DETERMINING THE EFFECT OF LIGHT CYCLES ON DOPAMINE LEVELS IN ZEBRAFISH RETINAL TISSUE UTILIZING AN HPLC ASSAY

A CAPSTONE BY

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ABSTRACT

This analysis utilizes a previously established catecholamine extraction method to analyze the dopamine concentration in zebrafish retinal tissue. High performance liquid chromatography (HPLC) with an electrochemical detector (Dionex ED40) was used to assay several standards and the retinal tissue samples using a constant concentration of DHBA as an internal standard. A total of six tissue samples were collected – three from 8 hours into the light cycle, and three from 8 hours into the dark cycle. Quantitatively analyzing these two sample sets allowed for a comparison of dopamine content at different points in the light cycle the fish had been acclimated to. The samples collected during the light cycle were found to have an average dopamine content of 0.3085463 μg per retina, and those collected during the dark cycle had an average dopamine content of 0.4429158 μg per retina. This quantitative analysis showed proof of a successful dopamine extraction with questionable accuracy. Discrepancies with previous analyses in this field will be investigated in future work by developing an assay more sensitive to dopamine metabolites to determine if those discrepancies are due to the breakdown of dopamine within the tissues.

CHAPTER 1 – INTRODUCTION

1.1 Chemistry and Physiological Significance of Dopamine and Catecholamines

Dopamine belongs to a class of compounds known as catecholamines. These compounds are classified by the presence of a catechol group, which is characterized by having a benzene ring attached to two hydroxyl groups, bonded to a terminal amine group. Catecholamines function physiologically as neurotransmitters and hormones. Dopamine, which is pictured below in Figure 1, has the simplest structure of the compounds classified in the catecholamine group.

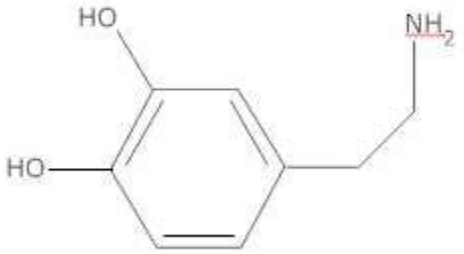

Figure 1: Chemical structure of Dopamine

Other compounds also classified as catecholamines include epinephrine and norepinephrin (also known as adrenaline and noradrenaline). Each of these compounds is derived from the amino acid tyrosine through a series of enzyme-regulated reactions. It begins with the production of tyrosine from its precursor amino acid, phenylalanine, or from its dietary consumption. The benzene ring of tyrosine is subsequently hydroxylated by the enzyme tyrosine hydroxylase to form dihydroxylphenylalanine (L-Dopa), the precursor to dopamine. The enzyme aromatic L-amino acid decarboxylase (also called Dopa decarboxylase) acts on L-Dopa and removes the carboxyl group from the carbon chain, creating the catecholamine dopamine. The beta carbon of dopamine is

hydroxylated in the presence of the enzyme dopamine β–hydroxylase to form norepinephrine. In the final step of the pathway for catecholamine synthesis, epinephrine is produced by the addition of a methyl group to the terminal amino group in a reaction catalyzed by phenylethanolamine N-methyltransferase (1). Each step of the pathway is shown on the following page in Figure 2. Because dopamine is unable to cross the blood-brain barrier, its precursors must be transported into the brain where the reactions in Figure 2 can take place. The dopamine and other neurotransmitters are subsequently packaged into synaptic vesicles from which they can be released upon the firing of the proper neurons (1).

Dopamine is located in many places throughout the body and functions in multiple ways which are dependent upon its location. Within the brain, dopamine functions in regulation of emotions, cognition, food intake, locomotion, positive reinforcement, feelings of pleasure, and endocrine activity. Additionally, dopamine present in retinal tissue plays a role in adaptation to light (2). Because dopamine functions in so many pathways, when it is present in incorrect levels it can cause the onset of multiple neurological disorders. When dopamine levels are not properly regulated within the brain and central nervous system, disorders such as Parkinson's disease, schizophrenia, Tourette's Syndrom, and hyperprolactinemia may result.

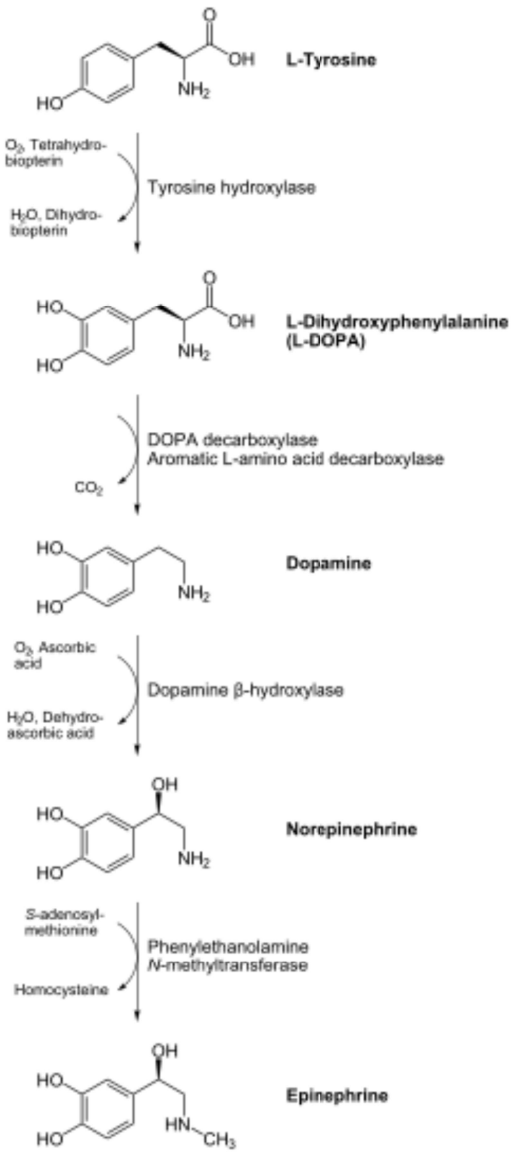

Figure 2: Pathway of Catecholamine Synthesis (1)

Parkinson's disease, which is characterized by progressive dysfunction of the motor system, is a direct result of the degeneration of dopaminergic neurons within the substantia nigra portion of the brain (3). This segment of the brain is a part of the larger basal ganglia system and functions in dopamine production via the pathway in Figure 2. The basal ganglia is the brain system responsible for controlled locomotion, and thus the destruction of neurons in this region cause symptoms such as the inability to coordinate motor functions and difficulty walking and controlling other muscles. L-Dopa is commonly used as therapy for this condition because of its ability to cross the blood-brain barrier and its easy conversion into dopamine (4). If the neurons in the substantia nigra are damaged by physical injury to the brain, symptoms similar to those of Parkinson's disease will follow immediately rather than progressively.

In addition to its roles in physically controlling bodily movement, dopamine is also notable for its various roles in emotional and psychiatric function of the body. Many mental conditions and disorders, such as drug addiction and schizophrenia, are believed to result from an imbalance of dopamine in the brain. This disorder is characterized by negative symptoms such as lack of emotions and energy, and decreased ability to use language as a communication tool. There are also positive symptoms associated with schizophrenia, some of the most prominent of which include hallucinations, delusions, and severely disruptive behavior. These symptoms have been shown to be associated with excessive levels and activity of dopamine and increased density of dopamine receptors in the cerebral cortex (5).

The multiple levels of dopamine functionality within the body have made learning more about its chemistry and the pathways it is involved in of prime interest for many

researchers. One such area of study has been discerning precisely how dopamine functions within the retinal tissue. Various experimental observations have been made in this field, including the determination that levels of dopamine and other retinal compounds fluctuate in accordance with the presence of a circadian rhythm or biological clock. In one such study where fish were acclimated to continuous darkness for a 56 hour period, Ribelayga, Wang and Mangel noted a cyclical pattern of dopamine concentration in the retinal tissue where the highest concentrations occurred during the daytime hours (even though light was not present to act as an external signal indicating that it was daytime), and the lowest concentrations occurred at night (6).

Though the presence of this circadian rhythm has been noted in the results of many studies, it has also been shown to be a rhythm that is easily manipulated by using light as an external timing cue. Exposing fish and other animals to different light and dark cycles has been shown to effectively retune the biological clock in such a way to align it with the light cycle the animal has been acclimated to. Kolbinger et al reported that when the fish were acclimated to a 6 hour light/6 hour dark cycle, the endogenous dopamine concentration varied with the shorter light cycle in a similar manner as it did in a 12 hour light/12 hour dark cycle (7). Additionally, studies such as one conducted by Weber et al have demonstrated that tyrosine hydroxylase, the enzyme that catalyzes the rate limiting step in the synthesis of dopamine, is upregulated in rats during the day, when dopamine concentration is typically lower (8).

1.2 – Using High Performance Liquid Chromatography as an Analytical Tool

High Performance (or High Pressure) Liquid Chromatography (HPLC) is a specialized form of column chromatography. This form of chromatography allows researchers to separate and purify individual components from solutions composed of a mixture of compounds based on the amount of time it takes for the component to flow through the column. Like other forms of chromatography, HPLC makes use of a stationary phase and a mobile phase that have differing polarities. Components of a solution are thus separated based on their respective polarities and how hydrophobic they are and whether their polarities are more similar to the stationary phase or the mobile phase.

In HPLC, the mobile phase (or eluent) can either be a pure solvent or a mixture of different solvents. The eluent runs through the sample after it has been injected prior to entry onto the stationary phase. Thus, at least some components of the sample being injected must be soluble in the mobile phase. The components that are most attracted to the mobile phase will elute from the column faster because they will be pulled through by the eluent flowing through the column.

The stationary phase in HPLC consists of a column usually packed with silica beads. There are many different types of columns that vary in the size of the silica beads, the size of the pores separating the beads, and also in the hydrophobic compound coating the beads. Typically, the hydrophobic coating is an alkyl chain of varying carbon length. Columns are classified by the length of the carbon chains composing the stationary phase. Some of the more common chain lengths are C_4, C_8, and C_{18}. When deciding which column type is appropriate for the components being analyzed, the size of the component

should be considered. Longer carbon chains like the C_{18} chain are more appropriate for separating smaller molecules that may have difficulty interacting with a short carbon chain, whereas the opposite is true for separating larger molecules (9). A C_8 column is used in this analysis because dopamine and its metabolic by-products are medium-sized compounds.

Columns are also classified by the diameter and length of the column itself. The column diameter is significant in that it affects how much solution can be loaded onto the column at one time. A smaller diameter allows less solution to load and may lead to increased sensitivity. The length of the column affects how quickly a solution's components will elute. It takes more time for a solution to flow through a longer column, so shorter columns (like the 50mm column used in this analysis) are typically used when a fast separation is desired (9). Components of a solution that have a greater affinity for the stationary phase than for the mobile phase will take more time to flow through the column and will thus have a longer retention time.

The two forms of HPLC are known as Normal Phase HPLC (NP-HPLC) and Reverse Phase HPLC (RP-HPLC). Normal phase makes use of a polar stationary phase and a non-polar or only slightly polar mobile phase. Reverse phase is used more commonly and utilizes a non-polar stationary phase, such as the one used in this analysis. A moderately polar mobile phase is usually used. This combination results in a longer retention time for the least polar components, especially those with a greater surface area to increase interactions between the component and the stationary phase. A shorter retention time is observed for more polar components, which interact more strongly with the mobile phase than the stationary phase.

The pH of the mobile phase is another factor that influences the retention of a solution's components. This is so because the pH of a solution affects how soluble different compounds are in that solution. For example, in this analysis an acidic pH is maintained in the mobile phase because catecholamines (the class of compounds being detected) are more readily soluble in solutions of a lower pH due to the fact they they are basic amines. Using a mobile phase with a low pH ensure protonation of the amine group of the dopamine and better extraction and solubility of dopamine in solution.

There are several factors that can be analyzed when determining the effectiveness of an HPLC separation. These methods all involve looking at the retention time of two or more components in the solution being analyzed. The basic idea is that if the retention times are too close together and the signals overlap, optimal separation of the components may not have been achieved. One factor that can be used is called the retention factor or the capacity factor, which is termed *k'*. This equation is useful in that the migration rate of any analyte through the column can be quantified and compared. The retention factor for analyte A is defined as:

$$k'_A = \frac{t_R - t_M}{t_M} \qquad \text{(Equation 1)}$$

where t_M represents the time it takes for the mobile phase to pass through the column, and t_R represents the time it takes (from injection) for analyte A to pass through (10). Typically, t_M will be the first peak observed on the chromatogram, as the solvent is the first substance to pass through the column. Thus, the value of k' should always be greater than one, but the higher the number the more distinct the two peaks will be.

The second factor that can be used when analyzing chromatograms is called the selectivity factor, represented as α. A column's selectivity factor is useful for comparing the k' values for two different components of a solution. By setting up a ratio of the two k' values which have a common t_R, the selectivity factor describes the separation of two different analytes, A and B, thus demonstrating a column's ability to distinguish between the two components.

$$\alpha = \frac{k'_A}{k'_B} \qquad \text{(Equation 2)}$$

For the above equation, species A is the component with the lesser retention time, while species B is retained in the column longer. Thus, like k', the selectivity factor will always be a value greater than one (10).

The final factor that can be determined, the resolution, is more comprehensive than the retention and selectivity factors in that it combines retention time data with data describing the physical appearance of the peaks created by two analytes. An analytical column's resolution, R, is defined by the following equation:

$$R = \frac{2(t_{RB} - t_{RA})}{W_A + W_B} \qquad \text{(Equation 3)}$$

Where t_{RA} and t_{RB} are the retention times of the two analytes being resolved and A elutes faster than B, and W_A and $_{WB}$ are the baseline widths of the respective peaks of analytes A and B, respectively, in units of the time being measured. Baseline resolution has been achieved with an R value of 1.5 or higher, meaning that analytes A and B have been completely separated by the column. With any value lower than 1.5, there is significant overlap between the signals of analytes A and B, resulting in an incomplete separation of the two (10).

When performing a chromatographic separation, one may need to make slight changes to the method to optimize any of the three factors described above. These changes can be achieved in several different ways. First, a longer column which has more "theoretical plates" could be used. Theoretical plates are an imagined concept where an analytical column is broken up into separate layers, and equilibration of a sample between the stationary and mobile phases occurs at each plate. Higher efficiency is achieved with increasing the number of theoretical plates used, which is comparable to increasing the length of the analytical column. However, this is not always the most practical way to optimize the resolution as new columns are typically expensive and the resolution only changes to the extent of the square root of the change in the theoretical plate count (11). Additionally, using a longer column causes increased run times for chromatographic separation. Other methods of optimization may be more desirable as they are more cost efficient and convenient.

The most common method of increasing the resolution of peaks is to change the composition of the mobile phase being used in the analysis. This could be accomplished by changing the polarity or the pH of the mobile phase, both of which will change the hydrophobicity and solubility of each component being analyzed and will thus change the retention times and resolution as well (11).

1.3 – Employing an Electrochemical Detector for Sample Analysis

Electrochemical detectors have an advantage over some other types of LC detectors in that they are universal for substances that are capable of being oxidized or reduced and they are also highly sensitive to low concentrations of analyte. In any chromatography system, the detector receives a signal from the analytical area that is consistent with the properties of the substance being analyzed. This signal may be the amount of light reaching the detector (in the case of UV-Vis Spectrometry), the angle of light striking the detector (which gives information about the refractive index of the analyte). In the case of electrochemical detection, that signal is the amount of current produced by the component when electrons are lost or gained by oxidation or reduction. Both dopamine and DHBA are detected by their oxidations. Oxidation occurs by applying a potential across the working and reference electrodes. This potential difference can be manually changed so that the output signal from the detector is appropriate. A potential difference of +1.00 volts was used in this analysis. The oxidized forms of each of these compounds are pictured below in Figures 3 and 4.

Figure 3: Dopamine Ortho-Quinone – the Oxidized Form of Dopamine

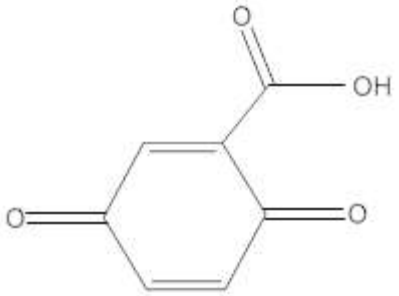

Figure 4: 3,6-Dioxy-cyclohex-1,4-enecarboxylic acid – the Oxidized Form of DHBA

When each of the above compounds are produced from the oxidation of their respective precursors on the working electrode, 2 electrons are released. The current produced as a result of the oxidations of these compounds is directly related to the signal read by the detector, and thus directly related to their concentrations as they are determined from the resulting chromatographs.

When deciding which type of electrochemical detection cell is appropriate for a particular analysis, there are two types commonly considered. The first type is a Coulometric detector. When using a Coulometric detector, 100 percent of the sample injected comes into contact with the working electrode and is either reduced or oxidized. Once the redox reaction has gone to completion, the current measured by the detector will eventually become zero and the total charge that is created is proportional to the mass of the component being detected. When an Amperometric detector is used, the solution to be separated is constantly running over the working electrode after the sample has been injected and elutes from the column. Thus, only some of the analytes will undergo the appropriate redox reaction as they pass through. The total current generated as the species is passing through is proportional to its concentration in the solution. With Amperometric detection, decreasing the flow rate of the injected sample allows a greater amount of the analye to undergo the redox reaction, producing a more intense signal and

a better chromatogram. For this reason, a flow rate of 0.2 mL/min was chosen in this analysis.

In this study, a previously designed method for extraction of catecholamines and subsequent HPLC (12) is utilized to quantify the concentration of dopamine present in the retinal tissue of *Danio rerio* at different time points of the light/dark cycle. These results can be compared to the known fluctuations of other compounds, such as tyrosine hydroxylase and DOPAC, when the zebrafish have been exposed to the same cycle.

CHAPTER 2 – EXPERIMENTAL METHODS

Adapted from (12)

2.1 - Chemicals Used

Dopamine (DA) and 2,5-dihydroxybenzoic acid (DHBA) used for the preparation of standard curves and subsequent quantification of each substance in the tissue samples were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-octanesulphonic acid sodium salt monohydrate (OSA) was purchased from Fluka (Buchs, Switzerland). Anhydrous citric acid, concentrated hydrochloric acid (37%w/w), ethylenediaminetetraacetic acid (EDTA), and HPLC grade methanol and acetone were purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). Sodium hydroxide pellets were purchased from Spectrum Chemical Manufacturing Corp., (Gardena, CA, USA). Sodium metabisulfate was purchased from Mallinckrodt Specialty Chemical Co. (Paris, KY, USA). Sodium chloride was purchased from JT Baker (Phillipsburg, NJ). Ultrapure water used for the eluent and all solutions was obtained from a Barnstead International E-Pure apparatus (Dubuque, IA, USA). Compressed helium gas for degassing the eluent before it combines with the injected sample was obtained from Messer MG Industries (Malvern, PA, USA).

2.2 - Preparation of Stock and Standard Solutions

Stock and standard solutions were prepared for DA and DHBA in order to create two standard curves. The DA stock was prepared by dissolving 10 mg of pure dopamine in a 1000 mL aqueous solution of ultrapure water containing 500 mg of sodium metabisulfate and 4.00 grams of sodium chloride, creating a 10 ppm stock solution. The solution was acidified with 5.0 mL of concentrated hydrochloric acid. These stock

solutions were stored in the dark and kept cool in a refrigerator which maintained a temperature of approximately 4 degrees Celsius. Stock solutions of DHBA were prepared using the same method, with 10 mg of pure DHBA replacing the DA. From each stock solution, five standards of varying concentrations (0.1, 0.2, 0.3, 0.5, and 1.0 ppm) were prepared by first filtering the stock solution through an Acrodisc 0.2 μm syringe filter obtained from Pall Gelman Science (Ann Arbor, MI, USA) and subsequently diluting the stock solution with an appropriate volume of ultrapure water to create a 100 mL standard solution. The standard solutions used to create the calibration curve were prepared by varying the concentration of dopamine from 0.1 ppm to 1.0 ppm in the same increments as the original DA standard solutions. The appropriate volume of 10ppm DHBA was added to each solution to give each standard a constant concentration of 0.2 ppm DHBA. All solutions were prepared using 100 mL Type A volumetric flasks.

2.3 - HPLC Analysis of Solutions

Chromatographic separation was achieved using an Acclaim 120 reversed phase column (C8, 3μm, 2.1 x 50 mm) from the Dionex Corporation. The mobile phase, or eluent, used for the separation was prepared in a solution of approximately 800 mL of ultrapure water mixed with exactly 100 mL of HPLC-grade methanol. Next, 12.50 grams of citric acid, 25 mg of EDTA, and 25 mg of OSA were dissolved in the solution. Once the components were completely dissolved, the solution was transferred to a 1 liter volumetric flask and diluted with ultrapure water until the mark was reached. The pH of the solution was adjusted by adding approximately 10 mL of 3M NaOH to the mobile phase, in order to achieve a pH of around 3.3. The compressed tank of helium gas was

connected to the HPLC and was run through the mobile phase before it passed through the column to ensure there were no gases dissolved in the solution that could potentially clog the column and disrupt the separation. The HPLC was set to perform chromatographic separation using an eluent flow rate of 0.2 mL/min and a 100 µL loop for sample injection. All separations were carried out at room temperature. The setup of this instrument is portrayed schematically in Figure 5 on the following page.

The chromatographic separation was observed using electrochemical detection. A Dionex Corporation gradient pump, model GP40, was used in conjuction with a Dionex Corporation electrochemical amperometric detector, model ED 40. The working electrode used in this analysis was composed of gold. The potential was set to +1.00 volts against the reference electrode, which was composed of silver-silver chloride.

Data from the electrochemical detection system was imported to the Dionex Corporation *PeakNet* software, version 5.11, where the chromatographs were plotted. The software was running on a Dell OptiPlex GX1 computer.

2.4 - Maintenance of Animals

The compounds of interest in this analysis were obtained from extractions of zebrafish (*Danio rerio*) retinal tissue. The zebrafish were obtained by the American University Biology department from a local supplier (Petsmart, Inc., Bowie, MD, USA) and the care and maintenance of the fish was strictly in line with the guidelines set forth by the National Institutes of Health and in the American University Animal Care and Use Committee.

Schematic of HPLC Instrument Used in Analysis

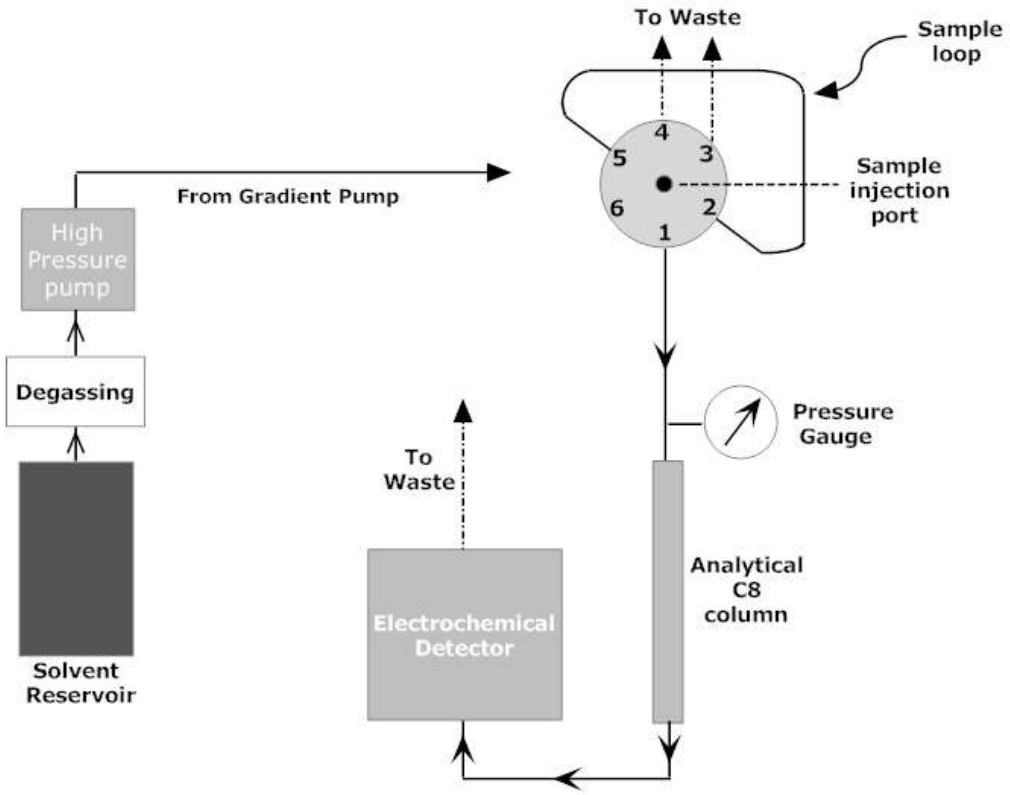

Figure 5: Diagram of HPLC System used in Sample Analysis

2.5 - Extraction of Catecholamines from Retinal Tissue

In order to obtain the retinal tissue samples, the zebrafish were sacrificed at the appropriate time in the light cycle and the retainae were dissected out. Samples were collected 8 hours into the dark time period, at approximately 0630 hours, and again 8 hours into the light time period, at approximately 1630 hours. Each sample consisted of ten retinae, and three samples were collected for each time period. Until the analysis was done, the retinae were stored in the dark in about 1 mL of methanol and kept at approximately -20 degrees Celsius. When the HPLC analysis was ready to be done, the tissue samples were homogenized into a solution.

A single solution was prepared for use in the homogenization of each sample. The solution contained 12.0 mg of DHBA, which functioned as an internal standard, dissolved in a mixture of 300 mL of concentrated HCl and approximately 500 mL of ultrapure water. The solution was transferred to a 1L volumetric flask and diluted with ultrapure water to the mark. After removing the methanol from each tissue sample, 1.00 mL of the homogenization solution was added to the sample with a micropipette. After sufficient mixing, each tissue mixture was subsequently centrifuged at 12.5 rpm for 15 minutes, and the supernatant obtained from the centrifugation was filtered through an Acrodisc 0.2 μm syringe filter and diluted to a volume of 10 mL. After filtering, the samples were injected into the HPLC and their DA and DHBA concentrations were measured using the HPLC Analysis procedure described earlier.

2.6 - Analytic Methods

The concentrations of DA, DHBA, and DOPAC in the retinal tissue samples were obtained through the use of several calibration curves. A separate curve was created for each of the above substances of interest by plotting five points of concentration versus the ratio of DA:DHBA peak area in the HPLC separation. DHBA, which was also added to the retinal samples to be analyzed, functioned as an internal standard throughout the experiment. Finding the ratio of DA:DHBA in the unknown tissue samples and comparing the experimental ratio to the equation of the line of best fit in the calibration curve allowed for determination of the DA concentration in each sample. The calibration curve and subsequent DA:DHBA ratios observed in the tissue samples can be found in the following discussion section. All plots for data analysis were constructed using Microsoft Excel 2003.

CHAPTER 3 – RESULTS & DISCUSSION

3.1 – Analysis of Dopamine and DHBA Standard Curves

Five dopamine standards were prepared with concentrations ranging from 0.1 ppm to 1.0 ppm as described in Section 2.2. Five DHBA standards of the same concentrations were also prepared. The potential applied to the working electrode was set to +1.00 Volts, as this had been previously determined to be the optimum potential for catecholamine analysis on the instrument used (12). Samples were injected into the instrument via a 100 µL injection loop and the system was set up so that the signal output would be read by an electrochemical detector (Dionex ED40). Using this setup, each of the dopamine standards was injected and allowed to run for ten minutes. The five resulting chromatographs are illustrated in Figures 6a through 6e. The dopamine peak areas for each run, which were used to create the dopamine standard curve (Figure 7) are displayed in Table 1. The line of best fit for this data was determined to have an equation of Y = 2E+06X–154778 and a correlation coefficient of 0.9873. The main result from this portion of the analysis is the demonstration of the linearity of dopamine standards and the observation that using this mobile phase, dopamine elutes from the column at approximately 2.45 minutes. This information was used to correctly identify the dopamine peak in the calibration curve in section 3.2.

TABLE 1: PEAK AREAS OF FIVE DOPAMINE STANDARDS

Dopamine Concentration (ppm)	Peak Area
0.1	214203
0.2	271069
0.3	470282
0.5	925296
1.0	22265446

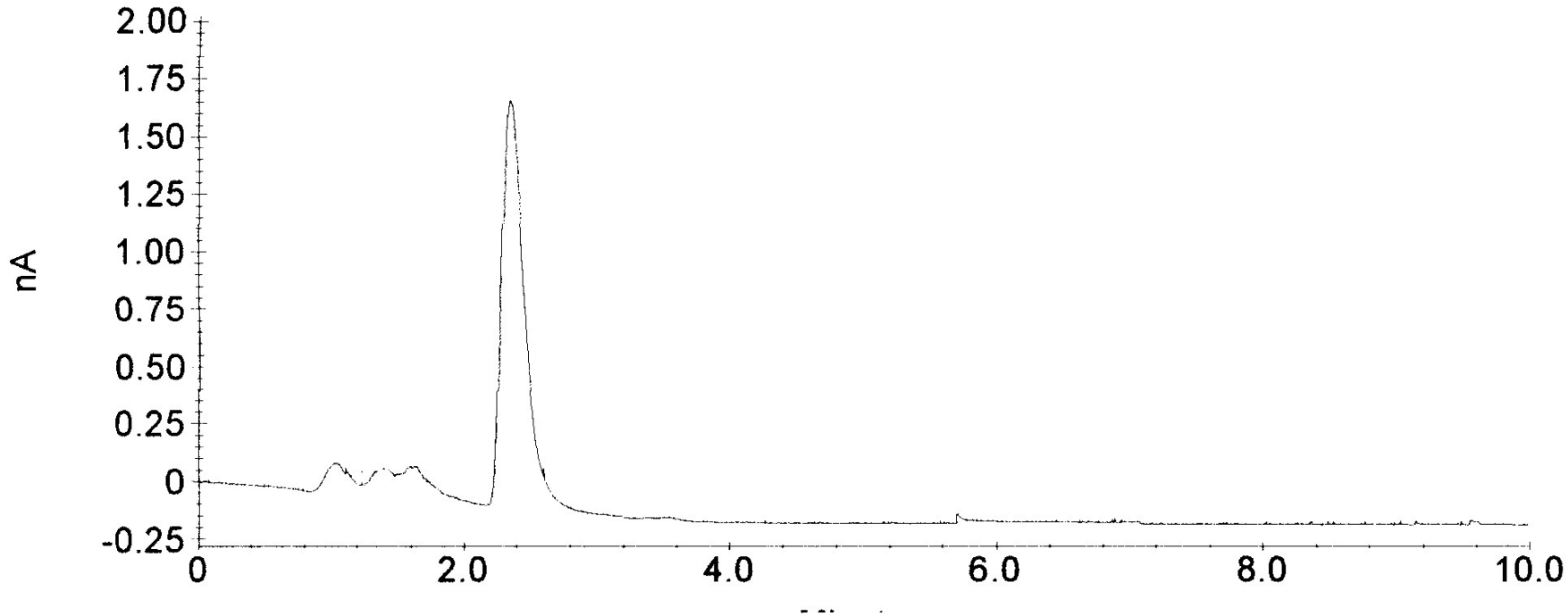

Figure 6a: Chromatograph for Dopamine Standard, 0.1 ppm

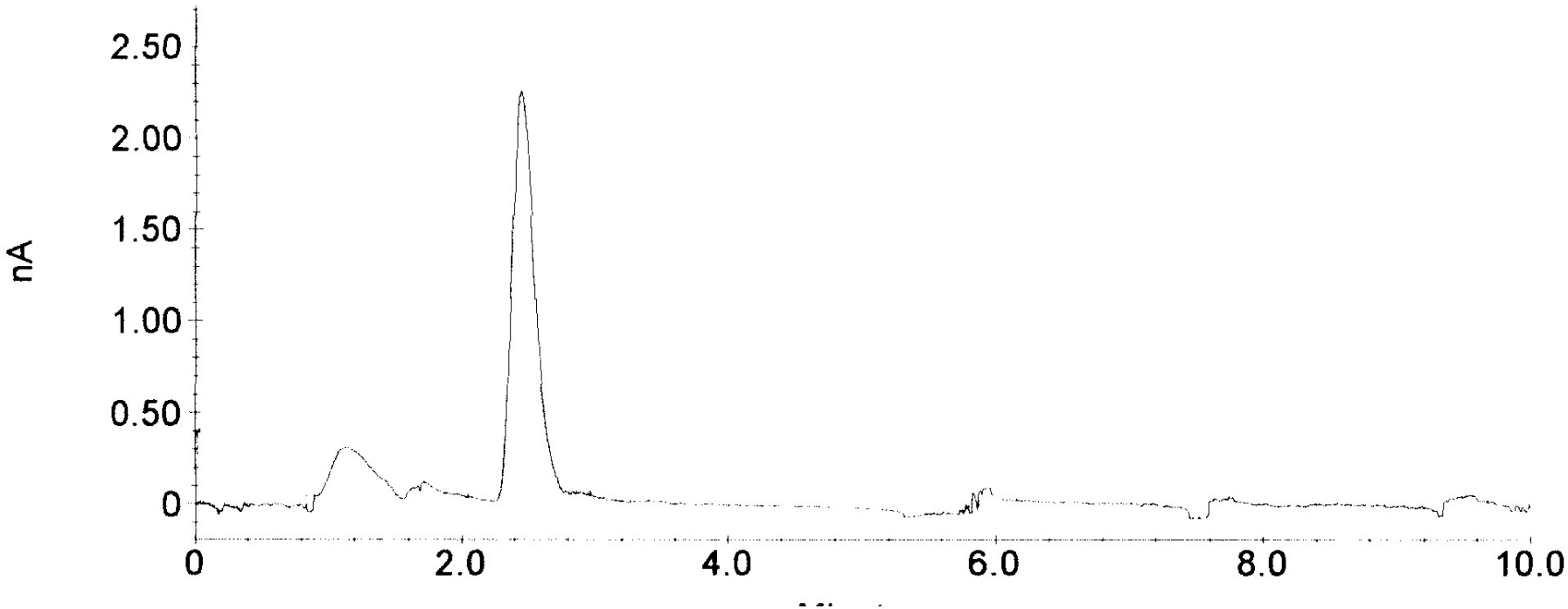

Figure 6b: Chromatograph for Dopamine Standard, 0.2 ppm

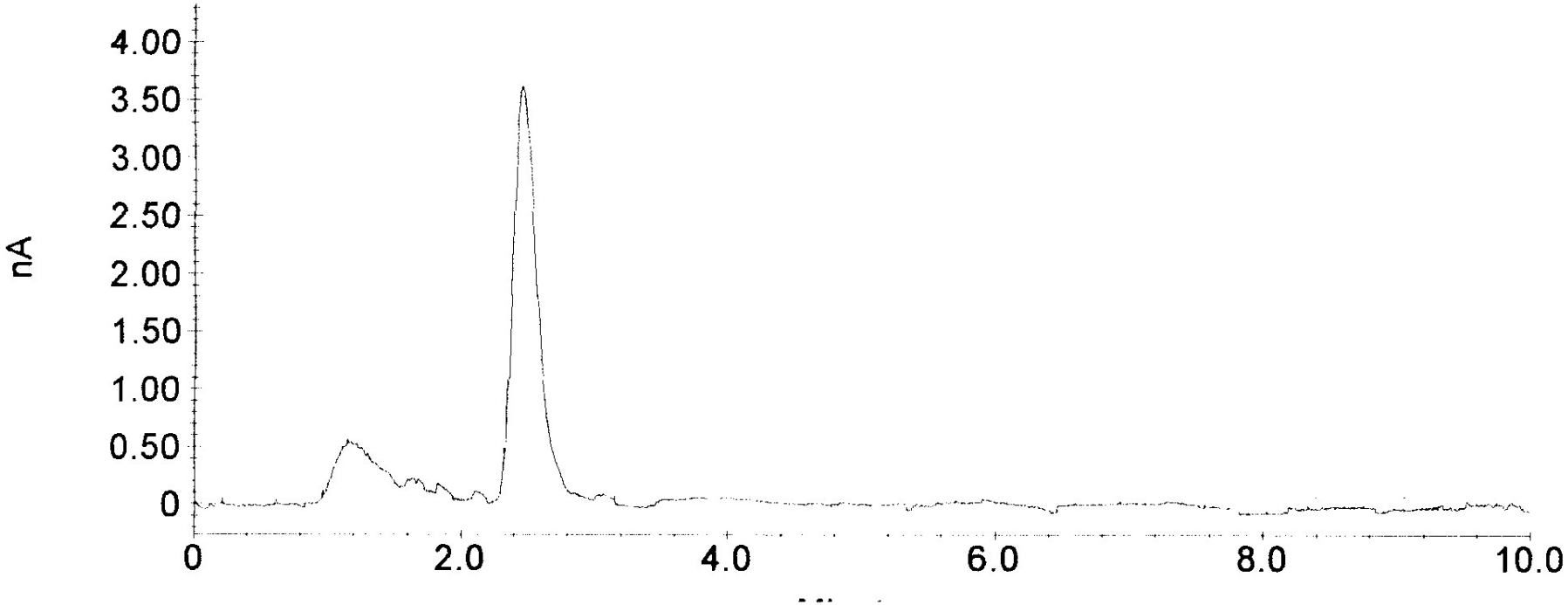

Figure 6c: Chromatograph for Dopamine Standard, 0.3 ppm

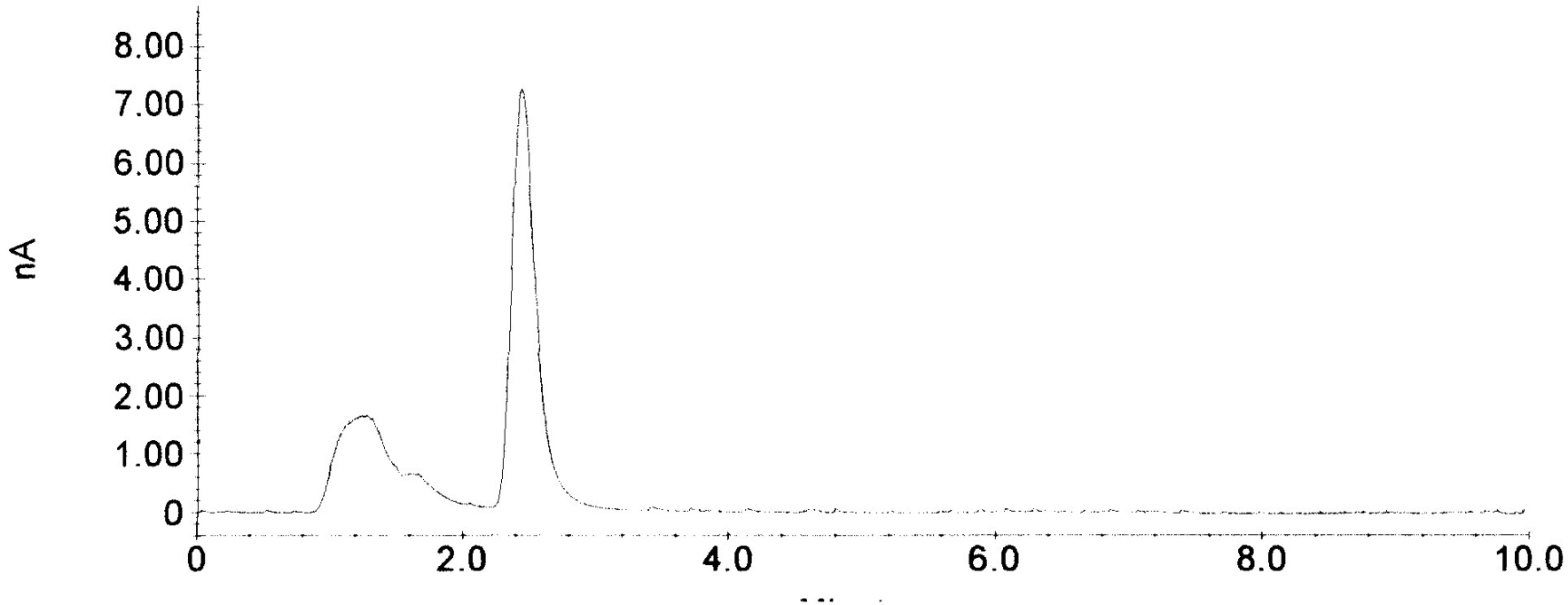

Figure 6d: Chromatograph for Dopamine Standard, 0.5 ppm

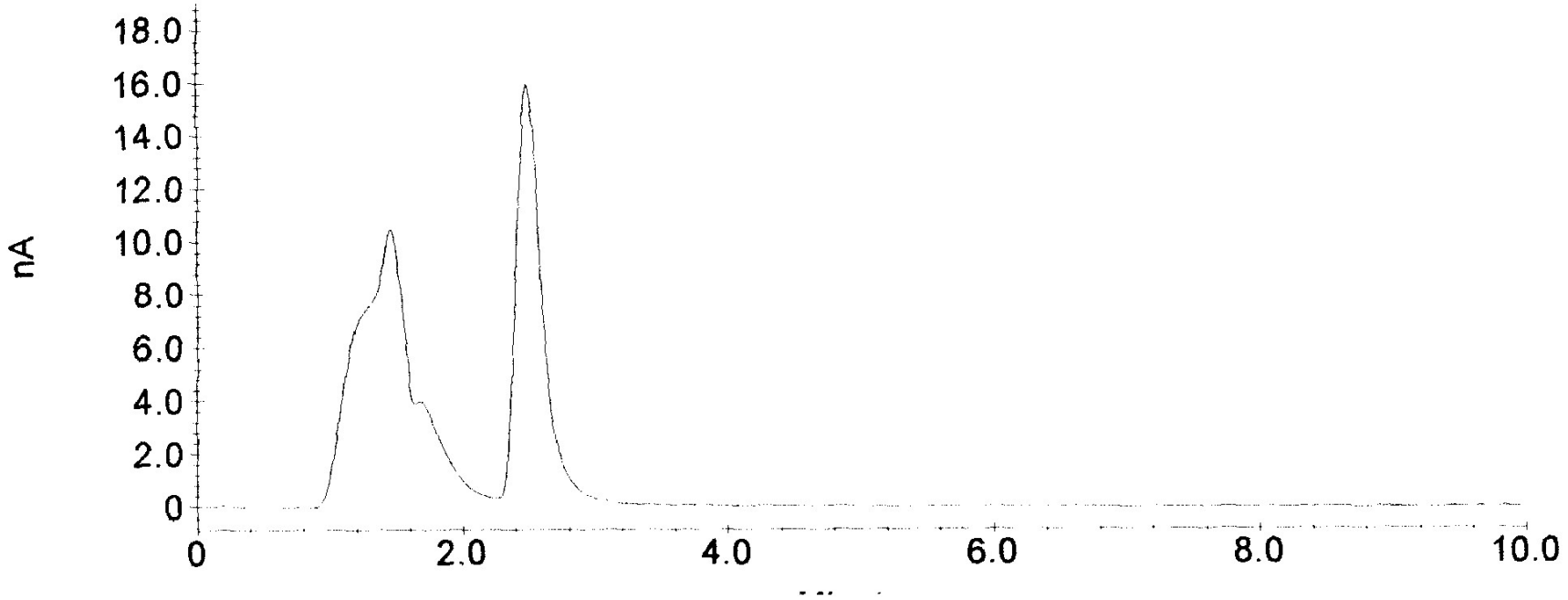

Figure 6e: Chromatograph for Dopamine Standard, 1.0 ppm

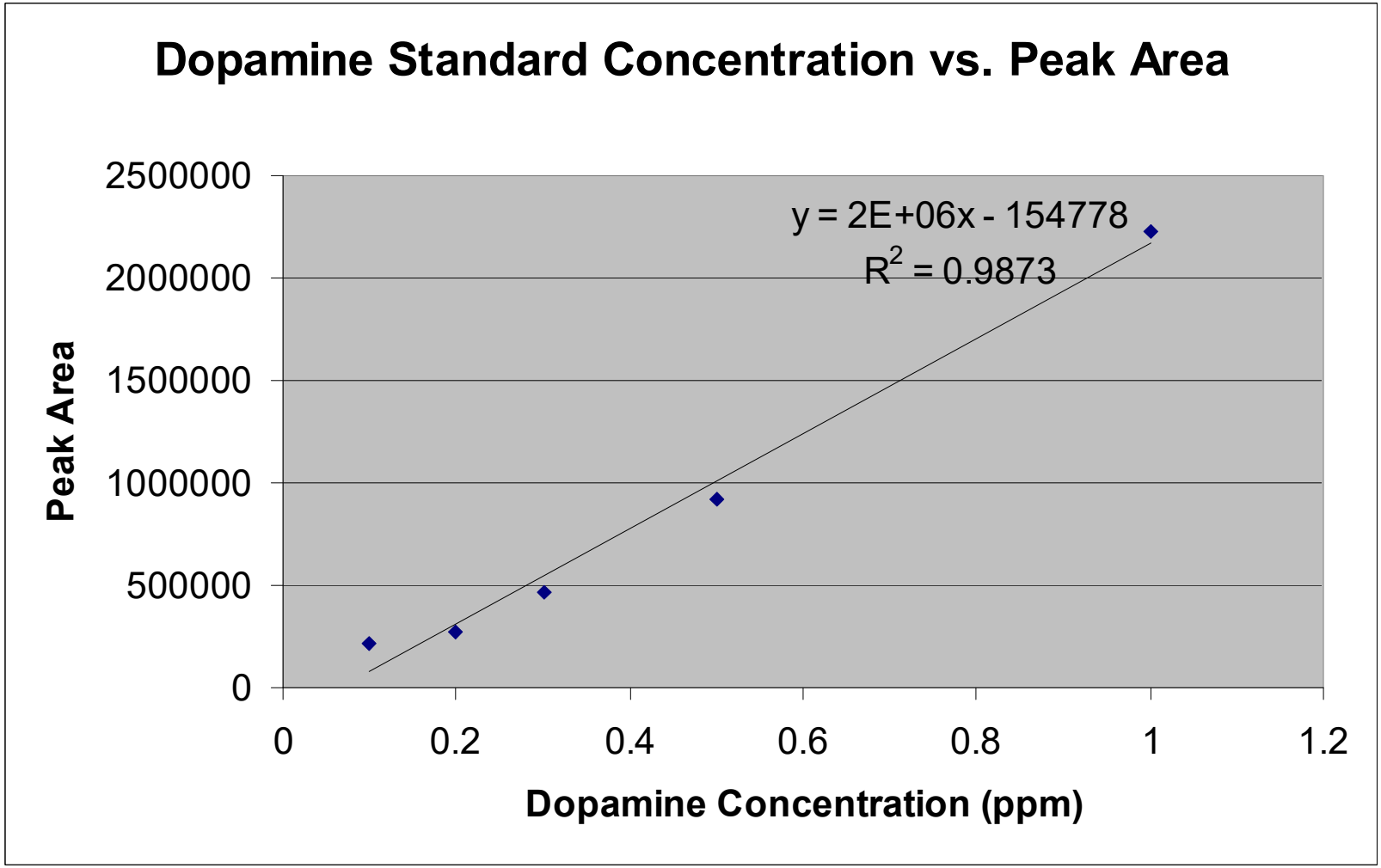

Figure 7: Analysis of Dopamine Standards for Linearity and Observation of Retention Time

Similarly, each of the DHBA standards was injected and allowed to run for ten minutes. The five resulting chromatographs are illustrated in Figures 8a through 8e. The DHBA peak areas for each run (Table 2) were used to create the DHBA standard curve (Figure 9). The line of best fit for this data was determined to have an equation of Y = 5E+06X+1E+06 and a correlation coefficient of 0.9804. The main result from this portion of the analysis is the demonstration of the linearity of DHBA standards and the observation that using this mobile phase, DHBA elutes from the column at approximately 3.00 minutes. This information was used to correctly identify the DHBA peak, used as an internal standard, in the calibration curve in section 3.2.

TABLE 2: PEAK AREAS OF FIVE DHBA STANDARDS

DHBA Concentration (ppm)	Peak Area
0.1	1294015
0.2	2131626
0.3	2858298
0.5	4024506
1.0	6136365

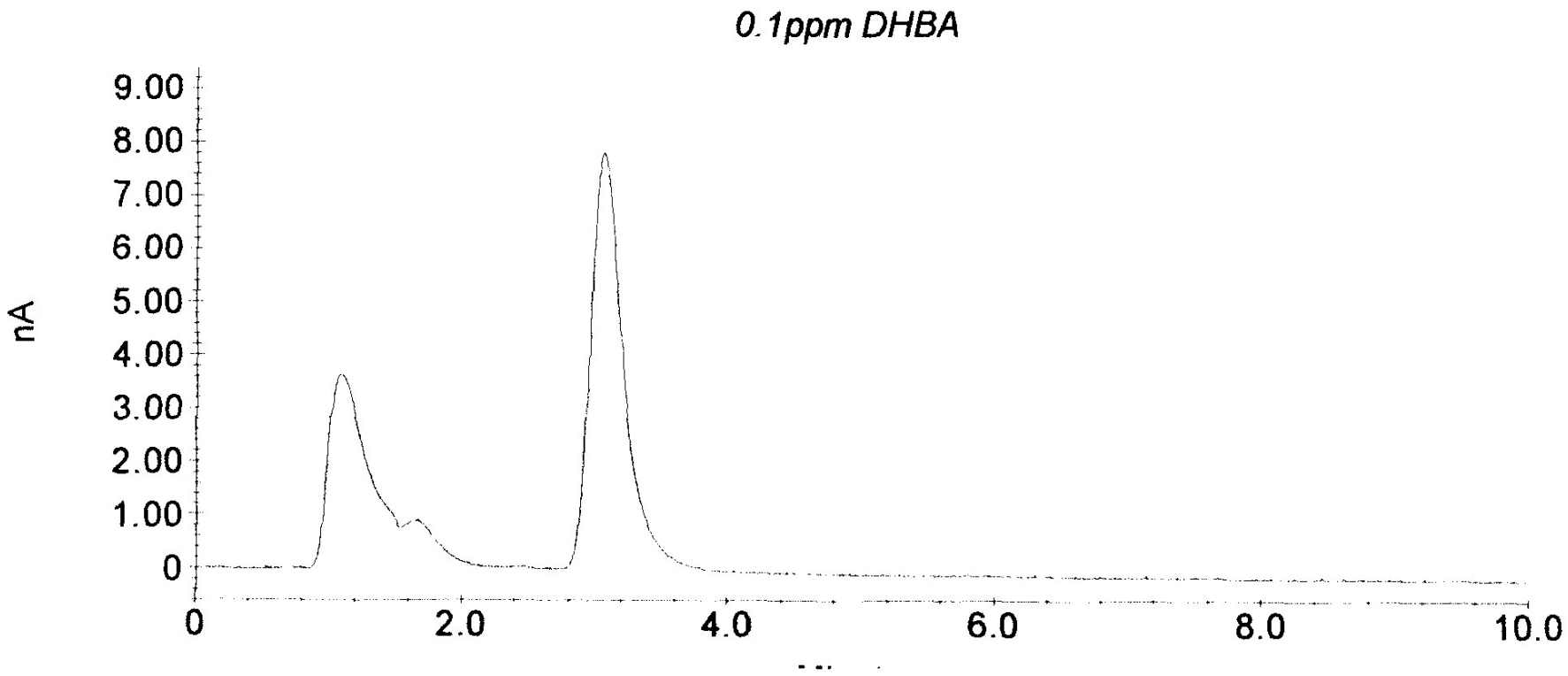

Figure 8a: Chromatograph for DHBA Standard, 0.1 ppm

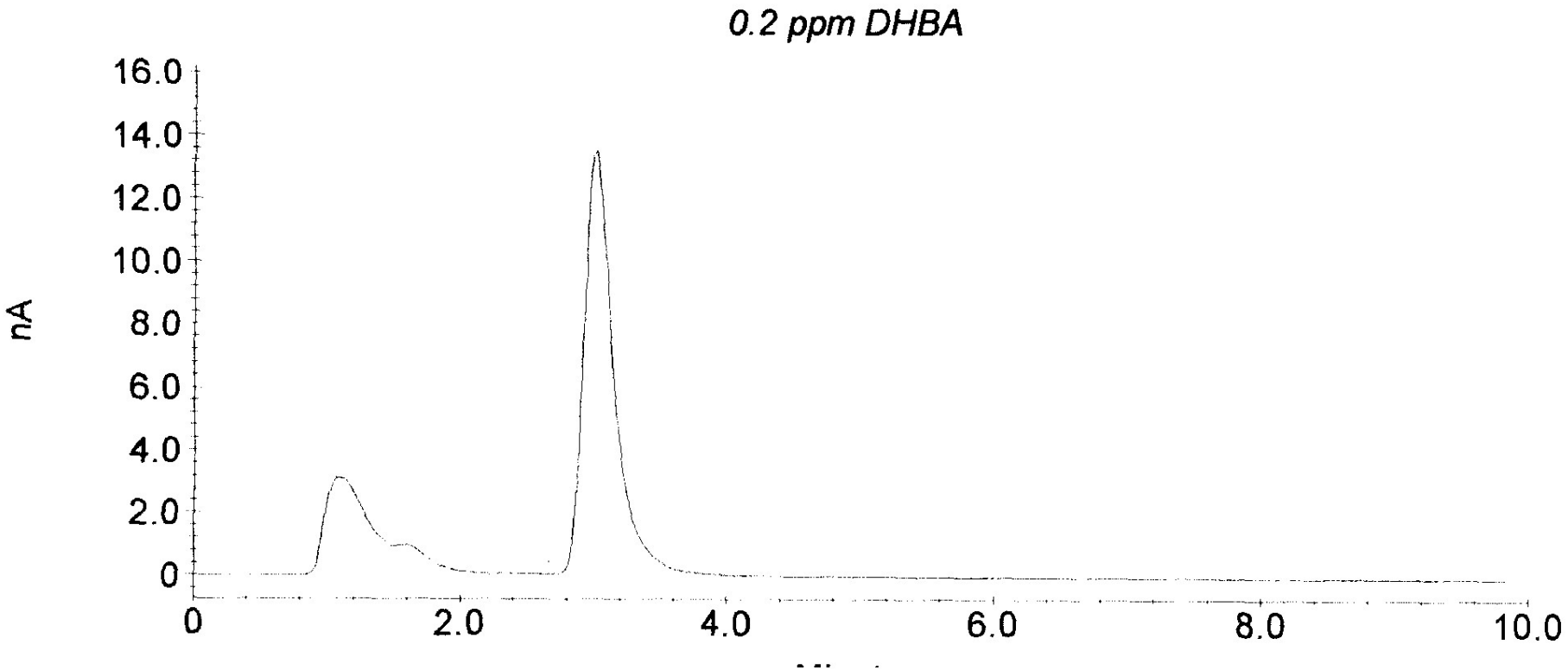

Figure 8b: Chromatograph for DHBA Standard, 0.2 ppm

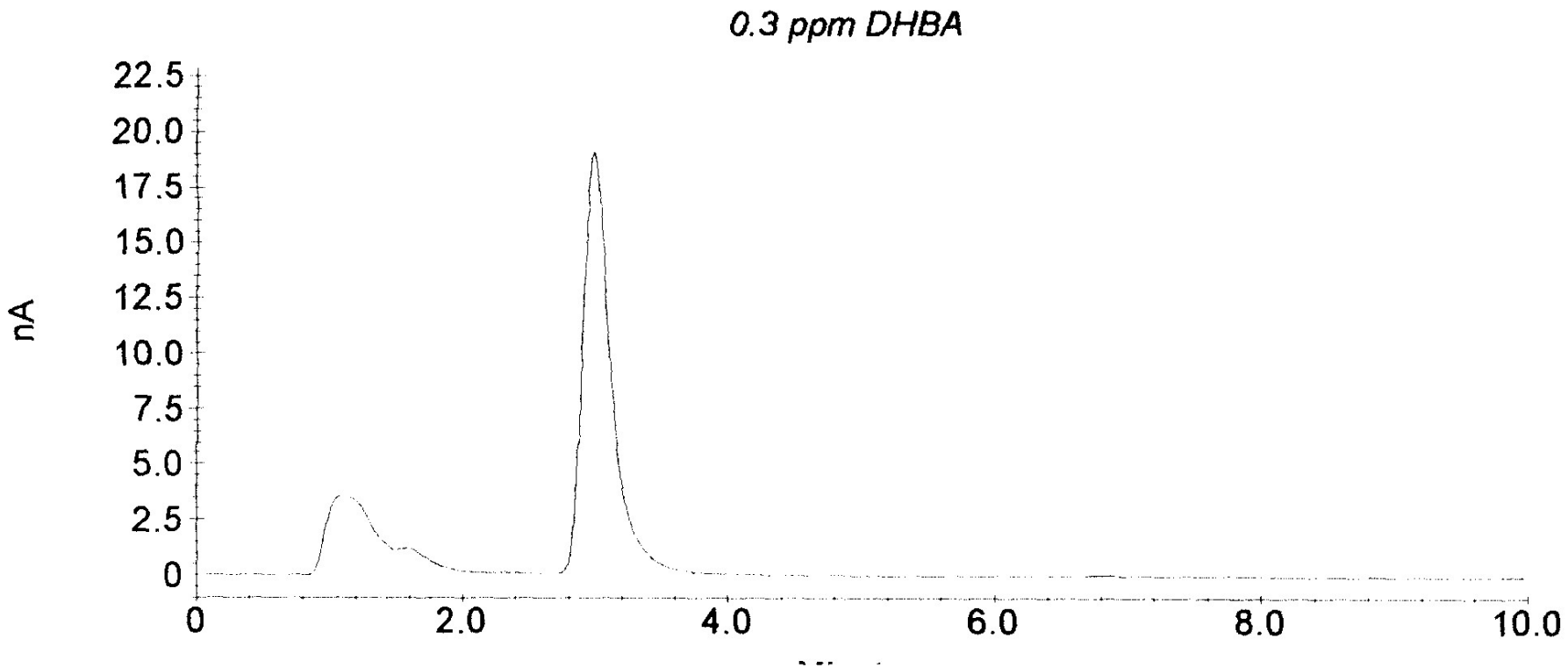

Figure 8c: Chromatograph for DHBA Standard, 0.3 ppm

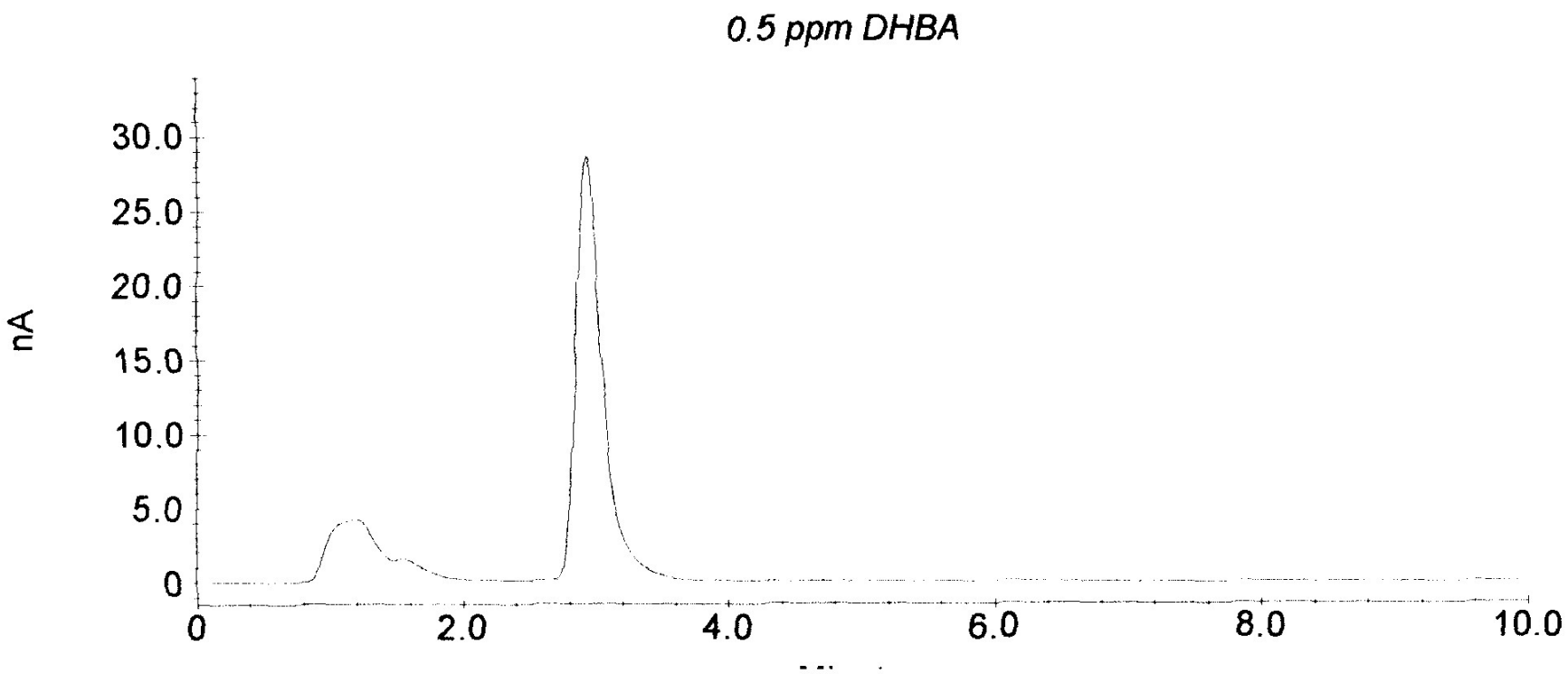

Figure 8d: Chromatograph for DHBA Standard, 0.5 ppm

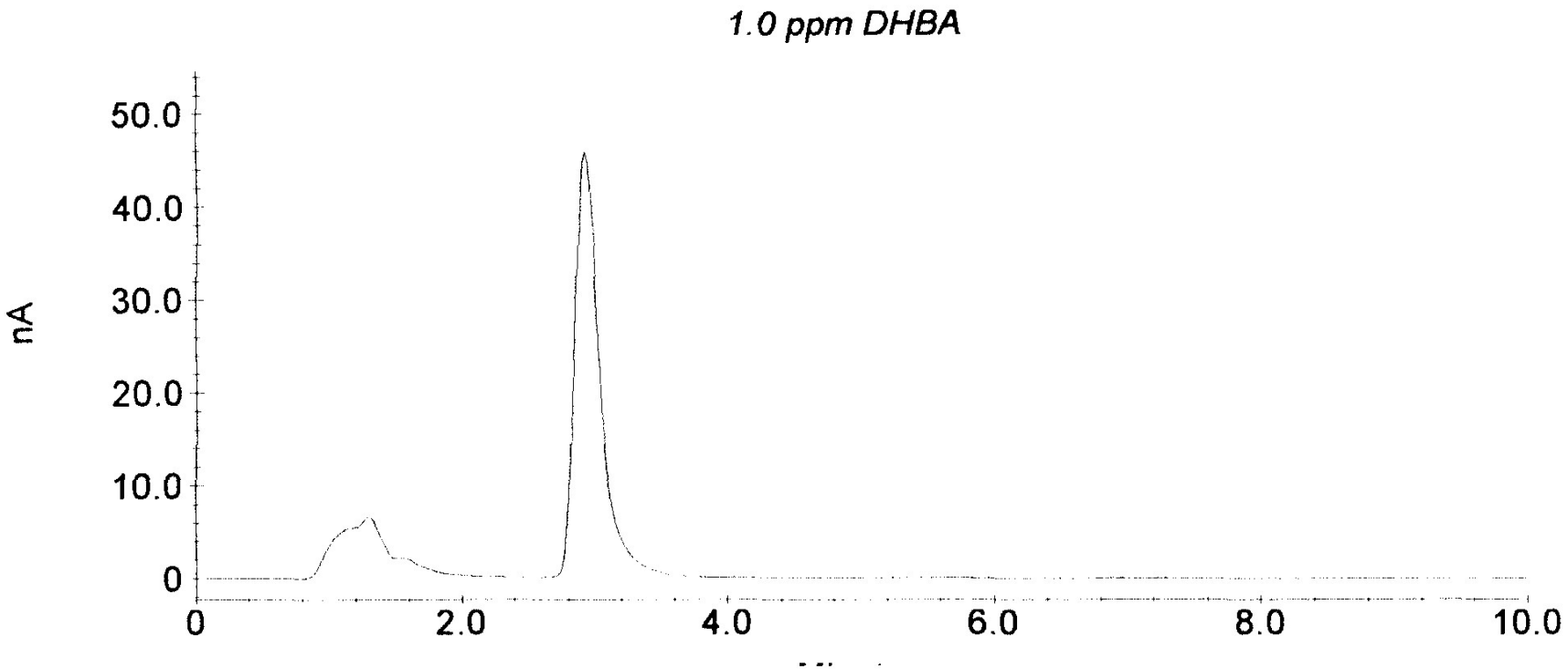

Figure 8e: Chromatograph for DHBA Standard, 1.0 ppm

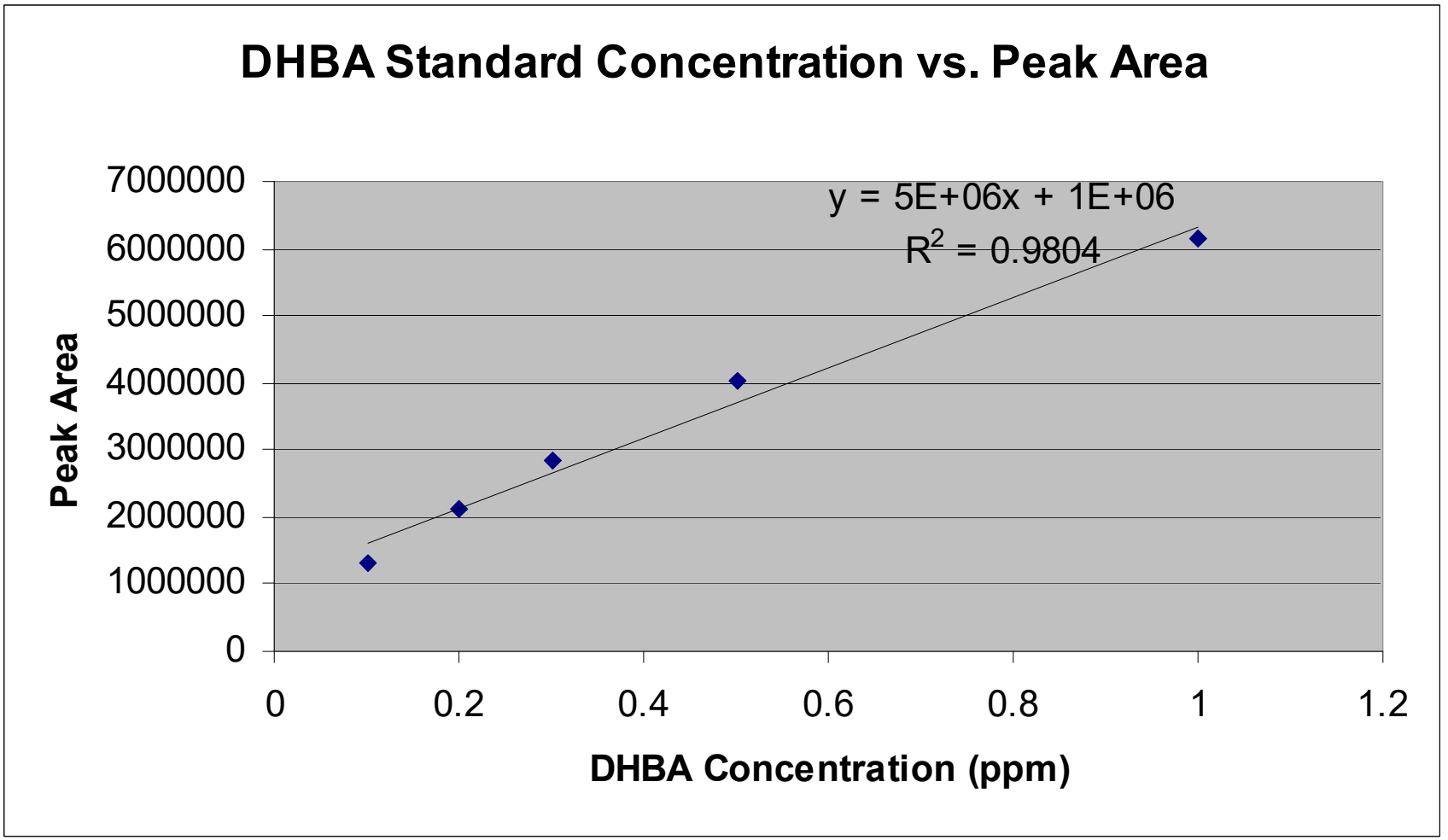

Figure 9: Analysis of DHBA Standards for Linearity and Observation of Retention Time

3.2 – Quantitative Determination of Dopamine in Zebrafish Retinal Tissue Samples

After demonstrating linearity between the concentrations of DA and DHBA and their respective peak areas, standard solutions of DA were prepared with a constant concentration of DHBA to act as an internal standard. The DA concentration in the solutions ranged from 0.1 ppm to 1.0 ppm, just as in the original DA standards, while the DHBA concentration remained at 0.2 ppm in each solution. The samples were run for ten minutes and the peaks were correctly identified as either DA or DHBA by comparing the retention times to those in the original standards. The chromatographs produced by these runs are illustrated in Figures 10a through 10e. The ratio of DA peak area to DHBA peak area was plotted against DA concentration to create a five point calibration curve to be used in DA determination in the tissue samples. The peak areas used to determine the ratio were automatically generated by the PeakNet software and are shown in Table 3. The calibration curve has an equation of $y = 14.79x - 0.3804$ and a correlation coefficient of 0.9999, demonstrating an almost exact linear relationship (Figure 11).

Table 3: Calibration Curve Data – Various DA Concentrations with a Constant DHBA Concentration of 0.2ppm.

Dopamine Concentration (ppm)	Ratio of DA:DHBA Peak Areas
0.1	1.143614661

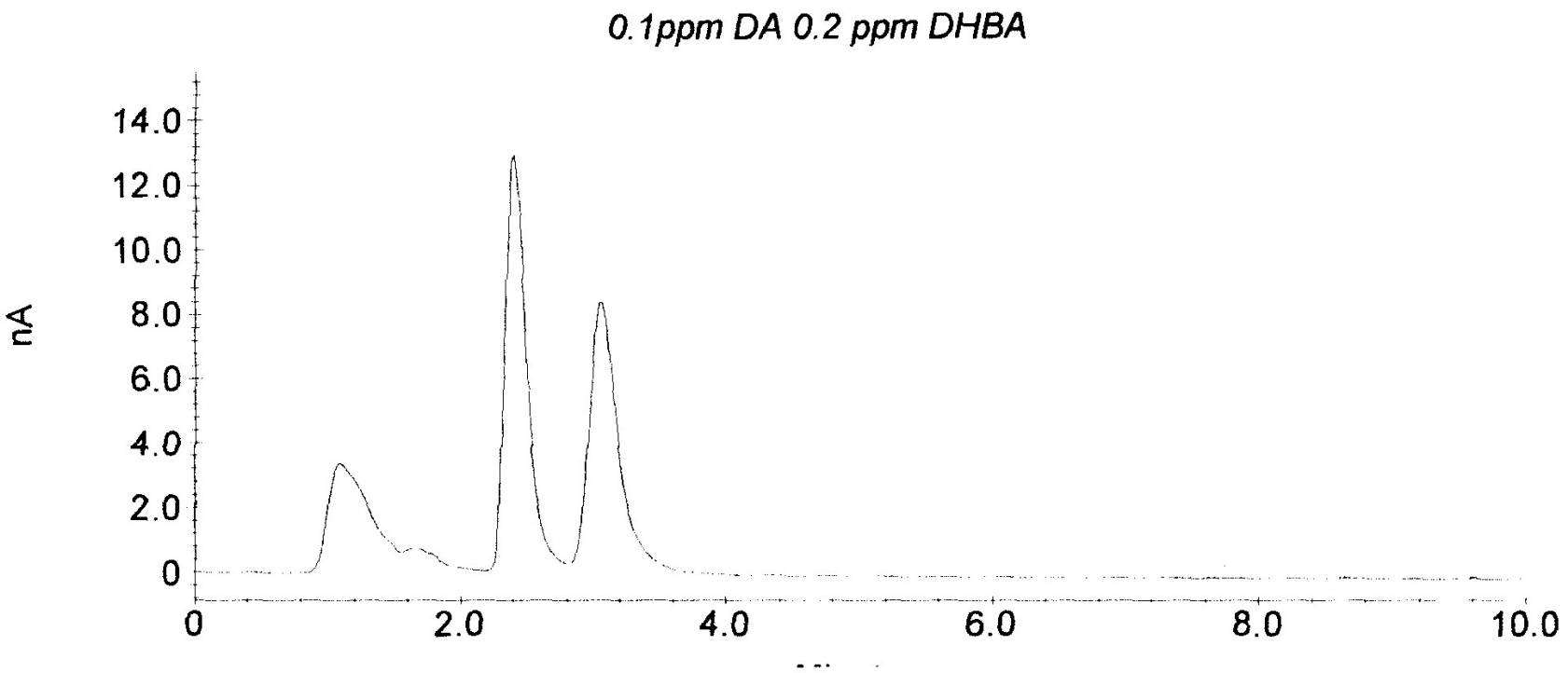

Figure 10a: Chromatograph for 0.1ppm DA, 0.2 ppm DHBA Standard

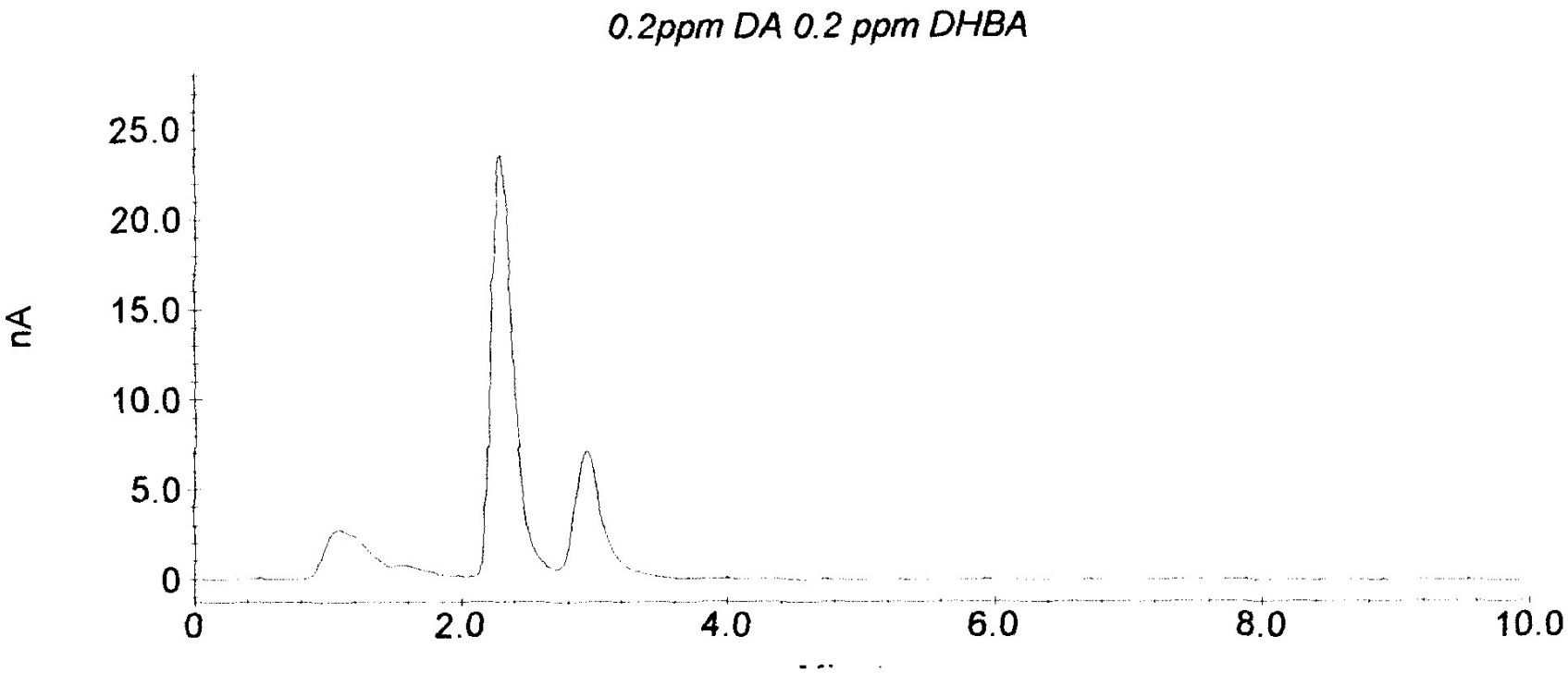

Figure 10b: Chromatograph for 0.2ppm DA, 0.2 ppm DHBA Standard

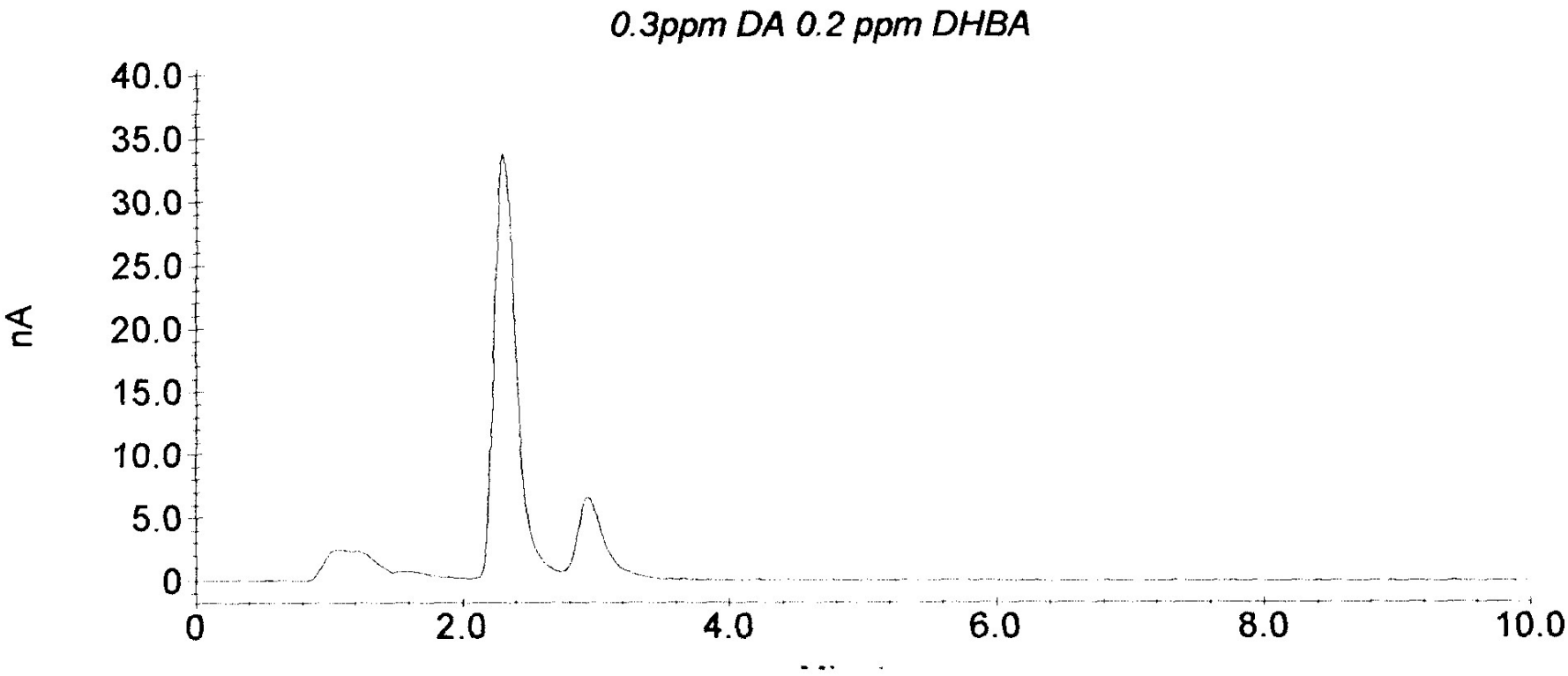

Figure 10c: Chromatograph for 0.3ppm DA, 0.2 ppm DHBA Standard

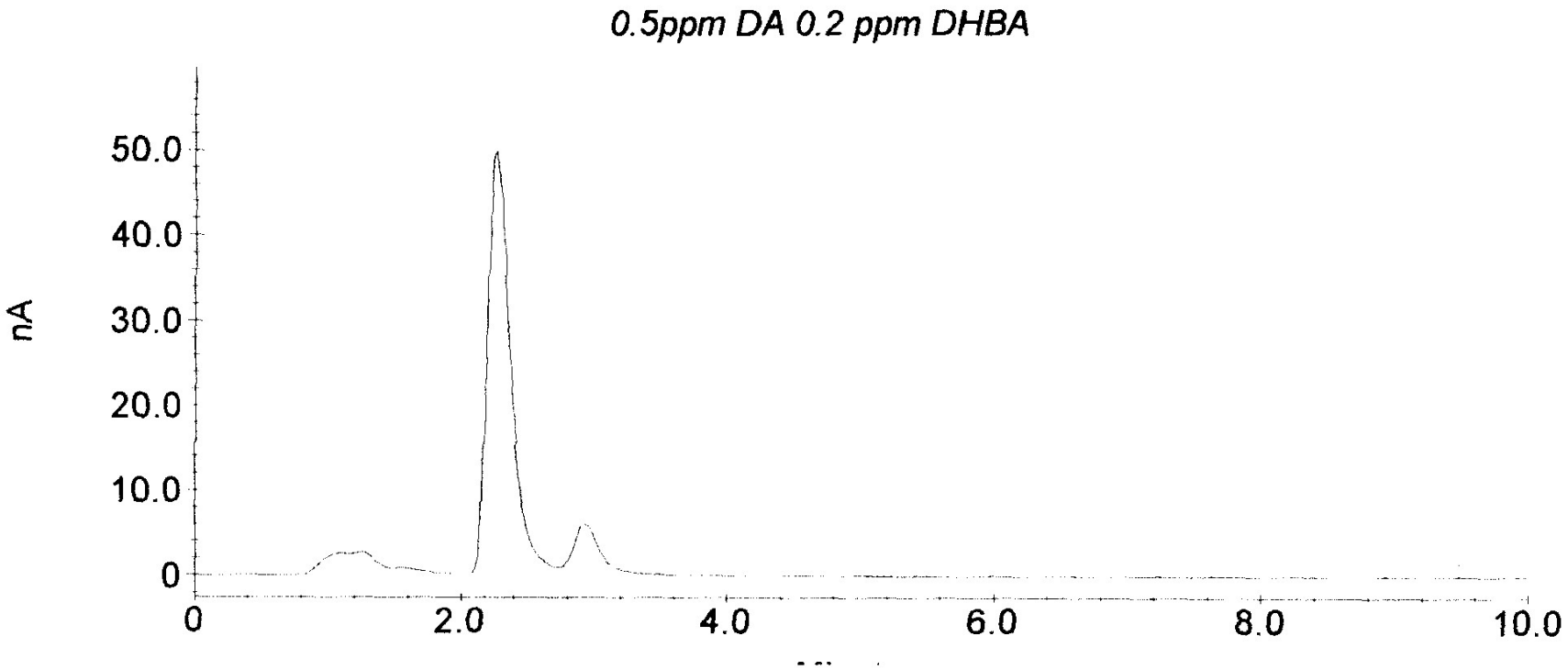

Figure 10d: Chromatograph for 0.5ppm DA, 0.2 ppm DHBA Standard

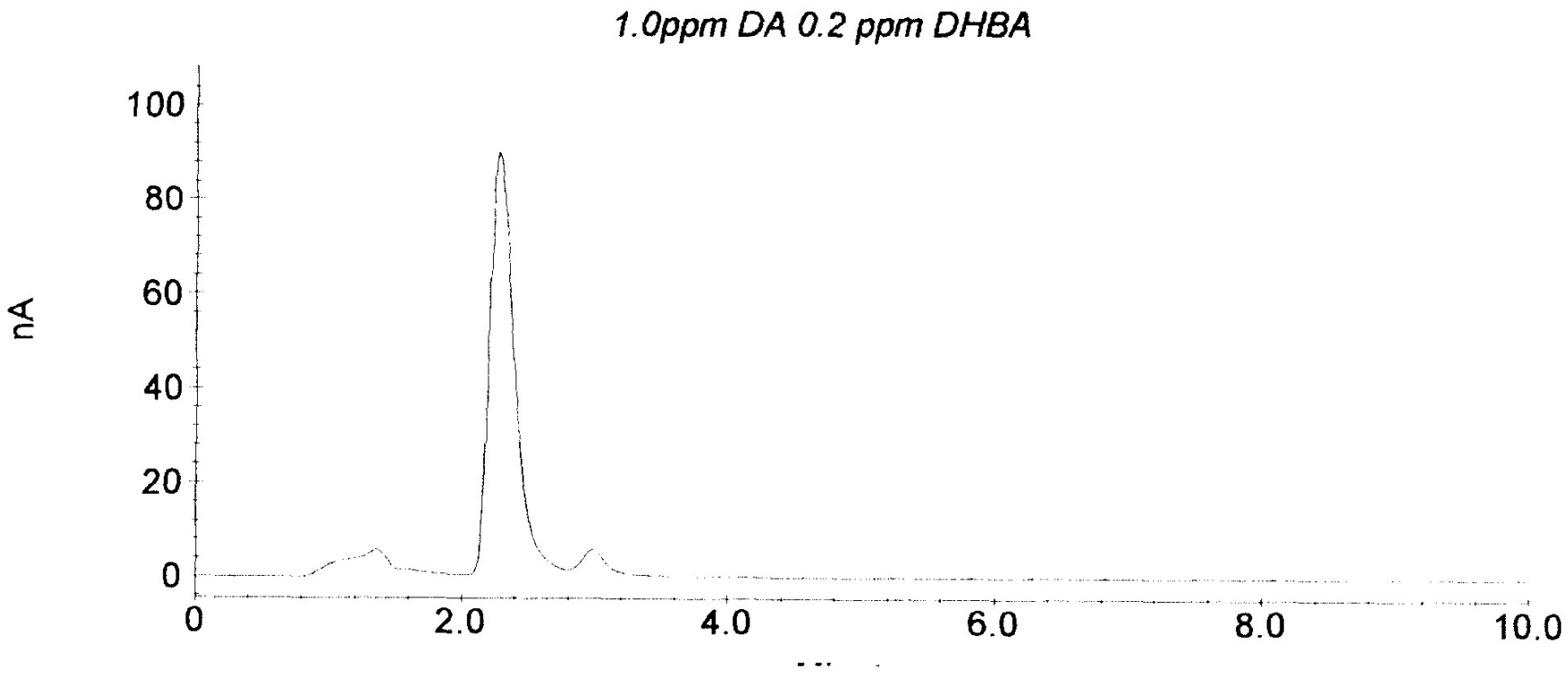

Figure 10e: Chromatograph for 1.0 ppm DA, 0.2 ppm DHBA Standard

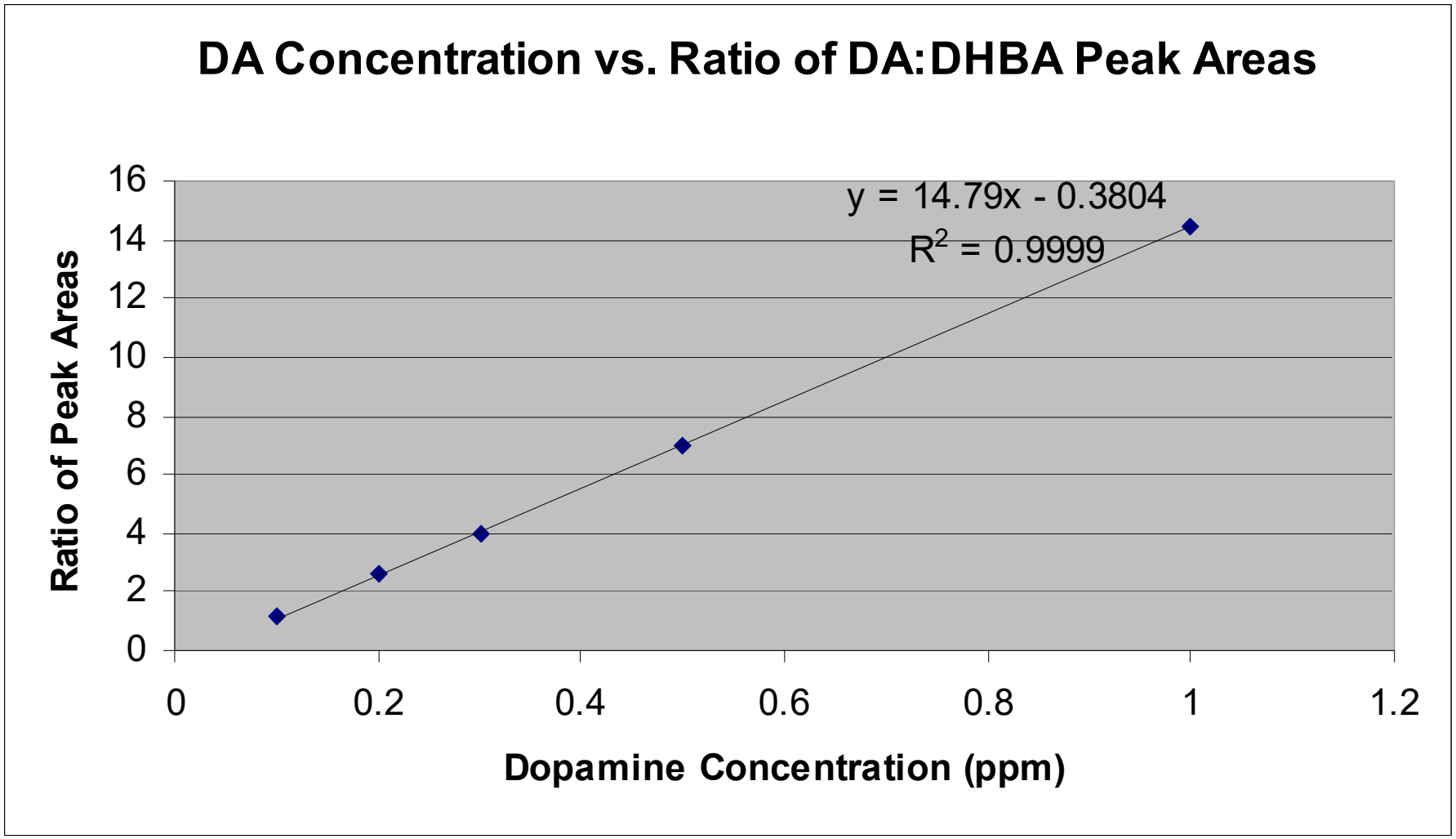

Figure 11: Calibration Curve for Dopamine Standards using an Internal Standard of 0.2ppm DHBA

The zebrafish retinae were obtained and processed as described in Section 2.5. The dopamine was extracted by homogenization in a solution of hydrochloric acid, and then centrifuged to remove larger tissue particles from the solution. Three samples were obtained from 8 hours into the light cycle, at approximately 1630 hours. These samples are referred to as L1, L2, and L3. Three additional samples were obtained 8 hours into the dark cycle. These samples are referred to as D1, D2, and D3. Each sample contained ten retinae, so the average results for tissue from each sample set are based on data for 30 retinae.

Each sample was injected into the HPLC and subject to the same mobile phase and conditions as the standard solutions. The samples were set to run for ten minutes each, and the chromatographs were analyzed for DA and DHBA. The resulting chromatograms for each trial of samples L1, L2, and L3 are illustrated in Figures 12a through 12c, and those for D1, D2, and D3 are shown in Figures 13a through 13c. Each sample was analyzed in duplicate so that an average value for the peak area ratios could be obtained. Although the peaks are not as visually obvious as those in the standard peaks, they are automatically detected by the PeakNet software when there is a change in current, and the peak area is automatically generated. The solvent response was unusually pronounced in these runs most likely because of the higher HCl concentration used to ensure complete DA extraction from the tissue. In these chromatographs, DA and DHBA eluted from the column in the same amount of time as they did in the standards,

DHBA peak areas were substituted for the “y” in the equation so that the DA concentration, x, could be determined. Before substituting peak area values into this equation, they were adjusted for the dilution factor since the homogenizing DHBA/HCl solution had a DHBA concentration of 1.2 ppm, six times higher than that of the DA standard solutions. The raw data and tissue sample DA concentrations are shown in Table 4.

The “L” sample set had an average DA concentration of 3.085463 ppm in the 10 mL extracted tissue solution. This equated to an average of 0.3085463 μg of DA per retina across these samples. The “D” sample set had an average DA concentration of 4.429158 ppm in the 10 mL tissue solution, equating to an average of 0.4429158 μg of DA per retina in these samples (Table 5). This data suggests that a greater amount of DA is produced in zebrafish retina during the dark hours of the cycle, since the solutions extracted at 0630 hours had a higher average concentration.

TABLE 4: RAW DATA FOR HPLC ANALYSIS OF RETINAL TISSUE SAMPLES AND DOPAMINE CONCENTRATION

Tissue Sample	Trial 1 Peak Areas			Trial 2 Peak Areas			Average DA/DHBA	DA/DHBA (Adjusted for Dilutions)	DA Concentration in Sample, ppm
	DA	DHBA	DA/DHBA	DA	DHBA	DA/DHBA			
L1	20253234	2911442	6.956427	18277505	3166062	5.772946	6.364687	38.188119	2.607743
L2	17721600	3798571	4.665333	12358823	2222987	5.559557	5.112445	30.674670	2.099734
L3	16543191	96424	171.5672	2725058	244407	11.14967	11.14967	66.89802	4.548913
D1	21630882	1298453	16.65896	20440416	2566916	7.963025	12.31099	73.86594	5.020037
D2	17863848	6325309	2.824186	18096246	2341704	7.727811	5.275999	31.655994	2.166085
D3	22659073	3247883	6.976567	8738827	380341	22.97630	14.97643	89.85858	6.101351

TABLE 5: AVERAGE RETINAL MASS OF DOPAMINE FOR EACH SAMPLE SET

Sample Set	**Average DA Concentration, ppm**	**Average Mass of DA in Total 1mL Sample, µg**	**Average Mass of DA per retina in sample, µg**
L	3.085463	3.085463	0.3085463
D	4.429158	4.429158	0.4429158

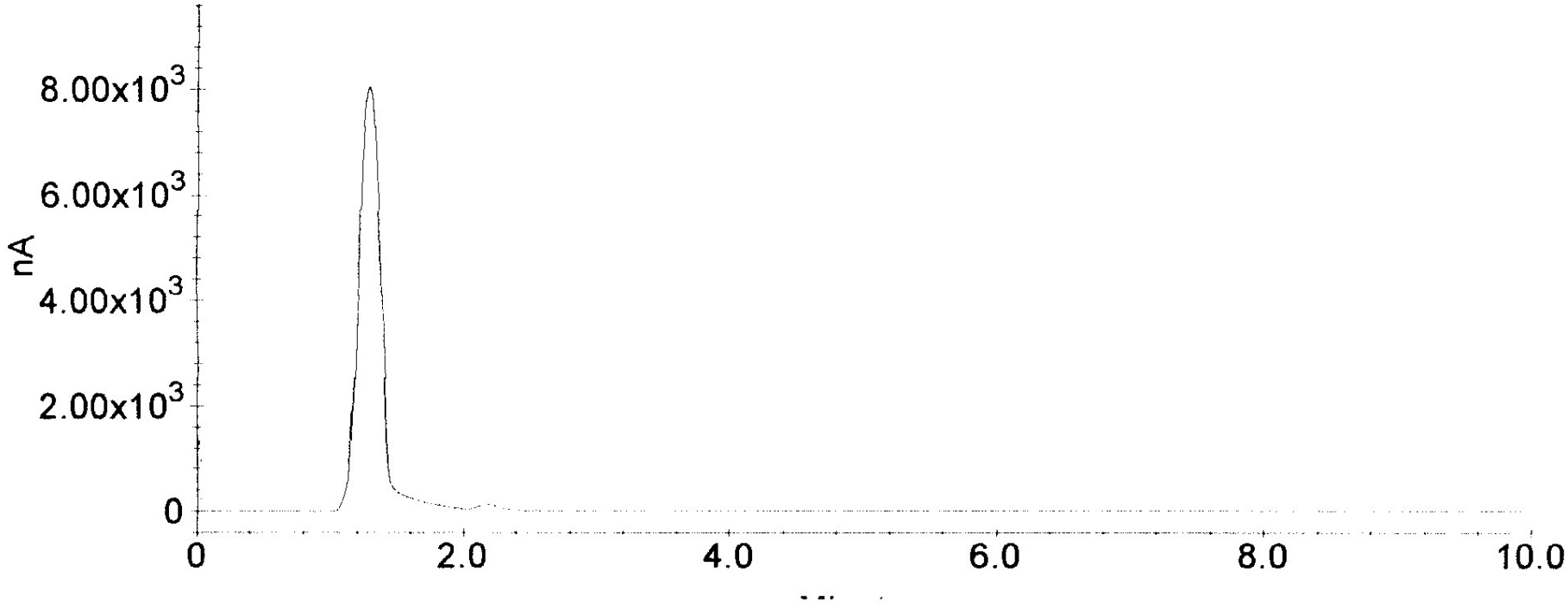

Figure 12a(i): Chromatograph for Sample L1 – Trial 1

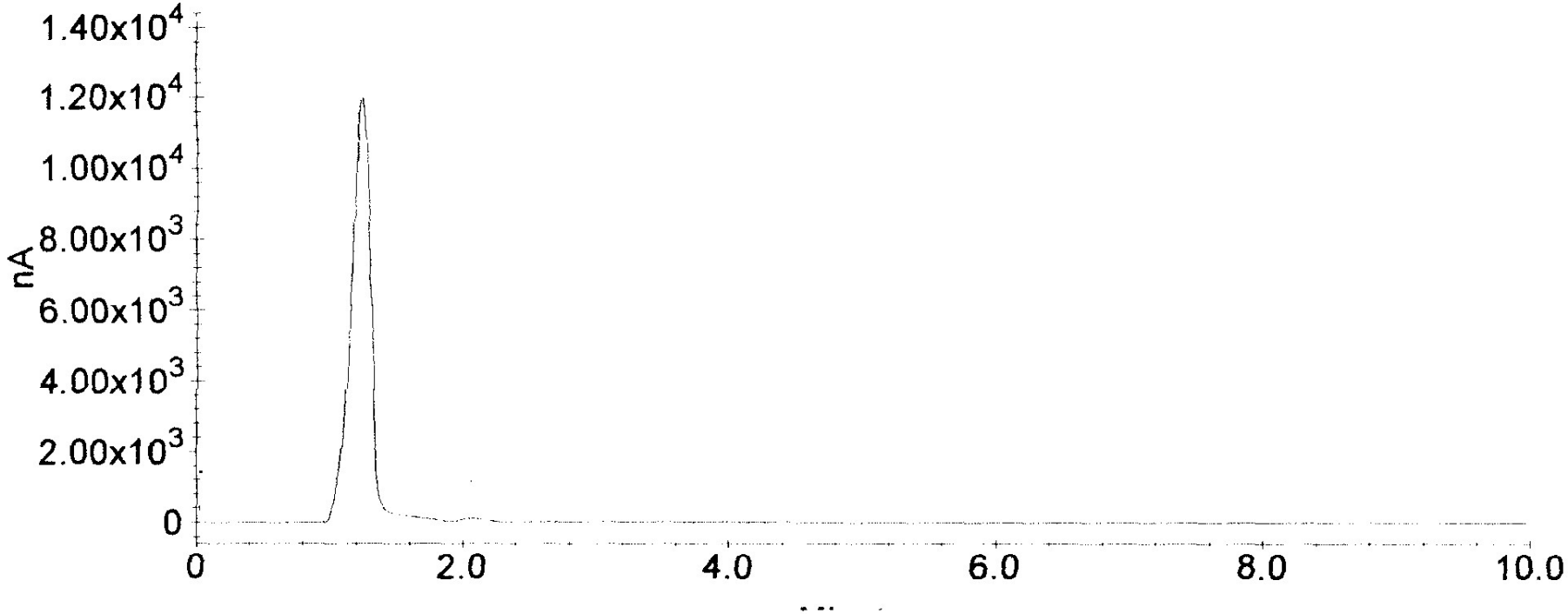

Figure 12a(ii): Chromatograph for Sample L1 – Trial 2

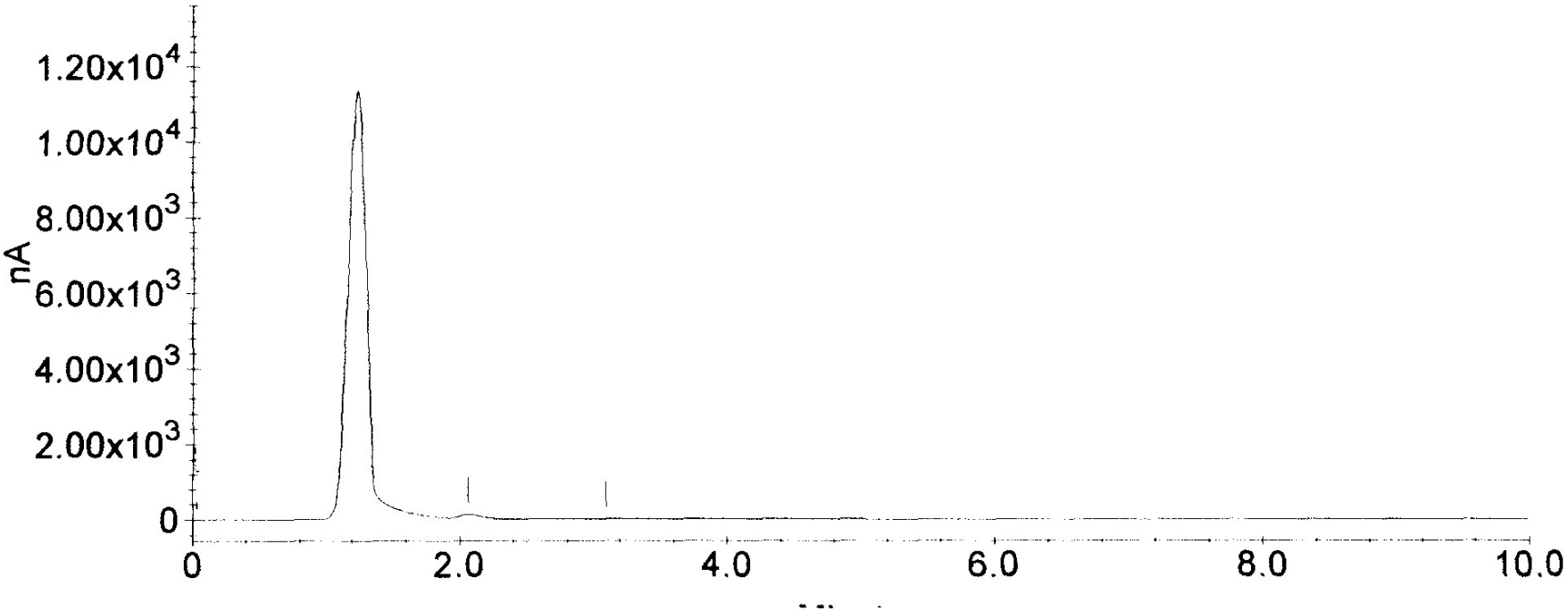

Figure 12b(i): Chromatograph for Sample L2, Trial 1

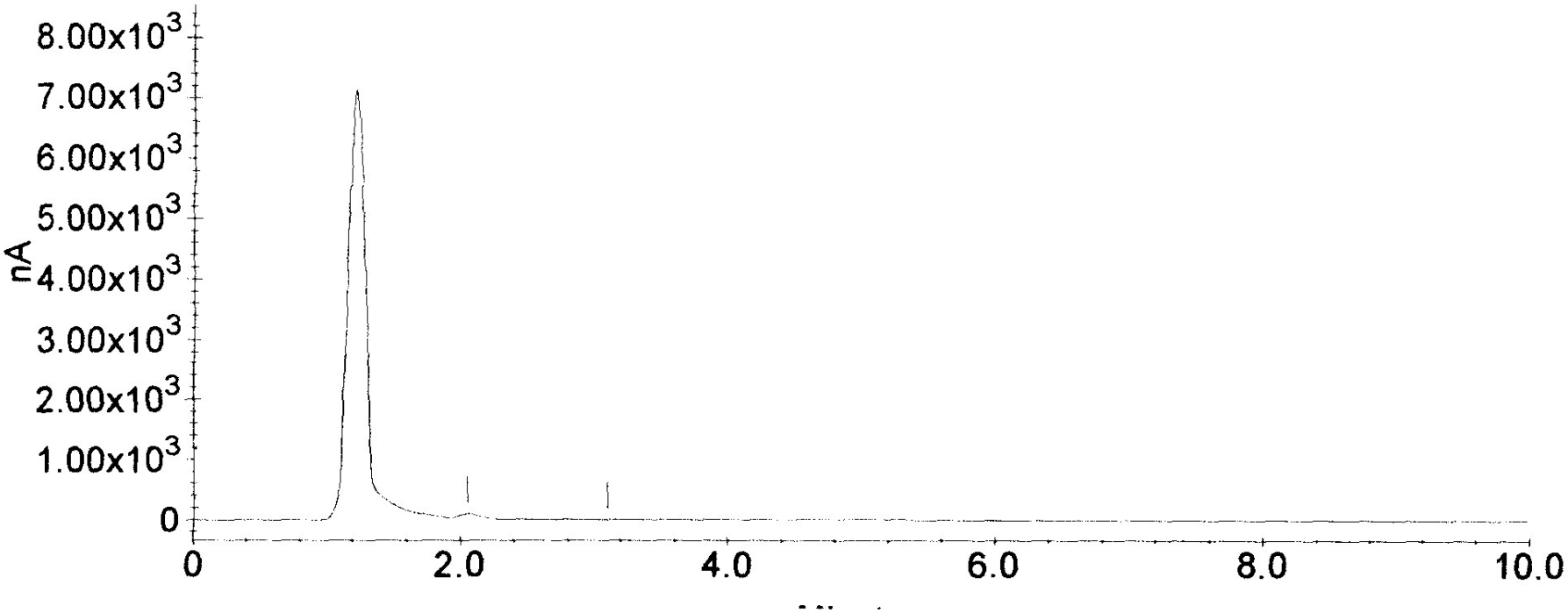

Figure 12b(ii): Chromatograph for Sample D2, Trial 2

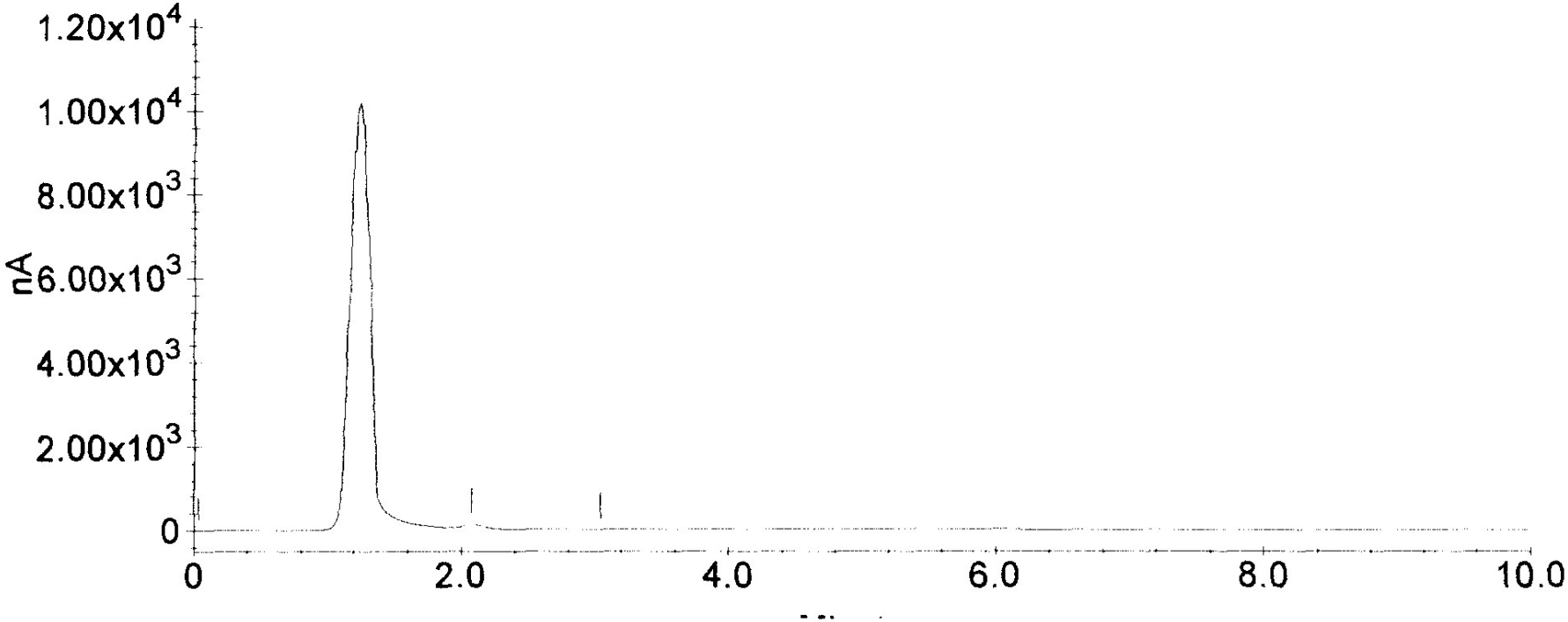

Figure 12c(i): Chromatograph for Sample L3, Trial 1

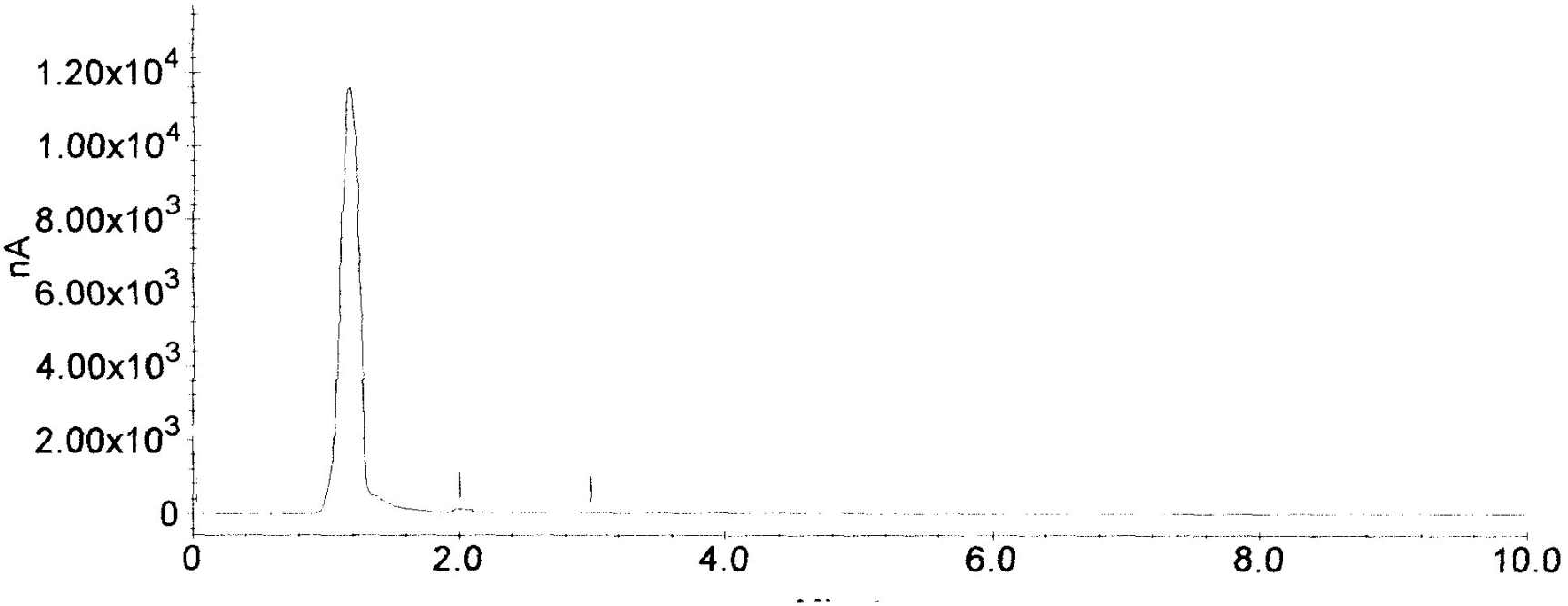

Figure 12c(ii): Chromatograph for Sample L3, Trial 2

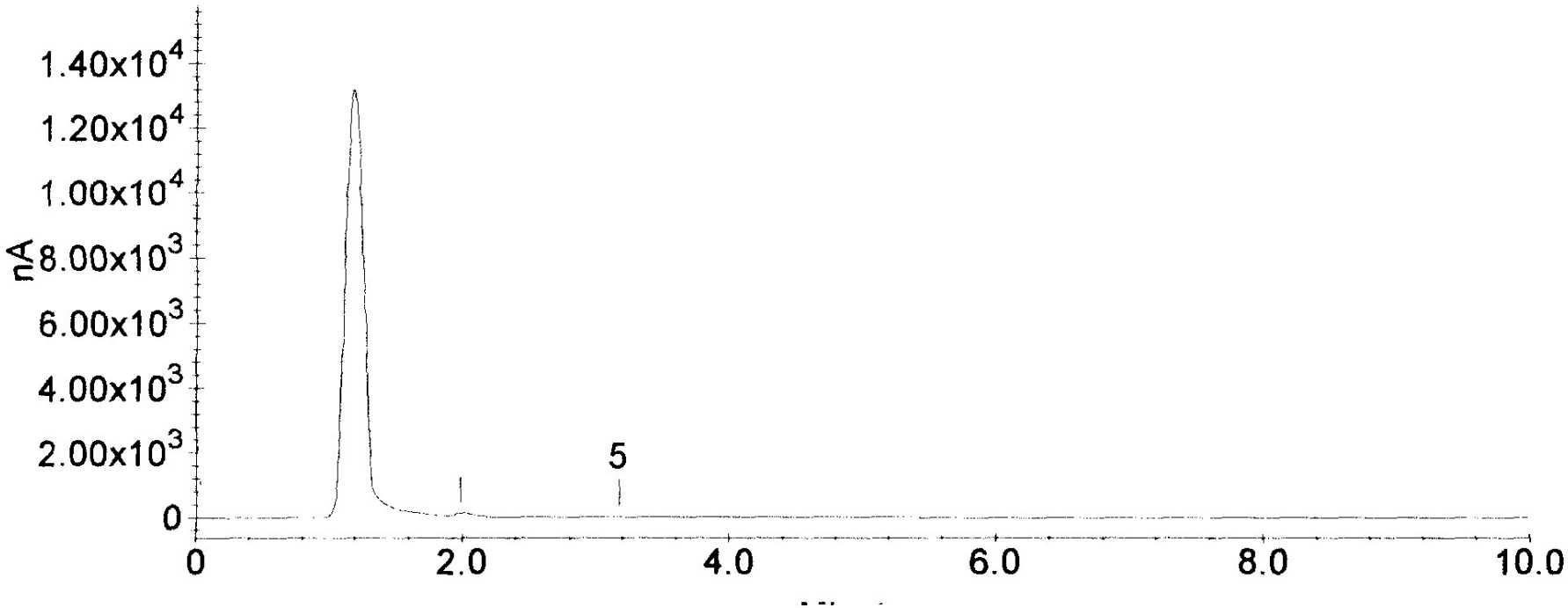

Figure 13a(i): Chromatograph for Sample D1, Trial 1

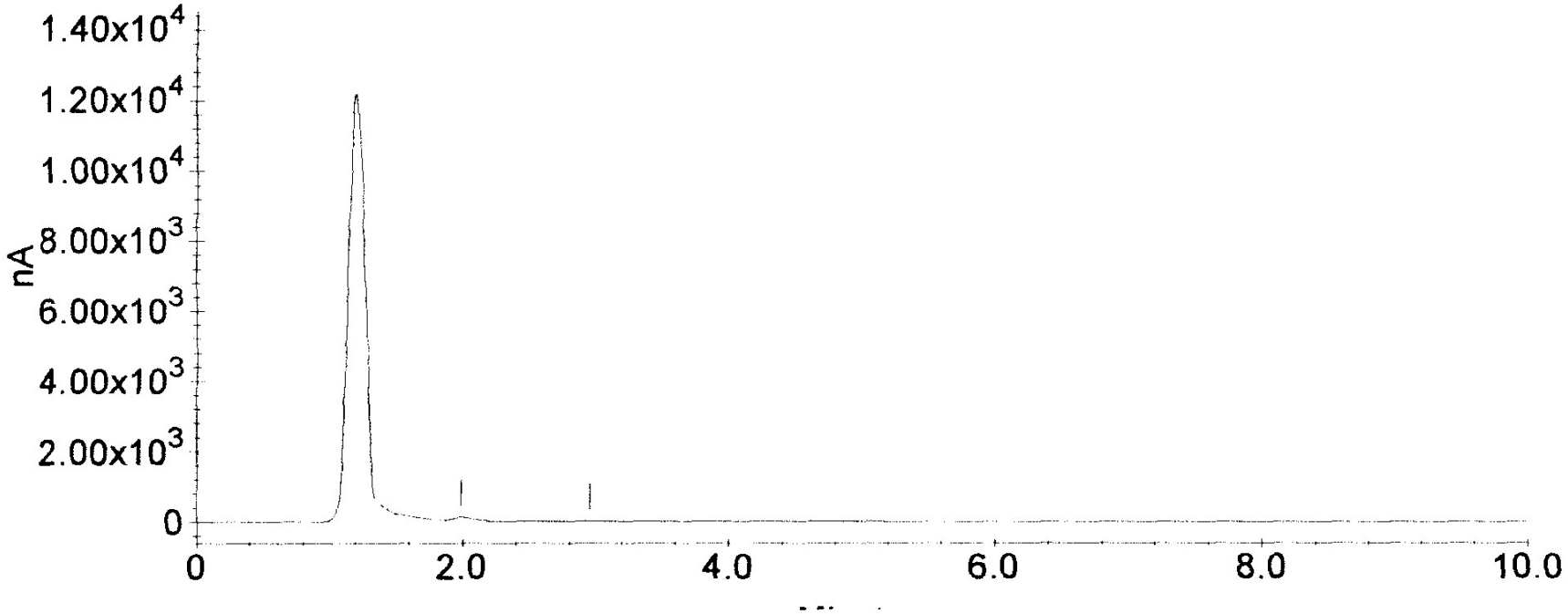

Figure 13a(ii): Chromatograph for Sample D1, Trial 2

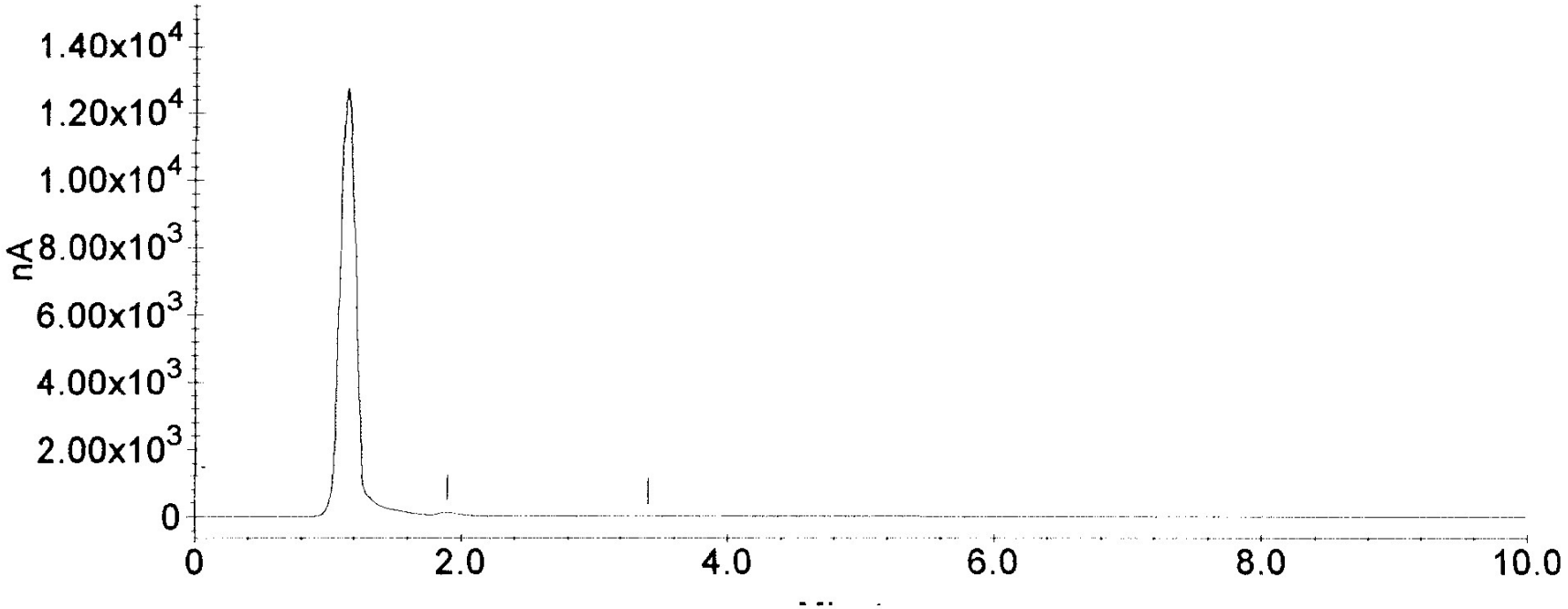

Figure 13b(i): Chromatograph for Sample N2, Trial 1

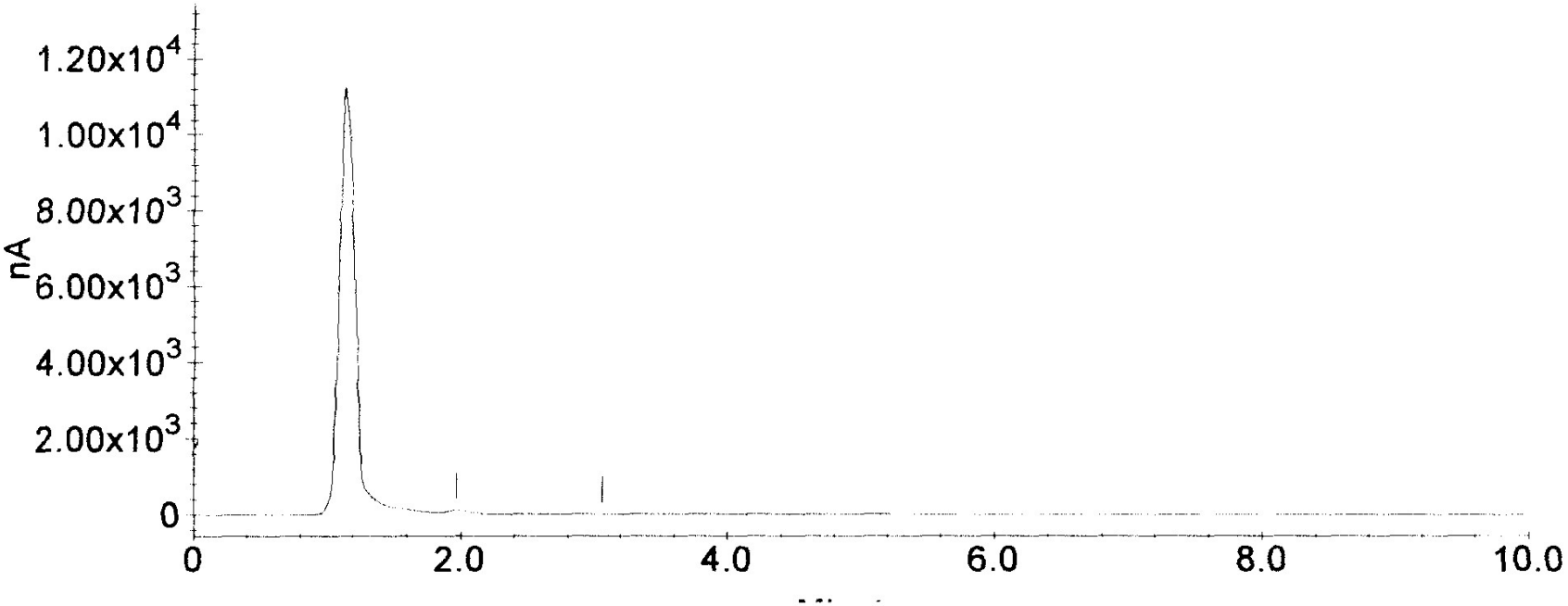

Figure 13b(ii): Chromatograph for Sample D2, Trial 2

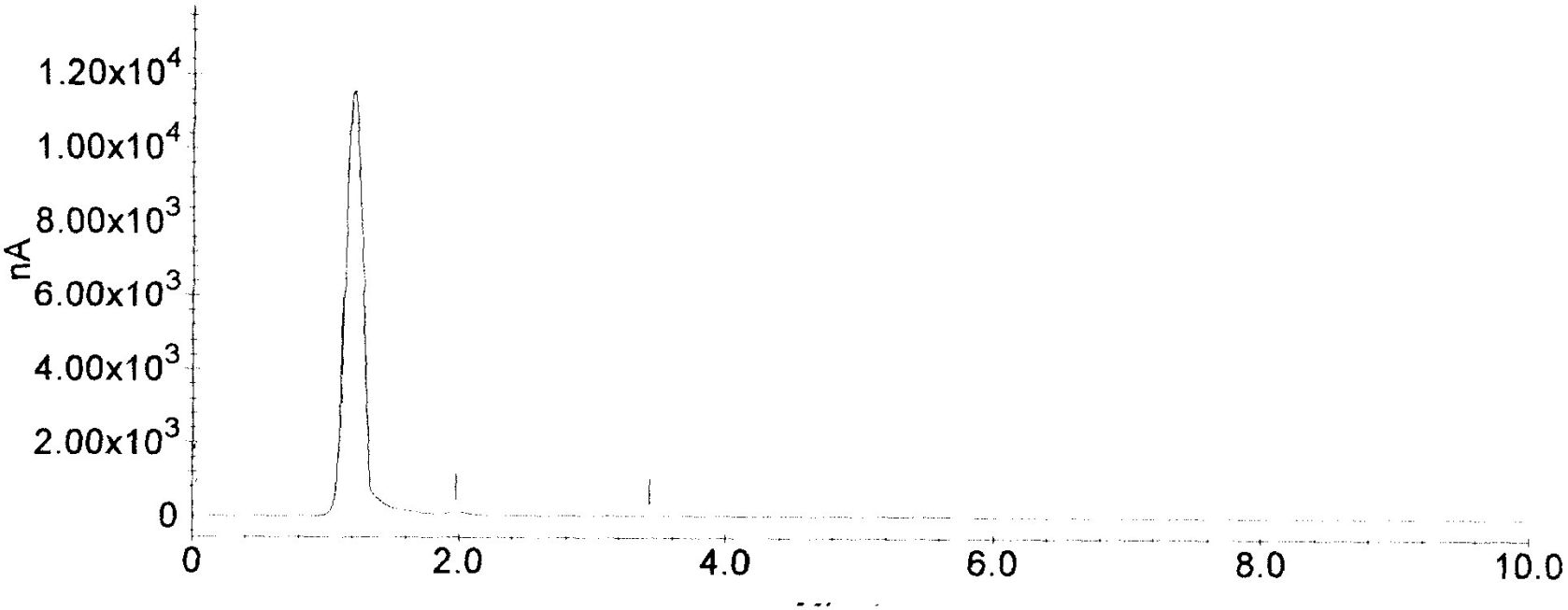

Figure 13c(i): Chromatograph for Sample D3, Trial 1

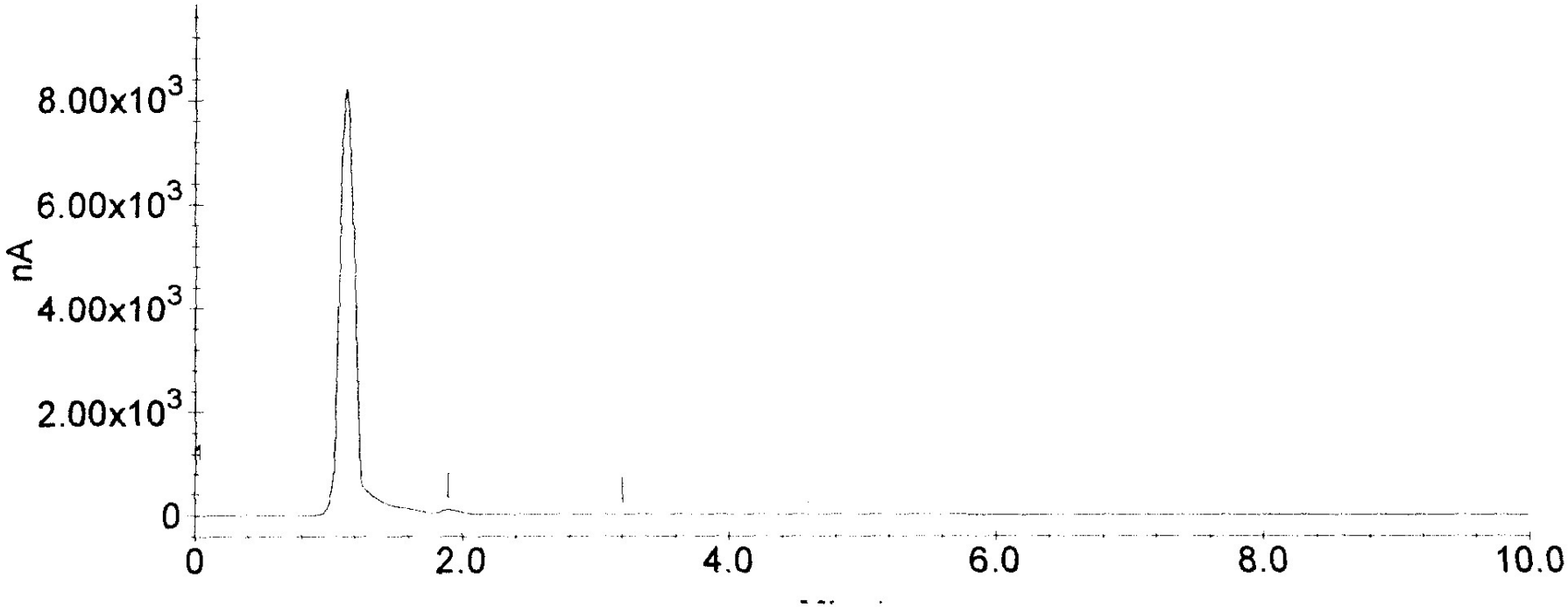

Figure 13c(ii): Chromatograph for Sample D3, Trial 2

3.3 – Qualitative Analysis of Dopamine Content Results

The HPLC analysis of the two sample sets of retinal tissue obtained yielded results indicating that retinal samples obtained 8 hours into the dark cycle had a higher DA concentration than those obtained 8 hours into the light cycle (0.4429158 μg versus 0.3085463 μg, respectively). These results fit into the well-expressed theory that retinal dopamine concentration changes in a cyclical manner, having different concentrations in daytime hours than nighttime hours. Previous studies have concluded that dopamine acts as an intrinsic signal for light; Kolbinger et al noted that endogenous dopamine content was high when measured during the dark phase and low when measured during the light phase (7). The researchers involved in the Kolbinger study referred to a study done by Kramer in which dopamine release was low in dark-adapted chicken retinae and high in light-adapted chicken retinae (13). This finding is in line with what Kolbinger et al found in that when dopamine release is low, it is being retained and HPLC assay would thus display a high DA concentration, and vice versa.

Data currently in the manuscript stage compiled separately by Limowski and Connaughton seemingly disagree with the results obtained in this study. In these studies, data suggested higher DA concentrations during the light hours than in the dark hours. The study by Connaughton goes a step farther by analyzing the retinal concentration of tyrosinase hydroxylase (TOH), the key rate-limiting enzyme in DA production. This data

along with the endogenous DA concentration, the results may show that DA release is low during the dark hours, which would support the finding of higher DA concentration during those hours. Additionally, it may provide an explanation for the fact that TOH levels change in a way that counters the DA concentration. TOH concentrations may be higher during the day because more DA is being produced, but that DA may not actually be released until the cycle reaches the dark hours, which would explain the noted increase in DA concentration during those hours. Moreover, the TOH concentration may be lower during the dark hours because it DA production is not being signaled.

Discrepancies in the various studies may also be a result of uncontrollable experimental error. DA is known to be regularly metabolized into DOPAC. The present study was unable to test for DOPAC concentration due to a lack of sensitivity to this compound. Future studies will need to develop a method that is sensitive to both DA and DOPAC in order to determine the extent to which DA is being metabolized in each sample analyzed. Additionally, due to time constraints, the fish were acclimated to the light cycle for only four days. This is a likely explanation for the seemingly inconsistent values obtained across the three L samples and the three D samples. More accurate results will be obtained in a future study by allowing the fish to acclimate to the cycle for at least one week prior to analysis and obtaining the samples over a period of three days, rather than using three samples obtained on the same day.

The overall accuracy of the results obtained will need to be tested with further analysis by increasing the sensitivity of the HPLC apparatus used.

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