5-2016

An Approach to Collect Non-Standard Microdialysis Compounds

Allison M. Schneider
University of Arkansas, Fayetteville

An Approach to Collect Non-Standard Microdialysis Compounds

An Honors Thesis submitted in partial fulfillment

of the requirements of Honors Studies in

Biochemistry

By

Allison Schneider

Spring 2016

Chemistry / Biochemistry

J. William Fulbright College of Arts and Sciences

The University of Arkansas

Acknowledgements

- Honors College, University of Arkansas
- Stenken lab members for their support and guidance, especially Valerie McKinney and Sarah Phillips
- My committee members, Dr. David Paul, Dr. Ravi Barabote, and Dr. Suresh Kumar, for spending time reviewing my project
- Dr. Julie Stenken for allowing me to work under her for two and a half years

Table of Contents

I. Abstract

Microdialysis is a sampling technique that is used to collect analytes through a semipermeable membrane. The collection occurs via the microdialysis probe where analytes can diffuse through the membrane and be collected by use of the outlet. Microdialysis is typically utilized with solutes that are hydrophilic and have a low molecular weight. It is unusual for hydrophobic solutes to be used, but pentane, a hydrophobic analyte, was studied in this research to see if non-standard microdialysis compounds can be collected. This is important because the pentane resembles hydrophobic signaling molecules in the brain, such as prostaglandins and leukotrienes and can ultimately lead to more information on the regulation of certain hormones in the body. The partition coefficient for pentane in safflower oil/air was calculated to determine if pentane could be successfully used in microdialysis. At the conclusion of this project, pentane was able to be collected and therefore non-standard microdialysis compounds can be collected.

The focus of this research switched from gas phase to solid phase chemistry to further broaden the study. Microdialysis was first completed with a 100 μM stock solution of imipramine hydrochloride. Microdialysis was completed again with the addition of C-18 Silica Magnetic Beads to increase the relative recovery of the analyte. The concentration of the analyte did decrease as expected because the beads adsorbed a portion of the analyte.

II. Introduction

A. Microdialysis Overview

Microdialysis sampling involves the collection of analytes through a semipermeable membrane. This method allows for continuous sampling from interstitial fluid of various tissues with minimum impact on surrounding tissues and the body as a whole. Microdialysis is able to collect drugs or endogenous compounds in a large amount of human organs and tissues.[6] These compounds are measured best in the tissue versus the bloodstream because biochemical events and effects from various drugs mainly happen in the tissue.[6] To collect solutes, the probe is inserted into a tissue and then the solutes diffuse through a semipermeable membrane in the tip of the probe; solutes can be sampled via the fluid that is passed at µL/min flow rates, as shown in Figure 1.[5] The probe is able to deliver and collect solutes or drugs simultaneously, which makes it unique versus other drug delivery devices.[9]

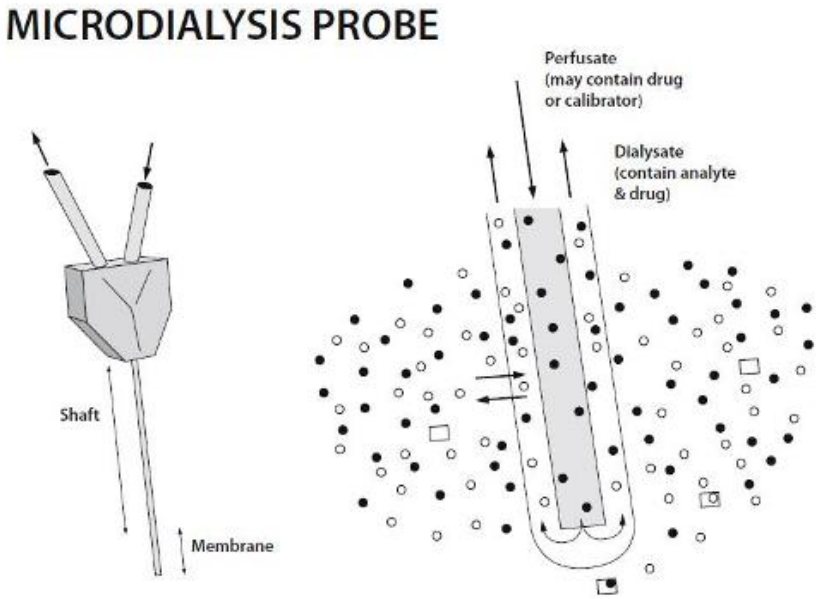

Figure 1. The probe with its inlet and outlet. "Microdialysis." Wikipedia. Web. 9 Apr. 2015

Microdialysis sampling has uses in many clinical applications.[6] The applications include uses in neurointensive care, reconstructive surgery, liver transplants, and abdominal surgery care.[6] It has also been used to analyze chemical

events during a foreign body reaction, which can occur when sensors or essentially anything is implanted into tissue or a biological system.[12] For example, sensors can be implanted to measure the glucose level in persons with diabetes.[9] This method is also a good sampling technique because it allows signaling molecules/neurotransmitters to be collected while the animal is alert and behaving normally.[10] This is important in order to relate the changes in concentrations of signaling molecules to apparent changes in behavior.[10] Microdialysis has the potential to assess brain stimulation for people battling with neurological diseases and could lead various treatments.[1] Microdialysis measurements in tissues are commonly used because it is noninvasive due to its small size, ease of use, and low complication rate.[6]

B. Microdialysis Background

The microdialysis probe has a semipermeable membrane that allows for substances to be delivered or collected through passive diffusion[1] across a concentration gradient.[3] Probes can have various molecular weight cutoffs (MWCO), meaning the probe can only work with molecules that do not surpass a certain weight. The probe used in this research had a 100 kDa MWCO. The MWCO is a maximum value and typically about ten percent of the MWCO can be sampled reliably.[12] The probe membrane is perfused with a fluid, which equilibrates with the fluid of the tissue outside the membrane[1] and is run at a particular flow rate, 1.0-2.0 μL/minute.[10] The analyte then travels from an area of high concentration to an area of low concentration; the high concentration of analyte is in the sample vial and goes to a lower concentration in the probe, which is then delivered to the collection vial. The microdialysis probe is connected to an inlet that links the probe to the gas tight syringe with the perfusion fluid

in it and to the outlet, which connects the sample vial to the collection vial. Depending on the experiment, air or high performance liquid chromatography (HPLC) water was utilized for the perfusion fluid.

C. Pentane and Safflower Oil Experiment

Microdialysis has been widely used for the collection of low molecular weight, hydrophilic solutes, but inconsistent results have been observed for low molecular weight, hydrophobic solutes, such as pentane.[14] Hydrophobic compounds are difficult to use because of poor solubility and/or binding to the microdialysis device.[5] The hydrophobic compounds resemble some of the signaling molecules in our body such as prostaglandins and leukotrienes. If it were possible to collect these types of molecules with microdialysis, then it would be a step forward in the regulation of certain types of hormones in the body.

i. Partition Coefficient

Microdialysis sampling is important in determining the partition coefficient of various compounds like pentane. A partition coefficient is the concentration ratio of a compound in two different phases in equilibrium, such as air and safflower oil

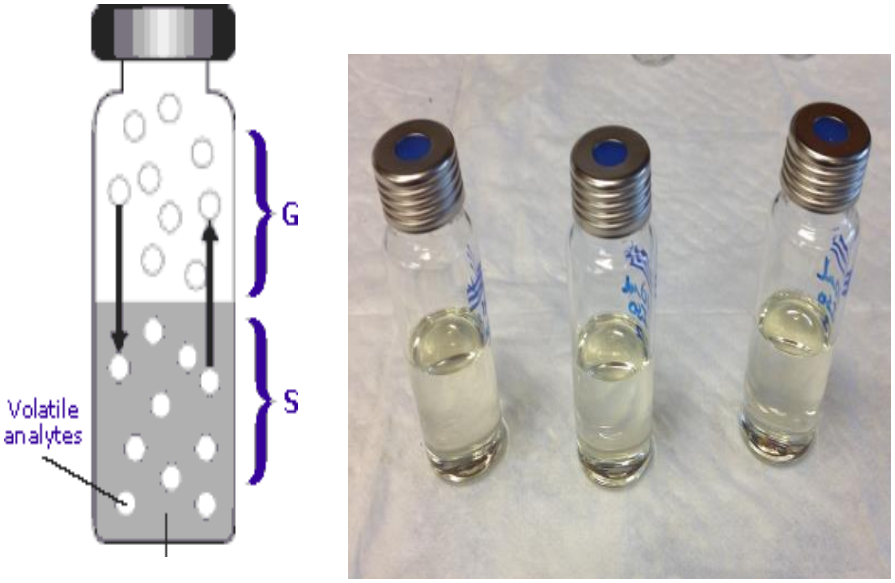

Figure 2. The partitioning of two phases. "A technique guide for static headspace analysis using GC." Web. 9 Apr. 2015.

that were chosen for this experiment. The partitioning is shown in Figure 2. Safflower oil was chosen because it was successful with dodecafluoropentane, another volatile compound.[8] The partition coefficient is necessary to know because it mirrors the partition that will happen to the analyte in the brain.[8] Microdialysis is the best method to use in calculating the partition coefficient because it has been widely used for extracellular measurements in vivo.[3] The partition coefficient can be found using Equation 1.[8]

$$K = \frac{[C]_2}{[C]_1} \quad (1)$$

K is the partition coefficient, $[C]_2$ is the concentration of the analyte in phase 2, and $[C]_1$ is the concentration of the analyte in phase 1. Pentane is a volatile compound, meaning it is easily evaporated at normal temperatures; therefore, headspace gas chromatography using a flame ionization detector (GC-FID) was chosen to measure the concentrations of the solutes in the collection vials. Pentane has a boiling point of 97 ºF.[11] Headspace sampling is done using a gas-tight syringe to extract the vapors above a liquid or solid; in this experiment, it is the extraction of the vapors above the safflower oil.[13] This method is common for the analysis of volatile compounds and is also used in forensic analysis.[13] Equation 2 shows how to find the concentration of pentane in the gaseous phase.

$$C_g = \frac{A-Y}{M} \quad (2)$$

C_g is the concentration of pentane in the gaseous phase, A is the average peak area, Y is the y-intercept from the calibration curve, and M is the slope from the calibration curve. Equation 1 can then be rewritten as Equation 3 below to find the concentration of pentane in the sample phase.

$$K \times C_g = C_s \quad (3)$$

K is the partition coefficient, C_g is the concentration of pentane in the gaseous phase, and C_s is the concentration of pentane in the sample phase.

ii. Gas Chromatography

Gas chromatography uses two different phases, mobile and stationary, to separate compounds. The separation is a result of the compounds partitioning between these two phases.[4] The sample being analyzed needs to be volatile; if not, pyrolysis GC or derivatization techniques can be used.[4] GC has a variety of detector options, but the flame ionization detector (FID) was used for this research. FID is the most commonly used detector because it responds to essentially all organic compounds.[2] Once the components of the compound reach the flame, charged species are created and collected at an electrode; a current is then produced that is proportional to the amount of carbons in the flame.[2] The time it takes for the compound to elute from the column is its retention time. The retention time is stated on the chromatogram, which is created automatically after each sample is run. Figure 3 shows the different parts of the GC.

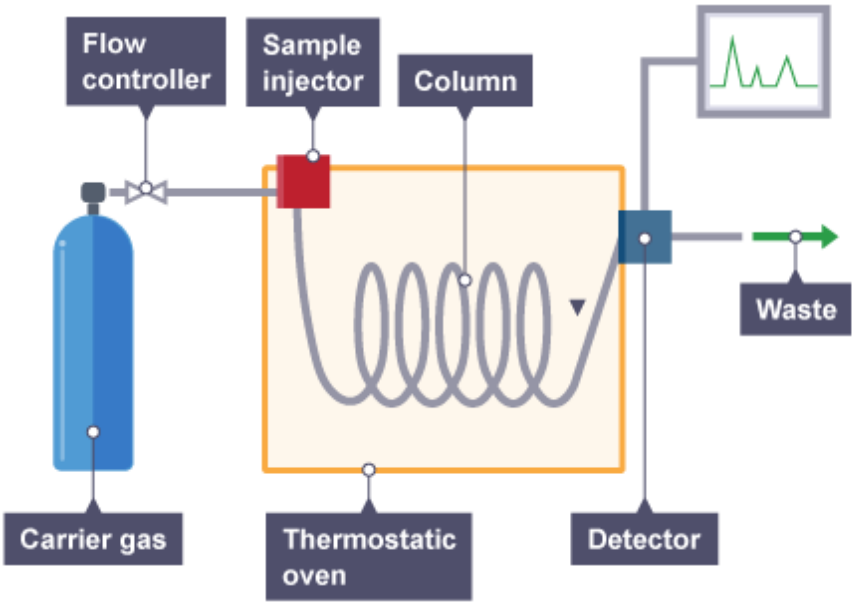

Figure 3. The gas chromatography instrument. "Gas

D. Imipramine Hydrochloride Experiment

The experiments with gas phase types of chemistry led to an interest in using a solid phase with microdialysis; therefore the switch from a volatile substance, pentane, to a solid substance, imipramine hydrochloride, was completed.

i. C-18 Magnetic Beads

The 1µm C-18 magnetic beads were combined with imipramine hydrochloride (IHCl) to see if the relative recovery (RR) of IHCl would increase during microdialysis. The RR can be calculated using Equation 3.[10]

$$RR = \frac{C_{outlet}}{C_{sample}} \quad (3)$$

C_{outlet} is the concentration of the analyte collected through microdialysis and C_{sample} is the concentration of the analyte outside the probe. By adding affinity agents (AAs), which in this case are the C-18 magnetic beads to the analyte that the probe is in, the RR can be increased.[10] The AAs increase the concentration gradient across the probe and push the mass transport to allow more analyte to diffuse into the probe.[10] AAs technically act like solid phase extraction devices. Small magnetic beads have a great binding capacity and reduce the rate of settling when compared to other AAs.[10] AAs have also been used to increase the recovery of neuropeptides.[7]

ii. Nanodrop Spectrophotometer

Every sample collected was analyzed using the Nanodrop instrument, which can be seen in Figure 4. To measure the analyte, this instrument only needs 2 µL of analyte, which is ideal because of the low amount of sample volume collected in microdialysis. A calibration curve was made from measurements on the Nanodrop using a 100 µM

IHCl stock solution. The analytes' absorbances determined on the Nanodrop were used to find the concentration of the analyte after microdialysis.

Figure 4. The Nanodrop Spectrophotometer.

III. Materials and Methods

A. Chemicals and Instruments

Pentane and imipramine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). 100% Pure LouAna Safflower Oil was purchased from Walmart. Microdialysis probes (CMA 20 Polyethersulfone membrane) that are 10 mm in length and 0.5 mm in diameter with a 100 kDa MWCO were purchased from Harvard Apparatus (Holliston, MA). The syringe pump and 1,000 µL gastight syringe were purchased from BASi (West Lafayette, IN). 250 mg of the 1 µm BcMag™ C-18 modified magnetic beads were purchased from Bioclone Inc. (San Diego, CA). The Nanodrop 2000 Spectrophotometer was purchased from Thermo Fischer Scientific Inc. (Waltham, MA). HPLC water was purchased from Fischer Chemical (Fair Lawn, NJ). The trifluoroacetic acid and acetonitrile was purchased from EMD Chemicals (Gibbstown, NJ). The VWR analog vortex mixer was purchased from Henry Troemner LLC (Thorofare, NJ). The Reichert Bright-Line hemacytometer was purchased from Hausser Scientific (Horsham, PA). A Shimadzu 2014 Gas Chromatograph was used with a Varian Factor Four VF-5ms capillary column with 5% phenylmethylpolysiloxane (Kyoto, Japan). Clear headspace vials (20.0 mL) and PTFE blue silicone screw caps were purchased from Thermo Fisher Scientific (Waltham, MA).

B. Pentane and Safflower Oil Experiment

i. Partition Coefficient

A calibration curve was made first by using 10 µL and 20 µL gastight syringes to inject 0.5 µL, 1.0 µL, 2.5 µL, 5.0 µL, and 10.0 µL of pentane into individual 20 mL headspace vials, which are $2.2 * 10^{-4}$ M, $4.3 * 10^{-4}$ M, $1.1 * 10^{-3}$ M, $2.2 * 10^{-3}$ M, and

3.0 *10^{-3} M concentrations respectively. Each concentration was made in triplicate samples, along with every sample analyzed throughout this research.

To see the partitioning effects of pentane, pentane was injected into safflower oil to determine the ratio of pentane in two phases, the air and the oil. Ten mL of safflower oil was measured into six headspace vials using a pipette and the caps were tightly secured. Three vials were injected with 10 µL of pentane using the 20 µL syringe and the other three vials were injected with 20 µL of pentane with a 50 µL syringe, which are 8.33 * 10^{-3} M and 1.67 * 10^{-2} M concentrations of pentane in oil respectively. The syringe must touch the oil when the pentane is injected. The solutions must sit for an hour to ensure full equilibrium. During this hour, the solutions need to be mixed via a vortex at the beginning, 30 minutes later, and at end of the hour.

Then 1.0 µL of the headspace of each sample for the calibration curve was injected into the gas chromatograph and detected by a flame ionization detector. In this experiment, the GC was comprised of a helium mobile phase and a flow rate of 1 µL per minute. The peak area was recorded and the data from the peak area and the calibration curve was applied to the calculation of the concentration of pentane and then the partition coefficient. If needed, the headspace vials may be reused for the calibration curve, but the amount of injections needs to be limited to a maximum of seven for the caps.

ii. Microdialysis

The syringe and microdialysis pump are assembled, as shown in Figure 5, with the blue inlet connecting the syringe and sample vial and the white outlet connecting the sample vial to the collection vial. The syringe is filled with air, which is this

experiment's perfusion fluid. Volumes of 10 µL and 20 µL of pentane were injected into sample vials and each sample was made in triplicate. The microdialysis probe,

Figure 5. Microdialysis set-up

which is connected to the inlet and outlet, was placed in the sample vial via the introducer. The flow rate was set at 1.0 µL per minute and the collection lasted for 40 minutes. The collection vials' headspaces were measured in the GC to determine peak area. The measured peak area and the calibration curve in part *Bi* was used to determine concentration of pentane after microdialysis. This concentration was compared to the concentration determined without using microdialysis.

C. Imipramine Hydrochloride Experiment

i. Microdialysis

A 100 µM stock solution of imipramine hydrochloride and HPLC water was created. A calibration curve was created using 100 µM, 80 µM, 60 µM, 40 µM, 20 µM, and a blank of just HPLC water. Samples (2 µL) of these solutions were placed on the Nanodrop to be analyzed in order to measure the solutions' absorbance at a wavelength of 210 nm. The stock solution was placed in the sample vial with the probe and the flow rate was set to 1 µL per minute for 20 minutes. HPLC water was used for the perfusion

fluid for this experiment. After microdialysis was performed, samples of 2 μL of the collection vial were analyzed using the Nanodrop. Microdialysis is run two more times for 20 minutes and then analyzed again. The average concentration of the stock solution is calculated using the calibration curve made from the standards.

ii. Microdialysis and C-18 Silica Magnetic Beads

The silica beads were prepared using the instructions included with the purchase of the beads. The washed beads were then mixed with 1/3 volume of the sample-binding buffer. A sample binding buffer and an equilibrium buffer were made with trifluoroacetic acid (TFA) and acetonitrile (ACN) as stated in the instructions. The sample binding buffer was 2% TFA in 5% ACN and the equilibrium buffer was 0.5% TFA in 5% ACN. Then, 1 μL of the bead solution was diluted with 100 μL of the binding buffer. 10 μL of the diluted solution was analyzed using a hemocytometer to determine the amount of beads per μL. The results indicated that there are approximately 3,460 beads per μL.

The diluted solution was placed in the sample vial and microdialysis was run with HPLC water as the perfusion fluid. The flow rate was set to 1 μL per minute and run for 20 minutes three times. Once microdialysis was complete, samples of the collection vial were analyzed using the Nanodrop to measure the absorbance. The concentration of the stock solution is calculated using the calibration curve and then compared to the concentration that was given without using the beads to determine which has a better relative recovery rate of imipramine hydrochloride.

IV. Results/Discussion

A. Pentane and Safflower Oil Experiment

i. Partition Coefficient

In Figure 6, the GC-FID chromatogram shows pentane with a retention time of 2.65 minutes with an end run time of 4.0 minutes. The partition coefficient of pentane in air and oil was consistently found to be between 24 and 27. This was found using the 10

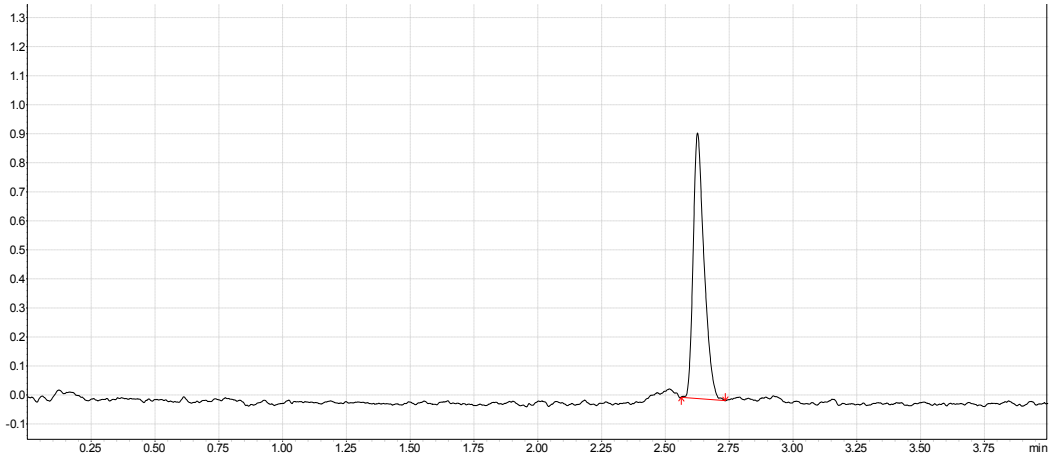

Figure 6. The chromatogram of pentane.

µL and 20 µL samples of pentane in safflower oil and the calibration curve shown in Figure 7. As mentioned earlier, safflower oil was chosen because it was successful with dodecafluoropentane, another volatile compound.[8] The total concentration of the 10 µL sample was 8.6 $*10^{-3}$ M and 1.7 * 10^{-2} M for the 20 µL sample. The 10 µL sample had an average peak area of 8069.33, a standard deviation of 384.66, and a percent relative standard deviation (RSD) of 5%.

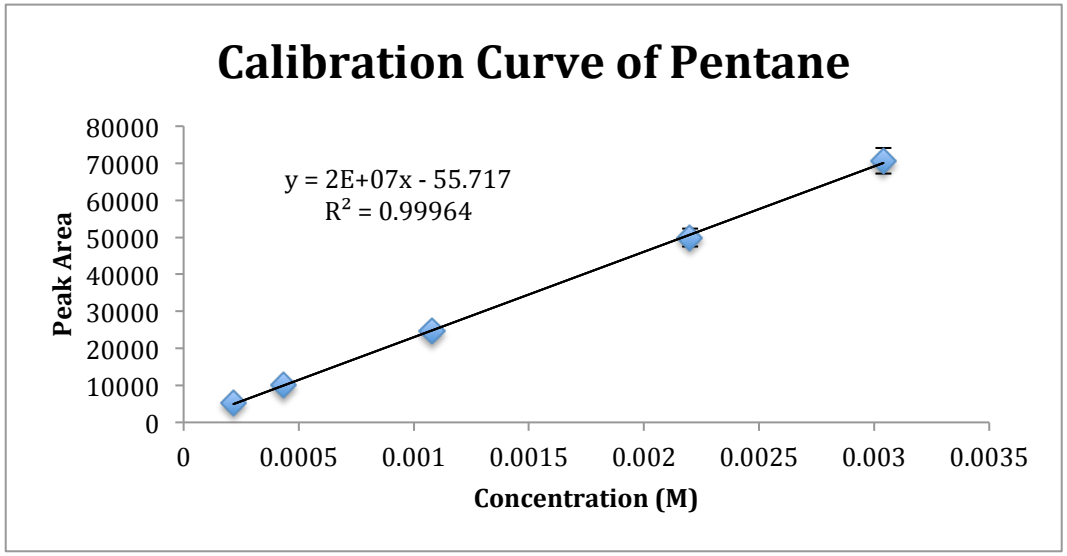

Figure 7. The calibration curve of pentane.

The 20 µL sample had an average peak area of 14454.67, a standard deviation of 783.66, and a percent RSD of 5%. The concentration of pentane in air for the 10 µL and 20 µL sample were 3.52 * 10^{-4} M and 6.20 * 10^{-4} M and the concentration of pentane in oil was 8.33 * 10^{-3} M and 1.67 * 10^{-2} M, respectively. These results mean that pentane partitions more into the safflower oil versus air and it is a great first step in determining if other hydrophobic solutes can be regulated by this technique.

ii. Microdialysis

Pentane was able to be sampled using microdialysis, but the concentration was much lower in the collection vial than the sample vial. Although it is normal for the concentration to get lower, the 10 µL sample of pentane and oil gave an average peak area of 2725.3, which correlates to a concentration of 1.39 * 10^{-4} M, which is lower than the concentration of this sample without microdialysis was 8.6 * 10^{-3} M. The standard deviation for the 10 µL sample was 328.05. The 20 µL sample gave an average peak area of 5053, which correlates to a concentration of 2.55 * 10^{-4} M, which is lower than the concentration of this sample without microdialysis, 1.7 * 10^{-2} M. The standard deviation for the 20 µL sample was 385.3. It was found that pentane can be collected

with microdialysis, but further research needs to occur to get results that allow for larger concentrations of this compound to be collected. Solutions for this problem could be trying different perfusion fluids or different types of microdialysis probes.

B. Imipramine Hydrochloride Experiment

i. Microdialysis

Figure 8 shows the calibration curve that resulted from 20 µM, 40 µM, 60 µM, 80 µM, and 100 µM solution of imipramine hydrochloride using a 100 µM stock solution; the average absorbance for each concentration analyzed by the Nanodrop Spectrophotometer was 0.031 AU, 0.070 AU, 0.115 AU, 0.157 AU, and 0.200 AU.

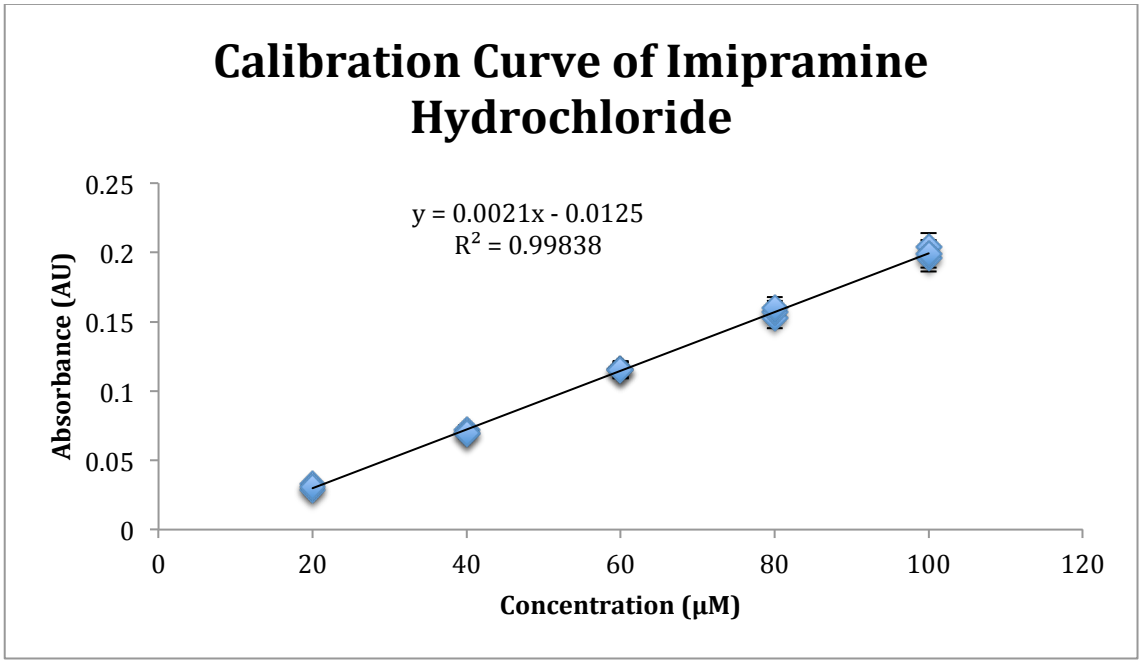

Figure 8. The calibration curve of imipramine hydrochloride.

The average absorbances for the three runs of imipramine hydrochloride with microdialysis were 0.091 AU, 0.106 AU, and 0.102 AU; the concentrations for these three runs were 49.29 µM, 56.43 µM, and 54.52 µM. The RRs for these runs were

calculated using Equation 3 and were 49.29%, 56.43%, and 54.52% because it was based off a 100 μM stock solution.

ii. Microdialysis and C-18 Silica Magnetic Beads

The RR of the samples with the magnetic beads should numerically be lower than the RR without the beads because the beads are absorbing part of the drug during microdialysis. The average concentrations of imipramine hydrochloride for the three trials ran were 50.50 μM, 51.86 μM, and 49.20 μM. These numerical values are also the values of the RRs, 50.50%, 51.86%, and 49.20%. The magnetic beads did absorb a portion of the drug, but it should absorb more than it did; without beads the RR averaged at 53.41% and with beads the RR averaged at 50.52%. The very similar values between the IHCl with and without beads could be due to the drug getting attached to the probe during microdialysis. The beads may also work better with different analytes. More experiments need to be executed to determine the best way to get the beads to work properly.

V. Conclusion

Microdialysis can be used with non-standard compounds. Hydrophobic analytes with low molecular weight, such as pentane can be sampled once a partition coefficient is created and reproducible. Further research on this topic needs to be completed to ensure accuracy and effectualness on live subjects. Silica magnetic beads showed that it could increase relative recovery in microdialysis of imipramine hydrochloride, but still needs further research to determine if it can increase even more. The slight increase in the RR gives potential for the use of the magnetic beads, but the difference needs to become more significant.

VI. References

1. Azeredo, Francine, Teresa Dalla Costa, and Hartmut Derendorf. "Role of Microdialysis in Pharmacokinetics and Pharmacodynamics: Current Status and Future Directions." *Clinical pharmacokinetics* 53.3 (2014): 205-12. Print.

2. Colón, Luis A., and Lisa J. Baird. "Detectors in Modern Gas Chromatography." *Modern Practice of Gas Chromatography*.John Wiley & Sons, Inc., 2004; 2004. 275-337. Print.

3. Darvesh, A. S., et al. "In Vivo Brain Microdialysis: Advances in Neuropsychopharmacology and Drug Discovery." *Expert opinion on drug discovery* 6.2 (2011): 109-27. Print.

4. Grob, Robert L. "Introduction." *Modern Practice of Gas Chromatography*.John Wiley & Sons, Inc., 2004; 2004. 1-21. Print.

5. Jensen, S. M., et al. "In Vivo and in Vitro Microdialysis Sampling of Free Fatty Acids." *Journal of pharmaceutical and biomedical analysis* 43.5 (2007): 1751-6. Print.

6. Khan, F., et al. "Effect of Perfusion Fluids on Recovery of Inflammatory Mediators in Microdialysis." *Scandinavian Journal of Immunology* 82.5 (2015): 467-75. Print.

7. Liang, Z., C. M. Schmerberg, and L. Li. "Mass Spectrometric Measurement of Neuropeptide Secretion in the Crab, Cancer Borealis, by in Vivo Microdialysis." *The Analyst* 140.11 (2015): 3803-13. Print.

8. McKinney, Valerie S. "In Vitro Microdialysis Sampling Collection of Volatile Organic Compounds (VOC's), Dodecafluoropentane (DDFP) and Isoflurane." M.S. University of Arkansas, 2015. Print.United States -- Arkansas:.

9. Mou, X., et al. "Modulation of the Foreign Body Reaction for Implants in the Subcutaneous Space: Microdialysis Probes as Localized Drug delivery/sampling Devices." *Journal of diabetes science and technology* 5.3 (2011): 619-31. Print.

10. Schmerberg, Claire M., and Lingjun Li. "Mass Spectrometric Detection of Neuropeptides using Affinity-Enhanced Microdialysis with Antibody-Coated Magnetic Nanoparticles." *Analytical Chemistry* 85.2 (2013): 915-22. Print.

11. National Center for Biotechnology Information. PubChem Compound Database; CID=8003, https://pubchem.ncbi.nlm.nih.gov/compound/8003 (accessed Apr. 9, 2016).

12. Sides, Cynthia R., and Julie A. Stenken. "Microdialysis Sampling Techniques Applied to Studies of the Foreign Body Reaction." *European Journal of Pharmaceutical Sciences* 57 (2014): 74-86. Print.

13. Snow, Nicholas H., and Gregory C. Slack. "Head-Space Analysis in Modern Gas Chromatography." *Trends in Analytical Chemistry: TRAC* 21.9 (2002): 608. Print.

14. Stenken, J. A., et al. "How Minimally Invasive is Microdialysis Sampling? A Cautionary Note for Cytokine Collection in Human Skin and Other Clinical Studies." *The AAPS journal* 12.1 (2010): 73-8. Print.