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Cytochrome P-450 Enzymes Contributing to Demethylation of Antidepressant Drugs in Vitro

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Identification of Drug Metabolites of Antidepressant Medications, Venlafaxine, Escitalopram, and Duloxetine in Rat Liver Microsome Using Compact Mass Spectrometry

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ABSTRACT

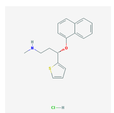
Antidepressants are commonly used for depression and anxiety. It is known that most drugs are metabolized by enzymes within the liver. The enzymes metabolize drugs by altering them to become more polar so that they can be excreted through the urine or bile. As a future pharmacist, I want to know how antidepressants are altered within the body before I distribute them. This study was done to determine the outcome of metabolism within three different antidepressants: venlafaxine (Effexor), duloxetine (Cymbalta) and escitalopram (Lexapro). These three drugs were mixed with rat liver microsomes and NADPH-generating system in phosphate buffer at pH 7.4. All samples were incubated for two hours at 37C. Each sample was subjected to the compact mass spectrometer fitted with a C18 reverse phase column. The results indicated that all three drugs underwent N-demethylation but not aromatic hydroxylation during metabolism. When looking at the area of the peaks within each metabolized drug, duloxetine metabolized the most out of all three drugs. Up to 30-70% of duloxetine was metabolized. Several controls were also incubated and analyzed, such as the mixture without the drug, the mixture without NADPH-generating system, the mixture without the rat liver microsome and a mixture of just the drug and buffer. Some of the controls without the drug or the microsome still showed MS peaks with the same molecular weight as the drugs or the metabolites. These peaks could indicate that there are contaminants within the solvent or the phosphate buffer that have similar molecular weights. The future goal is to repeat these experiments using HPLC/CMS to further separate the metabolites. Using different drugs would also explain the outcome of metabolism a little further, such as if a drug can be both demethylated and hydroxylated.

INTRODUCTION

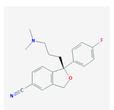
Depression is a serious mental illness that many college students experience. Between deadlines and relationships, college can be a very challenging time, making students feel very dispirited. It is hard to return from that feeling of anguish, so many students turn to antidepressant medications to feel better again. Duloxetine (Cymbalta), escitalopram (Lexapro) and venlafaxine (Effexor) are all antidepressant medications that inhibit serotonin-norepinephrine reuptake within the brain (Lam et al.). There are multiple dosages that can be prescribed, all based on severity of the depression. A blood cell count, test on hormone levels and a metabolic panel of the liver is also taken to decide the dosage for a patient. Cytochrome P450 (CYP) are enzymes of the liver that metabolize things that a person may ingest, including antidepressant medications. There are multiple different enzymes within the liver, all working together, but completing different steps in the metabolism process. CYP1A2, CYP2C19 and CYP2D6 are three of the multiple enzymes used within the liver. It isn't fully known how much of each enzyme is used within the liver, but it is known that each would be poor metabolizers if they had to work alone. Their greatest substrates are antidepressant drugs, including venlafaxine, escitalopram and duloxetine. As a college student that is surrounded by so many students in a low-mood, I feel it is necessary to determine how these enzymes in the liver metabolize the antidepressant medications given and to know that the reaction does not result in toxins being produced in the body. Also, as a pharmacy technician, I dispense antidepressants almost daily. Therefore, I'm very interested in knowing the details of how the liver metabolizes these medications.

Venlafaxine is a medication specifically to treat symptoms of low-mood, or depression, whereas duloxetine and escitalopram are to treat symptoms of low-mood and anxiety. However, duloxetine is also used to treat long-term pain from nerve diseases and diabetic nerve problems, as well as fibromyalgia. It has been discovered that the substrates for CYP2D6 are both venlafaxine and duloxetine, with escitalopram and sertraline (another similar antidepressant medication) as smaller substrates (Brachtendorf, et al.). CYP1A2 has been discovered to specifically catalyze in the presence of escitalopram and duloxetine, as well as somewhat in the presence of venlafaxine (Lobo, et al.). CYP2C19 is known for having the most involvement in metabolizing antidepressant medications due to having three main substrates and two other smaller substrates. The three main substrates are escitalopram, duloxetine and amitriptyline (another similar antidepressant medication) and the three partial substrates are venlafaxine and sertraline (Zhan, et al.).

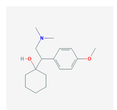
My proposal is to investigate how rat liver microsomes, which contain many CYP enzymes, react with each of the three medications, venlafaxine, escitalopram and duloxetine. The metabolism of each antidepressant medication highly depends on whether or not the cytochrome catalyzes a reaction, making the drug soluble. But what is this reaction? And are these man-made medications safe for our bodies after these reactions occur? By developing an assay to perform to help further the reaction and determine its affinity, I will find which of these drug metabolizes the most efficiently. By viewing the breakdown of the drugs with and without microsomes, I will determine how each drug is metabolized within the body.



Duloxetine



Escitalopram



Venlafaxine

MATERIALS AND METHODS

Venlafaxine, escitalopram, duloxetine, NADP, Glucose-6-Phosphate, and Glucose-6-Phosphate Dehydrogenase and rat liver microsome were purchased from Sigma Aldrich. Luna C18 reverse phase column (3 micrometer, 50 mm x 2 mm) was purchased from Phenomenex. Each drug's samples were metabolized and evaluated using an Advion Expression S Compact Mass Spectrometer equipped with an electrospray ionizer (ESI) and a Phenomenex Luna C18 column. The running time was 15 minutes per sample with a mobile phase of 95:5 acetonitrile:water and 2 mM ammonium acetate with a 0.2 mL/min flow rate. The injection volume was 5 μL. Three different concentrations of each drug were used along with four different controls. The drug concentrations were 100, 250, and 800 μM. For each drug, 65 μL of these concentrations along with a mixture of 35 μL NADPH-generating system and 25 μL microsomal suspension in 50 mM potassium phosphate buffer at pH 7.4 were added. The final concentration of NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and microsome were 1 mM, 5 mM, 0.5 U, and 0.2 mg/mL, respectively. The four controls had the following composition: (1) drug only, (2) drug with the microsome but without NADPH-generating system, (3) drug with NADPH-generating system only, and (4) NADPH-generating system and microsome without the drug. Samples were then incubated at 37°C for a period of 2 hours and then 250 μL methanol was added to stop the reaction. These samples were then centrifuged so that the supernatant could be taken and diluted to 1/20 (venlafaxine and escitalopram) or 1/100 (duloxetine) in the mobile phase before injecting the samples within the mass spectrometer. All injections within the mass spectrometer were performed in triplicates to ensure equal conditions for data collection and calculations.

CONCLUSION

According to the results, escitalopram, duloxetine and venlafaxine all underwent N-demethylation but not aromatic hydroxylation during metabolism in rat liver microsome. Even though duloxetine was only N-demethylated, it metabolized the most out of all three drugs up to 30-70%. In conclusion, it was confirmed that escitalopram, venlafaxine and duloxetine needed rat liver microsomes in order to metabolize. However, it was also confirmed that only escitalopram and venlafaxine needed NADPH-generating system as well, in order to metabolize. In further studies, repeating these experiments using HPLC/CMS techniques to further separate the metabolites would be ideal. Although duloxetine metabolized the most, it would be easier to understand how or why it metabolized the most if we were able to separate the metabolites further by using these techniques. Using different drugs would also explain the outcome of metabolism a little further.

RESULTS

Escitalopram

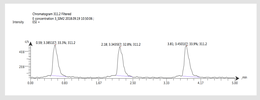


Figure 1: MS Chromatogram of N-Demethylation of Escitalopram

This figure shows three trials with their retention time and area at the molecular weight of 311.2 with the concentration of 800 μM. This is the molecular weight of escitalopram (325) with a methyl group subtracted from it (-14). Each sample was injected into the mass spectrometer three times in about a 1.5-minute interval. The demethylated metabolite shows up at 0.59 min, 2.18 min, and 3.81 min. These peaks were not visible without the presence of NADPH-generating system, meaning that escitalopram depends on this to metabolize.

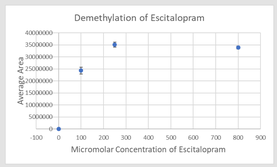


Figure 2: N-Demethylation of Escitalopram

This figure shows the average of all three trials (Figure 1) plotted against their concentrations: 0, 100, 250 and 800 μM. This plot resembles a hyperbolic curve.1-3% of the original concentration was metabolized.

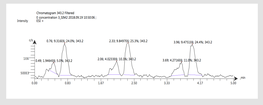


Figure 3: MS Chromatogram of Aromatic Hydroxylation of Escitalopram

This figure shows three trials with their retention time and area at the molecular weight of 343.2 with the concentration of 800 μM. This is the molecular weight of escitalopram (325) with a water group added to it (+18). Each sample was injected into the mass spectrometer three times in about a 1.5-minute interval. The metabolite does not show up at all. The peaks shown above have retention times that are later than the retention time of the drug at the molecular weight of 325, indicating that there is no hydroxylated metabolite.

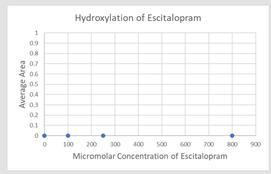


Figure 4: Aromatic Hydroxylation of Escitalopram

This figure shows the average of all three trials (Figure 3) plotted against their concentrations: 0, 100, 250 and 800 μM. This plot indicates no hydroxylation metabolites.

Venlafaxine

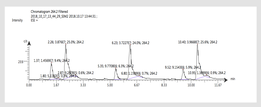


Figure 5: MS Chromatogram of N-Demethylation of Venlafaxine

This figure shows three trials with their retention time and area at the molecular weight of 264.2 with the concentration of 800 μM. This is the molecular weight of venlafaxine (278) with a methyl group subtracted from it (-14). Each sample was injected into the mass spectrometer three times every four minutes. The demethylated metabolite shows up at 1.37 min, 5.35 min, and 9.52 min. There were two peaks developed per round of injections, which means that one of the peaks was not the demethylated metabolite. Because the metabolite is known to show up at a retention time before that of the drug's (1.71 min, 5.69 min and 9.86 min), the first peak is the actual indication of demethylated metabolites. These peaks were not visible without the presence of NADPH-generating system. This means that venlafaxine is dependent on this in order to metabolize.

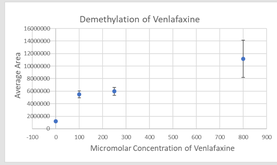


Figure 6: N-Demethylation of Venlafaxine

This figure shows the average of all three trials (Figure 5) plotted against their concentrations:0, 100, 250 and 800 μM. This plot resembles a hyperbolic curve. 0.9-1.5% of the original concentration was metabolized.

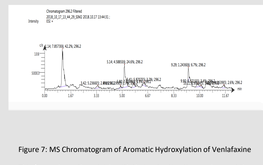


Figure 7: MS Chromatogram of Aromatic Hydroxylation of Venlafaxine

This figure shows three trials with their retention time and area at the molecular weight of 296.2 with the concentration of 800 μM. This is the molecular weight of venlafaxine (278) with a water group added to it (+18). Each sample was injected into the mass spectrometer three times every four minutes. The hydroxylated metabolite shows up at 1.14 min, 5.14 min, and 9.29 min.

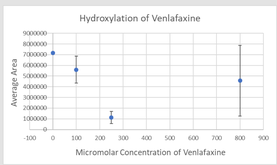


Figure 8: Aromatic Hydroxylation of Venlafaxine

This figure shows the average of all three trials (Figure 7) plotted against their concentrations: 0, 100, 250 and 800 μM. This plot indicates that no hydroxylation metabolites due to the inconsistency of the curve. This curve should be hyperbolic in order to indicate metabolism.

Duloxetine

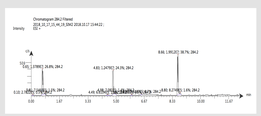


Figure 9: MS Chromatogram of N-Demethylation of Duloxetine

This figure shows three trials with their retention time and area at the molecular weight of 284.2 with the concentration of 800 μM. This is the molecular weight of duloxetine (298) with a methyl group subtracted from it (-14). Each sample was injected into the mass spectrometer three times every four minutes. The demethylated metabolite shows up at 0.65 min, 4.83 min, and 8.66 min. These peaks were not visible without the presence of NADPH-generating system, meaning that duloxetine is not dependent on it to metabolize.

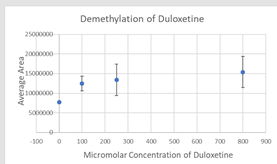


Figure 10: N-Demethylation of Duloxetine

This figure shows the average of all three trials (Figure 9) plotted against their concentrations: 0, 100, 250 and 800 μM. This plot resembles a hyperbolic curve. 33-72% of the original concentration was metabolized.

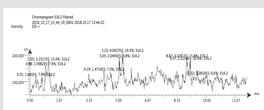


Figure 11: MS Chromatogram of Aromatic Hydroxylation of Duloxetine

This figure shows three trials with their retention time and area at the molecular weight of 316.2 with the concentration of 800 μM. This is the molecular weight of duloxetine (298) with a water group added to it (+18). Each sample was injected into the mass spectrometer three times every four minutes. There are no strong peaks that would indicate hydroxylated metabolites.

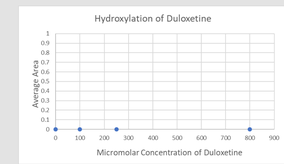


Figure 12: Aromatic Hydroxylation of Duloxetine

This figure shows the average of all three trials (Figure 11) plotted against their concentrations: 0, 100, 250 and 800 μM. This plot indicates no hydroxylation metabolites.

DISCUSSION

Escitalopram was the first drug tested, containing seven samples, three of different concentrations known as 100, 250 and 800 μM and four controls known as the mixture without the drug, the mixture without NADPH-generating system, the mixture without the rat liver microsome and just the drug alone. For each sample, the three peaks at escitalopram's molecular weight plus one for the original drug, minus fourteen (N-demethylation), and plus eighteen (aromatic hydroxylation) were selectively monitored and used to determine its metabolites. At the molecular weight of 311.2, three strong peaks with retention times of 0.59 min, 2.18 min, and 3.81 min indicated N-demethylated metabolites at 800 μM. Escitalopram's N-demethylation metabolized between 1-3%. At the molecular weight of 343.2, three small peaks with retention times of 0.76 min, 2.33 min, and 3.96 min that showed up later than the drug at a molecular weight of 325.2 indicated that there are no aromatic hydroxylated metabolites at 800 μM. Even though there is a small peak that forms before each peak that has the right retention times, it is still not thought to be the metabolites because the peak only appears in the 800 μM concentration and is not a clean peak. In the 100 and 250 μM concentrations, there are no peaks at the molecular weight of 343.2, there is only noise on each chromatogram. It is unknown what the peak within the 800 μM chromatogram is, but it could possibly be another component of the sample with the same molecular weight. Further controls would need to be studied to conclude which constituent contains the same molecular weight as the possible metabolite.

Venlafaxine was the second drug to be studied and interpreted by averaging the three peaks shown for each of the seven samples. At the molecular weight of 264.2, two strong peaks per injection displayed. Between the two of them, the first peak for each injection with retention times of 1.37 min, 5.35 min, and 9.52 min indicated N-demethylated metabolites at 800 μM. The second peak for each injection with retention times of 2.26 min, 6.23 min, and 10.40 min did not indicate N-demethylated metabolites due to the original drug at the molecular weight of 278.2 having retention times happening earlier than the these peaks. Metabolites must have retention times earlier than the drug's in order to actually prove metabolism since the metabolites are more polar than the original drug. Venlafaxine was N-demethylated between 0.9-1.5%. At the molecular weight of 296.2, three peaks with retention times of 1.14 min, 5.14 min, and 9.29 min indicated aromatic hydroxylated metabolites. However, when plotting the average of its peaks with each concentration injected, the plot did not represent a hyperbolic curve. A hyperbolic curve indicates the increase in the average area of the peak, eventually plateauing once it hits the saturation. Because the plot did not represent a hyperbolic curve, it can be assumed that aromatic hydroxylation did not actually happen within the metabolism of venlafaxine. It is unsure what these peaks are actually indicating that further studies with more controls would need to be completed in order identify them. Duloxetine was the last drug to be studied. At the molecular weight of 284.2, three strong peaks with the retention times of 0.65 min, 4.83 min, and 8.66 min indicated N-demethylated metabolites at 800 μM. Duloxetine's N-demethylation metabolized between 33-72%. At the molecular weight of 316.2, we did not detect strong peaks. Therefore, it is indicated that there was not any aromatic hydroxylation at 800 μM. Out of the three drugs, duloxetine metabolized more than escitalopram and venlafaxine. This is interesting and we are not sure why. When referring to the structure of duloxetine compared escitalopram and venlafaxine, duloxetine contains a secondary amine as opposed to tertiary amines of the other two drugs. Tertiary amines are known to be demethylated about 3-4 times as rapidly as the secondary amine (Brodie and Gillette), therefore, testing of another antidepressant containing a secondary amine would be necessary.

When analyzing the controls for each drug, the control that contained only the drug and microsome without the NADPH-generating system gave interesting results. For escitalopram and venlafaxine, the metabolites did not show up on the chromatogram in the absence of the NADPH-generating system. However, for duloxetine, the metabolites of N-demethylation did show up on the chromatogram in the presence of NADPH-generating system. This concludes that both escitalopram and venlafaxine need NADPH-generating system when metabolizing and duloxetine does not. NADPH is required for N-demethylation, so we are unsure why this is the case.

While injecting the samples of escitalopram into the compact mass spectrometer, each sample was injected three times within 1.5 minute intervals for a total of five minutes. The syringe was changed per sample, along with a wash of the needle and the column. After analyzing the data, it was then decided that in order to see a better separation between the three injections that the intervals needed to be stretched to every four minutes for a total of twelve minutes. In order to rely on retention times of the peaks appearing, it was practiced to inject the sample at exactly four minutes every time. There were a couple of times that samples were injected at a slightly later time, which could have affected the retention times slightly. Overall, this experiment ran very smooth and gave useful results.

ACKNOWLEDGEMENTS

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Abstract:

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Introduction:

Depression is a serious mental illness that many college students experience. Between deadlines and relationships, college can be a very challenging time, making students feel very dispirited. It is hard to return from that feeling of anguish, so many students turn to antidepressant medications to feel better again. Duloxetine (Cymbalta), escitalopram (Lexapro) and venlafaxine (Effexor) are all antidepressant medications that inhibit serotonin-norepinephrine reuptake within the brain (Lam et al.). There are multiple dosages that can be prescribed, all based on severity of the depression. A blood cell count, test on hormone levels and a metabolic panel of the liver is also taken to decide the dosage for a patient. Cytochrome P450 (CYP) are enzymes of the liver that metabolize things that a person may ingest, including antidepressant medications. There are multiple different enzymes within the liver, all working together, but completing different steps in the metabolism process. CYP1A2, CYP2C19 and CYP2D6 are three of the multiple enzymes used within the liver. It isn't fully known how much of each enzyme is used within the liver, but it is known that each would be poor metabolizers if they had to work alone. Their greatest substrates are antidepressant drugs, including venlafaxine, escitalopram and duloxetine. As a college student that is surrounded by so many

students in a low-mood, I feel it is necessary to determine how these enzymes in the liver metabolize the antidepressant medications given and to know that the reaction does not result in toxins being produced in the body. Also, as a pharmacy technician, I dispense antidepressants almost daily. Therefore, I'm very interested in knowing the details of how the liver metabolizes these medications.

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My proposal is to investigate how rat liver microsomes, which contain many CYP enzymes, react with each of the three medications, venlafaxine, escitalopram and duloxetine. The metabolism of each antidepressant medication highly depends on whether the cytochrome catalyzes a reaction, making the drug soluble. But what is this reaction? And are these man-made medications safe for our bodies after these reactions occur? By developing an assay to perform to help further the reaction and determine its affinity, I will find which of these drugs metabolizes the most efficiently. By viewing the breakdown of the drugs with and without microsomes, I will determine how each drug is metabolized within the body.

Materials and Methods:

Venlafaxine, escitalopram, duloxetine, NADP, Glucose-6-Phosphate, and Glucose-6-Phosphate Dehydrogenase and rat liver microsome were purchased from Sigma Aldrich. Luna C18 reverse phase column (3 micrometer, 50 mm x 2 mm) was purchased from Phenomenex. Each drug's samples were metabolized and evaluated using Advion Expressions Compact Mass Spectrometer fitted with an electrospray ionizer (ESI) and a Phenomenex Luna C18 column running for 15 minutes per sample with a mobile phase of 95:5 acetonitrile:water 2 μM ammonium acetate with a 0.2 mL/min flow rate. Three different concentrations of each drug were used along with four different controls. Each of the drug concentrations were 100, 250 and 800 μM. For each drug, 65 μL of these concentrations along with a mixture of 35 µL NADPH-generating system and 25 μL microsomal suspension in 50 μM potassium phosphate buffer at pH 7.4. The final concentration of NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and microsome were 1 μM, 5 μM, 0.5 U, and 0.2 mg/mL, respectively. The four controls had the following composition: (1) drug only, (2) drug with the microsome only, (3) drug with NADPH-generating system only, and (4) NADPH-generating system and microsome without the drug. Samples were then incubated at 37 degrees C for a period of 2 hours and then 250 μM methanol was added to stop the reaction. These samples were then centrifuged so that the supernatant could be taken and diluted to 1/20 (venlafaxine and escitalopram) or

1/100 (duloxetine) in the mobile phase before injecting the samples within the mass spectrometer. All injections within the mass spectrometer were performed in triplicates to ensure equal conditions for data collection and calculations.

Protocol for Venlafaxine:

1. Create NADPH-generating system by adding 0.492 mg of NADP in 50 μL of buffer to make 12.5 mM NADP, add 200 μL of buffer into the 100 U vial of G6P dehydrogenase, and remove 25 μL of the solution to make 12.5 U of G6P dehydrogenase. Create G6P by adding 0.88 mg of G6P in 100 μL of buffer to make 31.2 mM G6P all together. Measure out about 0.5 mg of NADP and about 1 mg G6P and add to vial with 160 μL of buffer. Mix well.
2. Create a stock of Venlafaxine with 4.4 mg/10 mL of buffer to equal 1.6 mM.
3. Dissolve 125 μL of Venlafaxine into 875 μL of KH2PO4 buffer (that has been already made) and place in test tube labeled, “Drug Conc. 1”.
4. Dissolve 313 μL of Venlafaxine into 687 μL of KH2PO4 buffer and place into test tube labeled, “Drug Conc. 2”.
5. Place stock 1000 μL of Venlafaxine into test tube labeled, “Drug Conc. 3”.
6. Dissolve 19 μL of microsomal solution into 125 μL of KH2PO4 buffer and place into test tube labeled, “Microsomal”
7. Add 200 μL of buffer into the 100 U vial of G6P dehydrogenase. Mix well. Remove 25 μL and add to the NADPH-generating system (step 1). Place the remainder into individual tubes of 25 μL and freeze, labelled, “12.5 U G6PD 1-7”
8. Take 35 μL of your dissolved NADPH and add to centrifuge tube #1 along with 65 μL of dissolved Venlafaxine (Drug Conc. 1). Suspend.
9. Take 25 μL of the dissolved microsomal suspension and add it to that centrifuge tube. Mix again suspend.
10. Take 35 μL of your dissolved NADPH and add to centrifuge tube #2 along with 65 μL of dissolved Venlafaxine (Drug Conc. 2). Suspend.
11. Take 25 μL of the dissolved microsomal suspension and add it to that centrifuge tube. Suspend.
12. Take 35 μL of your dissolved NADPH and add to centrifuge tube #3 along with 65 μL of dissolved Venlafaxine (Drug Conc. 3). Mix on a vortex mixer.
13. Take 25 μL of the dissolved microsomal suspension and add it to that centrifuge tube. Mix by suspension.
14. Take 35 μL of your dissolved NADPH and add to centrifuge tube #4, labeled, “No drug” along with 65 μL of KH2PO4 buffer. Suspend.
15. Take 25 μL of the dissolved microsomal suspension and add it to that centrifuge tube. Mix again by suspending.
16. Repeat the same steps for “Just drug”, “Just drug alone” and “No NADPH”.
17. Incubate all 7 tubes for 2 hours and then add 250 μL of methanol to each tube to stop the reaction and centrifuge them at 15,000 g x 20 minutes.
18. Extract the supernatants from the tubes and place into 7 new centrifuge tubes. Freeze until it is time to run in the MS.

19. Extract 10 μL of the supernatant from each tube. Put the 10 μL into 1 mL of MS solution (that has already been made).
20. Run the MS for all three experiments, follow the videos of Lee.

Protocol for Duloxetine:

1. Create NADPH-generating system by adding 0.492 mg of NADP in 50 μL of buffer to make 12.5 mM NADP, add 200 μL of buffer into the 100 U vial of G6P dehydrogenase, and remove 25 μL of the solution to make 12.5 U of G6P dehydrogenase. Create G6P by adding 0.88 mg of G6P in 100 μL of buffer to make 31.2 mM G6P all together. Measure out about 0.5 mg of NADP and about 1 mg G6P and add to vial with 160 μL of buffer. Mix well.
2. Create a stock of Duloxetine with 4.8 mg/10 mL of buffer to equal 1.6 mM.
3. Dissolve 125 μL of Duloxetine into 875 μL of KH2PO4 buffer (that has been already made) and place in test tube labeled, “Drug Conc. 1”.
4. Dissolve 313 μL of Duloxetine into 687 μL of KH2PO4 buffer and place into test tube labeled, “Drug Conc. 2”.
5. Place stock 1000 μL of Duloxetine into test tube labeled, “Drug Conc. 3”.
6. Dissolve 19 μL of microsomal solution into 125 μL of KH2PO4 buffer and place into test tube labeled, “Microsomal”
7. Add 200 μL of buffer into the 100 U vial of G6P dehydrogenase. Mix well. Remove 25 μL and add to the NADPH-generating system (step 1). Place the remainder into individual tubes of 25 μL and freeze, labelled, “12.5 U G6PD 1-7”
8. Take 35 μL of your dissolved NADPH and add to centrifuge tube #1 along with 65 μL of dissolved Duloxetine (Drug Conc. 1). Suspend.
9. Take 25 μL of the dissolved microsomal suspension and add it to that centrifuge tube. Mix again suspend.
10. Take 35 μL of your dissolved NADPH and add to centrifuge tube #2 along with 65 μL of dissolved Duloxetine (Drug Conc. 2). Suspend.
11. Take 25 μL of the dissolved microsomal suspension and add it to that centrifuge tube. Suspend.
12. Take 35 μL of your dissolved NADPH and add to centrifuge tube #3 along with 65 μL of dissolved Duloxetine (Drug Conc. 3). Mix on a vortex mixer.
13. Take 25 μL of the dissolved microsomal suspension and add it to that centrifuge tube. Mix by suspension.
14. Take 35 μL of your dissolved NADPH and add to centrifuge tube #4, labeled, “No drug” along with 65 μL of KH2PO4 buffer. Suspend.
15. Take 25 μL of the dissolved microsomal suspension and add it to that centrifuge tube. Mix again by suspending.
16. Repeat the same steps for “Just drug”, “Just drug alone” and “No NADPH”.
17. Incubate all 7 tubes for 2 hours and then add 250 μL of methanol to each tube to stop the reaction and centrifuge them at 15,000 g x 20 minutes.
18. Extract the supernatants from the tubes and place into 7 new centrifuge tubes. Freeze until it is time to run in the MS.

19. Extract 10 μL of the supernatant from each tube. Put the 10 μL into 1 mL of MS solution (that has already been made).
20. Run the MS for all three experiments, follow the videos of Lee.

Protocol for Escitalopram:

1. Create NADPH-generating system by adding 0.492 mg of NADP in 50 μL of buffer to make 12.5 mM NADP, add 200 μL of buffer into the 100 U vial of G6P dehydrogenase, and remove 25 μL of the solution to make 12.5 U of G6P dehydrogenase. Create G6P by adding 0.88 mg of G6P in 100 μL of buffer to make 31.2 mM G6P all together. Measure out about 0.5 mg of NADP and about 1 mg G6P and add to vial with 160 μL of buffer. Mix well.
2. Create a stock of Escitalopram with 5.2 mg/10 mL of buffer to equal 1.6 mM.
3. Dissolve 125 μL of Escitalopram into 875 μL of KH2PO4 buffer (that has been already made) and place in test tube labeled, “Drug Conc. 1”.
4. Dissolve 313 μL of Escitalopram into 687 μL of KH2PO4 buffer and place into test tube labeled, “Drug Conc. 2”.
5. Place stock 1000 μL of Escitalopram into test tube labeled, “Drug Conc. 3”.
6. Dissolve 19 μL of microsomal solution into 125 μL of KH2PO4 buffer and place into test tube labeled, “Microsomal”
7. Add 200 μL of buffer into the 100 U vial of G6P dehydrogenase. Mix well. Remove 25 μL and add to the NADPH-generating system (step 1). Place the remainder into individual tubes of 25 μL and freeze, labelled, “12.5 U G6PD 1-7”
8. Take 35 μL of your dissolved NADPH and add to centrifuge tube #1 along with 65 μL of dissolved Duloxetine (Drug Conc. 1). Suspend.
9. Take 25 μL of the dissolved microsomal suspension and add it to that centrifuge tube. Mix again suspend.
10. Take 35 μL of your dissolved NADPH and add to centrifuge tube #2 along with 65 μL of dissolved Duloxetine (Drug Conc. 2). Suspend.
11. Take 25 μL of the dissolved microsomal suspension and add it to that centrifuge tube. Suspend.
12. Take 35 μL of your dissolved NADPH and add to centrifuge tube #3 along with 65 μL of dissolved Duloxetine (Drug Conc. 3). Mix on a vortex mixer.
13. Take 25 μL of the dissolved microsomal suspension and add it to that centrifuge tube. Mix by suspension.
14. Take 35 μL of your dissolved NADPH and add to centrifuge tube #4, labeled, “No drug” along with 65 μL of KH2PO4 buffer. Suspend.
15. Take 25 μL of the dissolved microsomal suspension and add it to that centrifuge tube. Mix again by suspending.
16. Repeat the same steps for “Just drug”, “Just drug alone” and “No NADPH”.
17. Incubate all 7 tubes for 2 hours and then add 250 μL of methanol to each tube to stop the reaction and centrifuge them at 15,000 g x 20 minutes.
18. Extract the supernatants from the tubes and place into 7 new centrifuge tubes. Freeze until it is time to run in the MS.
19. Extract 10 μL of the supernatant from each tube. Put the 10 μL into 1 mL of MS solution (that has already been made).

20. Run the MS for all three experiments, follow the videos of Lee.

Experiment Tubes	Drug	NADPH	Microsomal Suspension	Buffer
Drug Conc. 1	65 μL	35 μL	25 μL	0
Drug Conc. 2	65 μL	35 μL	25 μL	0
Drug Conc. 3	65 μL	35 μL	25 μL	0
No drug	0	35 μL	25 μL	65 μL
No microsome	65 μL Drug Conc. 3	35 μL	0	25 μL
Just drug alone	65 μL Drug Conc. 3	0	0	60 μL
Without NADPH	65 μL Drug Conc. 3	0	25 μL	35 μL

Results:
Escitalopram

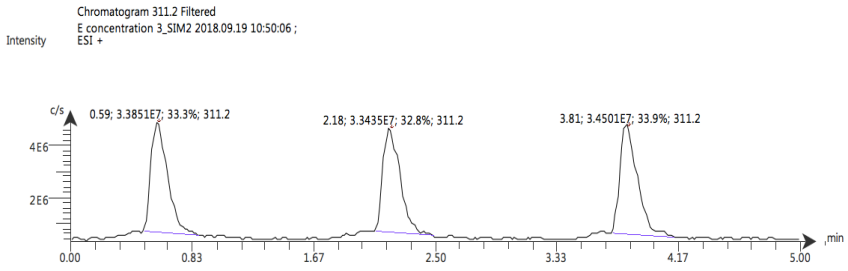

Figure 1: MS Chromatogram of N-Demethylation of Escitalopram

This figure shows three peaks with their retention time and area at the molecular weight of 311.2 with the concentration of 800 μM. This is the molecular weight of escitalopram (325) with a methyl group subtracted from it (-14). Each sample was injected into the mass spectrometer three times in about a 1.5-minute interval. The demethylated metabolite shows up at 0.59 min, 2.18 min, and 3.81 min. These peaks were not visible without the presence of NADPH-generating system, meaning that escitalopram depends on this to metabolize.

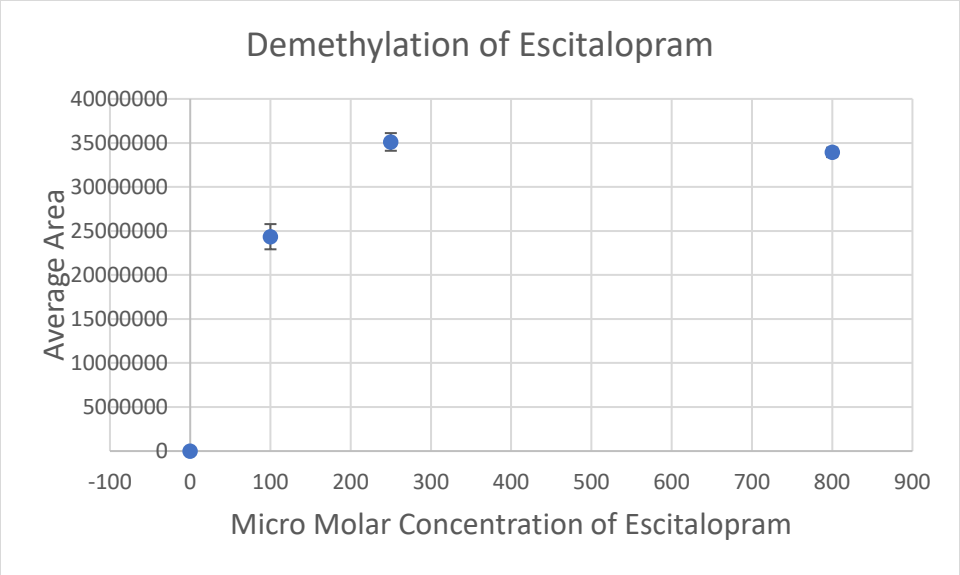

Figure 2: N-Demethylation of Escitalopram

This figure shows the average of all three peaks (Figure 1) plotted against their concentrations: 0, 100, 250 and 800 μM. This plot resembles a hyperbolic curve.1-3% of the original concentration was metabolized.

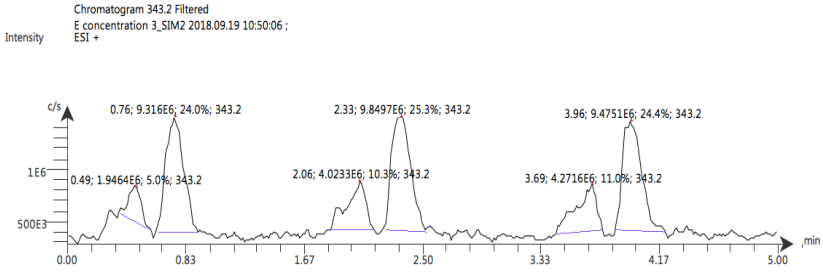

Figure 3: MS Chromatogram of Aromatic Hydroxylation of Escitalopram
This figure shows three peaks with their retention time and area at the molecular weight of 343.2 with the concentration of 800 μM. This is the molecular weight of escitalopram (325) with a water group added to it (+18). Each sample was injected into the mass spectrometer three times in about a 1.5-minute interval. The metabolite does not show up at all. The peaks shown above have retention times that are later than the retention time of the drug at the molecular weight of 325, indicating that there is no hydroxylated metabolite.

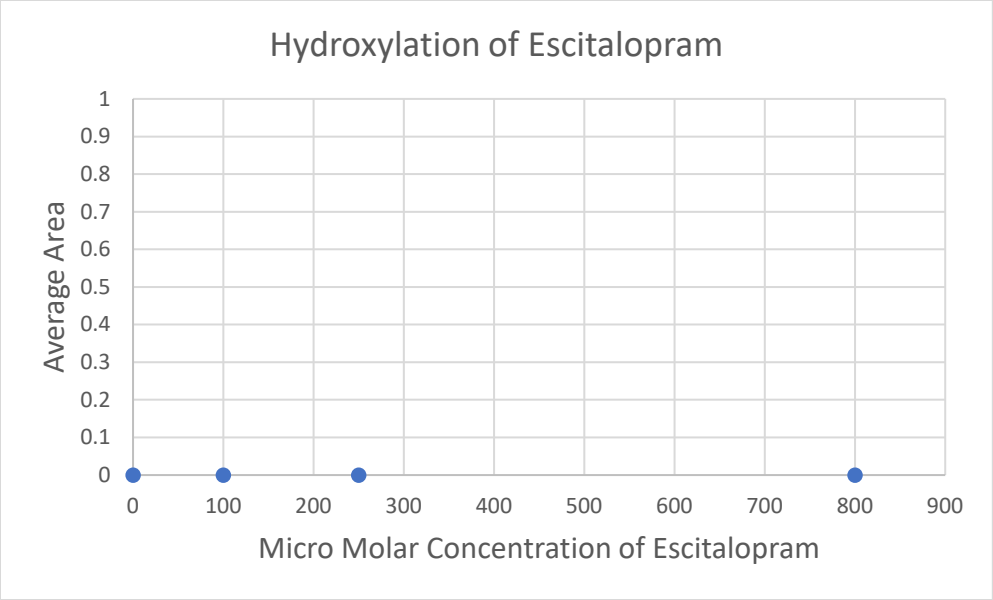

Figure 4: Aromatic Hydroxylation of Escitalopram

This figure shows the average of all three peaks (Figure 3) plotted against their concentrations: 0, 100, 250 and 800 μM. This plot resembles no hydroxylation metabolites.

Venlafaxine

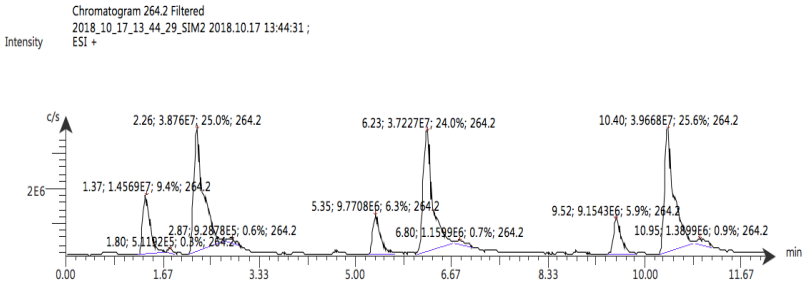

Figure 5: MS Chromatogram of N-Demethylation of Venlafaxine

This figure shows three peaks with their retention time and area at the molecular weight of 264.2 with the concentration of 800 μM. This is the molecular weight of venlafaxine (278) with a methyl group subtracted from it (-14). Each sample was injected into the mass spectrometer three times every four minutes. The demethylated metabolite shows up at 1.37 min, 5.35 min, and 9.52 min. There were two peaks developed per round of injections, which means that one of the peaks was not the demethylated metabolite. Because the metabolite is known to show up at a retention time before that of the drug's (1.71 min, 5.69 min and 9.86 min), the first peak is the actual indication of demethylated metabolites. These peaks were not visible without the presence of NADPH-generating system. This means that venlafaxine is dependent on this in order to metabolize.

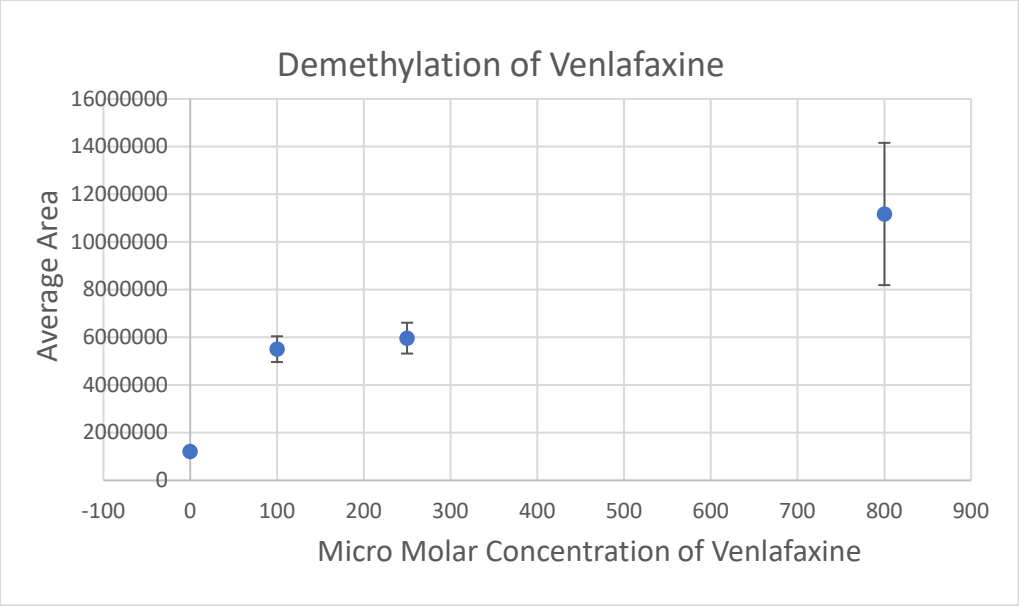

Figure 6: N-Demethylation of Venlafaxine

This figure shows the average of all three peaks (Figure 5) plotted against their concentrations: 0, 100, 250 and 800 μM. This plot resembles a hyperbolic curve. 0.9-1.5% of the original concentration was metabolized.

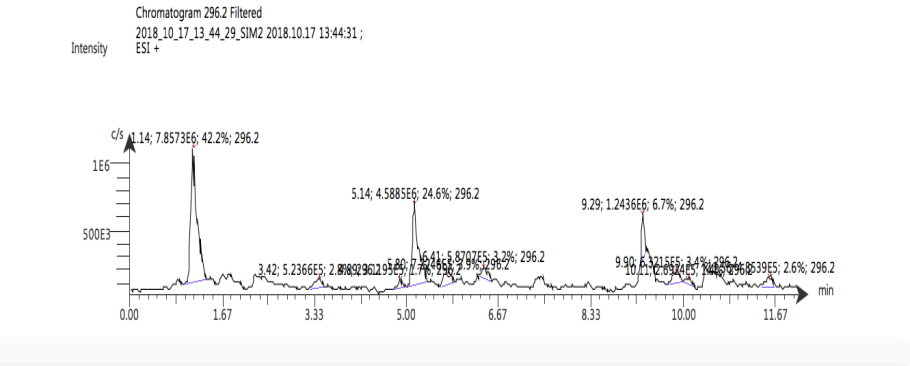

Figure 7: MS Chromatogram of Aromatic Hydroxylation of Venlafaxine

This figure shows three peaks with their retention time and area at the molecular weight of 296.2 with the concentration of 800 μM. This is the molecular weight of venlafaxine (278) with a water group added to it (+18). Each sample was injected into the mass spectrometer three times every four minutes. The hydroxylated metabolite shows up at 1.14 min, 5.14 min, and 9.29 min.

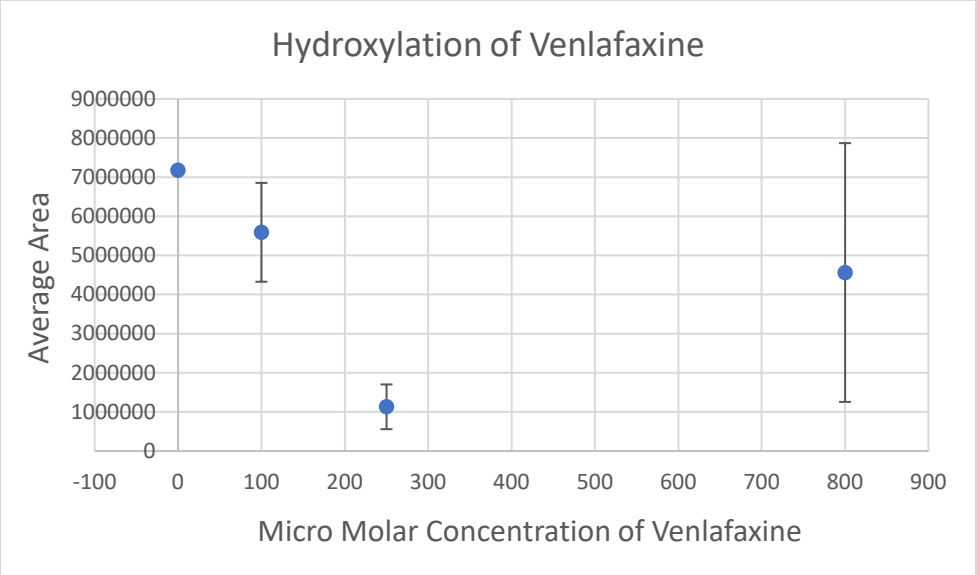

Figure 8: Aromatic Hydroxylation of Venlafaxine

This figure shows the average of all three peaks (Figure 7) plotted against their concentrations: 0, 100, 250 and 800 μM. This plot resembles no hydroxylation metabolites due to the inconsistency of the curve. This curve is supposed to be hyperbolic in order to indicate metabolism.

Duloxetine

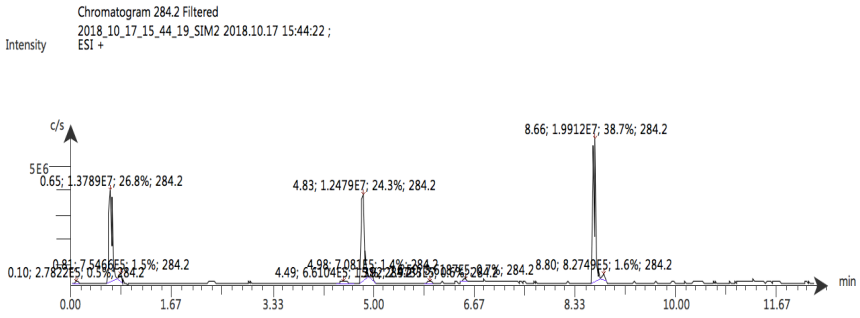

Figure 9: MS Chromatogram of N-Demethylation of Duloxetine

This figure shows three peaks with their retention time and area at the molecular weight of 284.2 with the concentration of 800 μM. This is the molecular weight of duloxetine (298) with a methyl group subtracted from it (-14). Each sample was injected into the mass spectrometer three times every four minutes. The demethylated metabolite shows up at 0.65 min, 4.83 min, and 8.66 min. These peaks were not visible without the presence of NADPH-generating system, meaning that duloxetine is not dependent on it to metabolize.

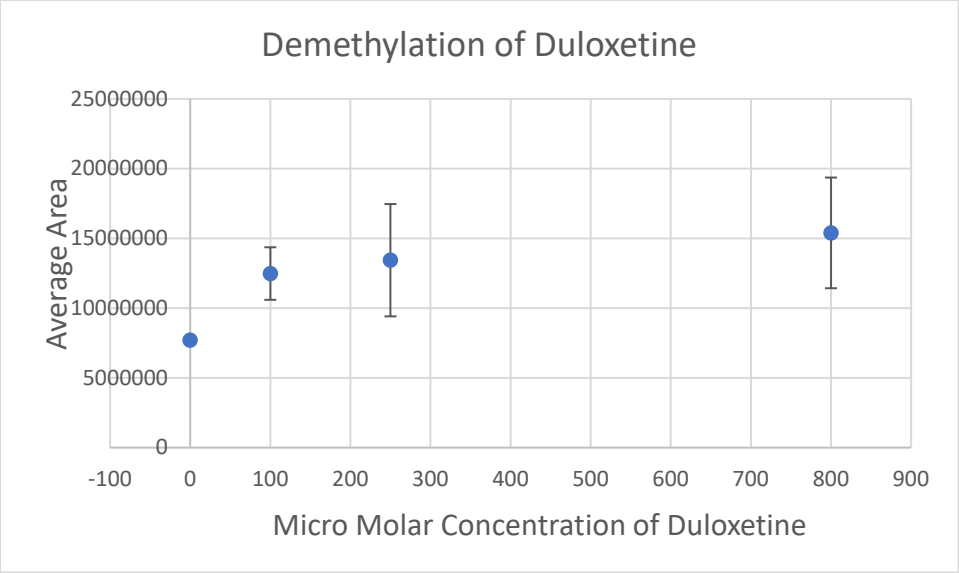

Figure 10: N-Demethylation of Duloxetine

This figure shows the average of all three peaks (Figure 9) plotted against their concentrations: 0, 100, 250 and 800 μM. This plot resembles a hyperbolic curve. 33-72% of the original concentration was metabolized.

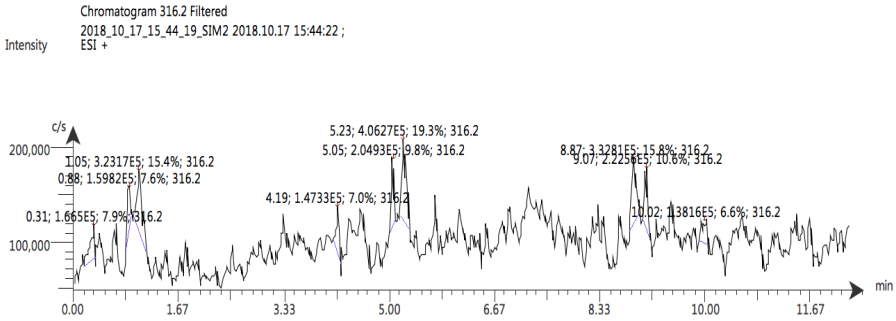

Figure 11: MS Chromatogram of Aromatic Hydroxylation of Duloxetine

This figure shows three peaks with their retention time and area at the molecular weight of 316.2 with the concentration of 800 μM. This is the molecular weight of duloxetine (298) with a water group added to it (+18). Each sample was injected into the mass spectrometer three times every four minutes. There are no strong peaks that would indicate hydroxylated metabolites.

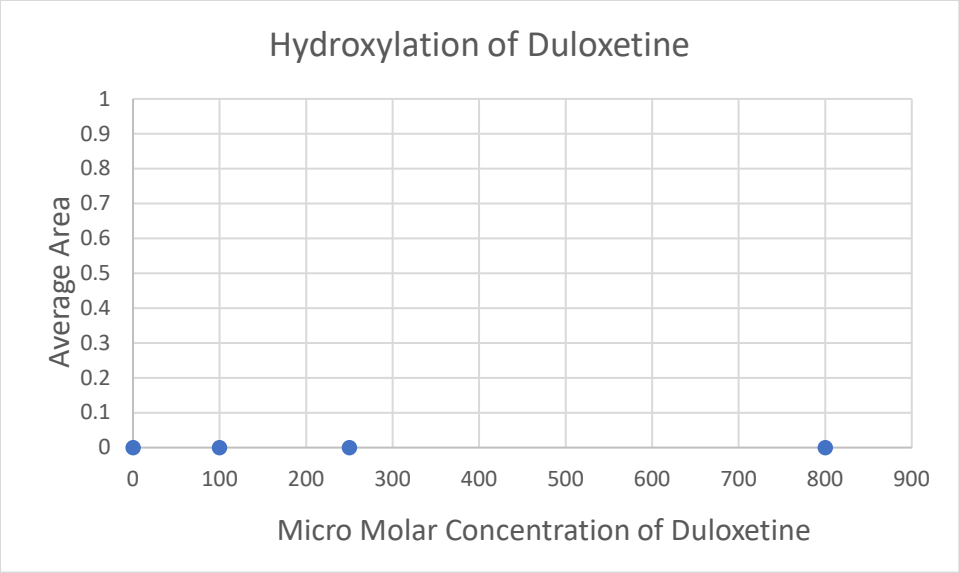

Figure 12: Aromatic Hydroxylation of Duloxetine

This figure shows the average of all three peaks (Figure 11) plotted against their concentrations: 0, 100, 250 and 800 μM. This plot resembles no hydroxylation metabolites.

Discussion:

Escitalopram was the first drug that was injected within the compact mass spectrometer, containing seven samples, three of different concentrations known as 100, 250 and 800 μM and four controls known as the mixture without the drug, the mixture without NADPH-generating system, the mixture without the rat liver microsome and just the drug alone. For each sample, the three peaks at escitalopram's molecular weight, its weight with fourteen subtracted (N-demethylation), and its weight with eighteen added (aromatic hydroxylation) were averaged and used to determine its metabolites. At the molecular weight of 311.2, three strong peaks with retention times of 0.59 min, 2.18 min, and 3.81 min indicated N-demethylated metabolites at 800 μM. Escitalopram's N-demethylation metabolized between 1-3%. At the molecular weight of 343.2, three weak peaks with retention times of 0.76 min, 2.33 min, and 3.96 min that showed up later than the drug at a molecular weight of 325.2 indicated that there are no aromatic hydroxylated metabolites at 800 μM. Even though there is a slight peak that forms before each peak that has the right retention times, it is still not thought to be the metabolites because the peak only appears in the 800 μM concentration and is not a clean peak. In the 100 and 250 μM concentrations, there are no peaks at the molecular weight of 343.2, there is only noise on each chromatogram. It is unknown what the peak within the 800 μM chromatogram is, but it could possibly be another component of the sample with the same molecular weight. Further controls would need to be injected to conclude which constituent contains the same molecular weight as the possible metabolite. Venlafaxine was the second drug to be injected and interpreted by averaging the three peaks shown for each of the seven samples. At the molecular weight of 264.2, two strong peaks per injection displayed. Between the two of them, the first peak for each injection with retention times of 1.37 min, 5.35 min, and 9.52 min

indicated N-demethylated metabolites at 800 μM. The second peak for each injection with retention times of 2.26 min, 6.23 min, and 10.40 min did not indicate N-demethylated metabolites due to the drug at the molecular weight of 278.2 having retention times happening earlier than the second peaks. Metabolites must have retention times earlier than the drug's to prove metabolism. Venlafaxine's N-demethylation metabolized between 0.9-1.5%. At the molecular weight of 296.2, three peaks with retention times of 1.14 min, 5.14 min, and 9.29 min indicated aromatic hydroxylated metabolites. However, when plotting the average of its peaks with each concentration injected, the function did not represent a hyperbolic curve. A hyperbolic curve explains the increase of concentration vs the average area of the peak, eventually plateauing once it has hit its highest concentration. Because the function did not represent a hyperbolic curve, it can be assumed that aromatic hydroxylation did not actually happen within the metabolism of venlafaxine. It is unsure what these peaks are indicating and further injections with more controls would need to be completed to narrow down what is happening at this molecular weight. Duloxetine was the last drug to be injected into the compact mass spectrometer with its seven samples, three times each. At the molecular weight of 284.2, three strong peaks with the retention times of 0.65 min, 4.83 min, and 8.66 min indicated N-demethylated metabolites at 800 μM. Duloxetine's N-demethylation metabolized between 33-72%. At the molecular weight of 316.2, only noise appeared on the chromatogram. Since no peaks were formed, it is indicated that there was not any aromatic hydroxylation at 800 μM. Out of the three drugs, duloxetine metabolized a lot more than escitalopram and venlafaxine. This is interesting and it is not indicated why. However, when referring to the structure of duloxetine compared escitalopram and venlafaxine, duloxetine only contains one methyl group coming off the nitrogen as opposed to two methyl groups coming off of the nitrogen like the other two drugs. This could be why duloxetine metabolized much more than the other two, but it is not certain. To further prove this hypothesis, testing of another antidepressant containing only one methyl group coming off the nitrogen would need to be metabolized, injected into the compact mass spectrometer and analyzed to see its percentage of metabolism of N-demethylation. When analyzing the controls for each drug, the control that contained only the drug and microsome and was lacking the NADPH-generating system gave interesting results. For escitalopram and venlafaxine, the metabolites did not show up on the chromatogram in the absence of the NADPH-generating system. However, for duloxetine, the metabolites of N-demethylation did show up on the chromatogram in the presence of NADPH-generating system. This concludes that both escitalopram and venlafaxine need NADPH-generating system when metabolizing and duloxetine does not. While injecting the samples of escitalopram into the compact mass spectrometer, each sample was injected three times within 1.5 minute intervals for a total of five minutes. The syringe was changed per sample, along with a wash of the needle and the column. After analyzing the data, it was then decided that to see a better separation between the three injections that the intervals needed to be stretched to every four minutes for a total of twelve minutes. In order to rely on retention times of the peaks appearing, it was practiced injecting the sample at exactly four minutes every time. There were a couple of times that samples were injected at a slightly later time, which could have affected the retention times slightly. Overall, this experiment ran very smooth and gave useful results.

Conclusion:
According to the results, escitalopram, duloxetine and venlafaxine all underwent N-demethylation but not aromatic hydroxylation during metabolism in rat liver microsome. Even though duloxetine was only N-demethylated, it metabolized the most out of all three drugs up to 30-70%. In conclusion, it was confirmed that escitalopram, venlafaxine and duloxetine needed rat liver microsomes to metabolize. However, it was also confirmed that only escitalopram and venlafaxine needed NADPH-generating system as well, to metabolize. In further studies, repeating these experiments using HPLC/CMS techniques to further separate the metabolites would be ideal. Although duloxetine metabolized the most, it would be easier to understand how or why it metabolized the most if we were able to separate the metabolites further by using these techniques. Using different drugs would also explain the outcome of metabolism a little further.

Acknowledgements:
A special thanks to Winona State University for supporting and funding this research with the Winona State Student Research Grant Program. An additional thanks to Dr. Lee Collier from Advion for guidance of endeavors in producing this project. Also, all assistance from other WSU students, faculty, and the chemistry department was very much appreciated.

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WINONA STATE UNIVERSITY
UNDERGRADUATE STUDENT RESEARCH & CREATIVE PROJECTS FINAL REPORT

Electronically submit complete final report ten (10) days following completion of project to Grants & Sponsored Projects (grants@winona.edu). Hover over fill-able fields for additional guideline and completion information.

Student Name:	Dani Schmaus	Student Email:	dschmaus14@winona.edu
Student Major:	Cell and Molecular Biology		
Faculty Sponsor:	Myoung Lee	Faculty Sponsor Email:	mlee@winona.edu
Title of Project:	Identification of Drug Metabolites of Antidepressant Medications, Venlafaxine, Escitalopram and Duloxetine, in Rat Liver Microsome Using Compact Mass Spectrometry		

Project Abstract:

Antidepressants are commonly used for depression and anxiety. It is known that most drugs are metabolized by enzymes within the liver. The enzymes metabolize drugs by altering them to become more polar so that they can be excreted through the urine or bile. As a future pharmacist, I want to know how antidepressants are altered within the body before I distribute them. This study was done to determine the outcome of metabolism within three different antidepressants: venlafaxine (Effexor), duloxetine (Cymbalta) and escitalopram (Lexapro). These three drugs were mixed with rat liver microsomes and NADPH-generating system in phosphate buffer at pH 7.4. All samples were incubated for two hours at 37C. Each sample was subjected to the compact mass spectrometer fitted with a C18 reverse phase column. The results indicated that all three drugs underwent N-demethylation but not aromatic hydroxylation during metabolism. When looking at the area of the peaks within each metabolized drug, duloxetine metabolized the most out of all three drugs. Up to 30-70% of duloxetine was metabolized. Several controls were also incubated and analyzed, such as the mixture without the drug, the mixture without NADPH-generating system, the mixture without the rat liver microsome and a mixture of just the drug and buffer. Some of the controls without the drug or the microsome still showed MS peaks with the same molecular weight as the drugs or the metabolites. These peaks could indicate that there are contaminants within the solvent or the phosphate buffer that have similar molecular weights. The future goal is to repeat these experiments using HPLC/CMS to further separate the metabolites. Using different drugs would also explain the outcome of metabolism a little further, such as if a drug can be both demethylated and hydroxylated.

The student-authored final report **MUST** include each of the following (check boxes to verify inclusion of each component):

☑ This report form, fully completed (page 1 of this form)
☑ A copy of the project end product, appropriate to the standards of the discipline

Applicant Signature:	Dani Schmaus	Date:	11/29/2018
Faculty Sponsor Signature:		Date:	11/29/2018

Submit complete reports electronically to Grants & Sponsored Projects (grants@winona.edu).

A deans' sub-committee makes decisions on Undergraduate Student Research & Creative Projects proposals.

Note that a copy of the project end product will be forwarded to Krueger Library for archival purposes.