

Quantitative in Vivo Monitoring of Primary Amines in Rat Caudate Nucleus Using Microdialysis Coupled by a Flow-Gated Interface to Capillary Electrophoresis with Laser-Induced Fluorescence Detection

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A method for monitoring primary amines in vivo using microdialysis coupled on-line with capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) with laser-induced fluorescence detection was explored. Dialysates were derivatized on-line with α -phthalaldehyde/ β -mercaptoethanol and automatically transferred to a separation capillary by a flow-gated interface. Analytes were detected on-column using the 2 mW, 354 nm line of a He–Cd laser for excitation. Dialysis probes were perfused at 79 nL/min, resulting in relative recoveries of nearly 100%, which allowed quantitative monitoring. On-line detection limits were in the 20–50 nM range, and the response was linear up to 50 μ M. Temporal resolution was between 45 s and 3 min and was limited by separation time or broadening of sample zones during transfer to the separation capillary, depending on the operational parameters. The system was applied to measurement of primary amines in the caudate nucleus of anesthetized rats. Using CZE for separation, it was possible to resolve and monitor several compounds, including aspartate and glutamate. The measured basal concentrations of aspartate and glutamate were 1.2 ± 0.1 and 5.0 ± 0.4 μ M, respectively, which agrees well with literature values. Increases in in vivo aspartate and glutamate were monitored with 90 s temporal resolution during K^+ depolarization using dialysis flow rates of 79 nL/min; however, temporal resolution of 45 s was possible at the expense of lower relative recovery if the dialysis flow rate was increased to 155 nL/min. The use of MEKC as the separation mode significantly increased the number of compounds that could be resolved and detected. Using MEKC to separate the dialysate samples allowed aspartate, glutamate, isoleucine, leucine, lysine, methionine, phenylalanine, taurine, tyrosine, and valine to be resolved and detected. The basal concentrations for these compounds using MEKC were 1.9 ± 0.2 , 4.1 ± 0.2 , 4.6 ± 0.7 , 2.6 ± 0.3 , 5.4 ± 0.4 , 1.8 ± 0.2 , 2.0 ± 0.2 , 11.3 ± 1.3 , 3.3 ± 0.9 , and 5.3 ± 0.3 μ M, respectively. The concentrations of these primary amines in the striatum were monitored after K^+ depolarization with 3 min temporal resolution. This is the first microdialysis system to generate high relative recoveries and good

temporal resolution simultaneously for multiple neurotransmitters.

Microdialysis sampling of extracellular fluids has emerged as an important technique for biomedical, pharmaceutical, and neuroscience applications.^{1,2} Advantages of microdialysis relative to other sampling approaches include less tissue damage and production of samples free of high molecular weight components such as proteins. In most cases, microdialysis is used for chemical monitoring by collecting dialysate in fractions and then analyzing the fractions by an appropriate technique such as HPLC,³ immunoassay,⁴ or capillary zone electrophoresis (CZE).^{5–8} It is also possible to couple the microdialysis probe to an on-line analytical method for convenient monitoring of concentration changes in close to real time.^{3,5,9–13}

A limitation of microdialysis is poor temporal resolution relative to many chemical events. The temporal resolution of microdialysis-based chemical monitoring is typically determined by the mass sensitivity of the analytical method utilized. Better mass detection limits allow more frequent sampling and better temporal resolution. The use of microscale separation techniques such as microbore LC and CZE has significantly improved the temporal resolution possible, allowing sampling rates of 45–120 s.^{11,12} The combination of microdialysis with on-line derivatization and fast separation by CZE seems to be an especially powerful approach to in vivo monitoring. For example, glutamate and aspartate have

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been monitored with 120 s temporal resolution by derivatizing dialysates on-line with naphthalene-2,3-dicarboxyaldehyde (NDA) and then automatically assaying the samples by CZE-LIF.¹¹ Many other compounds are potentially amenable to this approach. In the on-line approach, temporal resolution often becomes limited not by mass sensitivity but by separation time.

We have begun to investigate the coupling of CZE with microdialysis using a flow-gated interface.¹⁴ The system allows higher efficiency separations than previous on-line combinations of microdialysis and microscale separations, which increases the potential for monitoring multiple compounds.^{11,12} Another unique feature of this system is that it allows operation at low microdialysis flow rates while maintaining fast sampling times. For example, we have utilized flow rates as low as 40 nL/min while maintaining sampling rates less than 60 s.¹⁵ The use of low flow rates in microdialysis results in several advantages. At low flow rates, relative recovery for low molecular weight analytes approaches 100%, which allows for quantitative monitoring and eliminates the need for extensive calibration procedures. The higher relative recovery also increases the concentration of analytes, resulting in improved detection limits. In addition, low flow rates result in low absolute recovery (material removed per unit time), which is less likely to affect the tissue being studied. Finally, the low flow rate system is potentially compatible with smaller probes, which could lead to improved spatial resolution.

In our previous experiments with the flow-gated interface, a UV absorbance detector was used. It was found that poor sensitivity of this detection mode resulted in the method being useful for just a few compounds found in high concentrations. The goal of the present work was to combine the advantages of the flow-gated interface and its low flow rate capability with on-line derivatization in order to expand the applicability of the flow-gated approach. For this initial study, *o*-phthalaldehyde/ β -mercaptoethanol (OPA/ β -ME) was used for on-line derivatization of primary amines in the dialysate. Both CZE and micellar electrokinetic chromatography (MEKC) separation modes were explored. This is the first reported use of on-line derivatization with MEKC, and it was found that at least 20 compounds could be detected by this approach. Several amino acids, including the neurotransmitters glutamate, aspartate, and taurine, were identified and monitored with the system.

EXPERIMENTAL SECTION

Chemicals. All amino acids, derivatization reagents, and anesthetics were from Sigma (St. Louis, MO) and were used as received. All solutions were prepared with water purified and deionized using a Millipore Milli-Q water purification system (Milford, MA) and filtered through 0.2 μ m nylon membrane filters.

Capillary Electrophoresis. All separations were performed in 20 cm lengths of 25 μ m id 360 μ m od fused silica capillaries coated with polyimide (Polymicro Technologies, Phoenix, AZ). Each day, capillaries were rinsed for 10 min with 0.1 M NaOH, deionized water, and electrophoresis buffer. A Spellman 1000R CZE power supply (Plainview, NY) was used to apply voltage.

For in vivo measurements by CZE, the inlet-to-detector length was 15 cm, and applied voltage was -15 kV (90 s separation times) or -30 kV (45 s separation times). The migration buffer was 175

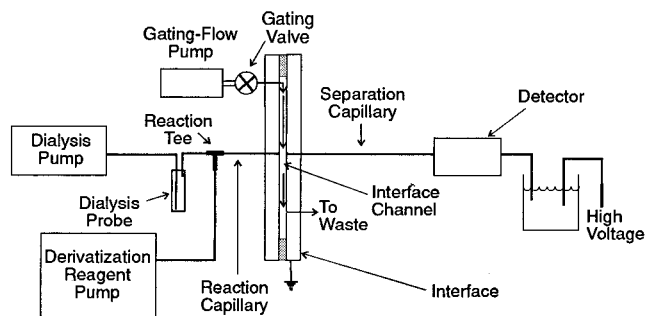


Figure 1. Block diagram of microdialysis/CZE-LIF system with on-line derivatization.

mM 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES), adjusted to pH 9.0 with 1 M NaOH. For MEKC separations, the capillary dimensions were the same and the applied voltage was -10 kV. For faster MEKC separations, the inlet-to-detector length was reduced to 10 cm, and the separation voltage was increased to -12 kV. The migration buffer for MEKC was 175 mM CHES at pH 9 with 100 mM sodium dodecyl sulfate. To allow the separation capillaries to reach the cathode, a 20 cm piece of 100 μ m i.d., 360 μ m o.d. fused silica capillary was connected to the end of the short separation capillary by butting the ends of the capillary inside a Teflon tubing sleeve (1 cm long, $1/16$ in. o.d., 0.01 in. i.d. from Alltech Associates, Deerfield, IL). This connection allowed the majority of the voltage to be dropped across the short capillary.¹⁶

On-Line Derivatization and Sample Injection. A diagram of the microdialysis/capillary electrophoresis system with on-line derivatization is shown in Figure 1. Microdialysate and the derivatizing agent were separately pumped into a 0.15 mm bore Teflon tee (Valco, Houston, TX) by a microsyringe pump at 79 nL/min. The tee dead volume was ~ 50 nL. Unless stated otherwise, the derivatization solution was 110 mM OPA and 220 mM β -ME in 25 mM borate buffer at pH 9.5. The derivatization solution was pumped through a 5.0 cm length of 75 μ m i.d., 360 μ m o.d. fused silica tubing to the reaction tee. The dialysis probe was connected to the reaction tee by a 2.5 cm length of 75 μ m i.d., 360 μ m o.d. fused silica tubing. The reaction capillary connecting the reaction tee to the interface consisted of 8.0 cm of 75 μ m i.d., 360 μ m o.d. fused silica tubing. The dialysis and derivatizing flows combined for a 158 nL/min flow rate through the reaction capillary, allowing for a reaction time of 2.2 min. All capillary-to-capillary connections were made by butting the capillaries together inside a 1 cm long Teflon tubing sleeve.

The separation capillary was coupled to the reaction capillary via a flow-gated interface, which allowed dialysate samples to be automatically injected onto the separation capillary. Operation of the interface has been described in detail elsewhere.^{14,17} Briefly, the interface consisted of a Lucite block that held the outlet of the reaction capillary and the inlet of the separation capillary aligned with a ~ 75 μ m gap between them. During a separation, a gating flow of electrophoresis buffer was pumped at 0.34 mL/min (Sage 341B syringe pump, Orion Research, Boston, MA) through the gap between the capillaries. This flow prevented derivatized dialysate from entering the separation capillary. To perform an injection, the gating flow was stopped by a pneumatic

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cally actuated gating valve. While the gating flow was stopped, the injection voltage was applied. In most cases, a 1 s delay was allowed between the stopping of the gating flow and application of the injection voltage. Once the injection was complete, gating flow was resumed, and the separation voltage applied. For *in vivo* measurements, dialysate injections were typically made with -1 kV for 5 s (CZE) or -100 V for 2 s (MEKC). Smaller injected amounts with MEKC were possible because of the better sensitivity of the lens system (Fluar objective) used for detection during MEKC experiments. The exact injection voltage and time were varied to get consistent performance in terms of theoretical plates and sensitivity. This was necessary because, with the flow-gated interface, the injected amount depends not only on the voltage and time but also on the space between reaction capillary and separation capillary, which varied from day to day.

Detection, Data Collection, and Data Reporting. Fluorescence detection was accomplished using an epillumination fluorescence microscope (Axioskop, Carl Zeiss, Hanover, MD) described elsewhere.^{18,19} The 354 nm line of a 2 mW helium-cadmium laser (Model 4210B, Liconix, Santa Clara, CA) was used as the excitation source. The excitation beam was reflected into the back of a $40\times$, 0.75 numerical aperture (NA) Neofluar objective (Zeiss) by a dichroic mirror with a 400 nm transition and focused onto the capillary. For MEKC runs, the objective was a $40\times$, 1.30 NA Zeiss Fluar oil immersion lens. (The Fluar lens became available during the course of the experiments and was therefore used only with MEKC.) The emitted radiation was collected by the objective, passed through the dichroic mirror, and filtered with a 450 nm interference filter with 25 nm bandwidth (Andover Corp., Salem, NH). Light passing through the interference filter was measured using a DCP-2 photometer system (CRG Electronics, Houston, TX), equipped with a R928 photomultiplier tube (Hamamatsu, Bridgewater, NJ). The output of the current-to-voltage converter was interfaced to an IBM-compatible computer. Data acquisition and automated control of the flow-gated system was accomplished with a National Instruments AT-MIO-16F-5 multifunction board (Austin, TX). Data collection rate was 10 Hz, which was sufficient to give at least 10 points over the narrowest peaks.

All mean values are reported \pm standard error of the mean with number of replicates n unless stated otherwise.

Microdialysis. Flexible loop (V-shaped) microdialysis probes (ESA, Bedford, MA) made from cellulose fibers (6 kD cutoff) were used. The flexible loop probes had $450\text{ }\mu\text{m}$ tip diameters and 2 mm tip lengths. The overall length from the tip of the probe to the outlet was 5 cm. Approximately 1 cm of the outlet was sleeved into a fused silica capillary of $150\text{ }\mu\text{m}$ i.d., $360\text{ }\mu\text{m}$ o.d. and sealed with cyanoacrylate cement. This modification facilitated using a butt connection with Teflon sleeves to the line transferring dialysate to the reaction tee. Dialysis probes were perfused with artificial cerebral spinal fluid (aCSF), consisting of 145 mM NaCl, 2.68 mM KCl, 1.01 mM MgSO_4 , and 1.22 mM CaCl_2 . The high K^+ perfusate solutions for stimulation experiments consisted of 2.62 mM NaCl and 145 mM KCl, and all other salts were the same. The dialysis flow rate was 79 nL/min unless stated otherwise. *In vitro* relative recovery was determined by comparing peak heights obtained when the dialysis probe was placed in a standards

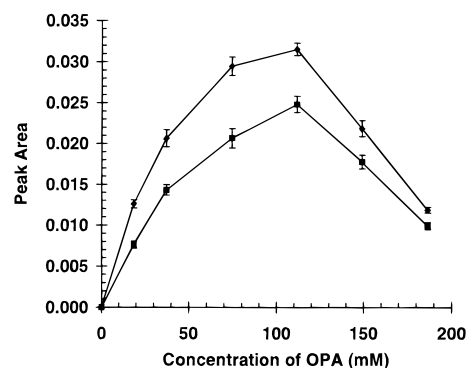


Figure 2. Effect of OPA concentration on the fluorescent signal of $5\text{ }\mu\text{M}$ aspartate (\diamond) and $4\text{ }\mu\text{M}$ glutamate (\blacksquare) in the on-line assay. Error bars represent ± 1 standard deviation. Peak areas are in arbitrary fluorescence units.

solution (all compounds at known concentrations between 2 and $20\text{ }\mu\text{M}$) at $37\text{ }^\circ\text{C}$ to those obtained when the probe was removed from the system and the same concentration of standards were pumped directly into the reaction tee.

Surgical Procedure. Male Sprague-Dawley rats weighing 250–350 g were anesthetized with subcutaneous injections of 100 mg/mL of chloral hydrate. The initial injection was 4.0 mL/kg. Booster injections of 2.0 mL/kg were given every 30 min until the animal no longer exhibited limb reflex. After surgery, the rat was kept unconscious with subcutaneous administration of 1.0 mL/kg chloral hydrate as needed. Once the rat was secured in the stereotaxic apparatus, the microdialysis probe was placed in the striatum to the coordinates $+0.02$ AP, -0.30 ML, -0.65 DV from bregma.²⁰ Basal level electropherograms were taken until they were stable, typically 1.5 h after insertion of the dialysis probe.

RESULTS AND DISCUSSION

On-Line Derivatization Conditions. Primary amines react rapidly with OPA in the presence of β -ME to produce fluorescent isoindole products.²¹ In addition, the derivatization agents themselves are not highly fluorescent. These characteristics made OPA an attractive derivatization agent for our on-line assays, where minimizing reaction time and interferences was desired. In developing the on-line system, it was important to assess the effect of OPA concentration and reaction time on signals for analytes.

Figure 2 demonstrates the effect of OPA concentration on the fluorescence intensity observed for derivatized aspartate and glutamate. For this experiment, a solution of $5\text{ }\mu\text{M}$ aspartate and $4\text{ }\mu\text{M}$ glutamate in aCSF was pumped directly into the reaction tee, which was interfaced with the CZE-LIF, at 79 nL/min. β -ME/OPA at a constant mole ratio of 2:1, dissolved in 25 mM borate buffer (pH 9.5), was also pumped into the reaction tee at 79 nL/min. A 2.2 min reaction and mixing time was achieved as described in the Experimental Section. As shown in the plot, signal intensity for the amino acids peaked at 110 mM OPA. This concentration was used for all further experiments. The decrease in fluorescence intensity at higher OPA concentrations may be related to instability of the isoindole derivative in the presence of large excess of OPA.²²

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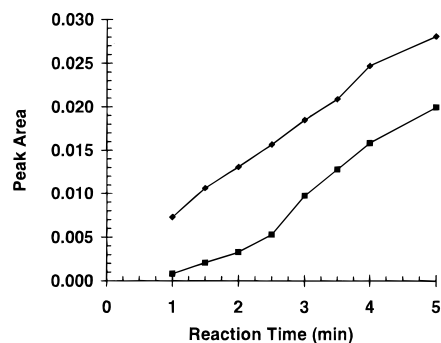


Figure 3. Effect of derivatization reaction time on the fluorescent signal of 5 μ M aspartate (♦) and 4 μ M glutamate (■) standards in the on-line assay. Peak areas are in arbitrary fluorescence units.

The effect of reaction time on fluorescence intensity for aspartate and glutamate was also investigated, and the results summarized in Figure 3. For this experiment, the conditions were similar to those for Figure 2, and OPA concentration was 110 mM. The reaction time was varied from 1 to 5 min by changing the length of the reaction capillary. As shown in the figure, the fluorescence intensity for both analytes steadily increased from 1 to 5 min reaction time. The long time required is surprising since the expected half-time for the OPA–amine reaction is around 6 s.²¹ Also, if we assume that complete mixing occurs in the average time it takes to diffuse across the diameter of the reaction capillary, then the samples should be mixed in about 5 s. Presumably, faster mixing and reactions could be achieved in smaller bore capillaries.

Detection Limits and Linearity. Detection limits were found to vary significantly with the choice of microscope objective. Some work used a Neofluar objective, which gave detection limits for amino acids of about 0.2–0.5 μ M in the on-line system. (Detection limits were calculated as the concentration that would give a peak height equal to 2 times the peak-to-peak noise of the electropherogram.) Later work used a Fluar objective, which had superior UV transmission characteristics and greater light collection efficiency. This lens resulted in a ~10-fold signal enhancement with no effect on the noise, as illustrated in the MEKC data for amino acids in Figure 4. Using this detector, the on-line detection limits for amino acids were approximately 20–40 nM. Detection limits were 2-fold worse in the on-line system than what could be obtained off-line, since the former required that the analyte be diluted 2-fold by the OPA/ β -ME stream during derivatization. Calibration curves for all of the identified amino acids were linear up to 50 μ M, with a linear correlation coefficient of at least 0.999.

Electrophoresis Conditions. To maintain high sampling rates and temporal resolution with the on-line system, it was important that both derivatization and separation be rapid. Maximizing separation speed required high electric field strengths (E). Figure 5 illustrates the effect of electric field strength on theoretical plates for 18 μ M aspartate in the on-line system using free solution CZE separation conditions similar to those used for in vivo measurements. The plates drop off significantly above 500 V/cm. Heating in the capillary, indicated by positive deviation from linearity in a plot of analyte velocity versus electric field strength, was not apparent until $E > 750$ V/cm (data not shown). Thus, the relatively low plates above 750 V/cm can be attributed

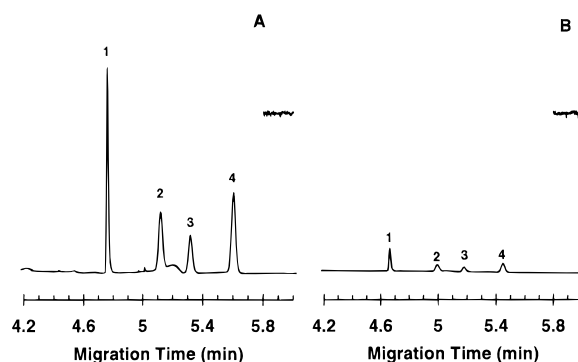


Figure 4. Comparison of Fluar (A) and Neofluar (B) objectives for MEKC-LIF. Peaks correspond to OPA derivatives of aspartate (1), leucine (2), phenylalanine (3), and isoleucine (4). Noise is shown in the inset of each figure at a scale 50 \times that for the electropherogram. Y-axis is in arbitrary fluorescence units and is the same for both electropherograms.

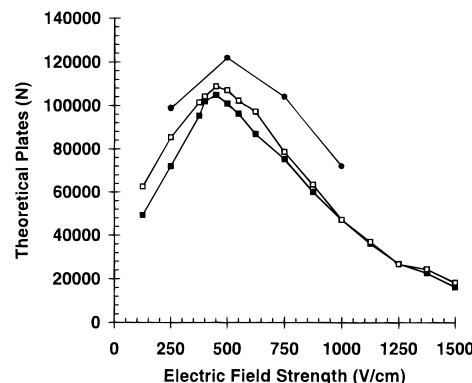


Figure 5. Theoretical plates (N) by CZE for aspartate as a function of electric field strength (E) under different experimental conditions: normal injection with sample in H₂O (□), normal injection with sample in aCSF (■), and minimized injection with sample in H₂O (●).

mainly to Joule heating, while the decline in plates between 500 and 750 V/cm is due to another source.

The lower than expected numbers of plates at moderate electric field strength are partially attributed to the fact that the injected sample from the dialysis probe is dissolved in the highly conductive aCSF, which creates sample overloading.²³ Samples dissolved in water instead of aCSF had slightly higher numbers of plates, especially at $E < 500$ V/cm, as shown in Figure 5. Another significant source of band broadening was injection volume. Figure 5 also shows data from an on-line injection, where the injection voltage and time were cut to the smallest values (0 V and 0.25 s) that allowed a peak for the 18 μ M aspartate with a signal-to-noise ratio > 5 when using the Neofluar lens. (Under these conditions, sample enters the separation capillary primarily by diffusion and flow from the reaction capillary.) With these injection conditions, the plate count was as high as 120 000 at 500 V/cm in a separation that took 2.7 min.

Based on these results, it was possible to choose conditions for in vivo experiments that yielded trade offs that best fit the goals of the experiment. Specifically, injections for in vivo measurements were usually at -1 kV for 1–2 s, which, as shown above, is too large to give optimum efficiency. However, the larger injections gave better signal-to-noise ratios, which was especially important for quantification in experiments that utilized the less

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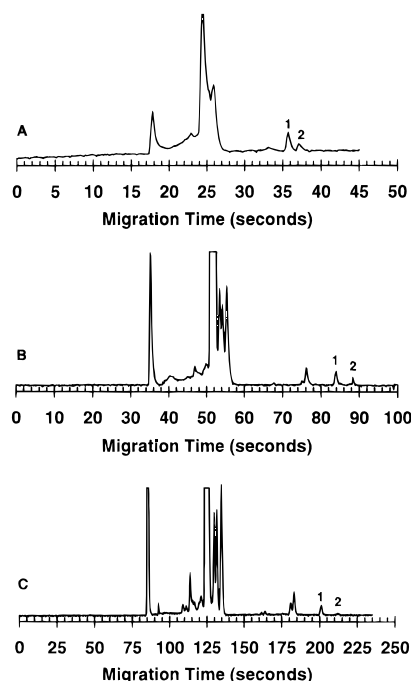


Figure 6. Effect of electric field strength on electropherograms obtained in vivo with the on-line system. Peaks 1 and 2 correspond to OPA derivatives of glutamate and aspartate, respectively. The electric field strengths were 1420 (A), 825 (B), and 405 V/cm (C). Injection was -1 kV for 3 s. Other conditions are given in the Experimental Section for CZE. Y-axis is in arbitrary fluorescence units and is the same for all electropherograms.

Table 1. In Vitro Recovery (%) of Primary Amines Using MEKC-LIF (Mean \pm Standard Error of the Mean, $n = 3$)

analyte	dialysis flow rate (nL/min)		
	57	79	111
aspartate	98.0 \pm 2.1	98.0 \pm 3.9	93.3 \pm 1.3
glutamate	98.1 \pm 2.2	98.3 \pm 2.9	91.2 \pm 1.6
isoleucine	98.2 \pm 1.9	98.8 \pm 1.6	91.2 \pm 1.2
leucine	98.2 \pm 1.9	98.7 \pm 1.3	91.7 \pm 2.6
lysine	98.8 \pm 2.3	98.6 \pm 1.9	93.4 \pm 1.6
methionine	97.9 \pm 1.9	96.7 \pm 3.6	91.1 \pm 2.7
phenylalanine	97.9 \pm 2.6	97.3 \pm 1.8	92.5 \pm 2.8
taurine	100.2 \pm 2.4	99.2 \pm 2.1	95.5 \pm 1.6
tyrosine	100.7 \pm 1.4	99.9 \pm 1.4	89.5 \pm 2.1
valine	99.2 \pm 1.9	98.4 \pm 3.0	94.3 \pm 1.4

sensitive Neofluar lens. Increasing electric field strength decreased resolution but gave faster analysis times, as illustrated by the in vivo electropherograms in Figure 6. As discussed below, glutamate and aspartate were the only compounds that were reliably resolved and identified by CZE. The zones due to glutamate and aspartate remain resolved, even at $E = 1420$ V/cm (see Figure 6), where Joule heating has a large adverse effect on efficiency. In this case, the separation is over in 35–40 s, allowing for the best temporal resolution in monitoring. In vivo, at $E = 825$ V/cm, we typically obtained averages of 44 000 plates for glutamate and 68 000 plates for aspartate in separations that required about 90 s.

Calibration of Dialysis Probes. In vitro relative recovery of the dialysis probe is summarized in Table 1. As shown, the relative recoveries are within experimental error of 100% when the dialysis flow rate is ≤ 79 nL/min. Thus, under these conditions, the concentration inside the probe is approximately equal

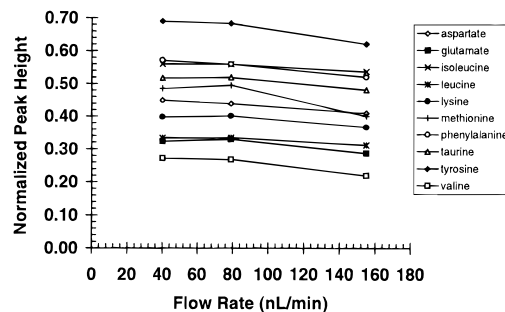


Figure 7. Effect of dialysis flow rate on normalized peak heights in vivo. Peak heights were normalized as described in the text. MEKC separation conditions as described in the Experimental Section were used. Each point is the average of three electropherograms. The relative standard deviations were from 1 to 9%; however, error bars were not included in the figure for clarity.

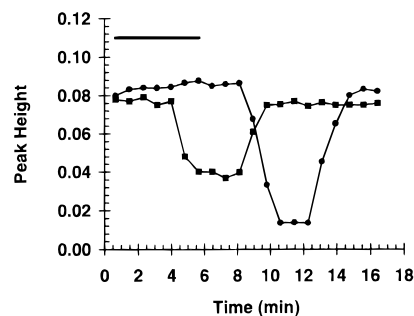


Figure 8. Response of the system to step changes in aspartate concentration with 45 s sampling rate. Initially, the probe was equilibrated with 10 μ M aspartate and was changed to a reservoir containing 5 μ M (155 nL/min) or 2.5 μ M (79 nL/min) aspartate at $t = 0$ min. The probe was switched back to the original reservoir at $t = 6$ min. The overall time the probe was in the 5 μ M/2.5 μ M solution is indicated by the bar. The delay in response is due to the dead volume of the system. The perfusion rates were (■) = 155 and (●) = 79 nL/min, while the temperature of the reservoir was 37 $^{\circ}$ C. Peak heights are in arbitrary fluorescence units.

to that outside the probe which suggests the possibility of quantitative in vivo monitoring. To confirm quantitative monitoring in vivo, we examined the peak height as a function of flow rate for several identified compounds (compounds measured using MEKC conditions) in vivo, as shown in Figure 7. (Since the peak height could vary because of slightly different reaction times at the different flow rates, the peak heights in Figure 7 were normalized to those obtained in vitro with the probe removed and a given concentration of analyte pumped directly into the tee at the same flow rate.) As the flow rate is decreased below 100 nL/min, the normalized peak heights do not increase significantly, which indicates that relative recovery is maximized, i.e., is near 100%, in vivo. These results are in agreement with our previous work for other small compounds¹⁵ and demonstrate that it is possible to quantitatively monitor compounds when using low flow rates without resorting to extensive in vivo calibration schemes.

Temporal Resolution. The temporal resolution of the system was evaluated in vitro by monitoring step changes in aspartate concentration, which were induced by moving the dialysis probe between reservoirs containing different concentrations of aspartate. Figure 8 illustrates the changes in aspartate peak height that were observed as the step changes were made while perfusing the probe at both 155 and 79 nL/min. The aspartate change was observed in the electropherograms 8 min after it was made at the probe when the flow rate was 79 nL/min, while the delay was

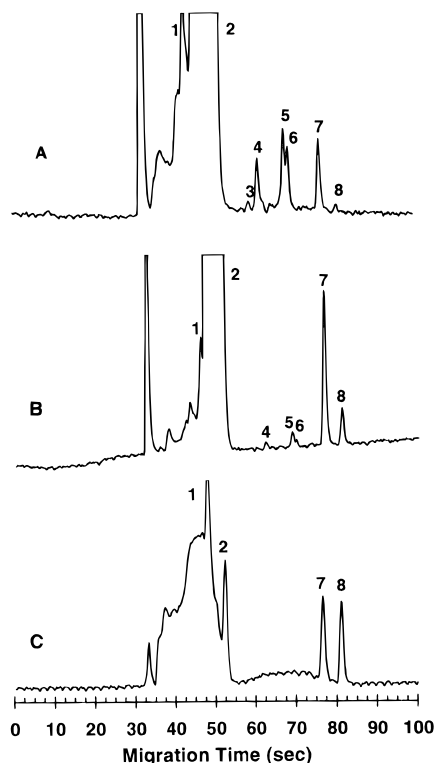


Figure 9. Comparison of typical electropherograms obtained in vivo before stimulation (A), in vivo during stimulation (B), and for 5 μ M standards of glutamine (1), glycine (2), glutamate (7), and aspartate (8) (C). Only glutamate and aspartate were consistently identified. $E = 825$ V/cm, and other conditions are given in the Experimental Section for CZE. Y-axis is in arbitrary fluorescence units and is the same for all electropherograms.

4 min with a perfusion rate of 155 nL/min. The delay was due to the time required for the analytes to flow through the dead volume from the probe to the interface. In addition to a delay in detecting the concentration changes, there is a broadening of the concentration pulses, as shown in Figure 8. At 79 nL/min, the step change in aspartate requires about 90 s to develop, and at 155 nL/min, the time is about 45 s. This effect is attributed to the time required to equilibrate across the probe and band broadening due to flow and diffusion during transfer from probe to interface. Thus, in this case where separations can be accomplished in 45 s, the temporal resolution is limited not by the separation time but by the sample broadening that occurs in the system. A possible approach to improving temporal resolution would be to decrease dead volume by decreasing the transfer and reaction capillary bore and/or length. With the system as designed, however, temporal resolution of 45 s for glutamate and aspartate can be obtained at the expense of slightly lower relative recovery by operation at 155 nL/min instead of 79 nL/min. The decrease in recovery is small, as can be seen by comparing peak heights in Figure 7.

In Vivo Detection of Glutamate and Aspartate by CZE-LIF. Initial in vivo experiments were performed using CZE for separation. Electropherograms obtained from on-line derivatization of dialysate from the rat caudate nucleus are compared to amino acid standards in Figure 9. The broad bands in the 35–50 s time range are associated with excess OPA and unresolved, neutral compounds. The following compounds were tested for matches in the in vivo electropherograms: glutamate, glycine, glutamine, aspartate, serine, leucine, isoleucine, taurine, alanine, and lysine. Of these compounds, only glutamate and aspartate

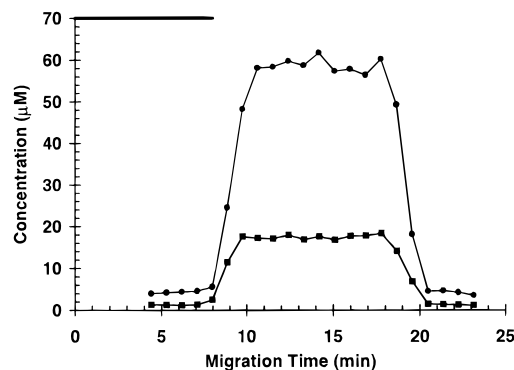


Figure 10. Concentration of glutamate (●) and aspartate (■) in vivo during K^+ infusion with the microdialysis probe, measured by CZE. The bar indicates the time that the probe contains 145 mM K^+ . The delay in response is due to the dead volume of the system.

were consistently found to be fully resolved and to have matching migration times. In addition to matching migration times, both peaks increased appropriately after the dialysate was spiked with glutamate and aspartate. The basal levels of aspartate and glutamate were 1.2 ± 0.1 and 5.0 ± 0.4 μ M ($n = 8$), respectively. These values and precision are in good agreement with previous reports.^{25–31}

Infusions of 145 mM K^+ through the dialysis probe caused the expected increase in glutamate and aspartate levels, as illustrated by comparing parts A and B of Figure 9. Results from monitoring the aspartate and glutamate overflow during a K^+ stimulation are illustrated in Figure 10. The timing of the increase in aspartate and glutamate concentration shows that they increased immediately after the high K^+ buffer reached the probe. (In vitro experiments found that the use of high K^+ buffer did not affect the injected amount of any identified amino acids.) The rise time of the increases suggest that they were occurring at least as fast as the system could monitor. The average concentration of aspartate and glutamate during stimulation was 4.8 ± 1.7 and 33.8 ± 12.3 μ M ($n = 5$), respectively. Although there is considerable variability among different groups, the percent increase and precision are similar to those reported previously using similar stimulations.^{29–31} The best previous temporal resolution for glutamate and aspartate by microdialysis was 120 s.¹¹

In Vivo Detection of Other Primary Amines Using MEKC-LIF. CZE was well-suited for rapidly resolving glutamate and aspartate in the dialysate samples. By manipulating the migration buffer and other separation conditions, it may be possible to resolve other compounds of interest as well. MEKC is another potentially powerful approach to resolving the OPA derivatives. This is especially true since many of the compounds that were unresolved by CZE had migration times that were near those

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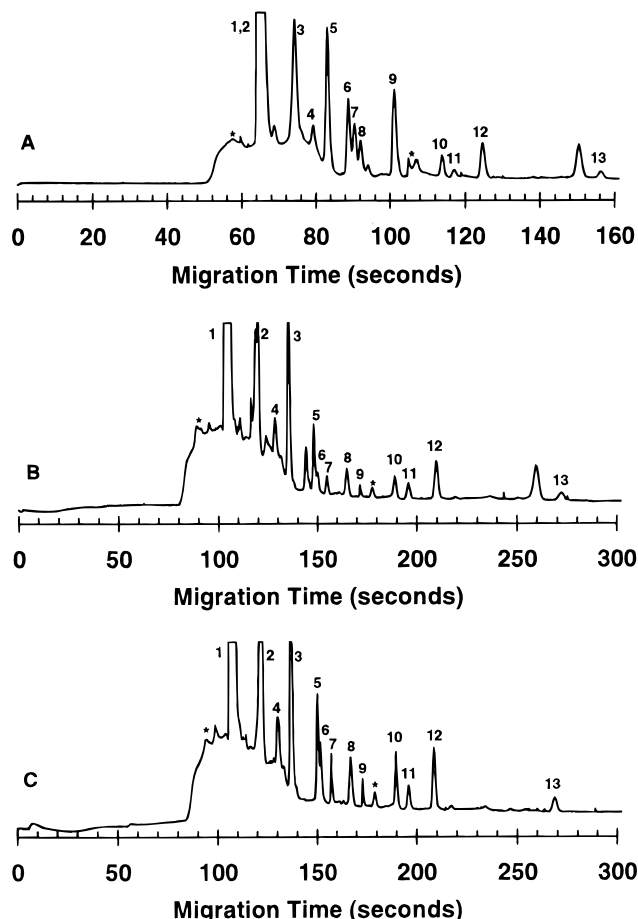


Figure 11. Comparison of MEKC electropherograms obtained in vivo with 10 cm effective length, $E = 600$ V/cm (A), in vivo with 15 cm effective length, $E = 400$ V/cm (B), and for standards (C). Conditions in part C are the same as those for part B, and other conditions are given in the Experimental Section. Note the different time scales for parts A and parts B and C. Peak identities and concentrations of standards are $15.2 \mu\text{M}$ glutamine (1), $14.4 \mu\text{M}$ serine and $12.1 \mu\text{M}$ threonine (2), $10.9 \mu\text{M}$ alanine and $12.2 \mu\text{M}$ glycine (3), $4.4 \mu\text{M}$ tyrosine (4), $17.0 \mu\text{M}$ taurine (5), $15.4 \mu\text{M}$ valine (6), $9.6 \mu\text{M}$ glutamate (7), $3.1 \mu\text{M}$ methionine (8), $4.4 \mu\text{M}$ aspartate (9), $4.1 \mu\text{M}$ leucine (10), $3.8 \mu\text{M}$ phenylalanine (11), $6.2 \mu\text{M}$ isoleucine (12), and $9.9 \mu\text{M}$ lysine (13). Impurities and reagent peaks are identified with an asterisk, while unidentified peaks are unlabeled. Y-axis is in arbitrary fluorescence units and is the same for all electropherograms.

expected for neutral compounds. Using MEKC, at least 20 peaks not associated with the reagents were resolved, as illustrated by the typical example in Figure 11B. Under conditions used for in vivo measurements, we obtained between 140 000 and 270 000 plates for resolved compounds. The results demonstrate the potential for detecting and monitoring a wide variety of compounds using this approach.

By matching migration times to standards (Figure 11B,C), the following compounds were identified in the electropherograms: aspartate, glutamate, isoleucine, leucine, lysine, methionine, phenylalanine, taurine, tyrosine, and valine. The following compounds were tested, but matching peaks could not be reliably found at basal conditions: γ -aminobutyric acid (GABA), tryptophan, cysteine, arginine, asparagine, and glutathione. The basal concentration levels for the identified compounds are shown in Table 2.

No attempt was made to vary the MEKC conditions in order to optimize resolution for particular compounds; therefore, it may be possible to develop MEKC conditions for many other com-

Table 2. Concentration of Primary Amines in Rate Caudate Nucleus Using MEKC-LIF (Mean \pm Standard Error of the Mean)^a

analyte	basal concn (μM)	stimulated concn (μM)
aspartate	1.9 ± 0.2	4.3 ± 0.9
glutamate	4.1 ± 0.2	17.6 ± 3.0
isoleucine	4.6 ± 0.7	5.7 ± 0.8
leucine	2.6 ± 0.3	3.2 ± 0.4
lysine	5.4 ± 0.4	6.2 ± 1.0
methionine	1.8 ± 0.2	3.1 ± 0.4
phenylalanine	2.0 ± 0.2	2.3 ± 0.2
taurine	11.3 ± 1.3	60.0 ± 5.4
tyrosine	3.3 ± 0.9	3.6 ± 0.8
valine	5.3 ± 0.3	10.6 ± 5.1

^a Stimulation was achieved by 145 mM K^+ infusion for 15 min at 79 nL/min ($n = 4$ for basal concentrations, and $n = 3$ for stimulated concentrations).

pounds. For example, in separations of standards, serine and threonine were not resolved, nor were alanine and glycine (see Figure 11C) under the conditions used; however, peaks matching these compound pairs were observed in the in vivo electropherograms. Within a given day, the resolution and pattern of peaks observed was stable; however, the retention times and resolution did vary from day to day. Especially problematic was variation of the migration times of taurine, valine, glutamate, and methionine. In some cases, taurine was well-resolved from valine, and in other cases, glutamate could overlap with either valine or methionine. Quantitative data are reported only for cases where resolution was >0.75 for a pair.

As for the CZE case, a number of extraneous background peaks appear in the standards and in vivo. For the MEKC separations shown in Figure 11B,C, the background peaks include the broad zone from 80 to 140 s, the peak at 180 s, and several small but perceptible peaks between 215 and 260 s. Most of the peaks appear to be the result of side reactions or minor products and not OPA/ β -ME itself, since injections of blanks had only a single reagent peak centered at ~ 120 s. Furthermore, the presence of extraneous peaks tended to be more noticeable with standards than for in vivo data. Compare, for example, the size of the broad zone in Figure 11C with that in part B. This may be because many more amines were present in the in vivo sample and they consumed the OPA, preventing it from being involved in side reactions. The presence of extraneous peaks may be a consequence of the large excess of OPA that was used. Reduction of the extraneous peaks, at the expense of sensitivity, may be attained by decreasing the OPA concentration (see Figure 2). In addition, prior purification of OPA by recrystallization may reduce this effect.³² The reagent peaks were problematic in that their magnitude could vary from day to day. For example, in some cases, the small peaks in the baseline between 210 and 250 s were larger than some of the analyte peaks.

For monitoring the amino acids, the separation time was shortened to ~ 3 min by decreasing the effective capillary length from 20 to 10 cm and by increasing electric field strength from 500 to 600 V/cm. Under these conditions, we obtained between 32 000 and 99 000 theoretical plates depending on the analyte. All of the identified compounds could be at least partially resolved

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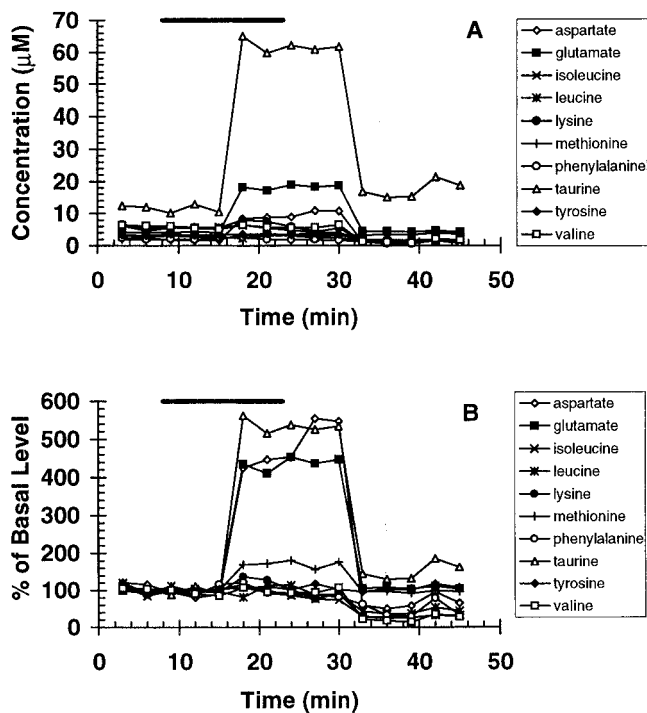


Figure 12. In vivo (A) concentration and (B) percent of basal levels of primary amines during K^+ infusion with the microdialysis probe, measured by MEKC. The bar indicates the time the probe contains 145 mM K^+ . The delay in response is due to the dead volume of the system.

under these conditions, as illustrated by the electropherogram in Figure 11A. To identify peaks and minimize the chance that unknown peaks were contributing to the signals in these electropherograms, data for every animal were always obtained under higher resolution conditions (longer columns) to determine the number of peaks present before utilizing faster, lower resolution runs.

The effect of a 15 min infusion of 145 mM K^+ on the concentration of the analytes is illustrated in Figure 12. The average concentrations obtained during the K^+ stimulation are summarized in Table 2. Some error may be expected in quantification, especially for valine, glutamate, and methionine, since they were not fully resolved under these conditions. It is apparent, especially when the concentration changes are plotted as percent increase from basal level (Figure 12B), that the three neurotransmitters (aspartate, glutamate, and taurine) are most

affected by the K^+ stimulation, as expected. The levels measured during K^+ infusion are in reasonable agreement with previously reported results.²⁹⁻³¹

CONCLUSIONS

We have described a system that couples microdialysis to CZE-LIF with on-line derivatization. While previous publications have reported a similar system using CZE-LIF,^{5,11} and others have coupled capillary LC on-line with microdialysis,¹² this system is unique in two respects. First, it is compatible with low perfusion flow rates, which results in higher relative recoveries. This results in several advantages, including higher concentration of analyte, quantitative monitoring without in vivo calibrations, less disturbance to the tissue, and potential compatibility with smaller probes for better spatial resolution. Second, the system allows higher theoretical plates, which increases peak capacity in the separation, which allows more compounds to be monitored simultaneously. The use of MEKC was especially powerful for resolving multiple amino acids in a reasonable time. The results are achieved without sacrifice in temporal resolution. Indeed, the use of CZE for the separation allowed glutamate and aspartate to be quantitatively monitored with the best temporal resolution to date without the need for in vivo calibrations. A possible improvement in the system is the use of other derivatization reagents, which may allow for improved sensitivity or different selectivity. Further optimization of MEKC or CZE conditions may allow them to be used for more compounds, such as other amino acid neurotransmitters. In addition, the use of smaller probes will be explored for better spatial resolution. Finally, improvements in the detection limit and smaller inner diameter capillaries may allow faster separations. Faster separations, combined with improved engineering of the transfer from dialysis probe to the electrophoresis, will allow better temporal resolution.

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