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Neurotransmitter sampling and storage for capillary electrophoresis analysis

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Abstract Quantitative analysis of signaling molecules from single cells and cellular materials requires careful validation of the analytical methods. Strategies have been investigated that enable single neurons and neuronal tissues to be stored before being assayed for many low-weight, biologically active molecules, such as serotonin, dopamine, and citrulline. Both metacerebral cell and pedal ganglia homogenates isolated from *Pleurobranchaea californica* have been studied by capillary electrophoresis with two complimentary laser-induced fluorescence detection methods. For homogenized ganglia samples, several cellular analytes (such as arginine and citrulline) are unaffected by standing at room temperature for days. Many other analytes in the biological matrix, including the catecholamines and indolamines, degrade by 20% within 10 h at room temperature. Rapidly freezing samples or preserving them with ascorbic acid preserves more than 80% of the dopamine and about 70% of the serotonin even after five days. In addition, serotonin and dopamine remain completely stable for at least five days by combining the ascorbic acid preservation and freezing at –20 °C. The timing of preservation is critical in maintaining the original composition of the biological samples. Using our optimum storage protocol of freezing the sample within 2 h after isolation, we can store frozen homogenate ganglia samples for more than four weeks before assay while still obtaining losses less than 10% of the original serotonin and dopamine. The nanoliter-volume single cell samples, however, must be analyzed within 4 h to obtain losses of less than 10% for serotonin related metabolites.

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1. Introduction

Simultaneous detection and quantitation of neurotransmitters, their metabolites and cofactors at the level of individual neurons provide fundamental information for neurophysiological understanding of the cellular processes occurring in the central nervous system. Capillary electrophoresis (CE) offers a number of advantages for measuring the chemical composition of individual cells. It provides rapid separations and high separation efficiencies with small sample volumes [1–2]. As many of the molecules of interest within a cell are in the picomolar to micromolar range, femtomole to zeptomole detection sensitivities are required. Laser induced fluorescence (LIF) detection in CE exhibits the best performance characteristics compared with other detection methods in terms of sensitivity, limits of detection and linearity [3].

Among neurotransmitters, the indolamines such as 5-hydroxytryptamine (5-HT or serotonin), the catecholamines such as dopamine (DA), and the gaseous radical nitric oxide (NO) are of particular interest because of their widespread distribution in central and peripheral tissues, broad spectrum of biological actions, and critical roles in diverse pathologies [4–5]. Two different CE-LIF systems have been developed in our group [6–11] to study these monoamines and the NO-related metabolites arginine (Arg) and citrulline (Cit). The LODs for these systems are ~ 20 attomole (10 nM) for 5-HT [7], ~ 240 attomole (120 nM) for DA [8], and ~ 50 attomole to 17 fmole (5 nM to 17 μM) for Arg and Cit [11] in neurons studied. However, for many experiments, a large number of samples are collected over a short period, which precludes the analysis of all samples in "real-time". In addition, samples may be collected at geographically distant locations and need to be shipped before assay. Thus, the current study is to determine the effects of sample storage on the quantitation of cellular analytes as determined by CE.

It is well known that biogenic amines are subject to oxidation in aqueous solution. The optimal storage conditions for cellular samples containing these and other compounds are not known. Literature reports on the degrada-

tion and storage conditions for solutions containing catecholamines or indolamines [12–20] are available, which indicate that temperature, solvent pH, antioxidant additives, storage container type and length of storage time can have dramatic effects on stabilities of biogenic amines [12–16]. However, there is significant variability in the storage conditions used by various investigators [17–20]. These amines have been stored at temperatures ranging from room temperature to –70 °C; in various acids and buffers, including perchloric acid, hydrochloric acid, formic acid and acetic acid, with molarities of 0.01 to 0.5; at pHs from less than 1 to more than 8; with additives of ethylenediaminetetraacetic acid (EDTA), sodium bisulfite, L-cysteine and ascorbic acid; in tubes made of polypropylene, soda-lime glass and borosilicate glass; and for periods of 12 h up to one year. Not surprisingly, past results have been highly variable so that optimum storage conditions for these samples are not always obvious from the literature. Another complicating factor is that the different analytical approaches and different sample types may require different preservation strategies. Most of these past studies were performed using standards [12–19], plasma or urine [20] as the matrix, and liquid chromatography with electrochemistry detection as the analytical method, so were not intended to preserve nanoliter cellular sample for assays with CE. The aims of the present study are as follows: (1) to determine the stabilities of 5-HT, DA, Arg, and Cit in pH 8.7 borate buffer, which is normally used for both CE systems under various preservation conditions; (2) to validate a storage protocol for cellular samples for CE-LIF; (3) to determine if a calibration curve will allow correction of the degraded analyte concentration for samples measured at different times after sample collection; and (4) to determine if the much smaller single cell samples degrade differently than the larger cellular homogenate samples.

2. Experimental

2.1 Chemicals and reagents

All chemicals used were of analytical or research grade. Two ~ 30 mM borate buffers with different pH were employed. The pH 8.7 borate buffer was prepared using 3.0 g boric acid (H_3BO_3; Fisher Scientific, Fair Lawn, NJ, or Sigma, St. Louis, MO), 9.2 g sodium borate ($Na_2B_4O_7 \cdot 10\ H_2O$; Fisher Scientific or Sigma) in 1.00 L ultrapure water (Milli-Q filtration system; Millipore, Bedford, MA). The pH 9.8 borate buffer was prepared using 0.03 g H_3BO_3 (Sigma), 0.97 g $Na_2B_4O_7 \cdot 10\ H_2O$ (Sigma) in 0.10 L ultrapure water (Millipore), with NaOH added dropwise to adjust to the desired pH and lauryl sulfate (Sigma) added to the buffer to a final concentration of 20 mM. Ascorbic acid was obtained from Fisher. All other chemicals were from Sigma. 5-HT and DA standards were prepared daily from solids in pH 8.7 buffer. Arg and Cit standards were prepared weekly in the pH 9.8 buffer. All solid standards were stored at 4 °C or frozen at –20 °C as appropriate when not in use.

2.2 Apparatus and methods

2.2.1 Wavelength-resolved CZE-LIF using native fluorescence. The laboratory-assembled CE system with 257 nm excitation and a CCD/ spectrograph for wavelength-resolved fluorescence emission spectra was employed for the monoamine study [7, 8]. This system has a home-built nanovial autosampler ideally suited for single cell assays [9]. This CE system allows sensitive detection and spectral identification of species that naturally fluorescence with 257 nm excitation, including tyrosine (Tyr), tryptophan (Trp), 5-HT, and DA. Briefly, a fused-silica capillary 800-mm-long, 50-μm-I.D./ 140-μm-O.D. (Polymicro Technologies, Phoenix, AZ, USA) was employed. The detection end of the capillary was directed into a sheath flow assembly, where the core stream was excited by a frequency-doubled, liquid-cooled argon-ion laser (Innova 300 FrED; Coherent, Palo Alto, CA) operating at 257 nm. The sheath flow buffer and the running buffer were the same pH 8.7 borate buffer. The sheath flow rate was at about ~ 0.5 mm/s and the laser power was 0.5 mW at the sheath flow cell. The collection optics were orthogonal to the excitation beam focusing the fluorescence emission to a f/2.2 CP 140 imaging spectrograph (Instruments SA, Edison, NJ, USA) and then onto a 1,024 × 256 detector-array, liquid-nitrogen-cooled scientific CCD (EEV 15–11; Essex, U.K.). Sample injection was performed electrokinetically at 2.1 kV (current ~ 2.4 μA) for 10.0 s from a 360 nL stainless steel microvial, and about 3 nL sample was injected into the capillary. The separation voltage was maintained at 21 kV (current ~ 25 μA). All experiments were at room temperature. Each day, the capillary was rinsed with 0.1 M NaOH, water and running buffer (pH 8.7), 5 min each. Between runs the capillary was rinsed for 5 min with the running buffer. Fluorescence emission from 260 to 710 nm was processed and viewed in MATLAB (the Mathworks, Natrick, MA, USA) on a PC.

2.2.2. CZE-LIF using a 350–356 nm Ar-Kr laser for excitation. A second laboratory-assembled CE system with fluorescamine (FA) derivatization, 350–356 nm LIF and a photomultiplier tube (PMT) was employed for Arg and Cit study. This system, similar to those described by Shippy [10] and Floyd [11], used a 810-mm-long 50-μm- I.D./ 365-μm-O.D. fused-silica capillary (Polymicro Technologies). The excitation source was a multimode argon-krypton (Ar-Kr) laser (Innova 70 Spectrum; Coherent, Palo Alto, CA) with 5 mW of the 350–356 nm output focused 40 cm from the injection end. Emission from FA-derivatized primary amine metabolites was collected, filtered, and focused onto a PMT detector (HC 125–03, Hamamatsu, Bridgewater, NJ). Sample injection was performed electrokinetically at 8 kV (current ~ 14 μA) for 8 s from a polypropylene microvial, with approximately 7.5 nL of sample introduced into the capillary. Separation potential was maintained at 20 kV (current ~ 45 μA). All experiments were at room temperature. Derivatized products were produced by combing 1 μL of FA-acetone solution (12 mg FA /1 mL acetone) with 7 μL of sample solution at room temperature for 2 min before analysis.

2.3 Cell isolation and sample preparation

Pleurobranchaea californica (200–500 g) were obtained from Sealife Supply (Sand City, CA) and kept in artificial seawater at 12–14 °C until use. The cell isolation procedure has been described previously [7–9]. For the single neuron work, a single metacerebral cell (MCC) was dissected from *Pleurobranchaea californica* under cold anesthesia in molluscan physiological saline. The MCC was isolated under a stereomicroscope using microscissors and glass micropipettes. In addition, several studies used the entire pedal ganglia. Isolated MCC neurons or pedal ganglia were placed in polypropylene centrifuge tubes (0.75 mL) and placed on dry ice immediately after dissection. The samples were then thawed and homogenized by the combined action of a stainless steel agitator and /or hypoosmotic buffer damage. Four MCC neurons were homogenized together in 2 μL and two pedal ganglia were homogenized together in 4 μL pH 8.7 borate buffer. The homogenization process required about 1 min. The homogenates were centrifuged at 10,000 × g for 10 min at 4 °C, then stored according to the protocols outlined below.

2.4 Storage protocols

2.4.1 Stability study of standards and homogenates. Pedal ganglia homogenates were prepared as above. Immediately after centrifugation, 2 μL of supernatant was used to prepare a 300 μL dilute homogenate solution composed borate buffer and added standards. The moles of standard compounds added to the final homogenate solution were 7.2, 48.0, 6.0 and 45.0 nanomole for Arg, Cit, 5-HT and DA, respectively. A second standard solution was prepared with the same standards but without the homogenate, as a control. Aliquots of 30 μL of the homogenates and in some cases, the controls as stated below, were used to evaluate the following experimental conditions:

I. Room temperature (RT) storage (20–25 °C): paired homogenates and controls
II. Heat (95 °C) for 5–10 min followed by RT storage: paired homogenates and controls
III. Freezing with dry ice or at –20 °C, followed by –20 °C storage
IV. Chemical preservation with ascorbic acid (2 mM) followed by RT storage
V. Ascorbic acid preservation followed by freezing storage

All aliquots were in their storage conditions within 5 min after centrifugation except the heated samples. Samples were studied at different time by CE for up to one week. To prevent evaporation of solution during the heating process, the stock aliquot was placed in a skirted vial and inverted in a 90 °C water bath for a period of 5 min. The inverted tube bases were then filled with cold water until tubes were cooled slightly below ambient before being righted and placed in a RT storage rack.

2.4.2 Degradation time-course. Pedal ganglia homogenates were prepared as in section 2.3. Immediately after centrifugation, 1 μL of supernatant was diluted to 200 μL final homogenate solution composed of borate buffer and added standards. The moles of standard compounds added to the final homogenate solution were 0.48, 0.2 and 3.0 nanomole for Cit, 5-HT and DA respectively. Then at various times (t = 0, 0.5, 1.0, 1.5, 3.0, 6.0, and 10.0 h), four 5 μL aliquots were removed, rapidly frozen on dry ice, and then stored at –20 °C. A sample "set" was composed of a group of seven aliquots collected as a function of time. Each of the four sets of frozen samples were then studied afterwards for up to one month. Within each set, the samples were analyzed randomly to avoid any systematic variations. This experiment was repeated twice using one additional *Pleurobranchaea californica* each time (4 pedal ganglia) to minimize single-animal variability in the data conclusions. In the second series, only one set of aliquots were collected for each animal, and the amount of DA added was decreased from 3.0 to 2.0 nanomoles.

2.4.3 "Single neuron" degradation study. In order to evaluate single cell preservation and to remove the cell-to-cell variability inherent in single-cell studies, 4 MCC neurons were dissected and placed in 2 μL pH 8.7 buffer as described in 2.3. Immediately after centrifugation, 360 nL was withdrawn for sheath-flow-CE-LIF measurement (t = 0.0 h) and the rest was stored in the polypropylene microvial in the freezer at –20 °C. At various times(t = ~ 0.3, 1.0, 2.5, 4.0, 6.0, 11.0, 25.0 and 75.0 h), the homogenate was thawed and centrifuged for 10 min at 4 °C, with 360 nL supernatant aliquots withdrawn and analyzed.

3. Results and discussion

It is well known that in aqueous solutions catecholamines, indolamines and their metabolites can undergo degradation due to oxidation. The goals of this study were to determine the stability of the monoamine neurotransmitters such as serotonin and dopamine and NO-pathway compounds Arg and Cit, and to determine a storage protocol for these compounds for both microliter-volume cellular homogenate samples and nanoliter-volume single cell samples. 5-HT and DA are natively fluorescent and were analyzed by the wavelength-resolved CE-LIF system, while the Arg and Cit were studied by a second CE system after FA derivatization.

3.1 Stability of 5-HT, DA, Arg and Cit under various storage conditions

In *Pleurobranchaea californica*, relatively few neurons in a ganglion containing thousands of neurons may contain a specific neurotransmitter; for example, there are relatively few 5-HT containing neurons in the cerebral ganglia. As a requirement of the present study is to make multiple measurements from a solution to determine analyte stability and only a single measurement can be performed for a single cell, we have chosen to use a homogenized ganglia spiked with appropriate compounds to produce neurotransmitter levels similar to what would be found in a positive neuron. With the presence of the rest of the cellular homogenate, it is possible to determine whether a homogenate-mediated effect on analyte degradation was observed. Percentage recoveries of 5-HT, DA, Arg and Cit under various storage conditions are shown in Fig. 1. Heating was used to eliminate any enzymatic activity that might exist in the biological matrix. The 100 percentages for 5-HT and DA were defined as the quantity measured at the first recorded data point, which were about 15 min after the cellular isolation (time 0 h) for RT storage and about 2 h for other storage strategies. The 100 percentages for Arg and Cit are defined similarly as the first measurement at 2 h. As shown in Fig. 1 A, at room temperature, Arg and Cit remained stable even after a week without any additive. 5-HT and DA, however, degraded quickly at room temperature. Faster degradation occurs in the homogenate samples compared to the controls. 5-HT in control solution was reduced by 20% by 7 h and 50% after 22 h; the 5-HT in homogenate solution was decreased by 80% in 22 h. After 3 d, only 3% of the original 5-HT remained in both samples, and it was undetectable by the fifth day. Compared with the 5-HT, DA decreased more gradually. About 80% of DA in homogenate solution and 90% in control solution was preserved within 1 d, and 30–50% recovery was observed for DA (30% in homogenate solution and 50% in control solution) by 5 d. Recoveries under heat-RT storage protocol are shown in Fig. 1 B. Again, the recoveries of Arg and Cit remained virtually 100% stable from the first measurement. The declines in the recoveries of 5-HT and DA remain essentially the same as with RT storage, except that there is no significant difference between the homogenate and control samples.

The observation that 5-HT and DA in homogenate-containing solutions degraded faster than in control solutions suggests that degradation factors other than simple oxidation by dissolved oxygen contribute to the loss of these species. These may be enzymatic in nature as the

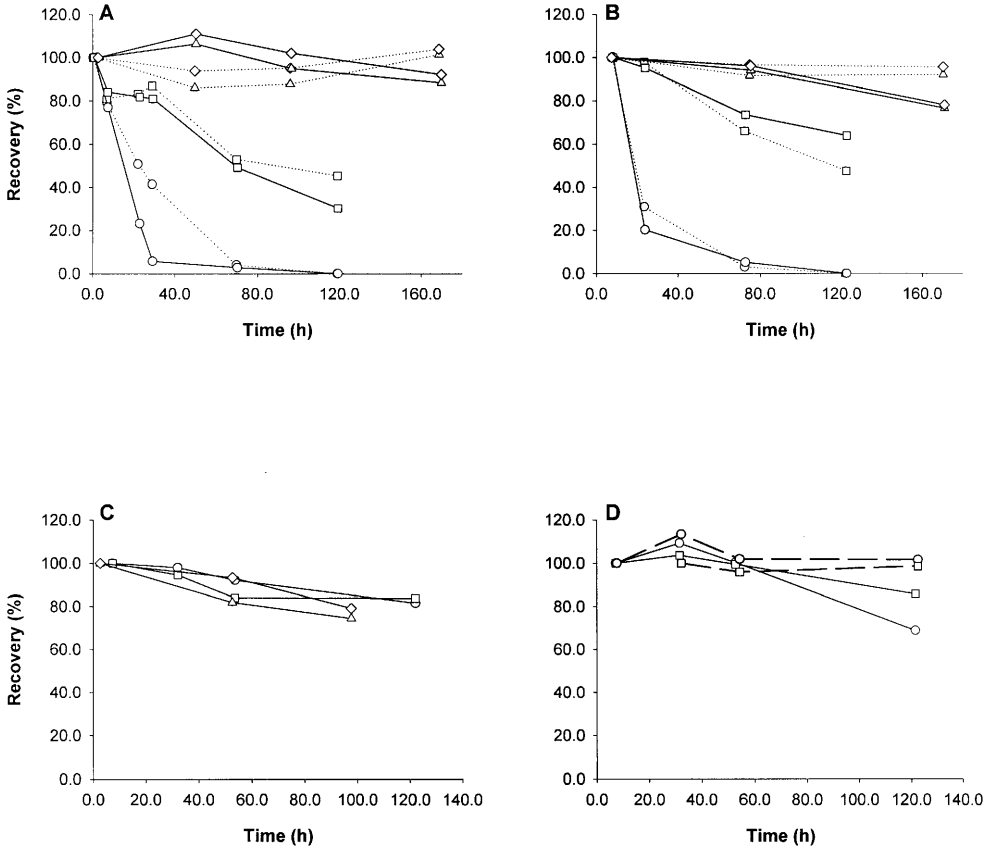

Fig. 1 Percentage recoveries of 5-HT (○), DA (□), Arg (△) and Cit (◇) under various storage conditions. (**A**) Room temperature storage; (**B**) heat-treatment followed by room temperature storage; (**C**) frozen storage and (**D**) ascorbic acid preservation with (solid line) and without (dashed line) frozen storage. Dotted lines in A and B are for the control samples; all others are for the homogenate samples. The first data point is defined as the 100% point for all measurements. Time 0 is defined as the time the first measurement was performed, which occurred as soon as possible after sample isolation and/or preparation

heat-treatment reduced the effect, so that the heated homogenate and control samples show no statistical differences in degradation.

For further investigations, only the homogenate samples were studied. The percentage recoveries with only –20 °C storage compared to ascorbic acid preservation with and without frozen storage are shown in Fig. 1 C and D, respectively. Recoveries of both 5-HT and DA improved considerably with these strategies. The recoveries of 5-HT and DA increased to ~ 80% at day 5 with freezing. Ascorbic acid preserved almost 100% of the DA and 5-HT for more than 2 d, and about 90% of the DA for 5 d. In addition, 5-HT and DA remained stable for at least 5 d under the combined ascorbic acid and freezing treatment. Thus, the optimum storage conditions used both ascorbic acid and freezing. The NO-pathway analytes, Arg and Cit, are stable and can be reliably measured after storage under most conditions tested here.

3.2 Degradation time-course at room temperature within 10 hours

While freezing preserves ganglia homogenate samples for several days, it may not always be feasible to freeze samples immediately after the isolation, such as when subsequent dissections, physiological measurements or other requirements preclude rapid freezing. Can one develop a correction for 5-HT and DA levels to account for reasonable delays in freezing? A degradation time-course experiment was undertaken for this purpose. For each experiment, the CE electropherogram was taken for samples frozen at 0, 0.5, 1, 1.5, 2, 3, 6 and 10 h after homogenization and freezing was assumed to preserve 100% of the original amount at the time of freezing. This assumption was tested by taking 4 sets of aliquots, freezing them, and analyzing them at day 1, day 4, day 15 and day 29 to determine whether there is detectable degradation from the frozen storage. Two additional experiments were performed with different animals to investigate whether degradation varies animal to animal. The percentage recoveries of 5-HT and DA for the 6 sets of measurements (4 sets from animal 1) are shown in Fig. 2. Most of the measurements are within the 1 sigma error range except several DA points at the fourth week measurement (data not shown), confirming that freezing for 29 d affects DA levels as previously determined. Freezing samples within 2 h after isolation preserves ~ 90% of the original 5-HT and DA, even after 4 weeks of storage, and even greater preservation is possible by the addition of ascorbic acid. However, although the measurements are reproducible, the average 5% uncertainty and the relatively small slope (20% for a 10 h delay) make reliable degradation correction curves for 5-HT and DA impractical for moderate delays in freezing as the uncertainty in the correction is similar to the magnitude of the correction.

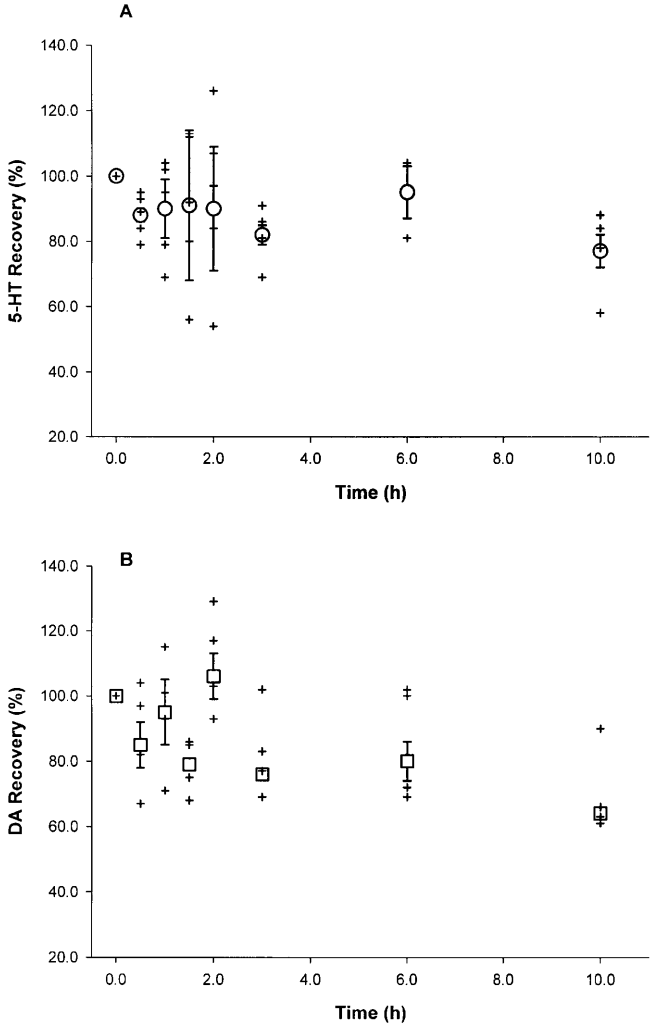

Fig. 2 Time-course degradation of 5-HT (**A**) and DA (**B**). The average percent recovery at various time-of-freezing is shown as symbols with error bars (-○- 5-HT; -□- for DA). The individual data points are presented by the cross hair symbols (+). The error bars are created as the sample standard deviation, N = 6 (for DA, N = 5, excluding the 4th week measurement)

3.3 Stability of 5-HT related metabolites in single neurons

Detection and quantitation of neurotransmitters from individual neurons are required for many neuronal studies. Using our methods, a single cell sample is stored in stainless steel microvial (~360 nL) and can be assayed only once. However, by homogenizing several cells in a few microliters, a stability study can be performed using the immediate freezing method. Individual MCC neurons are isolated, homogenized in small volume of buffer solution and then frozen immediately. Figure 3 shows the change of the concentrations of the native compounds, including 5-HT, one of its metabolites, n-acetyl serotonin (NAS) and the amino acids Trp and Tyr. The 100 percentages for each of these compounds are defined as the quantity from the first measurement, which was performed just prior to

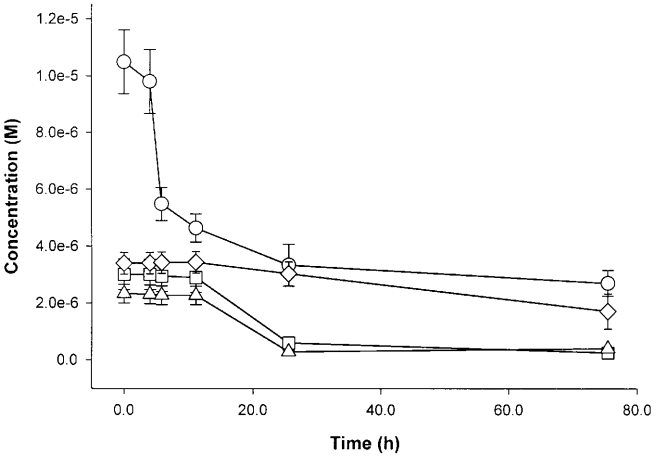

Fig. 3 Percentage recoveries of 5-HT (○), NAS (□), Trp (△) and Tyr (◇) versus time. 100% recovery is defined as the quantity measured at the first recorded data point (time 0). The error bars are created as the measurement error

sample freezing (time 0). Not surprisingly, Tyr is the most stable, with 60% remaining after 3 d. All the other compounds degraded by ~ 80% by the end of the third day. As before, 5-HT is the least stable, with only 40% observed after 11 h, while both Trp and NAS remained at ~ 90% stable for 11 h. However, there were no statistical differences in recoveries between time 0 and storage for less than 4 h for all compounds; therefore results for the less than 4 h intervals (t = ~ 0.3, 1.0, 2.5, 4.0 h) were combined and reported as the 4-h result. Our observations suggest that single neuron samples need to be analyzed within 4 h of freezing to reduce degradation from the cell matrix. Because of the small volumes and lack of sufficient numbers of MCC neurons, neither heat-treatment nor the combination of freezing with ascorbic acid addition were investigated.

Conclusions

We have investigated the degradation of 5-HT, DA, Arg and Cit for neuronal samples prior to capillary electrophoresis measurements. There is insignificant degradation of both Arg and Cit under all storage conditions tested for storage times of up to 5 d. In contrast, indolamines and catecholamines quickly degrade at room temperature and must either be frozen immediately or an antioxidant must be added after cell isolation, both of which greatly preserve such samples. One intriguing result from this study is that nanoliter-volume single-neuron homogenates are much less stable than the ganglia homogenates. The indolamines in < 2 μL volumes are only stable under freezing storage for 4 h. Possible reasons for the increased degradation include that the cell samples contain high levels of endogenous factors that degrade the transmitters, or that the smaller samples have a much greater relative surface area that increases the exposure of the indolamines and catecholamines to molecular oxygen.

Future work will investigate optimum combinations of freezing and additives for single cell samples. As each CE run, including rinsing and sample loading, is approximately 50 min long, only 5 samples can be obtained from a single animal or sets of experiments within this 4 h window. Current work also involves reducing the separation time to help alleviate this restriction.

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