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection is described. CE was chosen as the separation method because it is capable of highly efficient and often quite rapid separations of aqueous ionic species, and typical injection volumes are in the nanoliter range. The small sample volume requirements of CE make it possible to employ lower microdialysis flow-rates to increase analyte recovery without significantly compromising temporal resolution. To maximize the sensitivity, several different complexation approaches were investigated. The optimal method was then evaluated for monitoring KA in brain by microdialysis sampling following i.p. administration of tryptophan or kynurenine.

2. Experimental

2.1. Reagents

Kynurenic acid and zinc acetate were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide was purchased from Fisher Scientific (Fair Lawn, NJ, USA), and sodium chloride, potassium chloride, calcium chloride, magnesium chloride, sodium bicarbonate and sodium phosphate, dibasic were purchased from Acros Organics (Pittsburgh, PA, USA). All chemicals were used as received. Solutions were prepared in ultrapure water (Labconco, Kansas City, MO, USA) and filtered through a 0.2 μm syringe filter (Gelman Sciences, Ann Arbor, MI, USA) before use.

A stock solution of 150 μ*M* kynurenic acid, dissolved in filtered 1 m*M* NaOH, was prepared weekly. Solutions for analysis by CE–LIF or spectrofluorimeter, prepared fresh daily, were diluted to their final concentrations with ultrapure water or artificial cerebrospinal fluid (ACSF).

2.2. Capillary electrophoresis system

Separations were carried out using a laboratory-constructed CE system. Polyimide-coated fused-silica capillaries (50 μm I.D.×360 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA) and capillary lengths of 18–25 cm were used. A window for optical detection was created by removing a small section of the polyimide coating 10 cm from the injection end of the capillary. Injection and electrophoresis were driven by a CZE 1000r high-voltage power supply (Spellman Electronics, Plainview, NY, USA). Timed electrokinetic injec-

tions of 3 s at −12 kV were controlled by a timer box, which was constructed locally.

LIF detection was accomplished using a He–Cd laser (Model IK5351R-D, Kimmon Electric US, Englewood, CO, USA) with 5 mW at 325 nm for excitation. A He–Cd high reflector mirror (Newport, Irvine, CA, USA) was used to reflect the laser beam at a 90° angle onto a 1-mm fused-silica bi-convex lens having a focal length of 13 mm (Oriel, Stratford, CT, USA). The lens focused the laser beam within the center of the capillary, which was mounted on an x–y–z positioner with an adapter bracket (Newport). A 40× microscope objective (Nikon, Melville, NY, USA) was used to collect and refocus the fluorescent image from within the capillary. The image was spectrally filtered through a long-pass filter (348 nm, Oriel) and an interference filter (400 nm, Oriel). The emitted fluorescence was detected with an R1527 photomultiplier tube (Hamamatsu, Bridgewater, NJ, USA) operated at 1200 V supplied by a regulated power supply (Model 227, Pacific Photometric, Concord, CA, USA). The CE capillary and optics were enclosed in a light-tight black plexiglas box with slide-out sides and top to block out stray light and isolate the high voltage. Signal amplification and RC filtering were accomplished with a locally constructed circuit. Data collection was accomplished with a DA-5 data acquisition interface (Bioanalytical Systems, West Lafayette, IN, USA).

The background electrolyte (BGE) consisted of 40 m*M* zinc acetate, pH 5.5, unless stated otherwise. The CE capillaries were washed and conditioned with 0.1 *M* NaOH prior to use. A negative high voltage was applied at the detection end of the capillary while the injection end of the capillary was held at ground. Injection and separation voltages of ca. −12 kV (−600 V/cm) were employed in these studies.

2.3. Fluorescence studies

All fluorescence studies were conducted on a Shimadzu RF5000U spectrofluorophotometer. To determine the optimal excitation and emission wavelengths to be used, scans were performed from λ_{ex}= 200–500 and λ_{em}=300–650 nm. For the fluorescence intensity studies, the excitation wavelength was set at 325 nm and the emission wavelength was scanned from 300–650 nm.

The optimal concentration of zinc acetate added to the sample was also determined by fluorescence spectroscopy. Using a final concentration of 1 μ*M* KA, concentration ratios of Zn:KA from 0–10 000:1 were evaluated. The effect of pH on fluorescence intensity was also investigated. Samples were prepared by adding 100 μl of an 80 m*M* zinc acetate solution (pH 5.0–6.8) to 900 μl of a 1.1 μ*M* solution of KA. The final pH of the mixture was measured and recorded. For this study, the final concentrations of KA and Zn(II) were 1 μ*M* and 8 m*M*, respectively.

The time profile for complex formation was also obtained. KA (1 μ*M*) and zinc acetate (8 m*M*), pH 6.8, were mixed and placed in the spectrofluorimeter. The time for mixing (t_0) was 15 s. A 60-min time scan was conducted to monitor the stability of the fluorescent complex.

2.4. Complexation optimization

Three approaches were examined for the complexation of Zn(II) and KA prior to LIF detection. For pre-column complexation, zinc acetate, pH unadjusted, was added to a solution of KA in a sample vial prior to injection onto the capillary. Final concentrations of 100 n*M* KA and 0–800 μ*M* zinc acetate were studied. The CE run buffer consisted of 50 m*M* sodium acetate, pH 5.0.

For on-column complexation, the Zn(II) was included in the BGE so that complexation would occur during the separation. Zinc acetate solutions with concentrations from 35–75 m*M* were prepared, and their pH was adjusted between 5.0 and 6.8 using dilute acetic acid. For each new capillary, the BGE was allowed to equilibrate within the capillary for 30 min prior to making injections. Samples were injected electrokinetically (3 s at −12 kV), after which the separation voltage was applied.

In the final method, the use of pre-column complexation with inclusion of Zn(II) in the BGE was investigated. The zinc acetate BGE contained 40 m*M* Zn(II) at pH 5.5. The optimal ratio of zinc acetate to analyte for the pre-column step was also determined. A final KA concentration of 100 n*M* was used, and

concentration ratios of Zn:KA from 0–10 000:1 were evaluated.

2.5. Microdialysis sampling

Male Sprague-Dawley rats (250–350 g) were anesthetized with the inhalation anesthetic halothane and injectable ketamine/xylazine (90 mg/kg+10 mg/kg i.m.) and maintained under anesthesia for the duration of the experiment through additional doses as needed. The animal was positioned in the stereotaxic frame and its body temperature was maintained with a heating pad. Following disinfection, an incision was made in the scalp along the mid-line. Using stereotaxic coordinates, the guide cannula was implanted and held in position with epoxy and dental acrylic. Coordinates used were 4.8 mm posterior to bregma, 4.8 mm lateral to the midline and 5.8 mm ventral to the skull surface [26].

BAS 4-mm dialysis probes (Bioanalytical Systems) were perfused with ACSF (120 m*M* NaCl, 20 m*M* $NaHCO_3$, 3 m*M* KCl, 1.2 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$ and 0.25 m*M* Na_2HPO_4, pH 7.4) at a flow-rate of 500 nl/min. Prior to the start of the study, probes were calibrated in vitro by placing them in 250 n*M* KA that was stirred and maintained at 37°C. Dialysates (5 μl) were collected every 10 min; 0.3 μl of 150 m*M* zinc acetate was added, and the sample was injected onto the CE–LIF system.

The microdialysis probe was inserted into the guide cannula implanted in the hippocampus of the rat and was perfused for ca. 3 h prior to the dosing experiment described below. Samples were collected at 10 min intervals (5 μl final collection volume) throughout the experiment. Blank microdialysates were monitored for 1 h before any dosing of the animal was begun.

In one in vivo experiment, a bolus dose of tryptophan (75 mg/kg) in normal saline was administered by i.p. injection. Changes in KA levels were monitored for 8 h. In a second in vivo study, a bolus i.p. injection of kynurenine (75 mg/kg) in normal saline was administered to the rat, and changes in KA levels were monitored for 4 h. Dialysate samples were analyzed as described above for calibration.

3. Results and discussion

3.1. Fluorescence studies

To find the appropriate wavelength for use with CE–LIF, fluorescence studies were carried out using a spectrofluorophotometer. Previous LC–FD methods have used excitation and emission wavelengths of λ_{ex}=344 nm, λ_{em}=398 nm [1,3,23,24], and λ_{ex}= 254 nm, λ_{em}=404 nm [25]. Due to the availability of a 325 nm He–Cd laser in our laboratory, a fluorescence excitation study was done to investigate the applicability of this wavelength for excitation of the KA–Zn(II) complex. The excitation and emission spectra of KA and the KA–Zn(II) complex are shown in Fig. 1. One can see that the excitation spectrum is very broad and there is still a substantial signal at 325 nm, which validates the use of the He–Cd laser in this approach.

When the excitation and emission wavelengths had been established, the optimal concentration of zinc acetate to be added to the kynurenic acid sample was determined. Using a final concentration of 1 μ*M* KA, concentration ratios of Zn/KA from 0–10 000:1 were evaluated. The optimal ratio was determined to be approximately 8000:1 (Fig. 2).

Previous reports indicated that the fluorescence intensity of the KA–Zn(II) complex increased with increasing pH [23]. To verify this, the effect of pH on fluorescence intensity was evaluated over the range 5.0–6.8. The results showed an increase in

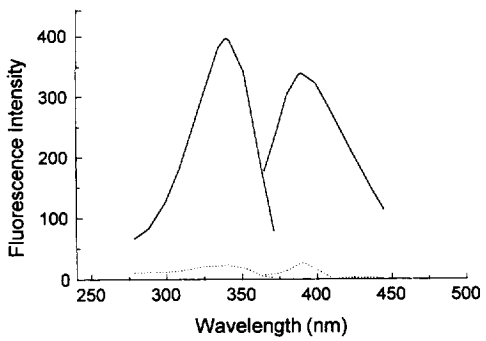

Fig. 1. Fluorescence excitation and emission spectra for KA (dotted line) and the KA–Zn(II) complex (solid line).

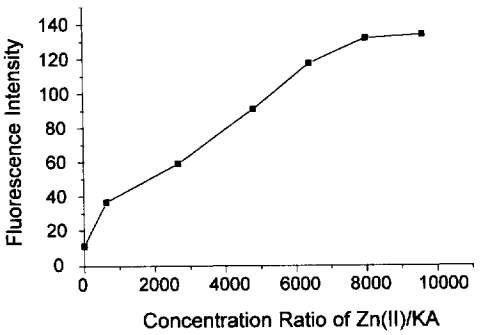

Fig. 2. Fluorescence intensity vs. Zn(II) concentration. Concentration of KA was 1 μ*M*.

fluorescence intensity with higher pH, which agreed with previous reports (Fig. 3). The maximum fluorescence was obtained at pH 6.8. At higher pH values, Zn(II) precipitated out of solution.

The rate of complex formation was also determined. It was found that the fluorescence intensity reached a maximum at t_0 (15 s), confirming that the complexation reaction is almost instantaneous. In addition, the fluorescence was found to be stable over the hour-long time scan.

3.2. Optimization of the complexation procedure

There have been several reports of the use of CE for the analysis of metal complexes. Pre-column complexation with 1,10-phenanthroline has been used for the determination of iron in rain-, lake- and tap-water [27]. One advantage of the pre-column approach is that the complex is formed prior to the separation. However, this approach is only applicable to complexes that have large association constants or are slow to form. In the second approach, referred to as on-column complexation, a complexing agent is added to the background electrolyte and the complex is produced after the sample is injected. On-column complexation has been used for the determination of metal ions [28,29] and peptides [30] by CE–LIF or CE–ED. This approach has the advantage that the sample is not diluted by the reagent prior to injection. However, complexation and separation conditions may be difficult to optimize simultaneously, and the complex must be stable throughout the entire separation process.

Three approaches to Zn(II) complexation with KA were examined in our attempt to achieve the best possible limits of detection (Fig. 4). Initially, pre-column complexation was investigated by combining zinc acetate (pH unadjusted) and KA in a sample vial prior to injection onto the capillary. The complex formed immediately and, therefore, could be directly injected onto the CE capillary. No difference in peak height was observed when additional reaction time was allowed for further complexation. Under these conditions, it was possible to detect free KA and KA–Zn(II) as separate peaks when KA was in excess. This confirmed that the complex was indeed being formed and could be separated from

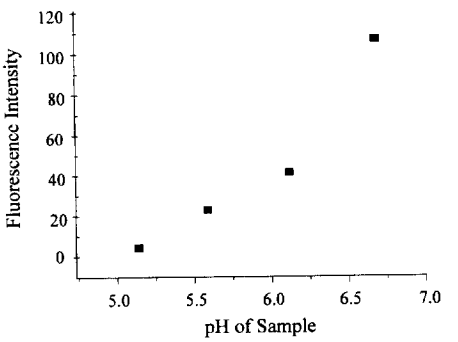

Fig. 3. Effect of pH on fluorescence intensity. Final concentrations of KA and Zn(II) were 1 μ*M* and 8 μ*M*, respectively.

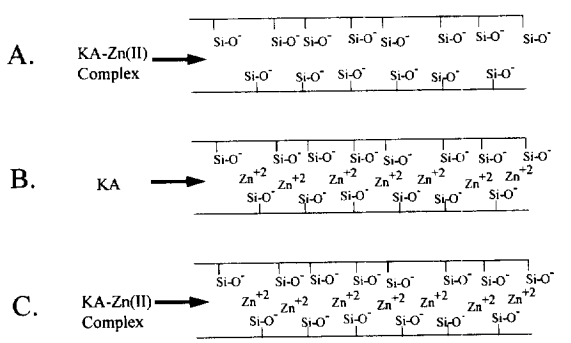

Fig. 4. Schematic of the three methods of zinc complexation investigated: (A) pre-column; (B) on-column and (C) pre-column complexation with a Zn(II) BGE.

KA by CE. The limit of detection (LOD) using this approach was approximately 800 n*M* for KA. Since the basal levels of KA in rat brain are much lower than 800 n*M*, additional complexation methods were investigated.

Next, on-capillary complexation was evaluated by including zinc acetate in the BGE. Equilibration of the zinc acetate-containing BGE with the fused-silica capillary results in complexation of the Zn(II) with the free silanol groups. KA was injected onto the capillary, and complexation occurred during the separation. Concentrations of zinc acetate in the BGE from 35–75 m*M* were studied; however, no significant change in peak height was seen. The peak height was also constant over the BGE pH range 5.0–6.0. At Zn(II) concentrations above 65 m*M* and pH values above 6.0, bandbroadening due to Joule heating became a problem; therefore, a BGE of 40 m*M* zinc acetate at pH 5.5 was employed for future studies. Using this approach, the LOD was decreased by approximately one order of magnitude to 100 n*M*; however, this was still not adequate for detecting basal levels of KA in vivo.

The limitations of these first two approaches may be that the KA–Zn(II) complex dissociates during the CE separation due to the equilibrium between free and bound ligand. Ultimately, the best method of complexation was determined to be a combination of these two approaches; the sample was first complexed pre-column by the addition of Zn(II) to the sample, and zinc was included in the BGE to stabilize the complex as it traveled toward the detector. Since the pH and concentration of the zinc acetate BGE were previously optimized for the on-column approach, 40 m*M* zinc acetate, pH 5.5, was utilized once again.

Utilizing on-column complexation and a Zn(II)-containing BGE, a limit of detection of 1 n*M* was achieved for KA diluted from the basic stock solution with water. This is close to the detection limit of 0.3 n*M* obtained by LC–FD [24]. However, when the stock solution of KA was diluted with ACSF rather than water, the LOD was significantly increased due to anti-stacking [31] and the high chloride ion content. For KA dissolved in ACSF, the LOD was 50 n*M*. Fig. 5 shows the effect of the sample matrix on the peak height for the KA–Zn(II) complex.

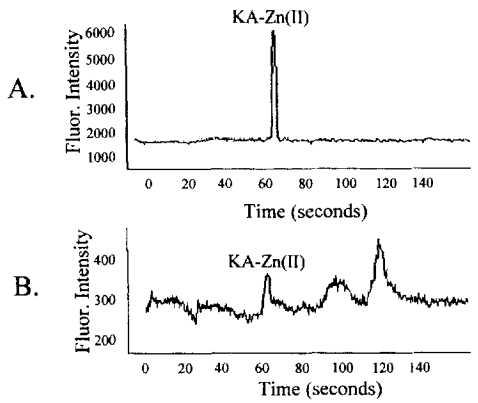

Fig. 5. Comparison of 75 n*M* KA diluted with (A) water and (B) ACSF. A final concentration of 800 μ*M* zinc acetate, pH unadjusted, was added to both samples. The decreased sensitivity in (B) is due to anti-stacking and the high chloride ion concentration.

3.3. *In vivo studies*

The next step was to evaluate the methodology for the determination of KA in vivo. Under physiological conditions, KA is essentially unable to penetrate the blood–brain barrier [32]. Its presence in the brain is therefore dependent upon its bioprecursor kynurenine, a major peripheral tryptophan metabolite, which can access the brain via the large neutral amino acid transporter [32]. The biosynthesis of KA from kynurenine is accomplished by kynurenine aminotransferase [24,33].

Although the mechanisms that regulate the ECF concentrations of KA in mammalian brain have not been elucidated, it has been shown that these KA levels can be manipulated experimentally. For example, the concentration of KA in striatal ECF is dramatically increased following peripheral and cerebral administration of its precursors, kynurenine and tryptophan [24]. In one study, a 1300-fold increase in ECF levels of KA was realized following i.p. administration of kynurenine and probenecid, a known inhibitor of organic acid transport [1]. These increases in KA could be blocked by central application of aminooxyacetic acid [24,33], veratridine [33] or glutamate [33].

To evaluate this methodology, kynurenine and tryptophan were administered i.p. to a rat, and their concentrations in the ECF were monitored by mi-

crodialysis. Using a flow-rate of 500 nl/min, the in vitro microdialysis probe recovery was 88±3.1% (n=2), which was considerably higher than that obtained by Swartz et al. [24] (11.3±0.4%, n=10) for LC studies. Unless noted, results have not been corrected for recovery. It was anticipated that the maximum concentration of KA in vivo would not exceed 1 μ*M*; therefore, the final concentration of Zn(II) in the sample was 8 m*M* to ensure the excess of Zn(II) ions needed for complete complexation.

Although basal levels of KA could not be detected in these studies, its increase following dosing with tryptophan and kynurenine could be monitored. In the first in vivo experiment, tryptophan (75 mg/kg) in saline was administered by i.p. injection. Microdialysis sampling was used to monitor KA levels in the hippocampus at 10 min intervals over an 8-h period (Fig. 6). A maximum concentration of 300 n*M* KA in the dialysate was reached at approximately 6 h.

Following a 100 mg/kg i.p. injection of tryptophan, Swartz et al. [24] also observed an 8–10-fold increase in striatal ECF KA at 6 h, which corresponds to a maximum concentration of ca. 150 n*M* (corrected for recovery of the probe). In our study, a smaller dose (75 mg/kg i.p.) of tryptophan produced a maximum hippocampal ECF concentration of KA of 345 n*M* (corrected for in vitro recovery) at approximately the same time after dosing.

In a second study, kynurenine (75 mg/kg) in saline was injected i.p. and the concentration of KA in rat hippocampus was monitored for 4 h (Fig. 7). In this case, the maximum concentration was reached at 3 h after dosing. With an identical i.p. dose of 75 mg/kg kynurenine, Swartz et al. [24] observed a maximum striatal concentration of KA at 2–2.5 h, which corresponded to 463 n*M* (corrected for recovery). In our studies, a similar maximum hippocampal ECF concentration of KA of 455 n*M* (corrected for in vitro recovery) was obtained. Fig. 8 shows electropherograms obtained before and after dosing with both kynurenine and tryptophan.

The CE methodology presented here has two potential advantages over the LC approach. The first

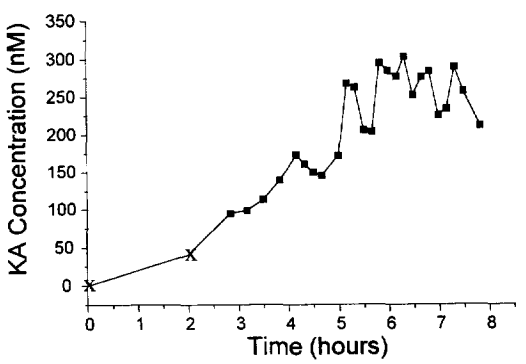

Fig. 6. Monitoring the increase of KA following an i.p. bolus dose of tryptophan (75 mg/kg). Points represented by an × signify dialysate samples with KA concentrations below the limit of detection for the current method.

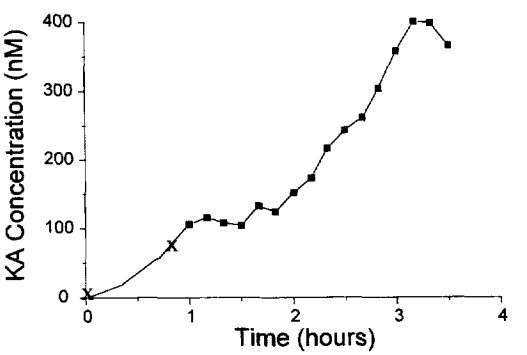

Fig. 7. Monitoring the increase of KA following an i.p. bolus dose of kynurenine (75 mg/kg). Points represented by an × signify dialysate samples with KA concentrations below the limit of detection for the current method.

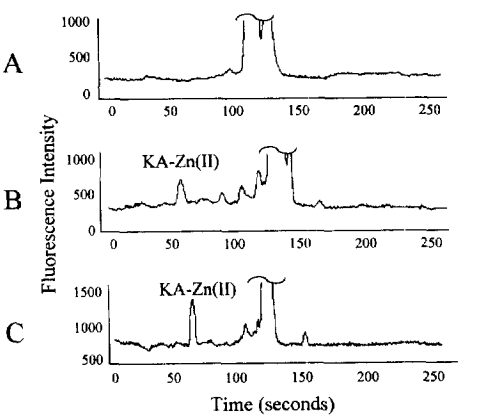

Fig. 8. Electropherograms (A) before dosing, (B) 6 h after a 75 mg/kg i.p. bolus dose of tryptophan and (C) 3 h after a 75 mg/kg i.p. bolus dose of kynurenine.

is the shorter analysis time. Using LC, KA had a retention time of 4.3 min [24], while in the CE method used for these studies, it had a migration time of just over 60 s. The second advantage is the small injection volumes needed, which make it possible to use slow flow-rates to obtain higher recoveries without sacrificing temporal resolution. The temporal resolution using the CE–LIF methodology was 10 min, while 30–60 min sampling intervals were used with LC [24]. If quantitation of additional neurotransmitters is desired, it is possible to analyze the same sample several times by other CE based methods without appreciable loss in volume.

In this example, 5 μl samples were analyzed, primarily because of the inability to achieve reproducible off-line injections of smaller volumes. However, the temporal resolution could be improved if the microdialysis system were coupled on-line to the CE–LIF system, which would permit reproducible analyses of submicroliter samples [13,14,16]. The temporal resolution that can be achieved with an on-line microdialysis–CE system is limited by the time needed to achieve the separation rather than the sample volume required for injection onto the CE system. Therefore, if such a configuration were employed, the sampling time intervals of the in vivo study could be significantly reduced.

A major disadvantage of the CE method is the mass detection limits. With LC, 30–50 μl could be injected on the column, resulting in a substantially larger mass loading and lower LODs. Another drawback of CE is that the injection is much more affected by the high ionic strength buffers. Future work will concentrate on the development of on-line preconcentration and desalting techniques that are compatible with CE–LIF and will bring the LODs closer to those of LC–FD.

4. Conclusions

In this report we have demonstrated the use of microdialysis in combination with Zn(II) complexation and CE–LIF for monitoring KA in vivo. Due to problems associated with the ionic strength of the sample matrix, KA could not be detected at physiological levels, but its increase could be monitored following i.p. dosing of the precursors tryptophan and kynurenine. One of the greatest potential advantages of this methodology compared to those currently in use is the increased temporal resolution. Microdialysis samples could be analyzed every 10 min, even when low flow-rates were used to increase analyte recovery through the probe. Future work will focus on improving the limits of detection for KA in a microdialysate sample matrix, which will greatly increase the usefulness of this technique. Increasing the temporal resolution through the on-line coupling of the microdialysis and CE–LIF systems will also be investigated.

Acknowledgments

This research received financial support from the National Institutes of Health Small Business Innovative Research (SBIR), grant 1 R43GM52272-01; the National Science Foundation EPSCoR; the Center for Bioanalytical Research (CBAR) and Bioanalytical Systems (BAS). The authors thank Dr. Malonne Davies, Dr. Hong Zuo and Dr. Kathy Kostel for technical assistance and Nancy Harmony for assistance in the preparation of this manuscript.

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