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Determination of tryptophan and kynurenine in brain microdialysis samples by capillary electrophoresis with electrochemical detection

Michael A. Malone[a,1], Hong Zuo[a], Susan M. Lunte[a,*], Malcolm R. Smyth[b]

[a]*Center for Bioanalytical Research, University of Kansas, Lawrence, KS 66047, USA*

[b]*School of Chemical Sciences, Dublin City University, Dublin, Ireland*

Abstract

Capillary electrophoresis with electrochemical detection (CEEC) is evaluated for the determination of tryptophan and kynurenine in microdialysis samples obtained from rat brain. These compounds were separated from all other electroactive metabolites of tryptophan. Limits of detection for both compounds were in the low attomole range. The response was linear for kynurenine between 4.9 and 980 fmol injected with a correlation coefficient of 0.9992 ($n = 12$). The system was evaluated for monitoring tryptophan and kynurenine in the extracellular fluid of the rat brain following systemic administration of tryptophan.

1. Introduction

Microdialysis is a sampling technique which allows continuous monitoring of substances from the extracellular space of tissue and organs with minimal perturbation of the physiological system [1,2]. An advantage of microdialysis sampling is that samples are protein-free and are therefore amenable to direct injection into the analytical system. In microdialysis, recovery of analyte through the probe increases with decreasing flow-rate; therefore, rates of 1 μl/min or less are typically employed. In order to obtain the best temporal resolution in microdialysis, highly sensitive techniques capable of analyzing small sample volumes are required.

Capillary electrophoresis (CE) is a highly efficient technique for the separation of charged analytes [3]. The integration of microelectrochemical detectors with capillary electrophoresis results in a highly efficient and sensitive system capable of analyzing ultrasmall sample volumes. The utility of capillary electrophoresis–electrochemistry (CEEC) has been demonstrated in the past for easily oxidizable compounds [4–6] and more recently for easily reducible compounds [7]. The latest advances in this field were recently reviewed by Ewing et al. [8]. CEEC has been employed for the analysis of amino acids and L-DOPA in microdialysis samples obtained from brain and blood, respectively [9,10].

In recent years there has been a surge of interest in the kynurenine pathway [11,12]. This pathway gives rise to a set of metabolites which account for approximately 90% of tryptophan metabolism in mammals. One of these,

* Corresponding author.

[1] Present address: Ciba-Geigy Ltd., Basel CH 4002, Switzerland.

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kynurenic acid, is believed to act as a non-selective antagonist of excitatory amino acid receptors and to attenuate the neuronal excitation induced by agonists of N-methyl-D-aspartate (NMDA). Basal levels of kynurenic acid have been reported to be in the low n*M* range in the extracellular fluid (ECF) of rats and slightly higher in humans [13,14]. It has been shown that kynurenic acid cannot cross the blood–brain barrier and, therefore, all kynurenic acid present in brain tissue is generated from kynurenine. Approximately 60% of kynurenine enters the central nervous system through the blood–brain barrier; the remaining 40% is synthesized directly in the brain from L-tryptophan [15].

The purpose of the present work was to evaluate the use of CEEC for the determination of compounds of the kynurenine pathway in brain microdialysis samples. The method was used to monitor both tryptophan and kynurenine in the brain following intraperitoneal (i.p.) administration of tryptophan.

2. Experimental

2.1. Reagents

Tryptophan, kynurenine, kynurenic acid, anthranilic acid, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, 5-hydroxyanthranilic acid, and xanthurenic acid were all purchased from Sigma (St. Louis, MO, USA) and used as received. All solutions were prepared in NANOpure water (Sybron-Barnstead, Boston, MA, USA) and passed through a membrane filter (0.2 μm pore size) before use. The CE separation buffer consisted of 20 m*M* sodium borate (pH 9) unless otherwise indicated. If a higher pH were required, the solution was adjusted with sodium hydroxide. All stock solutions were prepared daily in water and stored at 4°C.

2.2. Apparatus

The CEEC system and the construction of the Nafion decoupler which allows on-column electrochemical detection have been described elsewhere [16]. Electrophoresis was driven by a high voltage supply (Spellman Electronics, Plainview, NY, USA). Polyimide-coated fused-silica capillaries (360 μm O.D., 50 μm I.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA) and capillary lengths 70–90 cm were used. Sample introduction was accomplished using a laboratory-built pressure injection system. The injection volume was calculated to be 9.8 nl using the continuous fill mode by recording the time required for the sample to reach the detector. A Spectra-Physics (Data Jet) integrator (San Jose, CA, USA) was used for data acquisition.

Cylindrical carbon fiber microelectrodes were constructed using 33 μm diameter fibers (AVCO Specialty Products, Lowell, MA, USA). The fiber was bonded to a length of copper wire using silver epoxy (Ted Pella, Redding, CA, USA). Capillary tubes were pulled to a narrow tip with a Liste-Medical Model 3A vertical pipette puller (Greenvale, NY, USA). The carbon fiber was then inserted through the capillary until it protruded ca. 0.5 cm from the tip. UV-cure-glue (UVEXS, Sunnyvale, CA, USA) was applied to the tip at the junction of the capillary and the carbon fiber. The fiber was drawn back until the desired length (150–300 μm) protruded and sealed with Black and Decker thermogrip glue to fix the copper connecting wire in place.

2.3. Cyclic voltammetry

All cyclic voltammetry experiments were carried out using a Model CySY-1 computerized electrochemical analyzer (Cypress Systems, Lawrence, KS, USA). A three-electrode cell consisting of a carbon fiber or glassy carbon working electrode, a Ag/AgCl reference electrode and a platinum auxiliary electrode was used. All cyclic voltammograms were carried out in 20 m*M* sodium borate buffer (pH 9) using a scan rate of 100 mV s^{-1}.

2.4. Microdialysis

Male Sprague-Dawley rats (290–400 g) were anesthetized with urethane (1.5 g/kg i.p.) and maintained under anesthesia during the entire

experiment. The rats were placed in a stereotaxic frame on a heating pad at 37°C. CMA/12 3-mm dialysis probes (Bioanalytical Systems, West Lafayette, IN, USA) were implanted in the hippocampus of the rat brain at the coordinates 4.8 mm posterior to bregma, 4.8 mm lateral to the midline and 5.8 mm ventral to the skull surface.

The microdialysis probes were perfused with artificial cerebrospinal fluid (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* $NaHPO_4$) at a flow-rate of 250 nl/min. Probes were calibrated in vitro by placing them in a standard mixture of the tryptophan metabolites. The dialysates were collected and analyzed every 15 min and the relative recovery was calculated for each compound. All dialysates were directly injected into the CEEC system.

2.5. Tryptophan loading studies

Tryptophan (100 mg/kg i.p.) was systemically administered to rats. Baseline fractions were collected beginning ca. 3 h after probe implantation. At least four 15-min fractions were collected before any manipulation was attempted. Microdialysates were collected for 6 h following administration of tryptophan.

3. Results and discussion

3.1. Cyclic voltammetry

Cyclic voltammograms of tryptophan and its metabolites were obtained using both glassy carbon and carbon fiber electrodes. As can be seen from Table 1, the tryptophan metabolites exhibited a wide range of half-wave potentials. Kynurenic acid exhibited a very high oxidation potential (+ 1015 mV), and, in fact, was previously reported to be non-electroactive [17]. On the other hand, 3-hydroxykynurenine, 3-hydroxyanthranilic acid and 5-hydroxyanthranilic acid all exhibited very low oxidation potentials (as low as + 100, + 160 and +95 mV, respectively).

Table 1
The half-wave potentials of the tryptophan metabolites using a glassy carbon electrode

Compound	Half-wave potential(s) (mV) (vs. Ag/AgCl)
Tryptophan	590
Kynurenine	760
3-Hydroxykynurenine	100
Anthranilic acid	690
3-Hydroxyanthranilic acid	160 and 830
5-Hydroxyanthranilic acid	95
Kynurenic acid	1015
Xanthurenic acid	460

Experimental conditions given in Fig. 1.

One important consideration when using CEEC with a carbon fiber microelectrode is that the background current increases significantly at potentials above + 950 mV. Fig. 1 compares electropherograms obtained at + 900 and + 1000 mV. No response is obtained for kynurenic acid (Fig. 1A) at + 900 mV. At + 1000 mV kynurenic acid was detectable; however, an appreciable change in the quality of the baseline is evident (Fig. 1B). This large increase in background and baseline drift significantly decreases the *S/N* and makes it impossible to detect kynurenic acid at physiologically relevant levels in brain microdialysis samples. Since we were interested in the levels of the kynurenic acid precursors tryptophan and kynurenine in microdialysis samples, all subsequent studies employed a working potential of + 900 mV.

3.2. Separation optimization

Separation of all the compounds involved in the kynurenine pathway by CE was investigated. Initially, a 10 m*M* MES buffer at pH 7 was employed. However, both tryptophan and kynurenine migrated with the system (neutral) peak at this pH. If the buffer were changed to 10 m*M* sodium borate at pH 9, tryptophan and kynurenine were resolved from the system peak. Resolution could be further improved by increasing the ionic strength to 20 m*M*. Under these conditions, all the tryptophan metabolites were

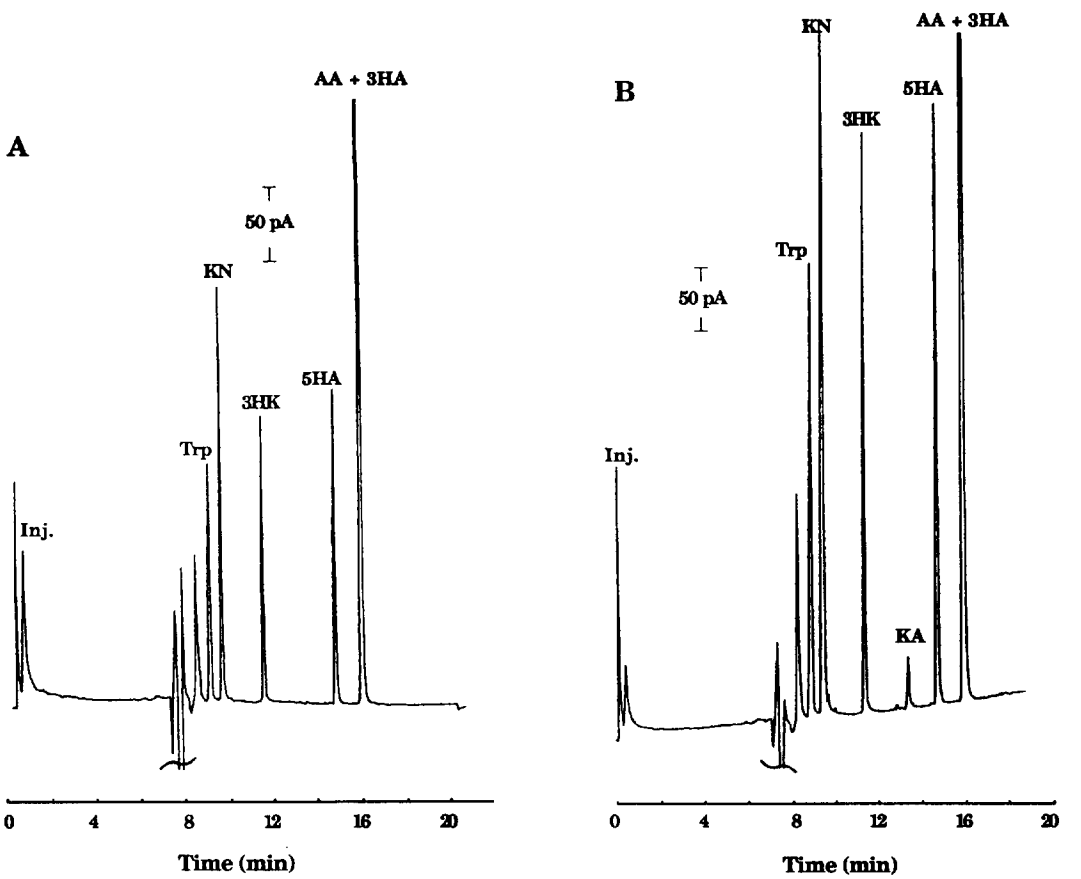

Fig. 1. Electropherograms of a standard mixture of tryptophan metabolites containing 1 μM each of tryptophan (Trp); kynurenine (KN); 3-hydroxykynurenine (3HK); anthranilic acid (AA); 3-hydroxyanthranilic acid (3HA); 5-hydroxyanthranilic acid (5HA) and 2 μM kynurenic acid (KA). Working potential of; (A) + 900 mV and; (B) + 1000 mV were applied. Separation conditions: 20 mM borate buffer, pH 9; capillary length, 80 cm, 50 μm I.D.; 20 kV.

resolved except anthranilic acid and 3-hydroxyanthranilic acid, which comigrated. These two compounds could be resolved at pH 9.6, but the overall separation efficiency was decreased. Xanthurenic acid exhibited an extremely long migration time at pH 9 due to its large negative electrophoretic mobility. Therefore, it is not shown in the electropherogram. A comparison of the separation at pH 9 and 9.6 is shown in Fig. 2.

3.3. Analytical characterization

Because kynurenine is important as a precursor to kynurenic acid, it was decided to use pH 9 for the separation of tryptophan and kynurenine in microdialysis samples. The limits of detection for all of the relevant tryptophan metabolites were determined at a detector potential of + 900 mV. A summary of the results is presented in Table 2. Very low limits of detection (LOD), generally in the attomole region, were obtained for most compounds. The LOD for kynurenic acid (determined at 1000 mV) was ca. 100 × higher than for the other compounds. As mentioned previously, this is a function of the higher baseline noise at the higher oxidation potential. In addition, + 1000 mV is not yet at the current-limiting plateau for kynurenic acid, so the response is not optimal.

The system was also evaluated for linearity and reproducibility. The detector response for kynurenine was linear between 0.5 and 100 μM

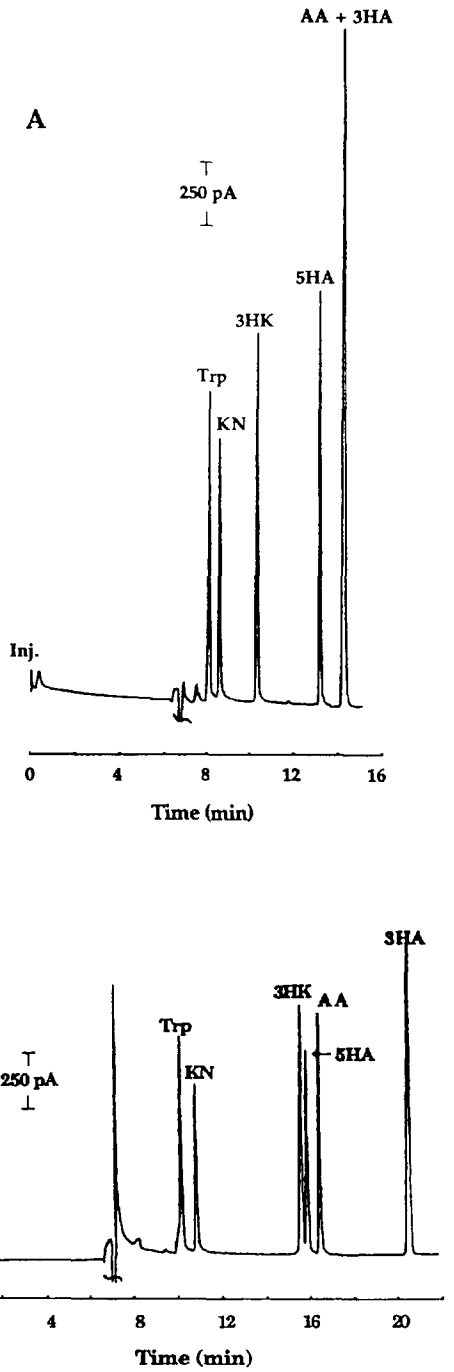

Fig. 2. Electropherograms of a standard mixture of tryptophan metabolites containing 5 μM each of Trp, KN, 3HK, AA, 3HA and 5HA. The electropherograms were run in a 20 mM sodium borate buffer at: (A) pH 9; and (B) pH 9.6. A working potential of +900 mV was used. All other conditions as in Fig. 1.

Table 2
Concentration and mass limits of detection of the tryptophan metabolites using CEEC

Compound	Concentration limit detection (nM)	Mass limit of detection (amol)
Tryptophan	4.8	46.0
Kynurenine	3.1	30.0
3-Hydroxykynurenine	0.4	4.2
Anthranilic acid	3.3	32.6
3-Hydroxyanthranilic acid	6.0	58.8
5-Hydroxyanthranilic acid	0.2	1.8
Kynurenic acid	22.2	218
Xanthurenic acid	0.6	5.9

(equivalent to 4.9–980 fmol injected) with a slope of 0.22 μM/nA and a regression coefficient of 0.9992 ($n = 12$). The reproducibility was determined by making repetitive injections of 10 μM (98 fmol injected) kynurenine. The R.S.D. was 4.54% ($n = 11$). No electrochemical activation of the carbon fiber was performed between injections since no passivation of the carbon fiber was evident following continuous use. However, electrochemical activation of the carbon fiber was necessary prior to initial use. Failure to do this resulted in slower electrode kinetics, which was evident in the cyclic voltammetric studies.

3.4. Analysis of rat brain dialysate using CEEC

In order to evaluate this method for monitoring tryptophan metabolites of the kynurenine pathway, the effect of intraperitoneal administration of tryptophan on the concentration of kynurenine in the ECF of the rat brain hippocampus was studied. In these studies, a very slow perfusion flow-rate of 250 nl/min was employed, resulting in high recovery values for tryptophan (72.5%, $n = 5$). This is much higher than the previously reported recovery of 19% using a perfusion flow-rate of 2 μl/min and LC analysis [18]. Recovery values for the other metabolites were also higher: kynurenine, 83.8%; 3-hydroxykynurenine, 43.2%; 5-hydroxykynurenine, 34.8%; and anthranilic acid, 62.4%.

It was found that immediately upon implantation of the microdialysis probe, the level of endogenous substances in the perfusate was high. Therefore, the probe was perfused for a period of at least 3 h prior to sampling, after which fractions were collected for an additional hour at 15-min intervals. Once the levels were determined to be steady, 100 mg/kg of tryptophan was administered i.p. The microdialysis experiment was continued for a period of 6 h.

Fig. 3 compares an electropherogram of dialysate samples collected (A) before tryptophan loading and (B) near the maximum ECF concentration of tryptophan. The increase of tryptophan and, subsequently, kynurenine in the ECF is clearly illustrated. It was found that the tryptophan concentration increased three- to four-fold following loading, and reached a maximum concentration of 2–3 μM ($n = 3$) at ca. 90 min after administration (indicated by arrow). This can be seen from Fig. 4A. The primary metabolite, kynurenine, reached a maximum concentration of 1.3 μM ($n = 3$) approximately 60 min after the maximum was reached for tryptophan, as can be seen in Fig. 4B.

Tryptophan and kynurenine were identified based on comigration with an authentic standard. The peaks are somewhat broader in the electropherograms of the microdialysate samples than in the electropherograms of the standards due to the discrepancy in ionic strength and pH between the dialysate samples (pH 7, 120 mM) and the run buffer (pH 9, 20 mM). Voltammetric characterization was also used to verify the identity of the kynurenine peak. This was accom-

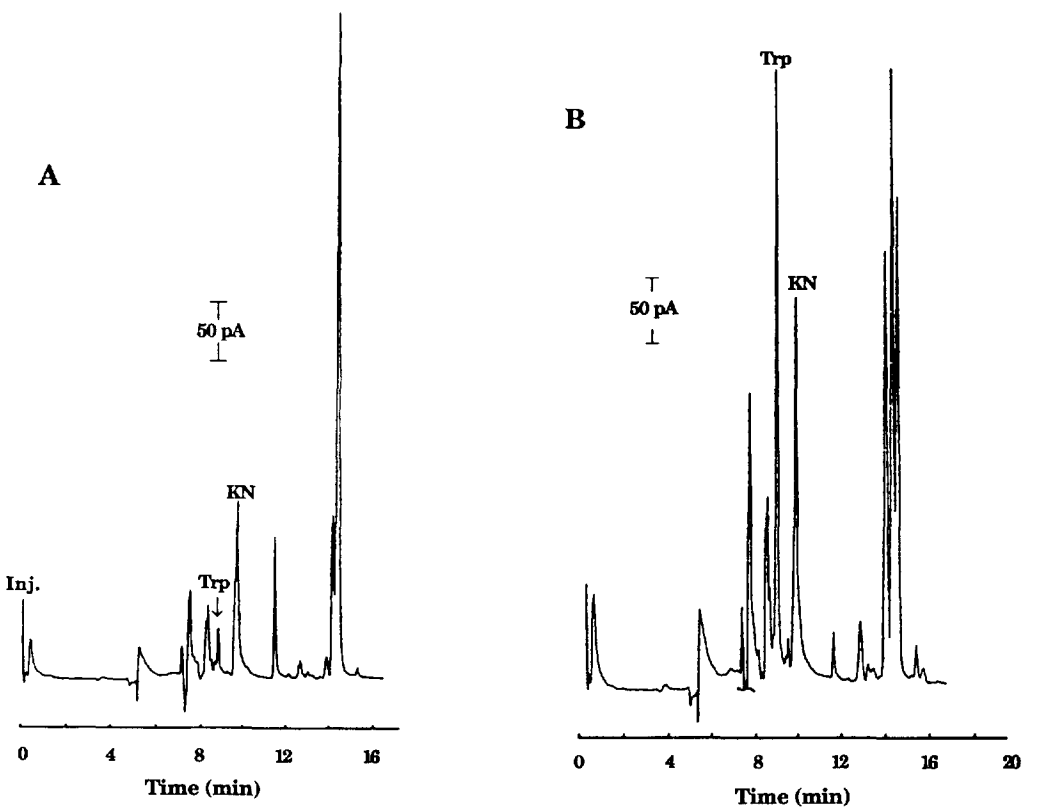

Fig. 3. Electropherograms of in vivo microdialysis samples taken from the hippocampus region of the brain of an anesthetized rat. (A) Dialysate sample taken before tryptophan administration; and (B) dialysate sample taken after tryptophan administration (100 mg/kg i.p.) near the maximum ECF concentration of Trp. The figure demonstrates the increase in both Trp and KN concentrations. Working potential = + 900 mV. Other conditions as in Fig. 1.

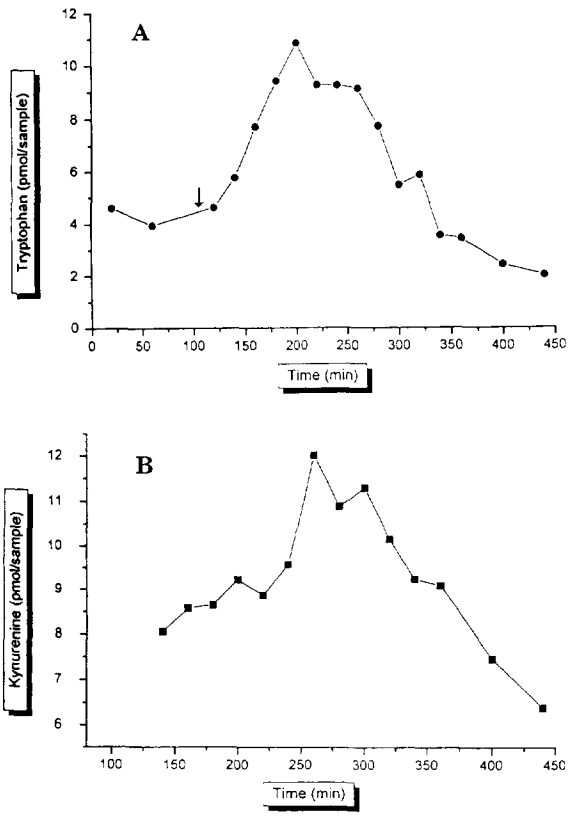

Fig. 4. A plot of ECF concentration vs. time for (A) tryptophan and (B) kynurenine for a tryptophan-loading experiment (100 mg/kg i.p.). The arrow indicates the time of Trp loading. Working potential +900 mV. All other conditions as in Fig. 1.

plished by calculating the current ratio of +800/+850 mV. The ratios were 0.65 and 0.62 for the sample and standard, respectively. In this particular set of experiments, the other tryptophan metabolites were not quantitated. However, it can be seen in Fig. 4B that the levels of 5-hydroxyanthranilic acid and 3-hydroxyanthranilic acid also increased.

4. Conclusions

In this report we have demonstrated the use of CEEC for monitoring in vivo microdialysis samples for kynurenine and tryptophan. The high sensitivity and low volume requirements of this technique make it possible to monitor these compounds with good temporal resolution and high recoveries. Future studies will focus on the development of improved methods for the separation and detection of the other tryptophan metabolites as well as alternative methods of detection for kynurenic acid.

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