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Trace-Level Amino Acid Analysis by Capillary Liquid Chromatography and Application to in Vivo Microdialysis Sampling with 10-s Temporal Resolution

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A sensitive method was developed to determine 16 amino acids, including all the neurotransmitter amino acids and neuromodulators, in physiological samples. Samples were derivatized with *o*-phthalaldehyde/*tert*-butyl thiol followed by two scavenging reactions that reduced the chemical background caused by excess derivatization reagent by ∼90%. A total of 250 nL of the derivatized sample was injected and concentrated onto a 50-μm-inner diameter capillary column packed with 5-μm reversed-phase particles and separated using gradient elution. Analytes were detected amperometrically at a cylindrical 9-μm carbon fiber microelectrode. The combination of on-column concentration, scavenging reactions after derivatization, high sensitivity electrochemical detection, and protocols to minimize amine contamination allowed detection limits of 90−350 pM (20−80 amol) for all the amino acids tested. This method was used to analyze in vivo microdialysate samples from probes implanted in the striatum of anesthetized rats. Probes were perfused at 1.2 μL/min and fractions collected every 10 s. The 200-nL fractions were diluted to 2 μL to facilitate sample handling for off-line analysis. The suitability of this method for simultaneous monitoring of all the major amino acid neurotransmitters with 10-s temporal resolution under basal conditions, during potassium stimulation, and during selective uptake inhibition of γ-aminobutyric acid is demonstrated.

Amino acids are an important class of neuroactive substances. Of particular interest are aspartate (ASP), glutamate (GLU), glycine (GLY), γ-aminobutyric acid (GABA), and taurine (TAU), all confirmed or suspected neurotransmitters, serine (SER), a neuromodulator, and arginine (ARG) and citrulline (CIT), the precursor and byproduct, respectively, of synthesis of NO, another neurotransmitter. Interest in these amino acids continues to increase as they have been implicated in physiological roles as diverse as learning, movement, and feeding as well as several diseases including Alzheimer's, Parkinson's, and epilepsy.[1] An important method of uncovering the function of these compounds is to correlate changes in their brain extracellular level with pharmacological manipulation or behavior. The most common approach to in vivo monitoring is microdialysis sampling coupled with assays of the amino acids.[2]

Temporal resolution in monitoring is important as neurotransmitter concentrations can change on the second time scale in response to stimuli. Temporal resolution by microdialysis is usually limited by the mass sensitivity of the analytical method used to quantify analytes because fraction collection times must be long enough to collect a detectable amount of analyte. Using HPLC for amino acid analysis, detection limits of 5−60 fmol are obtained and temporal resolution is limited to 5−30 min.[3−5]

The temporal resolution for monitoring some neuroactive amino acids has been improved considerably by using capillary zone electrophoresis with laser-induced fluorescence detection (CZE-LIF) for dialysate analysis.[6−10] Dialysate has been monitored as frequently as every 5 s for ASP and GLU using microdialysis on-line with CZE-LIF.[6] In addition, temporal resolution of 7 s to 2 min has been obtained for monitoring subsets of the amino acid neurotransmitters by fraction collection and off-line CZE-LIF analysis.[7−9]

Despite the attractive features of CZE-LIF, it has yet to be successfully used for simultaneous determination of all the neuroactive amino acids. A technique for simultaneous monitoring at high temporal resolution is desirable to detect unexpected changes in amino acids and to examine interactions of different neurotransmitter systems. This goal requires a method capable of analyzing nanoliter-volume dialysate samples with high sensitivity and sufficient resolving power to separate the trace-level

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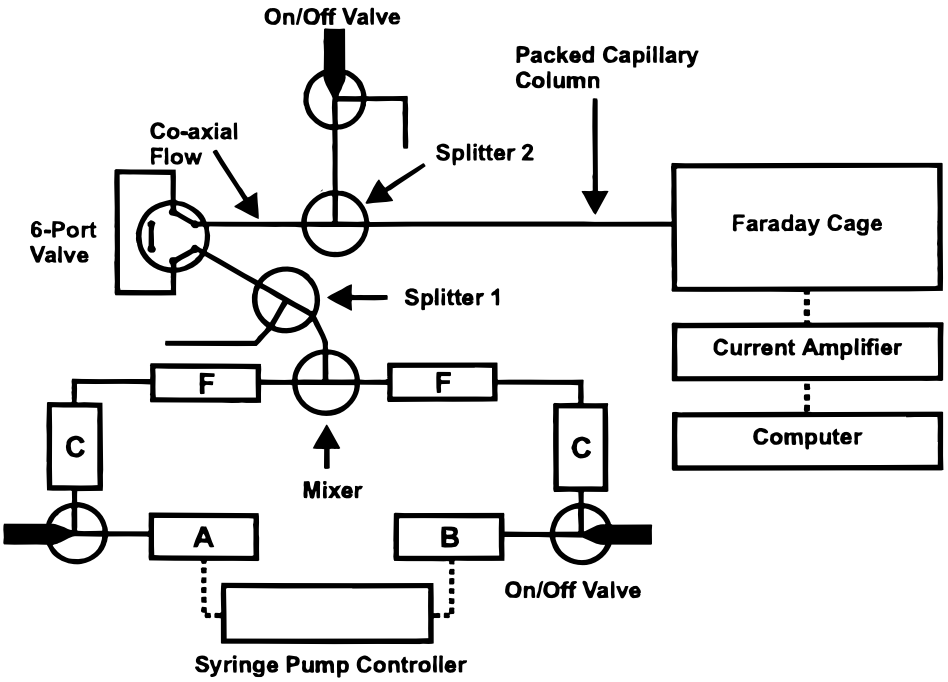

Figure 1. Schematic of capillary LC/EC instrument: A and B, high-pressure syringe pumps and gradient controller. The mobile phase exiting from the high-pressure syringe pumps passes through a set of high-pressure on/off valves, check valves (C), and 0.2-μm stainless steel filters (F) prior to mixing. After mixing, the mobile phase is carried to the injection valve by 0.005 in. $\times$ 5 cm stainless steel tubing.

neurotransmitters from the large number of endogenous amines found in the extracellular space. In this work, we explore the use of capillary liquid chromatography (LC) with electrochemical detection for this purpose.

The strategy used is to collect fractions at 10-s intervals (200 nL volume), dilute the fractions to 2 μL, derivatize samples with *o*-phthalaldehyde (OPA)/*tert*-butyl thiol,[11] preconcentrate the derivatized samples onto capillary LC columns, and separate the derivatized amines by gradient elution. The dilution step was necessary because 200-nL fractions are too small to be easily manipulated; however, after dilution it was feasible to use commercially available injection valves and microdispensers for sample manipulation and injection.

Success with this approach requires detection limits below 1 nM in order to quantify amino acids which can be present at low-nanomolar concentrations in dialysate after dilution. Existing methods of amino acid derivatization do not allow such low detection limits; therefore, improvement in derivatization was required. The primary difficulty in trace-level analysis with derivatization methods is that the large excess of reagent that is required to obtain a quantitative reaction also results in formation of numerous detectable compounds that interfere with detection of analytes. Several strategies have been devised to reduce the chemical background resulting from amine derivatization including purification of reagent,[12] chemical scavenging,[3,4,13,14] and electrochemical scavenging.[15] In this work, we describe a chemical scavenging scheme that greatly reduces the background from derivatization and allows analysis of amino acids at subnanomolar concentrations.

EXPERIMENTAL SECTION

Reagents. Amino acids, IAA, OPA, 2-methyl-2-propanethiol (*tert*-butyl thiol), nipecotic acid, and boric acid were of the highest purity grade from Sigma or Aldrich (St. Louis, MO). All buffer salts and solvents were obtained from Fisher (Fairlawn, NJ). The phosphate buffer salts were enzyme grade while other salts were certified ACS grade. Water, acetonitrile, and methanol were HPLC grade. Artificial cerebrospinal fluid (aCSF) contained the following: 145 mM NaCl, 2.68 mM KCl, 1.01 mM $MgSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$. The high K^+ was made similarly except KCl was 145 mM and NaCl was 2.68 mM. All solutions were made in clean glassware and filtered (see below).

Cleaning Procedures and Sample Vials. Volumetric flasks, centrifuge tubes, pipet tips, and sample microvials were washed using 1 N HCl followed by rinses with HPLC grade water and absolute ethanol. Vials were dried under a nitrogen stream and capped until use. Darkened, borosilicate reagent vials were soaked in a bath of 50% 5 N NaOH and HPLC grade methanol overnight prior to cleaning. Sample microvials (10 μL total volume) were made from 1000-μL pipet tips (Fisher) by sealing the small orifice by melting (not burning) using a Bunsen burner and solidifying on a dust-free metal surface. The tips were cut to a height of ~0.5 cm and cleaned.

Buffers and Mobile Phases. All buffers were made to be self-adjusting in pH after it was determined that the pH meter

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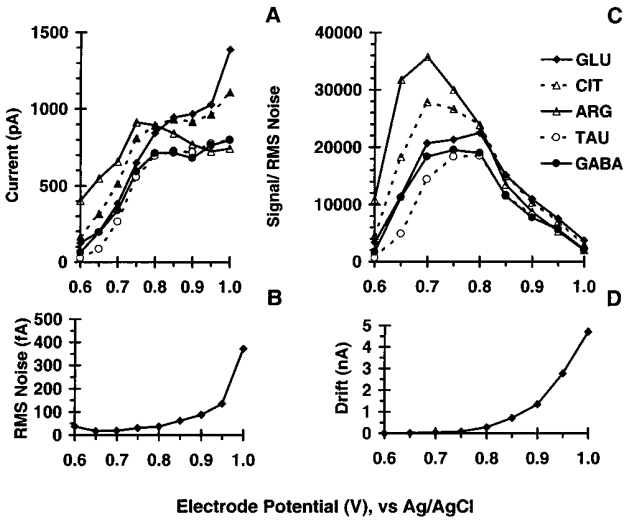

Figure 2. Electrochemical characteristics of OPA/*tert*-butyl thiol-derivatized amino acids and carbon fiber microelectrode. All amino acid concentrations were 1 μM, and injection volumes were 100 nL. (A) Electrochemical signal of amino acid derivatives as a function of electrode potential. (B) Root-mean-square noise of the electrochemical detector. (C) Signal-to-rms noise as a function of electrode potential. (D) Electrochemical drift (increase in background current between the ASP and GABA peaks). Derivatives were separated using a linear gradient of mobile phase A (50 mM phosphate pH 6.8 with 1 mM EDTA) and B (35% mobile phase A, 65% acetonitrile (v/v)) from 35 to 70% B and gradient slope of 2% B/min.

contaminated the buffers with low levels of amine. Solutions were filtered using 0.22-μm Teflon membranes (MSI, Fisher) and a cleaned, glass vacuum filtration system. Borate buffer used for derivatization was made to 0.4 M at pH 10.6 ± 0.1 using 12.37 g of boric acid, 7.53 g of NaOH (s), and 0.5 L of HPLC grade water. The phosphate-buffered mobile phase was made to 0.05 M phosphate buffered at pH 6.51 by mixing 4.73 g of NaH_3PO_4, 4.22 g of $Na_2H_2PO_4$, and 0.372 g of ethylenediaminetetraacetic acid (EDTA) in 1 L of HPLC grade water. Mobile phase A was 100% phosphate buffer while mobile phase B was phosphate buffer with 65% (v/v) acetonitrile. Mobile phases were prepared daily and degassed by He sparging.

Derivatization Solutions. A solution of 40 mM OPA and 50 mM *tert*-butyl thiol was made in a borate buffer containing 50% methanol (both solutions were filtered previously) by directly weighing necessary amounts of recrystallized OPA into a cleaned, glass vial prior to addition of the solvents.[14] The IAA was made to 1 M in methanol. A solution of 0.1 M cysteic acid and Na_2SO_3 was made in borate buffer. All solutions were stored in darkened, borosilicate glass vials. All samples were derivatized in previously cleaned polypropylene vials using an Eppendorf microdispenser and gel-loading pipet tips (Fisher). For microscale derivatization, 2-μL samples were derivatized as follows: 0.4 μL of OPA/*tert*-butyl thiol was added and allowed to react for 2 min, 0.4 μL of IAA was added and allowed to scavenge excess thiol for 4 min, and 0.4 μL of the cysteic acid/sulfite solution was added and allowed to react for 10 min. The final sample volume was 3.2 μL. For initial tests of the system, sample solutions were 500 μL and the reagents scaled up proportionately. If any buffer, solvent, or derivatization solution were observed to have particulates or dust, the solution was discarded and remade.

Capillary LC. Fused-silica capillaries of 48 μm i.d. × 40 cm (Polymicro Technologies, Phoenix, AZ) were fitted with end frits and packed with Alltima C8 particles (5 μm, 100 nm pore) in an acetone slurry (10 mg/mL) at 4000 psi using a pneumatic amplifier pump (Alltech Associates, Deerfield, IL).[16] Columns packed fully within 20 min and were then flushed with 90% (v/v) acetonitrile/water. After packing, the columns were trimmed to 36 cm and flushed with 100% B for 10 min prior to use. One gradient cycle was needed to clean the column after packing and flushing. A schematic of the pumping and injection system is shown in Figure 1. This design uses high-pressure syringe pumps, static mixing, and two mobile-phase splitters. The mobile phase was mixed and delivered at 8 μL/min by two 100 DM pumps (ISCO, Lincoln, NE). The first splitter carried 6 μL/min (27 μm × 170 cm capillary) flow to waste generating 2300 psi. The second splitter is closed during sample injection to conserve sample and opened during gradient elution to minimize gradient dwell times and sweep out the dead volume (~2.3 μL) of the injection valve (UW6, VICI, Houston, TX). To reduce the dead volume of the outlet port of the injection valve, the packed capillary column was inserted coaxially through the second splitting tee into the injection valve and positioned ~100 μm from the surface of the rotor, creating a thin annular region around the column head (360 μm) and valve pilot (400 μm). When the second splitter was open, the flow rate was ~1.7 μL/min, creating a gradient dwell time of 2 min. Careful positioning of the column is important to prevent scratching of the rotor surface and inner wall of the valve body.

Electrochemical Detection. Cylinder carbon fiber microelectrodes of 9 μm diameter × 1.5 mm length and Ag/AgCl reference electrodes were fabricated using published methods.[17] Prior to use, an electrochemical pretreatment was applied (0 to +1.8 V, at 1 V/s for 30 s).[18] Background current for these electrodes was typically ~20 pA after treatment. The electrochemical detector and collection electronics are similar to previous designs,[18] except a Stanford SR-570 low-noise current preamplifier (Sunnyvale, CA) was used in this work.

Microdialysis. In vivo experiments were performed in male Sprague−Dawley rats as described previously.[19] Microdialysis sampling was performed using side-by-side 4-mm probes constructed in-house.[20] Artificial cerebrospinal fluid was perfused at 1.2 μL/min using a microliter syringe pump (Harvard Apparatus 553206, South Natwick, MA). The microdialysis probe was placed in the striatum (AP = +0.2, ML = +3.0, DV = −6.5). Sample collection for these experiments began after basal levels were constant (~2 h). Microdialysis fractions of 200 nL each were collected at 10-s intervals into microvials containing 1.8 μL of aCSF. Fraction collection was performed manually by positioning the outlet of the dialysis probe into the collection vials during the experiment. After collection, microvials were covered using Parafilm and stored at −20 °C until assayed, typically within 12 h of collection.

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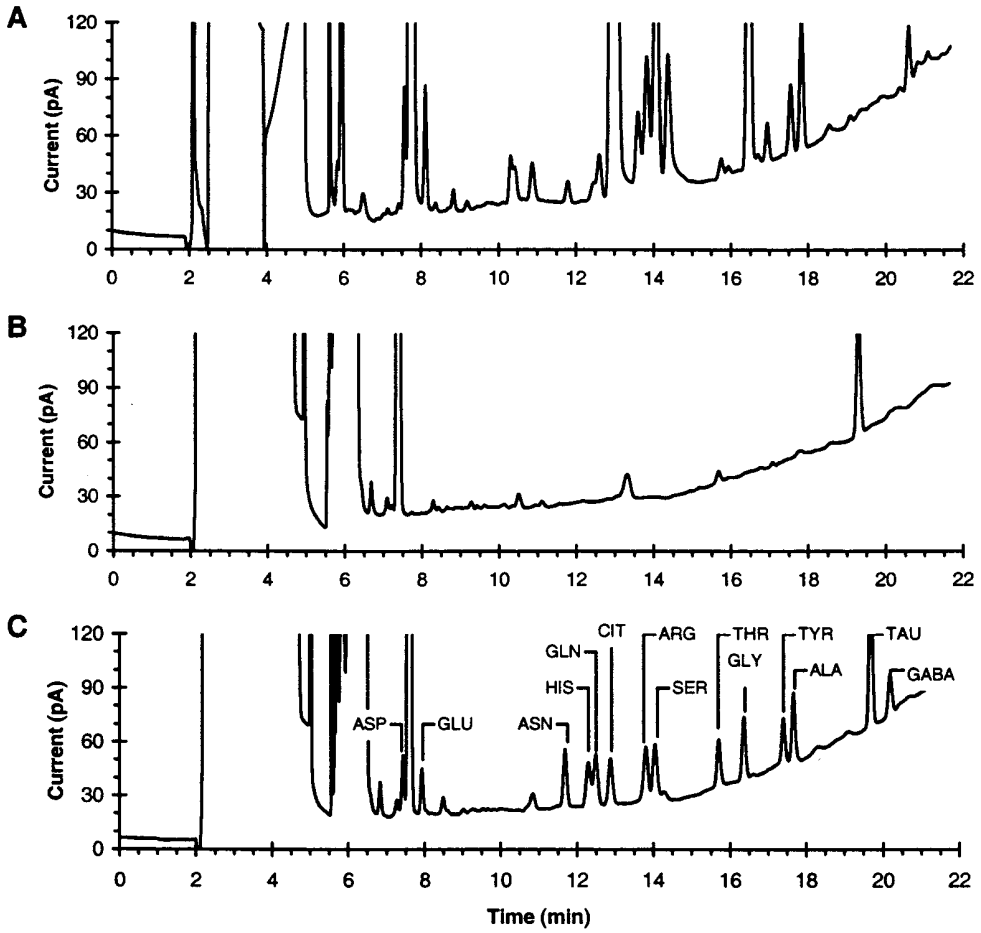

Figure 3. Illustration of effect of scavenging on blanks. (A) HPLC water without intentionally added amines after OPA/*tert*-butyl thiol derivatization only; (B) same sample after derivatization followed by scavenging reaction using IAA and then cysteic acid/sulfite; (C) 25 nM amino acid standard derivatized and scavenged as in (B). All injection volumes were 100 nL, and total sample volumes were 500 μL. Separation conditions as in Figure 2.

RESULTS AND DISCUSSION

Derivatization Reagent. The OPA/*tert*-butyl thiol reagent was used because the final amino acid derivative is highly lipophilic, which makes it ideal for preconcentration onto reversed-phase columns. Since the reagent is also highly soluble in aqueous solutions, the derivatized sample matrix is a weak mobile phase and permits sample injection volumes of up to 2 μL to be used without loss in chromatographic efficiency.[13]

High-Sensitivity Electrochemical Detection of OPA/*tert*-Butyl Thiol Derivatives. To select the optimal potential setting for electrochemical detection, the electrochemical signal, root-mean-square noise (rms), and drift caused by the gradient were all measured as a function of electrode potential (Figure 2). The electrochemical response of OPA/*tert*-butyl thiol derivatives was similar to results obtained by others using a glassy carbon electrode for most of the amino acids except ARG, which demonstrated a slight decrease in signal at the carbon fiber microelectrode at higher electrode potentials (Figure 2A). The rms noise of the detector increased drastically above +0.9 V due to the EDTA in the mobile phase. This increase in noise, combined with the large drift above +0.8 V, dictated the optimal detection potential to be +0.75 V.

Derivatization Conditions. In this study, the OPA concentration in the derivatization solution was 8 mM after addition to the sample, which proved to be sufficient OPA to quantitatively derivatize diluted microdialysate samples while creating minimal background. When the OPA concentration was less than 8 mM, the ASP and GLU peak heights decreased considerably, indicative of discrimination against these amino acids in the reaction.[21−23]

When a blank consisting of HPLC grade water that contained no intentionally added amines were derivatized according to conventional practice,[21] i.e., with no scavenging reactions, the resulting chromatogram was littered with peaks (Figure 3A). The size of the these peaks is greatly exaggerated by the relatively large injection volumes used for preconcentration. The source of these peaks could be contaminating amines or products from side reactions of OPA and *tert*-butyl thiol. To eliminate these background peaks, the thiol scavenger IAA was used to remove the excess thiol after sample derivatization.[3,4,13] The excess OPA was then scavenged by adding cysteic acid and sodium sulfite, a nucleophile that can be used to make OPA-based *N*-alkyl-1-isoindolesulfonates.[24] The resulting product was so hydrophilic it could not be retained on a C18 column (data not shown), making

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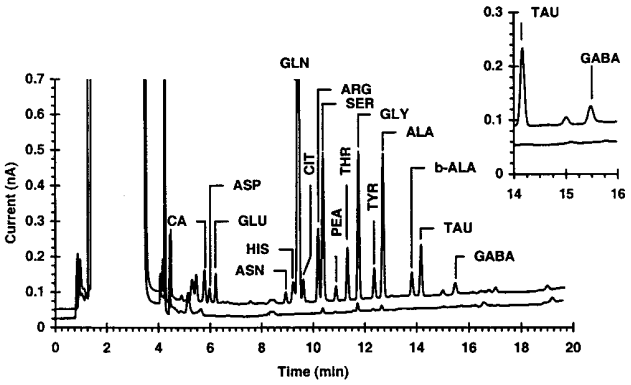

Figure 4. Microscale derivatization of aCSF (bottom trace) and an amino acid standard made to representative concentrations of a microdialysis sample (top trace). Samples (2.0 μL) were derivatized in a polypropylene microvial and 250 nL was injected onto the capillary column. Concentrations of amino acids (nM): CA (from scavenging reaction), ASP (10), GLU (20), asparagine (ASN) (20), histidine (HIS) (40), glutamine (GLN) (500), CIT (20), ARG (50), SER (100), *O*-phosphoethanolamine (PEA, 20), threonine (THR) (50), GLY (100), tyrosine (TYR) (20), L-ALA (100), β-ALA (25), TAU (50), and GABA (10). Inset is expanded view of GABA region. Separation pH was 6.51, with the same gradient as Figure 2.

it ideal for removing excess OPA. A large number of the peaks present in the OPA/*tert*-butyl thiol blank were greatly diminished after these reactions (see Figure 3B). In fact, if the background is considered as the total area of all peaks in the blank that elute in the range of interest, then the scavenging reactions reduced the background by ~90%. When a 25 nM amino acid solution was derivatized and excess reagent scavenged, a majority of the derivatives were free from interference (Figure 3C).

Despite the cleaning procedures used, some amine contamination did occur that could not be controlled. For example, the peak at 19.5 min in Figure 3B would interfere with TAU (see Figure 3C); however, this peak was eliminated when other batches of IAA were used. In some analyses, peaks that intereferred with SER and GLY were also observed in the blanks. In addition, the ASP derivative is partially resolved from a peak at 7.5 min that is the OPA/*tert*-butyl thiol derivative of cysteic acid. As shown in Figure 4, reducing the mobile phase pH to 6.51 improved the resolution of these two peaks.

Microscale Derivatization. The initial exploration of derivatization described above was performed on samples with 500 μL volume. When the samples were reduced to 2 μL in preparation for microdialysis studies, amine contamination became problematic. Experiments with microvials demonstrated a strong correlation between surface area-to-volume ratio and the degree of contamination, indicating surface contamination. When cleaning procedures and polypropylene microvials described in the Experimental Section were used, a majority of the contamination peaks were eliminated (Figure 4). The polypropylene sample vials were also advantageous since they did not adsorb the amino acids over the time scale used.

Analytical Figures of Merit. The use of scavenging reactions and 250-nL injection volumes allowed detection limits of ~300 pM for almost all amino acids tested. (Detection limits calculated using a signal-to-noise ratio of 3 and rms noise of 70 fA). Arginine and citrulline had slightly better detection limits (90 and 200 pM, respectively) due to better electrochemical signals for these derivatives. The similarity in detection limits for most of the amino acids indicates that no discrimination took place during the reaction and that most of these amino acids are free of side product interference which would artificially increase the derivative signal. In contrast, without scavenging, most of the amino acids coeluted with a background peak. For GLY and SER, small peaks from the background were still interfering. Since levels of these amino acids in diluted dialysate were >100 nM, the contribution of the background signal during microdialysis studies was negligible. When injection volumes greater than 250 nL were used, several additional peaks appeared in the chromatogram that were not removed with the scavenging reactions. Thus, this appears to be the limit to preconcentration that can be achieved with this approach.

The concentration detection limits reported here represent a 16-fold improvement compared to literature techniques capable of simultaneous, quantitative determination of ASP, GLU, GLY, TAU, GABA, SER, CIT, and ARG.[22] Combined with the inherent mass sensitivity of the capillary method (over 100-fold better than the HPLC method[22]), the technique is uniquely suited for analyzing small fractions collected from microdialysis. LODs of <10 nM for some of these amino acids by CZE-LIF have been reported;[25,26] however, these reports also indicate large variations in LODs for different amino acids with differences as large as 345-fold being reported. The uniformly low LODs obtained by this method are seen as an important advantage. In addition, the LC method is more amenable to simultaneous resolution of all of the amino acids of interest.

Calibration curves were linear up to 10 μM for each amino acid with correlation coefficients of 0.998 or better for all amino acids. Peak heights had 5−8% RSD for separately prepared samples ($n = 5$). Reproducibility measured in this way includes variability in sample preparation, derivatization, and injection. Preliminary experiments suggest that reproducibility can be improved by using an automated system for derivatization and injection. Retention time RSDs were <1% for multiple analyses in 1 day ($n = 5$).

High Temporal Resolution Monitoring during in Vivo Microdialysis. Using the methodology described above, the basal level of all the amino acid neurotransmitters were readily measured in 10-s microdialysis fractions as illustrated in Figure 5A and Figure 6. The basal amino acid concentrations and variability (see Figure 6) found in these studies are within the range of those found previously in striatum of anesthetized rats.[27−39]

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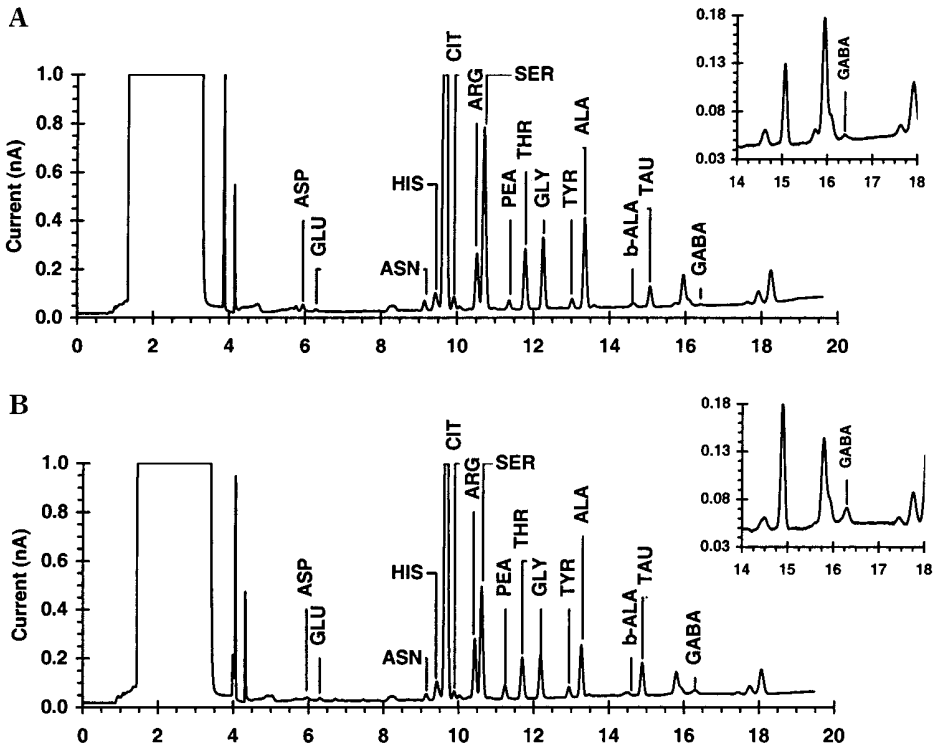

Figure 5. Chromatograms obtained from 10-s microdialysis fractions (200 nL) diluted to 2.0 μL prior to derivatization and scavenging. (A) Basal (B) Chromatogram obtained after the probe was perfused with 0.5 mM nipecotic acid for 20 min causing uptake inhibition selective for GABA. Known peaks are labeled. Insets highlight sections with GABA elution. Separation conditions are the same as for Figure 4.

In addition to the amino acids that were used in method development, several other compounds (unlabeled peaks in Figure 5) were detected in the dialysate. α-Aminobutyric acid and β-aminoisobutyric acid were identified as two of the three peaks eluting at 16 min while methionine (MET) and valine (VAL) were determined to be two of the three peaks eluting at 18 min by comparing the chromatograms to those for a 44 amino acid standard (Sigma Physiological Amino Acid Standard). The identity of the remaining peaks is unknown, but they were only present in the dialysate collected in vivo.

As a demonstration of monitoring during pharmacological manipulation by in vivo microdialysis, aCSF was supplemented with 0.5 mM nipecotic acid, an uptake inhibitor selective for GABA transporters.[40] After 20 min of treatment, GABA level increased 2.2-fold ($p < 0.001$, $n = 3$) relative to the basal level (Figure 5B and 6), which corresponds well to literature values for GABA uptake inhibition in the striatum.[41] Fluctuation in levels of the other amino acids (GLY, L-alanine (L-ALA)) were observed during GABA uptake inhibition; however, the changes were not statistically significant (see Figure 6), in agreement with previous work.[41]

To demonstrate the ability to temporally resolve concentration changes, we continuously collected 10-s fractions as the perfusate was changed from aCSF to aCSF supplemented with 150 mM K^+ for 60 s, a treatment that is expected to depolarize neuronal membranes and cause release of neurotransmitters. Large changes in the level of the neurotransmitter/neuromodulator candidates ASP, GLU, GLY, TAU, GABA, and SER, as well as L-ALA and β-ALA were observed (see Figure 6). (The changes in L-ALA and β-ALA are likely due to metabolic changes caused by the stimulation.) Figure 7 illustrates the temporally resolved changes from several consecutive fractions. As shown in Figure 7, all of the neurotransmitter candidates increased during stimulation and returned to baseline soon after while other amino acids showed no significant changes during the experiment. TAU exhibited unusual behavior in that its concentration increased slower and remained elevated longer than the other amino acids. This behavior has not previously been observed due to the long sampling times traditionally used in microdialysis studies. This delayed response is consistent with the hypothesis that TAU is not a neurotransmitter but is rather involved in osmotic regulation in the brain.[42] In addition to the temporal resolution shown, this experiment illustrates how experimental times can be reduced as the entire K^+ stimulation was performed in less time than it

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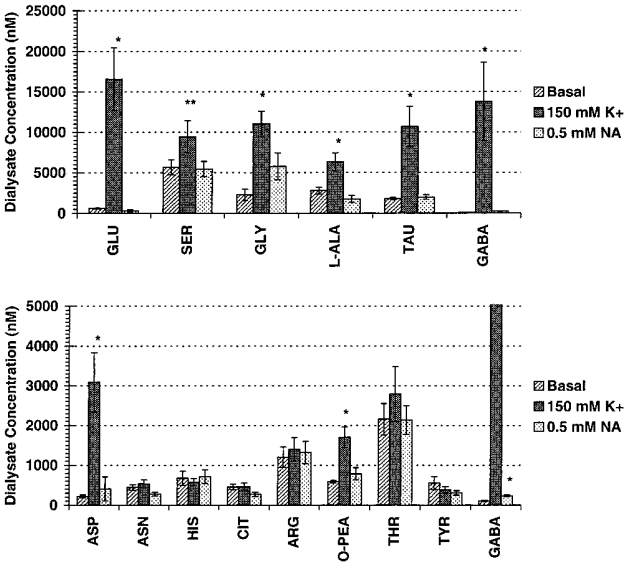

Figure 6. Results of 10-s monitoring during in vivo microdialysis of the striatum. Data are mean ± 1 SEM, $n = 3$; significant changes are noted by the asterisks (*, $p < 0.01$; **, $p < 0.05$). Basal levels correspond to aCSF (see Experimental Section) being perfused through the probe. For nipecotic acid data, aCSF was supplemented with 0.5 mM nipecotic acid. For K^+ stimulation, the K^+ concentration was elevated to 150 mM and data are for the maximal change (see Experimental Section). Upper graph shows the higher level analytes. Lower graph illustrates the lower level analytes. GABA is shown in both graphs because of a large change with K^+.

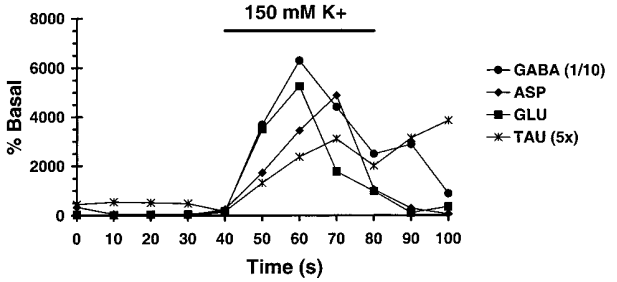

Figure 7. Temporally resolved changes in extracellular amino acid levels during K^+ stimulation. Data presented as percent of basal to normalize scale. The GABA scale was reduced 10-fold and TAU scale enhanced 5-fold because of the large differences in response. K^+-supplemented aCSF reached the probe at 40 s and was removed at 80 s. Amino acids not shown did not change during stimulation.

would take to collect one sample using conventional analytical methods.

Manually collecting 10-s fractions led to occasional sampling error that resulted in artificially low levels of neurotransmitter levels being reported in some fractions, such as fraction 8 (80-s point) in Figure 7. Errors of this sort arose when the probe outlet was not smoothly transferred from vial to vial. Automation of the fraction collection is currently being explored to minimize this error.

Other Applications. While well-suited for high temporal resolution monitoring during in vivo microdialysis studies, the high sensitivity of the method may also simplify other nanoscale applications such as single-cell analysis. Usually, great effort is exerted to avoid dilution of single cells by using nanoscale sample manipulation.[43−46] Using methods with low concentration and mass detection limits such as the one presented here, it may be possible to dilute small samples to volumes that are conveniently manipulated using ordinary microdispensers and injectors before analysis, thus avoiding specialized sample manipulation procedures.

CONCLUSIONS

We have presented a method capable of determining all the neuroactive amino acids in 200-nL microdialysis fractions collected at 10-s intervals allowing a 60-fold improvement in temporal resolution over previous methods for simultaneous determination of these compounds. Dilution of the 200-nL fractions to larger volumes greatly simplifies sample manipulation and allows for possible automation of this procedure using commercially available autosamplers. To achieve the detection limits necessary for quantification, it is necessary to eliminate amine contamination by thorough cleaning and minimize background peaks.

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