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Journal of Chromatography A, 914 (2001) 293–298

JOURNAL OF CHROMATOGRAPHY A

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Application of capillary electrophoresis with laser-induced fluorescence detection to the determination of biogenic amines and amino acids in brain microdialysate and homogenate samples

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Abstract

A procedure is described to derivatize 16 primary-amine-containing biogenic amines and amino acids in brain mixtures with the fluorogenic reagent 5-furoylquinoline-3-carboxaldehyde (FQ). These FQ-tagged compounds in the brain sample were resolved in less than 16 min based on micellar electrokinetic chromatography and laser-induced fluorescence. There was a linear relationship between the concentration of analyte and the fluorescence intensity, with correlation coefficients in the range of 0.96–1.00. The utility of this method for the quantification of the important inhibitory neurotransmitter γ-aminobutyric acid in microdialysates and brain homogenates from rats is illustrated.

Keywords: Derivatization, electrophoresis; Biogenic amines; Amino acids

1. Introduction

One of the major challenges for neuroscience researchers is to determine the relationship between brain structure and function [1]. Because neurotransmitters, several of which are amino acids or biogenic amines, play an important role in brain function, a better understanding of the chemical processes in the brain could lead to improved treatment of neurological and psychiatric disorders. Of the various analytical techniques currently in use, high-performance liquid chromatography (HPLC) has been widely applied with electrochemical or fluorescence detection [2–4]. However, the use of HPLC for analysis of neurochemicals in brain samples suffers from some disadvantages. The fluorescence methodology usually involves a tedious derivatization process. Electrochemical detection has relatively restricted concentration limits and not every compound is electrochemically sensitive. Furthermore, because many samples of interest come directly from living animals, the sample volumes are quite small and the concentrations of analytes are often low. Microbore liquid chromatography has been compared with conventional HPLC (250 mm× 4.6 mm I.D.) for analysis of neurotransmitters [5], but no resolution advantage was gained by the use of narrow bore columns compared to conventional columns [5,6].

Capillary electrophoresis (CE) with laser-induced

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)00539-8

fluorescence (LIF) is another choice for neurotransmitter measurement. LIF provides extremely high sensitivity, with detection limits approaching the single-molecule level [7,8]. This exquisite sensitivity makes CE–LIF an ideal technique for the analysis of biological samples, such as microdialysates from discrete brain areas, whose absolute amounts are very small. Also, the theoretical plate number of CE (10^5–10^6) is usually much higher than that of HPLC (10^3–10^4) [9]; this higher column efficiency allows for better resolution, which in some cases can result in shorter analysis times. This higher resolving power is also quite suitable for analysis of multiple compounds in biological samples. Last, the CE technique requires very low sample volumes, thereby allowing faster sampling rates to facilitate acquisition of dynamic pharmacological information.

CE with electrochemical detection has been applied to the determination of the catecholamine dopamine [10,11] and several excitatory amino acids [12]. Recently, several CE–LIF studies have been reported. Guzman and co-workers first analyzed glutamic acid and glutamine in brain microdialysis by CE–LIF [13–15]. The results showed that it was possible to label nanomolar solutions of several amino acids with the fluorescent dye fluorescein isothiocyanate and detect them with CE–LIF. Later, eight naphthalene-2,3-dicarboxaldehyde (NDA)-tagged amino acids in a rat brain homogenate [16] and 3 - (4 - carboxybenzoyl)quinoline - 2 - carboxaldehyde (CBQCA) derivatized amino acids in microdialysate samples [17] were separated and determined. In the past 4 years, in vivo monitoring of amino acids by microdialysis on-line coupled to CE–LIF has been developed [18,19]. Those results have shown high resolving power, good detection limits, and low sample volume requirements. However, the application of CE–LIF in neuroscience still needs further improvement. A major challenge is to increase the sensitivity and resolution of the analytical technique. Many compounds are not resolved and some of them are not detectable in brain samples with current analytical methods [19].

The goal of the present work was to develop a high-sensitivity analytical method for important biogenic amines and amino acids in brain samples, including brain microdialysates and tissue homogenate supernatants. After pre-column labeling with the fluorogenic reagent 5-furoylquinoline-3-carboxaldehyde (FQ), 16 neurochemicals containing primary amino groups were separated with CE–LIF in different sample matrices. The separation was based on a micellar electrokinetic chromatography (MEKC) buffer system. Biogenic amines and amino acids were resolved and detected in a single run and identified by co-migration with standards. Furthermore, we report the accuracy, precision, and linearity of the technique. The technique was then used to determine changes in the concentration of γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain, in rat brain samples after administration with the antidepressant/antipanic drug phenelzine (PLZ). The findings on GABA in the brain samples after drug treatment were similar to those found using HPLC. Minimal work-up procedures were involved and as little as 1 μl of sample was required for analysis.

2. Experimental

2.1. Reagents

All aqueous solutions were made with deionized water obtained from a Milli-Q system equipped with a 0.2-μm filter. Artificial cerebrospinal fluid (CSF) was made as 145.0 m*M* NaCl (Fisher, Edmonton, Canada), 3.0 m*M* KCl (Sigma, St. Louis, MO, USA), 1.5 m*M* $CaCl_2$ (BDH, Toronto, Canada), 1.0 m*M* $MgCl_2$ (Fisher), 2.0 m*M* NaH_2PO_4 (BDH), 2.0 m*M* Na_2HPO_4 (Fisher), and 2 m*M* dextrose (Fisher). The pH was adjusted to 7.3 using hydrochloric acid (Fisher).

Sodium dodecyl sulfate (SDS) was purchased from BDH. The amino acids, biogenic amines, and internal standard (*O*-methyl-L-threonine) were obtained from Sigma. Rhodamine 6G and β-cyclodextrin hydrate (β-CD) were provided by Aldrich (Milwaukee, WI, USA). Aqueous stock solutions of amino acids and biogenic amines were prepared at a concentration of 1 mg/ml; these solutions were diluted daily to the working concentrations with artificial CSF buffer before labeling. The final concentrations of amino acids and biogenic amines before injection varied from 10^{-6} to 10^{-4} g/l (about 10^{-8}~10^{-6} *M*).

FQ and potassium cyanide were from Molecular Probes (Eugene, OR, USA). The 200 m*M* stock solution of potassium cyanide was made in water and diluted to 25 m*M* in 10 m*M* borate buffer (pH 9.2) before derivatization. FQ solutions needed to be dried before storage. A stock solution of 10 m*M* FQ was prepared in methanol, and then 10-μl aliquots were placed into 500-μl microcentrifuge tubes. The solvent was removed under vacuum at room temperature using a Speed Vac (Savant Instruments, Farmingdale, NY, USA). The dried FQ was stored at −20°C, and was used without further treatment on the day of the experiment.

2.2. Instrumentation

The LIF detector and CE system are described elsewhere [20]. Briefly, the samples and standards were injected into a 40 cm×50 μm I.D.×140 μm O.D. or a 47 cm×15 μm I.D.×145 μm O.D. fused-silica capillary column. Light at 448 nm from an argon ion laser (Omnichrome, CA, USA) was used to excite the FQ-labeled amines. The fluorescence was filtered by a bandpass interference filter centered at 630 nm and detected by a photomultiplier tube (PMT) operated at 900 V. A Macintosh computer was used for instrument control, data acquisition and data processing. The signal from the PMT was conditioned with a laboratory-made current-to-voltage converter before digitization by the computer.

2.3. Surgical and sampling procedure for microdialysates

The microdialysis procedures used in the laboratory was described elsewhere [21]. Experiments were performed with male rats (Sprague–Dawley, 200–250 g). Briefly, a permanent intracerebral guide cannula was implanted into the region immediately dorsal to the caudate nucleus. The cannula was secured to the skull with four jeweler's screws and cranioplastic cement. In addition, a wire loop was embedded in the cement to attach the rat to the tether during the microdialysis procedures. A dummy cannula was inserted into the guide cannula to keep it free of debris. The skin was sutured rostral and caudal to the cemented area and the incision was sealed with an adhesive (Vetbond; 3M Animal Care Products). Following surgery, the rats were kept in a warm temperature-controlled environment until recovery from anesthesia.

Each rat was allowed to recover from surgery for at least 4 days and handled for 3 min on two separate occasions before microdialysis. On the day of microdialysis, the rat was placed in a round Plexiglas bowl (BAS) that contained a mixture of clean bedding and bedding from the rat's cage. After 5 min, the rat was attached to the tether. Following a 1-h habituation period, a microdialysis probe (3 mm membrane) was inserted into the guide cannula. The probe was perfused at the rate of 1 μl/min with artificial cerebrospinal fluid. After a 2-h stabilization period, three 25 min baseline samples were collected. Then, the vehicle or the agent of interest was administered intraperitionally (i.p.). Following the injection, 11 25-min samples were collected. The samples were kept on dry ice during the experiment and then transferred to an −80°C freezer for long-term storage.

2.4. Sampling procedure for tissue supernatants

Rats were injected with PLZ (15 mg/kg i.p.) or physiological saline vehicle and groups of five animals were killed by cervical dislocation and immediate decapitation at 4 h after injection. The brains were rapidly removed and homogenized in five volumes of ice-cold methanol, followed by centrifugation (10 000 *g* for 20 min) to remove the protein precipitate.

2.5. Derivatization procedures

Three experiments were conducted as described below. The amount of dye FQ was always in large excess.

In the first experiment, microdialysates were labeled to determine which amino acids and biogenic amines were present [22]. A 4-μl microdialysate sample or biogenic amine standard (10^{-6}~10^{-4} *M*) spiked with 1 μl internal standard (1 mg/ml, equivalent to 7.5 m*M* *O*-methyl-L-threonine) was mixed with 100 nmol dry FQ and 2 μl 25 m*M* KCN (pH 9.2). The mixture was reacted in the dark in a 65°C bath incubator (Fisher) for 16 min and then was diluted 200-fold before injecting in the capillary for

analysis. A blank solution of the same volume was derivatized using the same protocol.

In the second experiment to determine the linearity of the method, different volumes of standards (0, 0.6, 0.9, 1.2, 1.5 μl) and 1 μl internal standard (1 mg/ml, equal to 7.5 m*M* *O*-methyl-L-threonine) were mixed with artificial CSF buffer to a volume of 5 μl, and derivatized to yield 10^{-8}~10^{-6} *M* FQ-tagged amino acid and biogenic amine solutions.

In the third experiment, the concentrations of GABA in rat whole brain tissue homogenate supernatant samples and in striated microdialysates were measured by comparing the migration time and the peak intensity with rhodamine 6G as an internal standard. The samples were analyzed by HPLC at the same time [21].

2.6. Capillary electrophoresis

The running buffer for electrophoresis was 20 m*M* borate and 60 m*M* SDS at pH 9, or 15 m*M* borate, 45 m*M* SDS, and 5 m*M* β-CD at pH 8.5. The samples, blanks, and standards were injected electrokinetically at 100 V/cm for 5 s or 120 V/cm for 3 s; then the separation was performed at 200 or 400 V/cm. After each run, the capillary was flushed with 0.1 *M* NaOH followed by water and running buffer. The peaks for the analytes were identified by matching the migration time with those in the spiked sample and measured by comparing their height-ratios with the internal standard. Data were analyzed using MatLab and a two-point method was used to correct for variations in migration time [23].

3. Results and discussion

3.1. CE analysis of microdialysates

Brain microdialysis is valuable for collecting small amounts of samples from different tissues in freely moving animals [24]. Electropherograms of a brain microdialysate sample and a standard mixture are shown in Fig. 1. There are more than 20 peaks in the dialysate electropherogram; 16 co-migrated with standard FQ-labeled primary amines. This system is suitable not only for the detection of amino acids, but also for important neurotransmitter amines, such as dopamine (DA), 5-hydroxytryptamine (5-HT), and norepinephrine (NE), which are present at considerably lower concentrations than most amino acids.

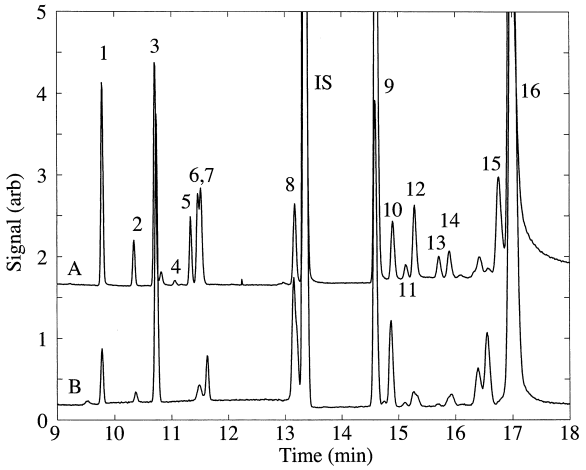

Fig. 1. Typical CE separations of neurotransmitters: (A) 16 biogenic amine or amino acids standards with internal standard (I.S.); (B) microdialysate sample spiked with internal standard. Experimental conditions: 16 min reaction time; 5 s×4 kV injection, 40 cm×50 μm I.D. capillary, E=205 V/cm; PMT voltage=900 V; running buffer 20 m*M* borate and 60 m*M* SDS. Peaks: 1=serine, 2=threonine; 3=glutamine; 4=glutamate; 5=dopamine (DA); 6=histidine; 7=asparagine; 8=alanine; 9=taurine (Tar); 10=aspartic acid; 11=γ-amino butyric acid (GABA); 12=glycine; 13=serotonin (5-hydrozytrptamine) (5-HT); 14=norepinephrine (NE); 15=methionine; 16=β-alanine. Variation in migration time was corrected using a two-point method based on the migration of components 1 and 16 [23].

The separation efficiency was high for this analysis. The number of theoretical plates was typically 120 000 for all peaks, except for peak 16 (β-alanine), which tailed in the electropherogram of the biogenic amine standards.

3.2. Calibration curve

To correct for variations in the injection volume, a modified amino acid, *O*-methyl-L-threonine (7.5 m*M*), was chosen to be an internal standard. This compound is not observed in the brain microdialysates, undergoes the same derivatization reaction as the biogenic amines and amino acids, migrates near the middle of the electropherogram, and does not co-migrate with any components in the brain microdialysates.

Table 1
Linearity of calibration curves for amino acids and biogenic amines[a]

Compound	Peak No. in Fig. 1	R^2	Slope (arbitrary)	Detection limit ($\cdot 10^{-9}$ M)
Ser	1	0.99	1.6	7
Thr	2	0.97	0.2	70
Gln	3	0.98	3	4
Glu	4	0.99	0.9	10
DA	5	0.97	0.15	80
His	6	0.99	10	1
Asn	7	0.98	7	2
Ala	8	1.00	1.4	8
Tar	9	0.99	100	0.1
Asp	10	0.96	2	6
GABA	11	0.98	8	2
Gly	12	0.97	10	1
5-HT	13	0.96	9	1
NE	14	0.96	0.6	20
Met	15	0.98	0.4	30
β-Ala	16	0.99	11	1

[a] Detection limit is defined as that amount of analyte that generates a peak that is three times larger than the noise in the background signal. Standard three-letter abbreviations for amino acids are shown; other abbreviations are listed in the caption to Fig. 1.

Linearity and detection limits are summarized in Table 1. The calibration ranges were selected according to the predicted concentrations in the dialysate samples to be determined. After normalization to the signal from the internal standard, there was a linear relationship between the concentration of analytes and the peak intensity, with correlation coefficients ranging from 0.96 and 1.00. The intercepts of the calibration curves did not significantly differ from zero, except for β-alanine, whose area was difficult to determine because of tailing. Detection limits were typically at the nanomolar concentration levels. Variations in sensitivity appear to be due to differences in reaction rate [22] and injection volume for each component.

3.3. Brain microdialysates and tissue homogenates

The influence of the antidepressant/antipanic drug PLZ on the profile of GABA in rat whole brain homogenates and striatal microdialysates was studied, and the results are shown in Figs. 2 and 3. The concentration of GABA in the brain supernatants increased threefold after drug treatment (Fig. 2), which agrees with previous gas chromatography and HPLC results [21,25,26].

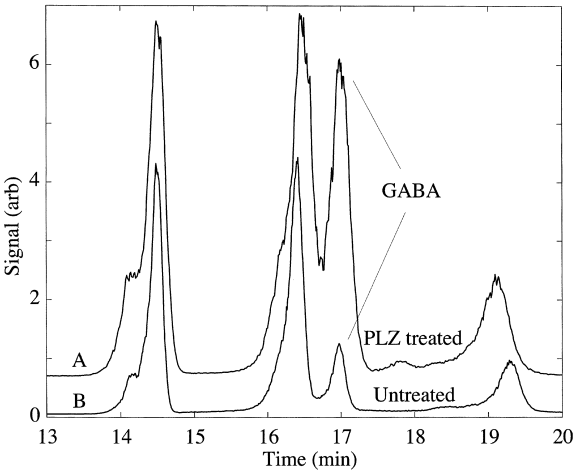

Fig. 2. Analysis of whole rat brain tissue homogenate supernatants for GABA: (A) sample from rat treated with PLZ; (B) sample from vehicle-treated rat with the same protocols as A. A 4-μl volume of 0.1 *M* NaOH was added to the samples, which were reacted 30 min at 65°C. A slightly longer capillary was used to generate this data compared with Fig. 1.

Similar data were obtained from brain dialysates (Fig. 3). The data at 50 and 75 min in Fig. 3

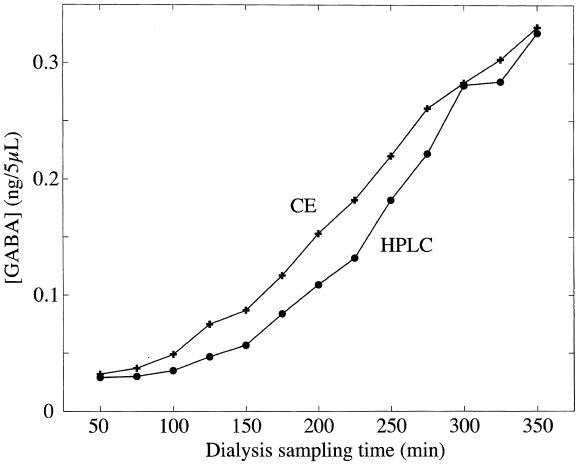

Fig. 3. Comparison of the determination of the concentration of GABA in striatal microdialysates by HPLC and CE. The two baseline samples are in the first 75 min, and the subsequent samples are after PLZ was administered to the rat.

correspond to background levels of GABA; PLZ was administered at 75 min, generating a sixfold increase in GABA levels over a 6-h period. Rhodamine 6G was used as an internal standard in this experiment. The reaction rate of *O*-methyl-L-threonine was different from other amino acids, and its use did not improve precision in this experiment. Rhodamine 6G does not take part in the labeling reaction, and the experimental precision improved by a factor of 2 compared to the use of *O*-methyl-L-threonine. The HPLC and CE results are identical for the initial and final GABA levels, but the CE data are systematically slightly higher for intermediate times. This difference in signal will require further investigation to identify its cause.

4. Conclusions

A rapid CE–LIF method has been developed for the simultaneous determination of several important brain biogenic amines and amino acids in a short time. Minimal work-up procedures and only small quantities of sample were required for analysis. The method generated a linear relationship between the concentration of 16 compounds of interest and the peak intensity. The assay was used for the analysis of ex vivo brain samples, including both brain microdialysates and homogenate supernatants. Results were similar to those obtained by HPLC analysis. The number of theoretical plates is typically 120 000.

Acknowledgements

This project was funded by the Natural Science and Engineering Research Council of Canada (N.J.D.) and the Medical Research Council of Canada (G.B.B.).

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