Published in final edited form as:
Anal Chem. 2005 August 15; 77(16): 5349–5355.

Microcolumn Separation of Amine Metabolites in the Fruit Fly

Tracy L. Paxon[a,*], **Paula R. Powell**[a,*], **Hyun-Gwan Lee**[b], **Kyung-An Han**[b,c], and **Andrew G. Ewing**[a,†]

a *104 Chemistry Research Building, Department of Chemistry, Pennsylvania State University, University Park, PA 16802*

b *InterCollege Graduate Program in Genetics, Pennsylvania State University, University Park, PA 16802*

c *208 Mueller Laboratory, Department of Biology, Pennsylvania State University, University Park, PA 16802*

Abstract

Electrophoretic resolution of fourteen biogenic amines and metabolites with similar mobilities is addressed by employing micellar electrokinetic capillary chromatography coupled to amperometric electrochemical detection. The present study describes the optimization of separation conditions to achieve resolution of analytes of biological significance within 20 minutes in a single separation. They include dopamine, epinephrine, norepinephrine, octopamine (OA), L-3, 4-dihydroxyphenylalanine, tyramine (TA), and serotonin as well as metabolites 5-hydroxyindolacetic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid, and 3-methoxytyramine in addition to N-acetylated metabolites including N-acetyl dopamine, N-acetyl octopamine (naOA), and N-acetyl serotonin. The optimized conditions used result in excellent reproducibility and predictable peak shifting, thus enabling identification of several metabolites along with their biogenic amine precursors in biological samples, specifically from the fruit fly *Drosophila melanogaster*. The separation method is sensitive, selective and quantitative as demonstrated by its capacity to detect changes in TA, OA, and naOA present in the head homogenates of the Canton-S and mutant *inactive*^{1} *Drosophila* lines. Quantitative analysis of metabolites in conjunction with their biogenic amine precursors in a single separation offers tremendous potential to understand the physiological processes and underlying mechanisms mediated by various biogenic amines in *Drosophila* and other animals.

INTRODUCTION

To understand fully the contribution of neuromodulators to neuronal function and dysfunction, it is essential to identify and quantify these small molecules in concert with the products and intermediates of their metabolism. The biogenic amines such as catecholamines [dopamine (DA), norepinephrine (NE), octopamine (OA), etc.] and the indoleamine serotonin (5-HT) are particularly important to study due to their roles in a variety of physiological processes including stress-induced responses, emotion, and adaptive behaviors such as learning, memory, and drug addiction. Quantitative analysis of the metabolites, or enzymatic degradation products, of these biogenic amines may reveal information regarding their release and turnover in the brain, providing valuable insight into particular neuronal activities that involve these neuromodulators.

† All correspondence should be addressed to Dr. Andrew G. Ewing, Email: age@psu.edu, Phone (814) 863-4653, Fax (814) 863-8081.
* Both authors contributed equally to the work presented herein

Despite a broad knowledge of fundamental metabolic pathways of individual biogenic amines, little is known about the physiological levels of metabolites and their regulation and significance in the brain. This is largely due to the unavailability of suitable analytical tools. Recent advances in innovative technologies have led to increased scientific focus in the area of metabolomic analysis, allowing the identification and quantification of numerous metabolites in a given biological sample. Analytical methods including fluorimetric, spectrophotometric, radioenzymatic, gas and liquid chromatography, and immunoassay have been employed for the analysis of biogenic amines and their metabolites in tissue and biological fluids; however, few technologies have found widespread acceptance, particularly in clinical laboratories.[1] Thus, it is of great interest to develop highly sensitive techniques to distinguish and monitor fluxes in the levels of biogenic amines and their metabolites with a high degree of resolution. Capillary electrophoresis (CE) is one of the most promising separation techniques for metabolome analysis.[2] Electrophoresis employing narrow inner-diameter (i.d.) capillaries enables sampling from extremely small volumes, is relatively simple, and allows rapid analysis times, while providing a high degree of spatial resolution.[3–5] Recently, CE with diode array detection has been employed clinically for the determination of catecholamine metabolites in patient urine,[6, 7] and has proven to be an effective approach as a diagnostic tool for metabolic disease.[8] In addition, micellar electrokinetic capillary chromatography (MEKC), a distinct separation mode of CE, has been employed for the metabolome challenge.[2] MEKC yields enhanced selectivity due to the addition of a surfactant in the electrophoresis buffer that improves the separation of similarly charged and sized biogenic amines and metabolites. Terabe *et al.* have successfully applied MEKC with laser induced fluorescence (LIF) detection to the analysis of the metabolome of bacteria cell extracts.[2] In the present study, we have developed MEKC technology for the separation of biogenic amines and metabolites in the fruit fly, *Drosophila melanogaster.*

Drosophila provides a powerful, genetically tractable model system for the study of diverse developmental and physiological processes. In particular, it is a highly attractive model to investigate the critical roles of biogenic amines involved in adaptive changes in neuronal activities that underlie learning, memory and drug addiction due to its short life cycle, relatively simple nervous system, and ease of molecular and genetic manipulation with completed genomic sequence. Multiple lines of evidence indicate that key molecules and signal transduction pathways involved in synaptic transmission, sensory processing, and higher-order brain functions are highly conserved between *Drosophila* and mammals.[9–12] Furthermore, the actions of biogenic amines in mammals are analogous to those in *Drosophila*.[13, 14] Specifically, catecholamines and indoleamines present in the mammalian central nervous system (CNS) and peripheral nervous system function as neuromodulators in *Drosophila* to mediate diverse physiological processes.[15, 16] While the synthesis of monoamines in invertebrates appears to follow similar pathways to those found in vertebrates, their major metabolic pathways are distinct from those in invertebrates. For example, in mammals catecholamines are metabolized by monoamine oxidases (MAO) for oxidative deamination whereas invertebrates typically utilize a variety of enzymatic routes including N-acetylation, γ-glutamyl conjugation, sugar conjugation, sulphation, β-alanyl conjugation, as well as oxidative deamination.[17] Multiple lines of evidence point to N-acetylation, the process by which N-acetyltransferase acetylates amino moieties, as the key monoamine deactivation pathway in insects, downplaying the mechanism of oxidative deamination inactivation.[14, 17–21] Recent findings, however, suggest that MAO may play a role in *Drosophila* metabolism, although the relative abundance of MAO metabolites compared to N-acetylated metabolites is not documented.[22] To assess the functional significance of distinct metabolic pathways, it is crucial to conduct neurochemical evaluation of the *Drosophila* samples by simultaneously analyzing multiple metabolic products of monoamines, specifically N-acetylated monoamines such as N-acetyl dopamine (naDA) and N-acetyl octopamine (naOA), as well as MAO metabolites including 5-hydroxyindoleacetic acid (5-HIAA) and 3,4-dihydroxyphenylacetic

acid (DOPAC). Improvements in current technology are needed to meet the challenge of measuring these metabolites.

The metabolite N-acetyl serotonin (na5-HT) and the products of γ -glutamyl conjugation have been identified in the sea slug *Aplysia californica* by CE with LIF detection.[23, 24] In addition, investigations of biogenic amines and N-acetylated metabolites have been reported on insects by utilizing high performance liquid chromatography (HPLC) analysis with electrochemical detection (EC).[19, 20, 25] Although these studies have provided significant insights into the metabolic pathways crucial for invertebrate monoamines, they offer limited quantitative information on relevant amines and metabolites. We have demonstrated the capacity of MEKC coupled to EC detection for optimized and reproducible separations of biogenic amine transmitters and precursors in *Drosophila* homogenates and, more importantly, its sensitivity to detect changes in monoamine quantities between control and transgenic animals.[26]

In this paper, we have developed a separation scheme to investigate monoamine metabolites present in the *Drosophila* CNS. This work utilizes the borate-complexation reaction and a sodium dodecyl sulfate (SDS) micellar buffer to elucidate several analytes, including biogenic amines, MAO metabolites, and N-acetylation products. We report the conditions required to achieve optimal, high-resolution separation of fourteen analytes of biological relevance and we have quantified several N-acetylation metabolites present in the *Drosophila* head. Furthermore, the comparative analysis of the monoamine and metabolite levels present in wild-type Canton-S and the mutant *inactive* (*iav*^{1}) flies demonstrates the sensitivity of the system. Thus, the borate-MEKC-EC system offers a valuable tool to distinguish and monitor fluctuations in metabolites and their biogenic amine precursors that underlie diverse physiological and behavioral processes in *Drosophila* and other animals.

EXPERIMENTAL SECTION

Reagents

All amine standards, L-3, 4-Dihydroxyphenyl alanine (L-DOPA), catechol (CAT), N-tris-[hydroxymethyl] methyl-2-aminoethanesulfonic acid (TES), sodium tetraborate, β-cyclodextrin (β-CD) and metabolite standards (unless otherwise indicated) were purchased from Sigma (St. Louis, MO). The naDA and naOA were obtained from the NIMH Chemical Synthesis and Drug Supply Program. SDS and a 48% aqueous solution of hydrofluoric acid (HF) were obtained from Aldrich (Milwaukee, WI). All chemicals were used as received. Separations were performed in 25 mM borate buffer containing 50 mM SDS and 2% 1-propanol, adjusted to pH 9.5. All standards were prepared as 10 mM stock solutions in 0.1 M perchloric acid and were diluted to the desired concentration with additional 0.1 M perchloric acid.

Drosophila strains and Homogenate Preparation

Canton-S wild-type and *iav*^{1} mutant *Drosophila melanogaster* strains were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN) and were maintained in the laboratory. Flies were cultured on standard cornmeal/agar medium and collected between 3-5 days after emerging from pupal cages. Flies were prepared for CE experiments based on a protocol previously described.[26] Specifically, flies were quick-frozen in a liquid nitrogen bath. The heads and bodies were separated by vortexing and the heads were then homogenized in 1 μL of 0.1 M perchloric acid (containing an internal standard of 10 μM CAT) per head. Samples were transferred to an Eppendorf centrifuge with a fixed angle rotor (Brinkman Instruments, Westbury, NY) and spun at 14,000 x rpm for 5 min at 4° C. The supernatant of all samples was subsequently centrifuged (14,000 x rpm, 4° C) through Amicon

Ultrafree-MC centrifuge filters (Fisher Scientific, Pittsburgh, PA). All resulting filtrates were dispensed into 3-μL aliquots and frozen until use.

Instrumentation and Analysis

The CE system with end-column amperometric detection utilized in this study was built in-house and has been described previously.[3, 27] Briefly, 45 to 50 cm of fused silica capillary with an outer diameter of 148 μm and i.d. of 13 μm (Polymicro Technologies, Phoenix, AZ) was used for all separations. Amperometric EC detection was carried out with a two-electrode format in which a 5-μm carbon fiber microelectrode was held between +0.70 and +0.75 V vs. Ag/AgCl reference electrode (World Precision Instruments, Sarasota, FL). Faradaic currents were amplified with a Keithley Model 427 current amplifier (Cleveland, OH) and collected with a LabView 5.1 interface (National Instruments, Austin, TX). Microsoft Excel (Redmond, WA) was used to generate the electropherograms and to perform statistical tests. Data analysis was performed using PeakFit Separation and Analysis Software (SPSS Inc., Chicago, IL) to estimate individual peak areas in all electropherograms.

Procedures

Capillaries were filled with separation buffer using a stainless steel reservoir with applied He pressure (100 psi). Buffer solutions were filtered with a 0.2 μm nylon filter (Alltech, Deerfield, IL) prior to use. Injections were performed electrokinetically at 5 kV for 5 s or at 5 kV for 3 s extracting approximately 1 nanoliter (nL) of homogenate from the thawed homogenate aliquot. To enhance microelectrode placement, the capillary i.d. was enlarged via HF etching as previously described.[27] Approximately 2 mm of the polyimide coating was removed from the capillary to expose the fused silica. The exposed portion of the capillary was placed in HF for 10 min at 200 psi, after which the same segment of capillary was placed in a sodium bicarbonate solution to neutralize the acid and was washed with water.

Safety Considerations

An in-house built safety interlock box was utilized to protect the user from high voltage. Since HF could cause severe burns, it was used with extreme care. HF was neutralized with sodium bicarbonate before disposal.

RESULTS AND DISCUSSION

Optimization of Separation Conditions for Biogenic Amines and Metabolites

Fourteen electroactive biogenic amines and metabolites were selected to gain insights into their potential roles in neurophysiology and behavior in animals, specifically *Drosophila*. A variety of parameters was examined in an effort to resolve these fourteen biogenic amines and metabolites. Previous work employed a 10 mM TES/30 mM SDS/2% 1-propanol separation buffer at neutral pH.[26] This buffer system provides excellent resolution of L-DOPA, NE, epinephrine (E), OA, DA, TA, and 5-HT (basic transmitters and a precursor); however, resolution is compromised when their metabolic compounds including 5-HIAA, homovanillic acid (HVA), 3-methoxytyramine (3-MT), DOPAC, naDA, naOA, and na5-HT are analyzed. As shown in Figure 1, when the aforementioned fourteen analytes are applied to MEKC-EC in the previously studied 10 mM TES/30 mM SDS/2% 1-propanol buffer,[26] only eleven of them are distinctly resolved with several analytes co-eluting. Thus, a new approach is needed to separate simultaneously the biogenic amines and metabolites selected.

The composition of the separation buffer, surfactant, pH, and use of an additive are important factors for providing the best separation selectivity; hence, a wide variety of parameters have been explored to resolve the selected analytes and these are summarized in Table 1. An acidic

buffer, acetic acid (pH 3.1), separates the biogenic amine and metabolite sample with poor resolution; therefore, a basic borate buffer has been employed. Six of the fourteen analytes examined in this study possess vicinyl hydroxyl groups which form a complex with the borate buffer as illustrated below.[4]

The borate complex formed by DA, NE, E, naDA, L-DOPA and DOPAC results in a modified net charge, thus affecting the partitioning behavior of the individual complexes with the SDS micelles. This improves resolution and yields adequate selectivity of the metabolites and their biogenic amine precursors. Increased separation times have been observed with increasing borate concentrations without significant improvement in resolution; hence, 25 mM borate was chosen to balance selectivity and separation time. Exploitation of SDS in this basic borate buffer enhances resolution as well. Various concentrations of SDS (ranging from 10 to 65 mM) have been evaluated with optimum resolution achieved using a concentration of 50 mM. Additionally, borate/SDS buffer has been studied at a variety of pH values from 8 to 12.5. Owing to partial ionization of phenolic hydroxyl groups in this pH range, separation resolution, peak profiles, elution time reproducibility, and elution time all vary with pH. The optimal resolution of structurally related analytes combined with the peak parameters occurs at pH 9.5. Other buffer conditions were investigated, including the addition of β-CD to the borate/SDS buffer, but were found to provide minimal to no enhancement.

In order to quantitate analytes of interest in biological samples, the use of an internal standard is imperative. The internal standard must be structurally similar to the biogenic amines and metabolites of interest, cannot be native to the biological sample, and must not coelute with any other analytes in the biological or standard sample. After testing multiple compounds for internal standards, CAT met the criteria mentioned above. Although CAT is non-ionic in most buffers used for CE, it is anionic in borate and thus has a distinct migration and elution time. Additionally, the use of 2% propanol enhances peak shapes and thus has been used in all separations presented. Ultimately, separation of the fourteen-analyte standards has been accomplished with optimal resolution and peak shapes in less than 20 min.

Identification of Biogenic Amines and Metabolites

The optimized separation of fourteen amines and metabolites is shown in Figure 2. The identities of individual peaks have been clarified by matching their elution times with that of each biogenic amine or metabolite individually analyzed under the same separation condition. Borate-complexed DA and E elute first followed by naOA, NE, OA, na5-HT, naDA, 5-HIAA, L-DOPA, and HVA. The internal standard, CAT, elutes after the DA metabolite HVA and before TA, 3-MT, 5-HT and DOPAC. This migration order is based largely on the hydrophobicity of each analyte and the partitioning behavior with micelles formed in the buffer. The peak corresponding to DOPAC (15, in Figure 2) is significantly smaller than those of the other analytes. As DOPAC is doubly negative when complexed with borate, it is discriminated against during electrokinetic injection. Moreover, DOPAC has slow oxidation reaction kinetics at the electrochemical detector also leading to a smaller signal.

The optimized separation conditions have enabled the identification and quantification of previously unresolved compounds, including L-DOPA, naOA, HVA, 5-HIAA, and DOPAC (Figure 1). OA is a major neurotransmitter and neuromodulator in the *Drosophila* CNS and is considered an insect counterpart of the mammalian neurotransmitter and neuromodulator NE; thus, the resolution of OA and the N-acetylated metabolite naOA is of utmost importance.

Under the current separation conditions, three different N-acetylated metabolites believed to be involved in monoamine inactivation have been separated from their biogenic amine precursors as well as from other metabolic compounds. Although several of these analytes have been studied by HPLC,[19, 25, 28] to our knowledge these fourteen analytes of biological relevance have not been resolved in a single separation previously. Moreover, CE separations of multiple N-acetylated metabolites in conjunction with their corresponding biogenic amines are novel.

Enhanced Reproducibility and Detection Limits

Reproducibility of migration times between experiments is often a problem with CE, raising questions regarding peak identification. We have addressed the issue of reproducibility by utilizing the same capillary and electrode for three consecutive separations of the standard mixture (Figure 3) with *Drosophila* homogenate samples run in-between (data not shown) to mimic peak shifting that may occur during a day of biological data collection.

As shown in Figure 3, all biogenic amines and metabolites applied are clearly resolved in 20 min for all three separations although an increase in migration time is observed for all analytes in each consecutive separation with greater changes taking place for 3-MT, 5-HT, and DOPAC. This change is likely attributable to the long residence time of the analytes in the capillary resulting from their increased interactions with the micellar phase, and may be augmented by adsorption of the biological molecules to the capillary walls. The increasing shift in migration time has been examined by calculating the standard deviation for three separations performed on the same capillary with the same electrode on the same day. The greatest changes are observed for the compounds with the longer residence time in the capillary; nevertheless, relative standard deviations are less than 1.6% for all compounds (0.25% for OA; 0.33% for E; 0.34% for naOA; 0.36% for na5-HT; 0.37% for DA; 0.47% for NE; 0.56% for naDA; 0.59% for 5-HIAA; 0.71% for HVA; 0.74% for TA; 0.77% for L-DOPA; 0.78% for CAT; 0.90% for 3-MT; 1.11% for 5-HT; and 1.51% for DOPAC). These small and consistent changes in migration times make it straightforward to identify the peaks in each separation of the standard mixture. Although identification and subsequent quantification of analytes in any complex biological sample can be quite challenging, it is feasible to identify and quantify peaks representing biogenic amines and metabolites of interest in biological samples by comparing electropherograms of standards separated before and after tissue homogenates analyzed under the same conditions. Subsequently, the inferred identities of individual peaks can be further confirmed by spiking the homogenate with known concentrations of each compound and then comparing it to the unspiked, original homogenate and the standard separations.

Analyses of the detector coulometric efficiency yield a slight decay in detector response (from 4 % for HVA to 31% for 3-MT with an average of 10 % for all analytes) after consecutive experiments, presumably due to fouling of the electrode from repeated exposure to biological samples. As an internal standard is employed, and pre- and post-standards are run with each homogenate, loss of detector sensitivity is closely monitored allowing the replacement of capillary and electrode as necessary. Calibration plots for all fourteen analytes are linear for concentrations from 250 nM to 5 μM with correlation coefficients of greater than 0.98. Utilizing these calibration plots, the detection limit of the system is as low as 3.4 attomoles (amol) for OA and 112.1 amol for L-DOPA (S/N = 3), with the remaining analytes falling in between. Previous studies have reported femtomole (fmol) amounts of biogenic amines in *Drosophila* head homogenates;[26] therefore, it is expected that the limit of detection with this borate-MEKC-EC system should be sufficient for the analysis of biogenic amines as well as metabolites in *Drosophila* head homogenates.

Identification of Biogenic Amines and Metabolites in Wild-type *Drosophila* Head Homogenates

To explore the feasibility and the capacity of the borate-MEKC-EC system to resolve biogenic amines and metabolites in biological samples, biogenic amines and metabolites in head homogenates of the wild-type *Drosophila* strain Canton-S have been separated and quantified. Analyses have been performed on male and female head homogenates indiscriminately as our previous studies indicate no statistical difference in basal monoamine levels between genders.[26]

Figure 4A-C shows three sections of a representative electropherogram of a Canton-S head homogenate. Several distinct peaks are consistently noted and have been tentatively identified as DA, naOA, OA, na5-HT, naDA, L-DOPA, and TA, indicated by numbers corresponding to those in Figure 2. DA is abundant and, although the peak is small and somewhat distorted by a noisy baseline, it is well resolved from the surrounding peaks (1, in Figure 4A); on the other hand, its N-acetylated metabolite naDA elutes at approximately 8.5 min (7, in Figure 4A) and its synthetic precursor L-DOPA at 10.0 min (9, in Figure 4B). Clearly present at approximately 7.3 min is a peak representing OA (5, in Figure 4A) and at 6.3 min is a peak representing naOA (3, in Figure 4A), a metabolite of the major *Drosophila* neurotransmitter. TA is a synthetic precursor of OA; however, it functions also as a neurotransmitter and a neuromodulator in insects as well as in mammals.[29] Although small, the peak representing TA is clearly resolved at 14.4 min (12, in Figure 4C) and thus quantifiable under these conditions. 5-HT is abundant in the adult fly brain as revealed by immunohistochemistry.[16] Indeed, using 10 mM TES/30 mM SDS/2% 1-propanol as a separation buffer, we have previously observed a peak for 5-HT with fair resolution.[26] In contrast, with the separation conditions described here, the peak corresponding to 5-HT is not consistently detectable in the fly. The reason for this loss is not clear at this time. Nonetheless, the N-acetylated metabolite of 5-HT, na5-HT, is distinctively resolved at 7.8 min (6, in Figure 4A). Finally, the internal standard CAT adopted in this study elutes at 10.8 min (11, in Figure 4B) and does not appear to interfere with the surrounding peaks. The CAT peak is easily quantified, allowing accurate calculation of the biogenic amines and metabolites present in *Drosophila* head homogenates.

There are multiple peaks with undefined identity and these likely represent electroactive species present in the *Drosophila* head homogenate, possibly including other N-acetylated compounds such as N-acetyltyramine as well as numerous small neuropeptides (less than 3000 Daltons) that contain electroactive amino acids. These molecules are also potentially important for *Drosophila* brain function, but are beyond the scope of the present work. The separation conditions that are optimized here for the simultaneous analysis of fourteen biogenic amines and metabolites clearly depict four biogenic amines and three N-acetylated metabolites from the *Drosophila* head homogenates with good resolution.

Other publications have also reported separations of specific biogenic amines and their metabolites from various *in vivo* preparations. For example, DA has been consistently detected in *Drosophila* samples not only by MEKC-EC[26] but with HPLC-EC[25, 30–33] and GC-MS[34] methods. Variability is reported in amount of neurotransmitters with each of these methods and can be attributed not only to variation in fly strain, but also to slight differences in environmental factors such as temperature and humidity as well as generational differences among the same fly strain. Furthermore, variability within an order of magnitude is common in biological analyses, particularly when differing methods of sample preparation or extraction are employed. It is therefore imperative to perform control experiments to minimize generational and environmental factors. Several analyses utilizing HPLC-EC have identified naOA and OA in the cockroach, *Periplaneta american*,[19, 20, 28] as well as in *Drosophila*.[25] Although observed in the cockroach head homogenates, the N-acetylated metabolites na5-HT and naDA have not been previously observed in *Drosophila*.[19, 20, 28] HPLC requires

significantly larger sample volumes (μL) than CE [nL–attoliter (aL)] and thus involves higher dilution of samples or larger sample sizes. Our study employing narrow i.d. capillaries for borate-MEKC separation is a significant advance in the analysis of these neurochemicals, particularly for biological samples whose volumes are often limited, and allows for a more accurate assessment of the contents of an insect brain. The measurement of naDA, na5-HT, and naOA in the cockroach brain has demonstrated that N-acetylation rather than oxidative deamination is the primary route of monoamine catabolism in that insect.[19, 20, 28] The abundant presence of N-acetylated metabolites in our analysis of *Drosophila* head homogenates is consistent with this hypothesis. More importantly, the apparently undetectable levels of oxidative deamination products 5-HIAA, HVA and DOPAC in the same homogenates strongly indicates that monoamine metabolism in insects, or at least in *Drosophila* occurs primarily through N-acetylation.

MEKC-EC Comparison of Canton-S wild-type and Inactive *Drosophila* Head Homogenates

One of the significant advantages of the MEKC-EC method is the quantitative information that can be collected for each analyte. To address whether changes in biogenic amines and metabolites caused by various physiological conditions can be quantified by this method, we have examined the *Drosophila* mutant iav^1. The iav^1 mutant was initially isolated due to its reduced locomotor activity and its genetic lesion has recently been mapped to the gene coding for the TRPV channel subunit IAV.[35] Although the underlying mechanism is unclear, the iav^1 mutant has been shown to contain low levels of TA and OA, [30, 36, 37] thus it serves as an excellent model to assess the sensitivity of the borate-MEKC-EC system.

Accordingly, three independent preparations (fly cultures one, two, and three in Table 2) of Canton-S and iav^1 head homogenates have been analyzed by borate-MEKC-EC. For each preparation, three (fly culture three) or four (fly cultures one and two) different homogenate aliquots have been separated, the peaks corresponding to the biogenic amines and their metabolites under study have been identified and quantified, and their average value per head is reported for comparison. Moles of each biogenic amine or metabolite in the homogenates have been calculated by comparison to standard separations before and after the separation of the homogenate and normalized to the internal standard, CAT. These data have been pooled and the average amount ± SEM for each of five neurochemicals is presented for comparison.

The data in Table 2 exhibit a great deal of variation between fly cultures. This could be the result of several factors including variability in sample handling, separation variability, and subtle differences in the environment of each fly culture. The internal standard, CAT, has been used in an attempt to minimize differences in methodology concerning the sample handling and separation. If the changes in analytes observed between cultures are real, then this would indicate the technique could be further used to evaluate environmental factors affecting fly cultures. Here we focus on the comparison of wild-type and mutant flies.

The femtomole amount of naOA, TA and OA detectable in the iav^1 head homogenates are significantly reduced when compared to those in the wild-type Canton-S head homogenates. Interestingly, the most prominent change is observed for TA which is reduced to levels below the detection limit in two independent iav^1 cultures (Figure 4D). The amount of TA observed in wild-type flies is close to the limit of detection in the borate-MEKC-EC system. Thus, trace amounts of TA could be present in the iav^1 head homogenates, but these are below the detection limit of our current methodology. Statistical analysis by one-way ANOVA reveals that the amount of TA ($p = 0.007$), OA, and naOA (p 0.01) in the iav^1 fly head homogenates is statistically different from that in the wild-type head homogenates.

In order to prove the authenticity of the chemical changes in the iav^1 mutant head homogenates, several peaks that should remain unchanged have been quantified. The sustained DA and naDA

(Table 2) and na5-HT and L-DOPA (data not shown) are not significantly different from those in the wild-type head homogenates ($p > 0.5$, one-way ANOVA). These data reveal that not all electroactive species in the fly are altered as a result of the *iav*^{1} mutation, and demonstrate the sensitivity of borate-MEKC-EC to separate and quantify three important N-acetylated compounds vital to understanding neurochemical processes in the fly, *Drosophila*.

CONCLUSIONS

The borate-MEKC-EC technique reported here offers significant advantages for the separation and identification of biogenic amines and metabolites in *Drosophila melanogaster* and other biological systems. Importantly, we have accomplished the resolution of fourteen compounds of biological relevance in a single separation. Optimized separation parameters provide consistent resolution of standards containing DA, E, naOA, NE, OA, na5HT, naDA, 5HIAA, L-DOPA, HVA, CAT, TA, 3MT, 5HT and DOPAC within 20 minutes. The separation reproducibility, despite peak shifting upon exposure to biological solutions, is less than 1.6% (RSD) for all these compounds, allowing straightforward identification of biogenic amines and their metabolites. Limits of detection as low as 3.4 amol for OA make the borate-MEKC-EC system sensitive for volume limited sample analysis. We have succeeded in identifying and quantifying several neuromodulators and three N-acetylation metabolites in Canton-S wild-type *Drosophila*. The borate-MEKC-EC method has also been used to examine the *Drosophila iav*^{1} mutant, where quantitative comparison of metabolite and biogenic amine levels between the wild-type fly and the mutated fly have been made in these genetically distinct animals.

Acknowledgements

This work was supported, in part, by grants from the National Institutes of Health (K.-A.H.; A.G.E) and the National Science Foundation (A.G.E.). Dr. George A. Brine from the NIMH repository is gratefully acknowledged for generously providing N-acetylated compounds. Nick Kuklinski and Marc Maxson are acknowledged for helpful statistical discussions.

References

1. Peaston RT, Weinkove C. Ann Clin Biochem 2004;41:17–38. [PubMed: 14713382]
2. Terabe S, Markuszewski MJ, Inoue N, Otsuka K, Nishioka T. Pure Appl Chem 2001;73:1563–1572.
3. Wallingford RA, Ewing AG. Anal Chem 1988;60:258–263. [PubMed: 3354836]
4. Wallingford RA, Ewing AG. J Chromatogr 1988;441:299–309. [PubMed: 3410921]
5. Wallingford RA, Curry PD, Ewing AG. J Microcolumn Sep 1989;1:23–27.
6. Siren H, Karjalainen U. J Chromatogr A 1999;853:527–533. [PubMed: 10486762]
7. Siren H, Mielonen M, Herlevi M. J Chromatogr A 2004;1032:289–297. [PubMed: 15065807]
8. Presto Elgstoen KB, Jellum E. Electrophoresis 1997;18:1857–1860. [PubMed: 9372280]
9. Fernandez-Chacon R, Sudhof TC. Annu Rev Physiol 1999;61:753–776. [PubMed: 10099709]
10. Korsching S. Curr Opin Neurobiol 2002;12:387–392. [PubMed: 12139985]
11. Panda S, Hogenesch JB, Kay SA. Nature 2002;417:329–335. [PubMed: 12015613]
12. Waddell S, Quinn WG. Trends Genet 2001;17:719–726. [PubMed: 11718926]
13. Mercer, A. R. In Arthropod Brain: Its Evolution, Brain, Structure, and Functions; Gupta, A. P., Ed.; Wiley-Interscience: New York, 1987, pp 399–414.
14. Roeder T, Seifert M, Kahler C, Gewecke M. Arch Insect Biochem Physiol 2003;54:1–13. [PubMed: 12942511]
15. Blenau W, Baumann A. Arch Insect Biochem Physiol 2001;48:13–38. [PubMed: 11519073]
16. Monastirioti M. Microsc Res Tech 1999;45:106–121. [PubMed: 10332728]
17. Sloley BD. Neurotoxicology 2004;25:175–183. [PubMed: 14697892]
18. Dewhurst SA, Croker SG, Ikeda K, McCaman RE. Comp Biochem Physiol B 1972;43:975–981. [PubMed: 4662578]

19. Downer RG, Martin RJ. Life Sci 1987;41:833–836. [PubMed: 3613843]
20. Martin RJ, Downer RG. J Chromatogr 1989;487:287–293. [PubMed: 2498374]
21. Roeder T. Annu Rev Entomol 2005;50:447–477. [PubMed: 15355245]
22. Yellman C, Tao H, He B, Hirsh J. Proc Natl Acad Sci U S A 1997;94:4131–4136. [PubMed: 9108117]
23. Park YH, Zhang X, Rubakhin SS, Sweedler JV. Anal Chem 1999;71:4997–5002. [PubMed: 10565288]
24. Stuart JN, Zhang X, Jakubowski JA, Romanova EV, Sweedler JV. J Neurochem 2003;84:1358–1366. [PubMed: 12614336]
25. Monastirioti M, Linn CE Jr, White K. J Neurosci 1996;16:3900–3911. [PubMed: 8656284]
26. Ream PJ, Suljak SW, Ewing AG, Han KA. Anal Chem 2003;75:3972–3978. [PubMed: 14632107]
27. Sloss S, Ewing AG. Anal Chem 1993;65:577–581.
28. Martin RJ, Bailey BA, Downer RG. J Chromatogr 1983;278:265–274. [PubMed: 6421859]
29. Berry MD. J Neurochem 2004;90:257–271. [PubMed: 15228583]
30. McClung C, Hirsh J. Curr Biol 1999;9:853. [PubMed: 10469593]
31. Neckameyer WS. Dev Biol 1996;176:209–219. [PubMed: 8660862]
32. Neckameyer WS, Woodrome S, Holt B, Mayer A. Neurobiol Aging 2000;21:145–152. [PubMed: 10794859]
33. Ramadan H, Alawi AA, Alawi MA. Cell Biol Int 1993;17:765–771. [PubMed: 8220304]
34. Watson DG, Zhou P, Midgley JM, Milligan CD, Kaiser K. J Pharm Biomed Anal 1993;11:1145–1149. [PubMed: 8123727]
35. Gong Z, Son W, Chung YD, Kim J, Shin DW, McClung CA, Lee Y, Lee HW, Chang DJ, Kaang BK, Cho H, Oh U, Hirsh J, Kernan MJ, Kim C. J Neurosci 2004;24:9059–9066. [PubMed: 15483124]
36. Chentsova NA, Gruntenko NE, Bogomolova EV, Adonyeva NV, Karpova EK, Rauschenbach IY. J Comp Physiol B 2002;172:643–650. [PubMed: 12355233]
37. O'Dell KM. Heredity 1993;70:393–399. [PubMed: 8496068]

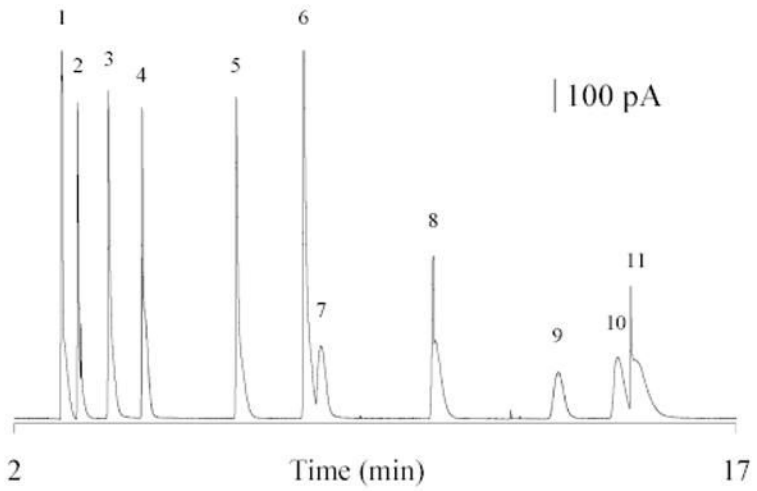

Figure 1.

A single separation of 100 μM standards shown with the following peak identification: L-DOPA and naOA (1), naDA (2), na5-HT (3), HVA, 5-HIAA and DOPAC (4), NE (5), E (6), OA (7), DA (8), TA (9), 3-MT (10), 5-HT (11). Separation was performed in a 13-μm i.d. capillary with a separation potential of 550 V/cm. The working electrode was held at +750 mV vs. a Ag/AgCl reference electrode.

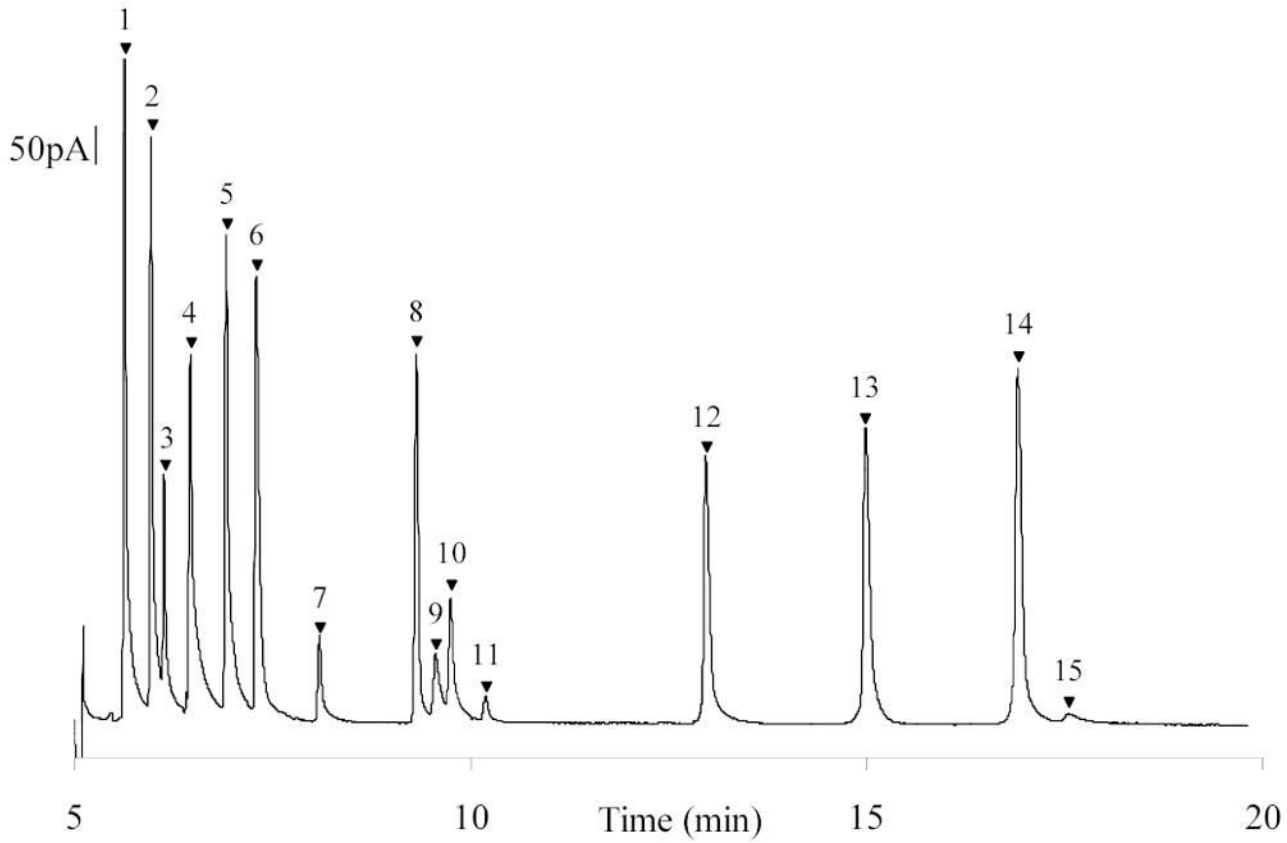

Figure 2.

A single optimized separation of 100 μM standards (10 μM CAT, internal standard) shown with the following peak identification: DA (1), E (2), naOA (3), NE (4), OA (5), na5HT (6), naDA (7), 5-HIAA (8), L-DOPA (9), HVA (10), CAT (11), TA (12), 3-MT (13), 5-HT (14), DOPAC (15). Separations were performed in a 13-μm i.d. capillary with separation potentials of 333 V/cm. The working electrode was held at +750 mV vs. a Ag/AgCl reference electrode.

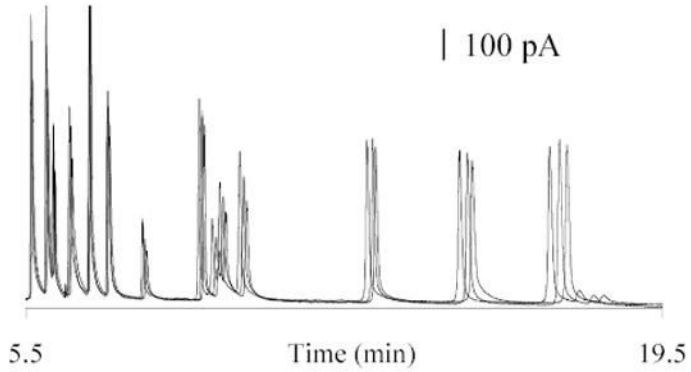

Figure 3.

Three standard separations of 100 μM amines, metabolites and internal standard. The peak order is as in Figure 2. Separations were performed on a 13-μm i.d. capillary with separation potentials of 333 V/cm. Fly homogenate samples were run between standard separations. The working electrode was held at +750 mV vs. a Ag/AgCl reference electrode.

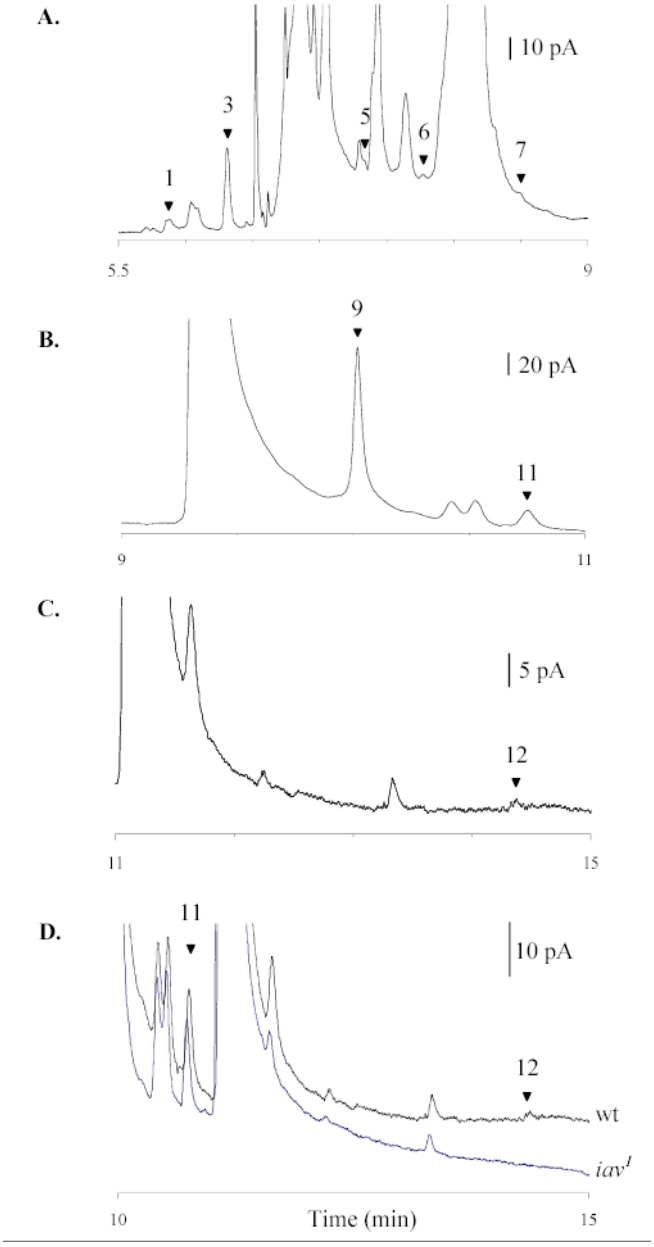

Figure 4.

(A) Enlargement of 5.5 min to 9 min of a MEKC-EC separation of a *Drosophila* head homogenate highlighting DA (1), naOA (3), OA (5), na5HT (6), and naDA (7). (B) Enlargement of 9 min to 11 min of the separation emphasizing peaks for L-DOPA (9), and CAT (11). (C) Enlargement of 11 min to 15 min of the electropherogram showing TA (12). (D) Comparison of Canton-S (wt) and *iav*^{1} *Drosophila* head homogenates highlighting the internal standard CAT (11) and TA (12). The numbers correspond to those in Figure 2. Field strength for the separation was 333 V/cm. The working electrode was held at +750 mV vs. Ag/AgCl.

Table 1

Optimization of Separation Conditions

Optimization Parameters	Conditions	Affect on Resolution
Field Strength	Capillary length	Improved with increased field strength
	Applied potential	
Buffer	Phosphate, Acetic Acid, MES	Acidic and Neutral: Poor; unstable baseline
	TES	Basic: 14 analytes resolved
	Borate	
Borate Complexation (mM)	10, 25, 30, 40, 50	No significant improvement
Additives	β–CD	Minimal – No enhancement
	SDS	Increased with increased SDS conc.
SDS Micelles (mM)	10, 30, 35, 40, 50, 55, 65	Increased with increasing SDS concentration
pH	8.0 to 12.5 (in 0.5 pH unit increments)	Very sensitive to pH
Internal Standards	Dihydroxybenzylamine	Coelutes with DA
	Isoproterinol	Coelutes with E
	Ascorbic Acid	Coelutes with peak in fly sample
	Acetaminophen	2 peaks, 2nd peak coelutes with OA
	Catechol	Excellent internal standard

Summary of the optimization parameters, conditions attempted, and the affect on resolution of standards containing fourteen biogenic amines and metabolites using a 13-μm i.d. capillary. 2 % 1-propanol was employed in all separations to enhance peak shape.

Table 2

Quantification of Biogenic Amines and Metabolites in Wild-type and Inactive Drosophila Head Homogenates

	TA (fmol)		OA (fmol)		naOA (fmol)		DA (fmol)		naDA (fmol)	
fly culture	**wt**	***iav*^{1}**	**wt**	***iav*^{1}**	**wt**	***iav*^{1}**	**wt**	***iav*^{1}**	**wt**	***iav*^{1}**
1	37.2	0.2	311.0	36.5	142.0	34.9	79.5	64.3	732.8	424.9
2	9.9	0.0	173.5	32.5	74.9	38.7	70.9	55.0	397.4	756.7
3	3.7	0.0	84.6	24.2	42.8	16.1	38.0	39.7	527.4	341.3
Avg ± SEM	17.0 ± 10.3	0.1 ± 0.1	189.7 ± 65.9	31.6 ± 3.1	86.6 ± 29.2	31.4 ± 5.5	62.7 ± 12.7	53.0 ± 7.2	552.5 ± 97.6	527.1 ± 115.0
	p = 0.007		p = 0.002		p = 0.010		p = 0.360		p = 0.900	

Separation of 3 separate fly culture homogenization trials showing quantification of OA, naOA, TA, DA, and naDA in wild-type (wt) and inactive (*iav*^{1}) fly populations. n = 4 for fly cultures one and two; n = 3 for fly culture three. An average of the three trials ± standard error of the mean (SEM) and p-values determined by one-way ANOVA are reported. Mass amounts are given per fly head.