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Capillary Electrophoresis Mass Spectrometry based Detection of Drugs and Neurotransmitters in *Drosophila* Brain

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Abstract

Capillary electrophoresis coupled to mass spectrometry has been used to determine the in vivo concentrations of the neuroactive drug, methylphenidate, and a metabolite in the heads of the fruit fly, *Drosophila melanogaster*. These concentrations, evaluated at the site of action, the brain, have been correlated with orally administered methylphenidate. *Drosophila melanogaster* has a relatively simple nervous system but possesses high order brain functions similar to humans; thus it has been used as a common model system in biological and genetics research. Methylphenidate has been used to mediate cocaine addiction due to its lower pharmacokinetics, which results in fewer addictive and reinforcing effects than cocaine; the effects of the drug on the nervous system, however, have not been fully understood. In addition to measurements of drug concentration, the method has been used to examine drug dose dependence on the levels of several primary biogenic amines. Higher in vivo concentration of methylphenidate is observed with increasing feeding doses up to 25 mM methylphenidate. Furthermore, administered methylphenidate increases the drug metabolism activity and the neurotransmitter levels; however, this increase appears to saturate at a feeding dose of 20 mM. The method developed for the fruit fly provides a new tool to evaluate the concentration of administered drug at the site of action and provides information concerning the effect of methylphenidate on the nervous system.

INTRODUCTION

A wealth of studies have been carried out on animal models including rats, mice, baboons, and monkeys to investigate neurochemical changes in the central nervous system associated with drug action and addiction.^{1–5} The smaller invertebrates such as *Drosophila melanogaster* (fruit fly) have also been heavily used as a model in neuroscience and to study drugs of abuse.^{6–8} We have used the fly model to study the effects of methylphenidate on the actions of cocaine at dopamine neurons.⁹

Methylphenidate abuse is more limited than that of cocaine, which is considered highly addictive.^{10,11} The half-life of methylphenidate in the human brain, based on the duration of dopamine transporter blockage, is longer than that of cocaine (75–90 min vs. 15–25 min, respectively).¹² Since the clearance of the stimulant from the brain is necessary before it is possible for an individual to fully experience the reinforcing effects of the drug again, frequent repeated administration and overall abuse of methylphenidate is limited in

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comparison to cocaine. This makes it important in model systems to understand the concentration of methylphenidate, or drug in general, in the brain after administration.

Experimental designs in model systems used to examine the effects of drugs like methylphenidate typically use drug administration via injection or feeding. In these studies the amount of drug used for modeling and experimentation is usually taken as the dose given and not the concentration of the drug in the brain or cells. Although the concentration of a drug or metabolites at the site of action is the important factor in determining the action of that substance on tissues, cells, and receptors, and the pharmacokinetics in the case of drugs, little data can be found for these measurements.^{13–15}

In this paper, we present a strategy using capillary electrophoresis coupled to mass spectrometry for analysis of methylphenidate and neurotransmitter concentrations in the fly brain. By feeding drug at different levels, we have examined the dose dependence of administered drug on brain concentration of methylphenidate. Furthermore, we investigate a metabolite of methylphenidate as well as its effect on the levels of several neurotransmitters in fly brain.

EXPERIMENTAL SECTION

Materials

Methylphenidate hydrochloride was purchased from Sigma-Aldrich (Stockholm, Sweden) and the internal standard, methylphenidate-D₉ 100 µg/mL, was purchased from LGC Standards AB (Borås, Sweden). N-acetyl dopamine and N-acetyl octopamine were obtained from The National Institute of Mental Health chemical synthesis and drug supply program (Research Triangle Park, NC, USA). Other chemicals were purchased from Sigma-Aldrich (Stockholm, Sweden). Water was obtained from a MilliQ (MQ) water purification system (Millipore, Merck, Darmstadt, Germany). Stock solutions of methylphenidate and biogenic amines 2 mM were prepared in 5% formic acid (v:v) and further diluted with 5% formic acid to the desired concentration before use. An 82.6 µM solution of methylphenidate-D₉ was prepared by diluting the stock solution with 100% methanol and used as an internal standard. All standards were stored at –20°C until analysis.

Fly cultures and sample preparation

Transgenic *Drosophila* flies (TH-GFP) were cultured on standard potato meal/agar medium. One to four-day-old male flies were selected from the medium and transferred into the yeast paste containing a known amount of methylphenidate. The drug-containing food was replaced daily to ensure the flies were fed with the same methylphenidate concentration for three days. The flies were then transferred into a 15-mL centrifuge tube, quickly frozen in liquid nitrogen, and vortexed to detach the heads from the bodies.¹⁶ Thirty fly heads were collected and homogenized in 60 µL of concentrated formic acid with the internal standard, methylphenidate-D₉, and subsequently centrifuged at 20000 × g for 20 min. The supernatant was transferred into a new vial (PCR tube, 200 µL), gently dried and reconstituted in 10 µL of 10 mM ammonium formate. The solution was further centrifuged at 20000 × g for 30 min and subsequently injected into the CE-MS system.

Capillary electrophoresis

Capillary electrophoretic separation was performed on a Beckman P/ACEMDQ system (Beckman Coulter, Brea, CA, USA) using 80 cm long fused silica capillary (50 µm ID, 350 µm OD; Polymicro Technologies, Phoenix, AZ, USA). Prior to analysis, the capillary was conditioned consecutively with 1 mM NaOH, MQ water, and separation electrolyte, each solution for 5 min. Samples were introduced into the CE system using hydrodynamic

injection at a pressure of 10 psi for 5 s. The CE analysis was carried out with a +20 kV separation voltage, with the cathode outlet of the capillary connected to the MS interface. Separation was obtained in 50 mM citric acid electrolyte ($\text{pH} \approx 2.1$).¹⁷

Electrospray ionization mass spectrometry

The CE was coupled to a micro-ToF-Q II Mass Spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a CE-MS electrospray interface (CE-Sprayer, Agilent Technologies, Santa Clara, CA, USA). Isopropanol/water was used as sheath liquid (70:30, v/v) with a flow rate of 3 $\mu\text{L}/\text{min}$ provided by an external syringe pump (KD Scientific, Holliston, MA, USA). Analysis was performed in positive mode (+4500 V). The nebulizer gas was kept at a pressure of 0.4 bar and the drying gas maintained at 180°C at a flow rate of 4 L/min.

Mass spectra were acquired over the range of m/z from 50 to 500 with the scan rate of 1 Hz. Characteristic ions for each compound were selected for quantification: m/z 234.15 for methylphenidate ($\text{M}+\text{H}$), m/z 243.21 for methylphenidate- D_9 ($\text{M}+\text{H}$), m/z 104.07 for γ -aminobutyric acid (GABA) ($\text{M}+\text{H}$), m/z 137.07 for dopamine ($\text{M}-\text{NH}_3$), m/z 136.08 for octopamine ($\text{M}-\text{H}_2\text{O}$), m/z 121.07 for tyramine, m/z 196.09 for N-acetyl metabolites ($\text{M}+\text{H}$), and m/z 250.15 for the hydroxylated metabolite of methylphenidate ($\text{M}+\text{H}$). All compounds are listed as nominal masses below. MS/MS data were acquired for all signature masses with collision energies between 20 eV and 30 eV, mass isolation width 1 Da and in-source collision induced decay (ISCID) 5 eV.

RESULTS AND DISCUSSION

CE-MS method and validation

The goal of this work has been to develop a method to simultaneously determine neuroactive small molecular weight compounds, including methylphenidate, neurotransmitters and two neurometabolites in invertebrate brain. An acidic electrolyte, citric acid (50 mM), was chosen to prevent the degradation of biogenic amines as well as to eliminate their adsorption to the capillary surface.^{17–19} This provided reproducible peak areas, and stable signals.

To validate the reliability of the method, the performance of the whole analytical procedure has been tested, where possible, including the carryover, repeatability, limit of detection, and recovery of the sample preparation. To check for contamination by carryover from previous samples, drug-free fly head samples have been analyzed following a sample with a high oral dose of methylphenidate. No carryover is observed as indicated by no peak detected for methylphenidate in the electropherogram of the control sample. Limit of detection (LOD) and limit of quantification (LOQ) values for methylphenidate have been calculated based on the standard deviation of noise within the migration area of the drug in the control sample (3 and 10, respectively) and the peak height of the analyte in the sample spiked with 17.9 μM methylphenidate. The LOD obtained for the method is 5.12 μM and the LOQ is 15.3 μM . To examine the recovery of methylphenidate through the sample preparation process, 100 μM methylphenidate was spiked into the drug-free sample at the beginning of the process and quantitatively compared to the sample spiked after the process. The recovery through sample preparation was found to be above 85%. The repeatability of the analysis shows that for the measurements of methylphenidate at all concentrations examined, the relative standard deviation (%RSD) is about 30%. The relative quantification of neurotransmitters and metabolites provides RSDs ranging from 20 to 35% except for the N-acetyl metabolites in the control sample and tyramine at a 25 mM feeding dose, for which the variation is higher (about 50%). The data demonstrate a reliable, sufficiently sensitive,

and reproducible analysis for measurement of the in vivo methylphenidate, neurotransmitter and metabolite concentrations.

Identification of methylphenidate and neurotransmitters in *Drosophila* heads

Identification of methylphenidate and its metabolites has been carried out by use of selected ion monitoring for detection. Here, both the MS and MS/MS spectra of the compounds obtained in standards and in 30-head samples after three-day administration of 20 mM methylphenidate have been examined. Methylphenidate has a migration time at 8.3 min with a symmetric and sharp peak (Figure 1A). The characteristic MS ion is m/z 234 ($M+H$) corresponding to the intact molecular ion. In the MS/MS mode, the molecular ion m/z 234 produces product ions at m/z 174, corresponding to loss of the carboxylate group ($-COOCH_3$), and at m/z 84, corresponding to the piperidinium group. The spectral patterns obtained from the standard (Figure S1, supporting information) and head sample (Figure 1B) are identical. For the internal standard, methylphenidate D_9 , the MS/MS pattern is similar to that of methylphenidate; however, it differs by 9 Da (Figure 1C). Fragmentation of the methylphenidate D_9 molecular ion at m/z 243 resulted in product ions at m/z 183 (m/z 174+9) and 93 (m/z 84+9), indicating that the nine deuterium atoms are located at the piperidinium residue.

CE-MS of fly head samples has been furthermore used to identify neurotransmitters, including GABA, tyramine, as well as octopamine, dopamine and their metabolites N-acetyl octopamine and N-acetyl dopamine, respectively. Electropherograms with selected ion monitoring are shown in Figure 2A. Most of the peaks are sharp and symmetric, with the exception of the N-acetyl metabolites where N-acetyl dopamine and N-acetyl octopamine overlap. Some effort has been made to modify the buffer electrolyte in order to separate these metabolites. However, we found it difficult to improve the separation owing to the chemical similarity of these compounds and the limited choice in electrolyte solution while maintaining compatibility with MS detection. The separation efficiency has also been examined for these analytes. The peak efficiencies ranged from 3000 to 80000 theoretical plates. Although these are not excellent compared to what is expected in capillary zone electrophoresis with a simple electrolyte, this is compensated by the capability for structure identification and efficient mass separation in the quadrupole-ToF mass spectrometer. Thus, simultaneous detection of these compounds from the heads of *Drosophila melanogaster* is possible with co-migrated peaks resolved in the mass spectrometer based on their different mass/charge values, although caution needs to be used in absolute quantitation as there could be ion suppression effects.

The neurotransmitters have been identified by MS/MS analysis of the fly head sample as shown in Figure 2B, and also by comparison of the MS/MS spectra to standards. Most of these neurotransmitters and metabolites show identical MS/MS spectra to the standards, with the exception of the N-acetyl metabolites (Figures S2 and S3, supporting information). Although they have the same molecular mass, m/z 196, the fragmentation probabilities of N-acetyl octopamine and N-acetyl dopamine are not similar. From the standard, the specific ions for N-acetyl octopamine are m/z 196 ($M+H$), 178 ($M-H_2O$), 154 ($M-CH_2CO$), and 136 ($M-CH_4CO_2$), and for N-acetyl dopamine these are m/z 196 ($M+H$), 154 ($M-CH_2CO$), and 137 ($M-CH_5CNO$). The N-acetyl metabolite peak appears to contain both N-acetyl dopamine and N-acetyl octopamine as the MS/MS pattern of m/z 196 in the fly sample contains all of the above fragments. Separate detection of these neurotransmitters can be accomplished by use of selected ion monitoring for detection in the electropherograms, taking advantage of the specific masses, m/z 178, 136 for N-acetyl octopamine and m/z 137 for N-acetyl dopamine; however, these ion peaks are not detected at high enough levels in the fly brain for accurate analysis. Thus, the m/z 196 fragment representing both N-acetyl metabolites has been used for quantification.

Determination of drug at the site of action: methylphenidate concentration in *Drosophila* heads

CE-MS has been used to determine the in vivo methylphenidate concentration in the heads of flies previously administered methylphenidate at different doses. Figure 3 shows the dose dependence of the in vivo methylphenidate concentration when the flies have been fed with doses up to 25 mM. The in vivo level consistently increases from 80.1 μM at a feeding dose of 5 mM to 374.3 μM at 25 mM. These doses correspond to levels of 1.9 to 8.7 mg/kg, and that is within the range of oral administration dose examined in rats for pharmacokinetics and bioavailability (0.75–10 mg/kg).^{20,21} The accumulated methylphenidate in the heads is about 2% of the concentration in the oral doses. Interestingly, the concentration in the fly head increases by 150% when the oral dose is increased from 5 mM to 10 mM, but then increases only approximately 30% for feeding doses from 10 mM to 25 mM. This might be due to the saturation of transport of methylphenidate into the brain. Alternatively, this might indicate another mechanism for methylphenidate clearance occurring at higher feeding concentration. The non-parametric ANOVA analysis followed by post-hoc analysis (Bonferroni test) shows a significant difference between 5 mM and all the other doses, and between 25 mM and the other doses except 20 mM ($p < 0.05$). However, the difference between the concentrations from 10 to 20 mM is not significantly different at this p value. Overall, these results show a drug dose dependence of the in vivo methylphenidate concentration following the feeding dose as expected. Most importantly, we can use this to determine the actual concentration of drug at the site of action; the fly brain.

Hydroxylated metabolite of methylphenidate in *Drosophila* heads is different

Methylphenidate metabolism can occur via several different pathways, such as hydroxylation, oxidation, carboxyl-esterification, and trans-esterification resulting in various different metabolites.²² The action of the metabolites might be highly important in the pharmacological effects of the drug on the nervous system owing to both pharmacokinetics of the original drug and any effects of the metabolites. Thus, we have also checked for and, in one case, quantified the methylphenidate metabolites. Interestingly, a metabolite with the mass m/z 250 is detected. The compound appears to be a product of methylphenidate, as it is not detected in control fly samples without the drug. MS/MS analysis has been used to investigate the structure of the metabolite (Figure 4). Two major ions, m/z 250 and 100, are shifted correspondingly from those of methylphenidate, m/z 234 and 84. The 16 Da difference suggests that the metabolite is the hydroxylated product of methylphenidate. In addition, the fragment m/z 100 of the metabolite corresponds to the piperidinium fragment of methylphenidate, m/z 84, plus 16 Da, indicating hydroxylation of the piperidinium group in the metabolite structure. This structure is different from previous findings showing that the hydroxylated metabolite of methylphenidate is p-hydroxymethylphenidate. Interestingly, different metabolite patterns of methylphenidate are found in different animals and humans.^{20,23–26} The p-hydroxymethylphenidate metabolite has been found to be one of the principal forms in rats and dogs; however, its abundance percentage in total metabolite composition is not similar. In humans, the main metabolite of methylphenidate is ritalinic acid and metabolism by hydroxylation is not a dominant pathway. We propose that the hydroxylated product of methylphenidate in *Drosophila* is different from the other animal models studied to date, suggesting a difference in the metabolism pathway. Additionally, other metabolites such as ritalinic acid and lactam are not detected with the examined drug doses and the conditions used here. This might suggest that the hydroxylation is the most favorable metabolism pathway for methylphenidate metabolism in the fly brain thus limiting the other pathways within the feeding doses used.

The concentration of the metabolite with respect to the oral doses used has also been calculated using the internal standard, methylphenidate-D₉ (Figure 5). The concentration

increases from 16.1 to 35.5 μM with feeding doses from 5 mM to 20 mM indicating that higher drug doses result in higher accumulated metabolite as expected. As the level of the metabolite at 25 mM feeding dose is not significantly different from that at 20 mM ($p < 0.05$), it seems likely that the factors governing the metabolic rate become limited at this concentration level. In addition, the concentration of the metabolite ranges from one fifth to one ninth that of the *in vivo* parent drug, thus accounting for 11 to 20% of the *in vivo* methylphenidate concentration.

Effects of methylphenidate on neurotransmitter levels in *Drosophila* heads

The effects of oral doses of methylphenidate on the neurochemistry in the fly head are important in understanding the mechanism of action of this drug. Variation of important neurotransmitter and metabolite levels in the fly head, including dopamine, octopamine, tyramine, N-acetyl metabolites of catecholamines, has been examined here by peak comparisons to the internal standard at various oral doses of the drug (Figure 6). One of the most striking observations is that the relative amount of tyramine and octopamine increases significantly when the oral dose is increased from 5 mM to 20 mM. Octopamine and tyramine levels are about 1.5 and 3.5 times higher compared to control flies, respectively, under these conditions. When the oral dose is increased from 20 to 25 mM, however, these substances are statistically unchanged ($p < 0.05$). In the biosynthetic pathway, tyramine and octopamine concentrations depend on the activity of tyrosine decarboxylase as this is the rate-limiting enzyme in tyramine and octopamine synthesis. It has been shown that unfavorable conditions such as temperature, mechanical, or chemical stimuli can change the amount of biogenic amines in the brain by changing the activity of the corresponding enzymes.^{27–29} Tyramine and octopamine content have been observed to change dramatically under stressful conditions due to the variation in tyrosine decarboxylase enzyme activity.^{30,31} This has been suggested to be a crucial mechanism for invertebrates to develop appropriate stress reactions that enable their adaption to unfavorable environments. Tyramine has also been shown to be important in cocaine sensitization for flies deficient in tyramine and octopamine biosynthesis.³² Following cocaine exposure, tyramine levels in the fly have been found to increase owing to the active regulation of tyrosine decarboxylase activity. In fact, under extreme sensitization, tyrosine decarboxylase activity is elevated approximately 80% compared to controls.

The trend of increasing tyramine and octopamine levels with increasing methylphenidate doses might be explained by a similar drug-dependent stimulation of tyrosine decarboxylase enzyme activity. Increasing methylphenidate dose might cause a higher tyrosine decarboxylase activity leading to elevated tyramine and octopamine levels necessary for sensitization. However, at *in vivo* concentrations of 20 mM or above, the neurotransmitter concentrations level out which might imply that either the highest sensitization for the drug has been reached, or that the tyrosine decarboxylase activity reaches its highest activity. Moreover, the drug dose dependence pattern of these neurotransmitters is similar to that of the hydroxylated methylphenidate metabolite, for which the concentration of the compound is statistically unchanged between 20 and 25 mM ($p < 0.05$). Alternatively, an equally plausible mechanism is that methylphenidate causes an increase in tyrosine synthesis. This explanation has the merit that leveling off of both tyramine and octopamine levels is explained by saturating tyrosine decarboxylase. Therefore, it seems convincing to assume that the action of methylphenidate in the fly head in this regard is maximized at an oral dose in the range from 20 to 25 mM. This is consistent with *in vivo* work suggesting that the saturation of drug action is in this concentration range.⁹ On the other hand, the trend for dopamine concentration appears to decrease slightly in the fly brain following oral methylphenidate administration. This difference is not significant ($p < 0.05$), but might be interesting.

In contrast to the compounds described above, the relative concentration of the N-acetyl metabolites (Figure 6) and GABA (Figure S4, supporting information) are not significantly different across the oral drug doses used here. The level of the N-acetyl metabolites trends towards decreasing in drug-treated compared to control samples, and perhaps increases slightly at higher drug doses, but this change is not statistically significant ($p < 0.05$). This small trend is not unexpected as methylphenidate is not directly involved in the efflux of these neurotransmitters via transporters or their biosynthetic pathway.

CONCLUSIONS

We have developed an analytical method based on CE-MS to simultaneously determine the in vivo concentrations of methylphenidate, metabolites, and neurotransmitters in *Drosophila* heads. The procedure is reliable, reproducible and sufficiently sensitive to detect and quantify the drug and some major neurotransmitters in fly brain samples. The method provides us with the ability to gain insights into the actions of methylphenidate in the nervous system at various orally administered doses of the drug. This method provides a direct means to measure the concentration of a drug at the site of action and in very small, fruit fly brain, samples and it represents an important step forward in quantitative studies in vivo. Furthermore, we were able to relate the in vivo concentration of methylphenidate to the content of major neurotransmitters and metabolites in the fly heads and they are dose dependent, although the nonlinear dependence suggests saturation of the drug clearance mechanism at higher doses. The data presented are useful to gain better understanding of the overall mechanisms of methylphenidate abuse. They provide insight in the pharmacokinetics of the drug as well as its adverse impact in the nervous system of the *Drosophila* model system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Identification of methylphenidate in a 30-fly-head sample following oral administration of 20 mM methylphenidate for 3 days before analysis. **A)** CE-MS selected ion monitoring electropherogram at m/z 234, **B)** MS/MS spectrum of methylphenidate with the piperidinium residue on the right side of the red mark in the methylphenidate structure, and **C)** MS/MS spectrum of the internal standard methylphenidate D₉. The molecular ions of methylphenidate, m/z 234 (M+H), and methylphenidate D₉, m/z 243, were selected for MS/MS at the collision energy 20 eV, isolation width 1, and ISCID 5 eV.

Figure 2.

CE-MS of neurotransmitters and metabolites for a 30-fly-head sample following oral administration of 20 mM methylphenidate for 3 days before analysis. **A)** Electropherograms with selected ions: GABA m/z 104, tyramine m/z 121, octopamine m/z 136, dopamine m/z 137, N-acetyl metabolites m/z 196. **B)** MS/MS identification of neurotransmitters in the fly heads. The MS specific ions of neurotransmitters were selected for MS/MS at the collision energy 20 eV, isolation width 1, and ISCID 5 eV; GABA m/z 104,;M. A non-parametric ANOVA followed by post-hoc analysis (Bonferroni's tyramine m/z 121, octopamine m/z 136, dopamine m/z 137, N-acetyl metabolites m/z 196.

Figure 3.

The in vivo concentration of methylphenidate in fly heads corresponding to various oral methylphenidate administration levels up to 25 mM. The internal standard, methylphenidate D₉, was added at 2.5 μM. Error bars are mean ± SEM for $n = 9$ –12 samples for each level of administration and ranged from 5 to 33 μM. Anon-parametric ANOVA followed by post-hoc analysis (Bonferroni's test) showed significant differences between each feeding dose and the 5 mM dose with *** = p -value < 0.001 and * = p -value < 0.05. The methylphenidate concentration at the 25 mM dose is also significantly different than that at 10 mM (@@@ = p < 0.001) and at 15 mM (@ = p < 0.5). The numbers in the bars are the mean value.

Figure 4. Identification of the hydroxylated metabolite of methylphenidate in a 30-fly-head sample following oral administration of 20 mM methylphenidate for 3 days before analysis. **A)** CE-MS selected ion monitoring electropherogram m/z 250, **B)** MS/MS spectrum of the methylphenidate metabolite. The MS specific ion for the metabolite m/z 250 (M+H) was selected for MS/MS at the collision energy 20 eV, isolation width 1, and ISCID 5 eV.

Figure 5.

Concentration of the hydroxylated metabolite of methylphenidate in a fly head corresponding to various oral doses of methylphenidate up to 25 mM. Error bars are mean $\pm$ SEM for $n = 9$ –12 samples at each level of administration and ranged from 2 to 4 μM . A non-parametric ANOVA followed by post-hoc analysis (Bonferroni's test) showed significant difference between these concentrations. The significance levels are represented by symbols: @@@ or ***, $p < 0.001$, @@ or **, $p < 0.01$ and @ or *, $p < 0.05$. The significance for concentration of metabolite at a dose of 5 mM methylphenidate versus other doses is indicated with * and for the 15 mM dose with @. The numbers in the bars are the mean value.

Figure 6.

Relative quantification of neurotransmitters dopamine, octopamine, tyramine and N-acetyl metabolites (N-acetyl dopamine and N-acetyl octopamine) in fly heads corresponding to various oral doses of methylphenidate. The comparisons were made using the ratio of the peak area of the analyte and that of the internal standard, methylphenidate D_9 . Error bars are mean $\pm$ SEM for $n = 9-12$ samples for each level of administration.