Laboratory 2 – Drug metabolism

1. Metabolism of Drugs by the Glutathione S-transferases

a) Background

The GST Drug Metabolizing Enzymes

The Glutathione S-transferase (GST) superfamily of enzymes play a critical role in the detoxification of numerous endogenous and xenobiotic compounds, as well as responding to oxidative stress. GSTs are conjugating enzymes that transfer reduced glutathione (GSH) to various substrates including environmental toxins, cancer chemotherapeutics, and various hydroperoxides (Hayes et al., 2005). In addition to their role in metabolism, various GST enzymes have been shown to play vital roles in biosynthesis, cell signalling, and cell survival and apoptosis (Allocati et al., 2018).

The mammalian GSTs comprise three families based on their subcellular location, the cytosolic GSTs, mitochondrial GSTs, and microsomal GSTs. The cytosolic GSTs are further divided into seven classes that have distinct, but overlapping substrate specificities - alpha, mu, pi, theta, zeta, omega, and sigma, with multiple genes within each class. Following the formation of homo- or heterodimers of the same class, the proteins become catalytically active and can metabolize substrates. The mitochondrial GSTs, class kappa, which are also found in the peroxisomes, also form dimers, but are structurally more similar to the prokaryotic disulphide-bond-forming oxidoreductase and 2-hydroxychromene-2-carboxylate isomerases than the mammalian cytosolic GSTs, and are likely a product of convergent evolution to fulfill a similar cellular role (Blackburn et al., 2011). The microsomal GSTs are also known as MAPEGs, membrane-associated proteins involved in eicosanoid and glutathione metabolism. They are found in the endoplasmic reticulum, form trimers, and are structurally unrelated to cytosolic GSTs, despite having GSH conjugating activity (Hayes et al., 2005; Oakley, 2011).

GSTs are expressed throughout the body, with differing amounts of each class present in various tissues, however most GST families are expressed highly in the liver, kidney, and small intestine, with transcriptomic data showing that the liver is host to ~12% of all GSTs expressed in the human body (Buratti et al., 2021; Mohana and Achary, 2017).

In order to test the ability of GST (or other drug metabolizing enzymes), we typically use tissue lysates, or further purified fractions such as the S9 fraction, cytosol, or microsomes depending on what is being tested and where the specific enzyme of interest is found.

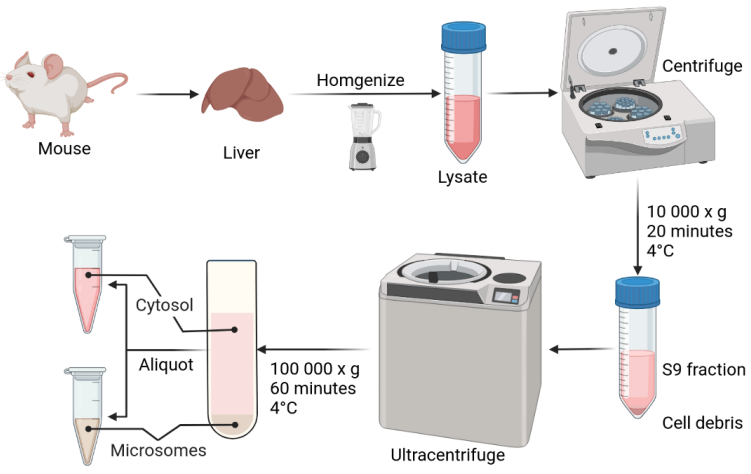


Figure 1. Preparation of mouse liver subcellular fractions. Made in biorender.com

Tissue lysates are produced by collecting tissue from an organism and blending it into a slurry of tissue and buffer using mechanical means – a blender for large amounts, a rotor/stator homogenizer for medium to small pieces, or a Dounce homogenizer for really small (and soft) pieces of tissue. These lysates can be converted to the S9 fraction by centrifuging them at 10 000 x g for 20 minutes at 4°C. After centrifugation, the supernatant will be the S9 fraction which is made up

of the endoplasmic reticulum and cytosol of the cell. The pellet will be cellular debris containing cell membranes, mitochondria, lysosomes, nuclei, etc. To separate the S9 into microsomes (endoplasmic reticulum) and cytosol, the S9 is centrifuged at 100 000 x g in an ultracentrifuge for an hour at 4°C. After ultracentrifugation, the supernatant will be the contents of the cytosol (in buffer), and the pellet is the microsomes. The microsomes are then typically resuspended in 3x volume of buffer at an appropriate pH.

In our experiment, we are particularly interested in the cytosolic GSTs, so we'll be using mouse liver cytosol purchased from a commercial vendor (Sekisui Xenotech). This cytosol consists of separate pools of male (n=1637) and female (n=800) CD1 mice, and is provided at 10 mg/mL in a buffer consisting of 50 mM Tris-Cl, 150 mM KCl, and 2 mM EDTA.

Sex and Metabolism

Women have historically underrepresented in clinical trials; however, we know that there are significant differences in drug metabolism between men and women. In recent years there has been a push to ensure more equal representation, however the effect of sex on drug metabolism and pharmacokinetic remains a concern. There are a number of physiological reasons for these differences including pharmacokinetic factors such as the volume of distribution typically being smaller in women; and pharmacodynamic factors, such as differences in receptor number, binding, and signalling pathways (Soldin and Mattison, 2009).

The CYP450 phase I conjugating enzymes show a sex-dependent difference in activity, with these enzymes typically being more active in men, however there are some exceptions. CYP2D6, the enzyme responsible for metabolizing >20% of prescription drugs, such as codeine is more active in women. However, this particular enzyme also shows substantial genetic polymorphism in many populations. Many phase II conjugating enzymes also show differing activity between sexes, with UGTs, sulfotransferases, and methyltransferases all having greater activities in men than in women.

Sex is the property or quality by which organisms are classified as female or male on the basis of their reproductive organs and functions. Although sex is usually categorized as female or male, there is variation in the biological attributes that comprise sex, and how those attributes are expressed.

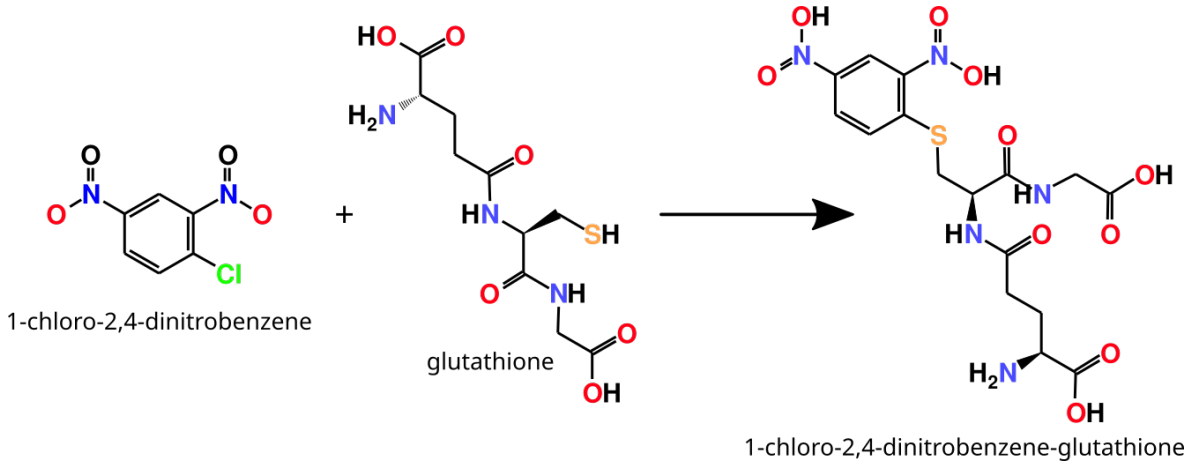
Gender is a social construct that is largely culturally determined, and encompasses the societal expectations, behaviours, expressions and identities of women, men, and gender diverse people. It influences perceptions of both self and others, and how people interact in society. Gender identity is not confined to the binary man/woman, but rather exists on a continuum and can change over time.

These sex differences are also seen in animal models, however they are not always the same as what are seen in humans, so caution is needed when trying to translate work done in animals to humans.

The Beer-Lambert Law

The Beer-Lambert law correlates the absorbance at a specific wavelength of a substance with its concentration, how strongly it absorbs light at a given wavelength per molar concentration, and how far the light beam passes through the sample. This is represented by the formula: $A_\lambda = \varepsilon c l.$ Here A is the absorbance at a particular wavelength, ε is the molar attenuation coefficient ($M^{-1}cm^{-1}$), c is the concentration (M), and l is the pathlength (cm). The molar attenuation coefficient (also commonly called the molar extinction coefficient) is determined empirically for a particular chemical by calculating the slope of the absorbance vs. concentration plot. This is specific for a particular wavelength and is sensitive to buffer composition and things that might affect the absorbance of a compound.

In this lab we will investigate the effect of sex and pH on the activity of mouse cytosolic GSTs. Total GST activity can be assessed using the general substrate 1-chloro-2,4-dinitro benzene (CDNB).



Following the conjugation of glutathione to this molecule during conjugation, the metabolite can be detected using absorbance at 340 nm, and the amount produced determined using the Beer-Lambert law and the extinction coefficient ε= 9600 M^{-1} cm^{-1} (González et al., 1989; Habig and Jakoby, 1981; Habig et al., 1974).

b) Experimental Design

Preparation of phosphate buffers

In order to test the effect of pH on our reactions, we need to make buffers at the appropriate pH. In the lab we have **0.500 M stock solutions** of potassium phosphate monobasic (KH_2PO_4), and potassium phosphate dibasic (K_2HPO_4). Calculate how much of each stock you would need to make **0.100 L** of buffer at pH 5.5 and pH 7.5. Remember that the **pK_a** values for phosphoric acid are 2.12, 7.21, and 12.32. Although buffers are best when the pKa is within ~ ±1 of the pH, there is some wiggle room and it is generally still ok if you're within ~ ± 2.

$$pH - pK_a = log\left(\frac{[A^-]}{[HA]}\right) \qquad C_1V_2 = C_2V_2$$

- **Buffer pH 5.5:** 0.1 M potassium phosphate buffer, pH 5.5
  - K_2HPO_4: _____ mL → ___ mL + ___ µL
  - KH_2PO_4: _____ mL → ___ mL + ___ µL
- **Buffer pH 6.5:** 0.1 M potassium phosphate buffer, pH 6.5 **[made by teaching staff]**
  - K_2HPO_4: __3.263__ mL → 3 mL + 263 µL
  - KH_2PO_4: __16.737__ mL → 16 mL + 737 µL
- **Buffer pH 7.5:** 0.1 M potassium phosphate buffer, pH 7.5
  - K_2HPO_4: _____ mL → ___ mL + ___ µL
  - KH_2PO_4: _____ mL → ___ mL + ___ µL

Procedure:

1. Calculate the amounts of potassium phosphate monobasic (KH_2PO_4), and potassium phosphate dibasic (K_2HPO_4) needed to make your buffer.
2. To a 100 mL beaker labeled with the identity of your solution, names, and date; add ~50 mL of pure reverse osmosis water (RO H_2O).
3. Add the appropriate amount of K_2HPO_4 or KH_2PO_4 using an appropriately sized serological pipette and/or micropipette, depending on the volume and directions of your TAs.
4. With the assistance of your TA, check the pH of your solution, and adjust it up with potassium hydroxide, or down with phosphoric acid, as needed.
5. Carefully pour your pH adjusted buffer into a 100 mL graduated cylinder and remove the stir bar.
6. Using pure RO H_2O, bring your buffer to a total volume of 100 mL.
7. Transfer your solution back into your labelled beaker.
8. Wash the stir bar and graduated cylinder in the sink by rinsing 3x with tap water and 2x with pure RO H_2O, then placing in the drying area or rack to dry.

GST Activity Reaction Setup

We will do our assays in triplicate, doing a separate assay for each sex, at different pHs. Each group will do an assay using either males or female mouse cytosol at your assigned pH. This way we will generate data on the GST activities at all three pHs for both males and females during the lab session (your TAs will use the pH 6.5 buffer to demonstrate the procedure.

A number of stock solutions are needed to prepare this assay. Due to the small amounts of chemical that need to be measured, the exact volume being made can only be calculated after the amount of drug weighed is known.

- 50 mM 1-chloro-2,4-dinitro benzene (CDNB) in DMSO (MW: 202.58)
    - Due to the hazardous nature of this chemical the Instructor or TAs will prepare this solution.
    - Only a very small volume of 50 mM CDNB is needed for our assay, but the minimum mass we should weigh on our balance is ~1 mg. How much of this stock solution would 1mg make?

Warning: CDNB is dissolved in DMSO, and is hazardous. It is toxic, corrosive, and hazardous to both human health and the environment.

Although we are using it in very small amounts, proper personal protective equipment (PPE) like gloves and a lab coat are essential when handling this stock solution!

It is occasionally used medically to induce a type IV hypersensitivity reaction and is sometimes used as an indirect measurement of T-cell activity in a patient.

- **10 mM Glutathione** (**GSH**) in 0.1 M potassium phosphate buffer (MW: 307.32 g/mol)
    - This must be made separately for each pH being tested ($CV = \frac{m}{MW}$)
    - Mass in your tube: _____ mg, volume buffer needed to make 10 mM solution: _____ µL.
    - Store on ice until ready to use

- **20 mM Cibacron blue** (GST inhibitor) in 0.1 M potassium phosphate (MW: 840.11 g/mol)
    - This must be made separately for each pH being tested
    - Mass in your tube: _____ mg, volume buffer needed to make 10 mM solution: _____ µL.
    - Store on ice until ready to use

You will use **ONE** of the following:

- **0.05 mg/mL female CD1 mouse liver cytosol** in buffer
    - Cytosol is provided from the vendor at 10 mg/mL
    - The working stock (0.05 mg/mL) must be prepared using your buffer
    - Combine 2 µL of 10 mg/mL stock with _____ µL of buffer. Mix gently. Store on ice.

- **0.05 mg/mL male CD1 mouse liver cytosol** in buffer
    - Cytosol is provided from the vendor at 10 mg/mL
    - The working stock (0.05 mg/mL) must be prepared using your buffer
    - Combine 2 µL of 10 mg/mL stock with _____ µL of buffer. Mix gently. Store on ice.

Procedure:

NB: Each reaction will be done in triplicate – you will have filled a total of 9 wells (3x3 grid) when done.

1. Place a UV-transparent microplate on ice, and label the SIDE with your section, group, and date.
2. For each of your three reactions at your chosen pH combine the 1-chloro-2,4-dinitro benzene, liver cytosol, buffer, and cibacron blue (if needed) according to the chart below.

Component	Initial concentration	Final concentration	Standard reaction (µL)	Inhibitor control (µL)	No GSH control (µL)
1-chloro-2,4-dinitro benzene	50 mM	0.5 mM			
Liver cytosol	0.05 mg/mL	5 ng/µL			
Buffer	0.1 M	---			
Cibacron blue	20 mM	2 mM	---		---
Glutathione	10 mM	1 mM	10	10	0
Total volume	---	---	100	100	100

3. Record your plate load order in your worksheet.
4. Set up the plate reader for your run with the assistance of the instructor and/or TAs. Make sure to name the plate with your group & pH.
5. Incubate for 2 minutes at 37°C in the plate reader.
6. The instructor will very quickly add 10 µL of 10 mM GSH to each well except the no-GSH negative control wells. This must be done very quickly and accurately in order to get useable data.
7. Immediately place back in the plate reader, mix briefly but thoroughly using shaking function of the plate reader, then start the read.
    - It is critical that this be done quickly – the reaction starts the instant the GSH is added, and you are losing data every second the plate goes unread.
8. The reaction is monitored every **10s** for **5 minutes at 340 nm**.

Data analysis

The instructor will perform the data analysis for the lab and will upload a file containing the analysed data to Canvas which you can then use to complete your lab worksheet. The protocol given below is for your information only.

1. Save your data on the SpectraMax iD3 plate reader as a .sda file.
2. Export the data as a text file with the same name using the "column" format.
3. Create a spreadsheet with separate tabs for raw data, load order, cleaned data, slopes, and analyses.
4. Copy your data into the "raw data" tab.
5. Copy the plate loading diagram into the plate load order tab.
6. Copy the time data into the "cleaned" tab, and convert it into seconds.
7. Copy the absorbance data into the "cleaned tab" and give each well a descriptive name.
8. Copy the cleaned data (time & absorbances for each well) into the "slopes" tab.
9. Now we need to do linear regressions and determine the slopes for every well.
    1. Export the "slopes" tab as a .csv file named "gst.csv".
    2. Open R studio and import gst.csv
    3. Run the "slopes.R" script
    4. Open the resulting "gst_slopes.csv"
10. Copy and paste the sample, slope, intercept, and r^2 columns into the appropriate sections.
11. Use the equation of the line to calculate initial and final concentrations of the metabolite and determine the number of nmoles present in your 100 µL well volume.
12. Determine the rate of metabolism in nmol/min/mg for all samples
13. Create a graph comparing the average rates for all samples and determine the effect of sex and pH on total cytosolic GST metabolism.

c) References

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Laboratory 2: Drug metabolism

Instructor GST Metabolism data analysis guide

1. Export kinetic data as time format from Softmax Pro 7 software.
2. Open exported data in Excel and label this worksheet as “raw data”.
3. Copy time data into a new worksheet and convert from minutes:seconds to seconds
4. Copy this data into a new worksheet “slopes” under a column labeled “time”
5. Paste the group names (e.g. group1_5.5_male_std1) as headers into the “slopes” worktab.
6. Copy data from “raw data” into “slopes” under the appropriate column based on the student plate load order.
7. Export the “slopes” sheet as a CSV file (“gst.csv”, and save to downloads folder).
8. Open R and script “slopes.R”
    a. Run script.
    b. This script will clean our data and do a linear regression on all wells so that we can quickly determine the slope and intercept of each line of best fit.
    c. Script will save file “gst_slopes.csv”
9. Open file and copy data into analysis sheet
10. Calculate the average µmol/min/mg, standard deviation, and %CV for each well of sample (54 wells total for all treatments).
    a. Using the equation of the line, calculate the absorption at Ti (slope*time i[0s])+intercept
    b. Using the equation of the line, calculate the absorption at Tf (slope*time f[300s])+intercept
    c. Calculate the initial concentration: (Calculated absorption at Ti)/(molar extinction coefficient * pathlength)
        i. Note that you may need to correct for the pathlength when using a plate reader and not a 1 cm cuvette.
    d. Calculate the final concentration: (Calculated absorption at Tf)/(molar extinction coefficient * pathlength)
    e. Calculate the change in concentration (concentration final – concentration initial)
    f. Convert this concentration from M to mM.
    g. Calculate the number of mmol in 100 µL (conc [mM] * 0.0001 µL/L)
    h. Calculate the number of nmoles in 100 µL (mmol/100µL * 1 000 000 nmol/mmol)
    i. Calculate nmol/min (nmol / (time [s]/60))
    j. Calculate nmol/min/mg (nmol/min / 0.0005 mg)
    k. Calculate the average nmol/min/mg from the triplicates for each sample. Any rates that are less than zero can be set to zero, it is not possible to have a negative rate of metabolism in this assay, and negatives are due to experimental error.
    l. Calculate the standard deviation and percent variance for each sample.
11. Create a graph of the average nmol/min/mg data
12. If desired, a one-way ANOVA can be performed to examine if the differences between any of the groups are statistically significant.
    a. In a separate worksheet labeled “stats”, copy the sample names to a column “g” (18 sample names – 3 for each of the 6 conditions)
    b. Copy the activity data to a column “activity”
    c. Export stats tab as CSV file “gststats.csv” to downloads folder
    d. Run “gstStats.R”
    e. Copy data to stats page and write out meaning by group. Note: not all of these students have taken a statistic course at this point in time (but will eventually as part of their degree).

slopes.R

```
# import data from csv file
gst <- read.csv("~/Downloads/gst.csv")

# extract column names
cols <- names(gst)

#remove the time column
colsn <- cols[cols!="time"]

# add ~ t to each column name in prep for using lapply
colsnt <- paste(colsn, '~ time')

# do linear regression on all samples and get summary data
# slope.lm <- lapply(colsnt, function(x) summary(lm(x, data = gst)))

# do linear regression on all samples and get extracted slope, intercept, r2
m <- lapply(colsnt, function(x) summary(lm(x, data = gst))$coefficients[2,1])
b <- lapply(colsnt, function(x) summary(lm(x, data = gst))$coefficients[1,1])
r2 <- lapply(colsnt, function(x) summary(lm(x, data = gst))$r.squared)

# create a list of columns for dataframe
data.list <- list(colsn, m, b, r2)

#create dataframe
frame <- as.data.frame(do.call(cbind, data.list))

#give columns descriptive names
colnames(frame) <- c("sample", "slope", "intercept", "r2")

#coerce data.frame to all-character
frame2 <<- data.frame(lapply(frame, as.character), stringsAsFactors=FALSE)

#export data as a .csv file
write.csv(frame2, "~/Downloads/gst_slopes.csv", row.names=FALSE)
```

gstStats.R

```
# import data from csv file
gststats <- read.csv("~/Downloads/gststats.csv")

# do analysis of variance by group
gst.aov <- aov(activity ~ g, data = gststats)

# display summary
TukeyHSD(gst.aov)
```

 Name:

Laboratory 2 – Drug Metabolism Worksheet [35 points total]
Due: In lecture, the day after lab.

Preparation of Phosphate buffers [14 points]

1. How much 0.5 M potassium phosphate monobasic (KH_2PO_4), and 0.5 M potassium phosphate dibasic (K_2HPO_4) are needed to make 0.1 M potassium phosphate buffer at pH 5.5 and 6.5. Give your answers in the spaces provided.

   a. H_2PO_4 has three pK_a values: **2.12, 7.21, and 12.32**. Circle the one that you should use to make the buffers we are using in today's lab. **[1 point]**

   b. Buffer pH 5.5: 0.1 M potassium phosphate buffer, pH 5.5
      i. Calculate the concentration needed of the conjugate acid and the conjugate base **[2 points]**

      ii. Calculate the volumes needed of the conjugate acid and the conjugate base to make your buffer. **[2 points]**

      K_2HPO_4: _____ mL → _____ mL + _____ µL

      KH_2PO_4: _____ mL → _____ mL + _____ µL

c. Buffer pH 7.5: 0.1 M potassium phosphate buffer, pH 7.5

i. Calculate the concentration needed of the conjugate acid and the conjugate base **[2 points]**

ii. Calculate the volumes needed of the conjugate acid and the conjugate base to make your buffer. **[2 points]**

K_2HPO_4: _____ mL → _____ mL + _____ µL

KH_2PO_4: _____ mL → _____ mL + _____ µL

2. What pH buffer did your group make? [1]: _____

3. What was the purpose of dividing our volumes of 0.5 M potassium phosphate monobasic (KH_2PO_4), and 0.5 M potassium phosphate dibasic (K_2HPO_4) into mL and µL when making our solutions? [1]

4. Why is it important to routinely calibrate the pH metre? [1]

5. Why don't you bring your solution to its final volume before adjusting the pH? [1]

6. Why don't we need to sterilize this solution? [1]

GST Activity Reaction Setup [14 points]

7. What is the purpose of doing our assay in triplicate? [1]

8. 1-chloro-2,4-dinitro benzene (CDNB) is hazardous. What do the symbols on the bottle mean? [2]

Stock solution calculations:

9. What is the mass of GSH in your tube? [1]: _____

10. How much buffer do you need to add to make a 10 mM stock given that the MW of GSH is 307.32 g/mol? [1]

11. What is the mass of cibacron blue in your tube? [1]: _____

12. How much buffer do you need to add to make a 20 mM stock given that the MW of Cibacron blue is 840.11 g/mol? [1]

13. How much buffer did you need to add to your 2 µl of stock protein to make your 0.05 mg/ml solution of CD1 mouse cytosol? [1]: _____

14. Using the provided information, complete the reaction table below. You have been supplied the initial and final concentration of each reagent, as well as the final reaction volume. Using the same formulas we discussed in lab 1, determine how much of each reagent is required in µL. [5]

Component	**Initial concentration**	**Final concentration**	**Standard reaction (µl)**	**Inhibitor control (µl)**	**No GSH control (µl)**
1-chloro-2,4-dinitro benzene	50 mM	0.5 mM			
Liver cytosol	0.05 mg/ml	5 ng/µL			
Buffer	0.1 M	---			
Cibacron blue	20 mM	2 mM	---		---
Glutathione	10 mM	1 mM	10	10	0
Total volume	---	---	100	100	100

15. Record your plate load order below **[1]**

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Data analysis [7 points]

16. Based on the information provide in lecture, your lab manual, and by your TAs, write a hypothesis about what effect biological sex and buffer pH will have on the activity of the cytosolic GSTs in our assay. [2]

17. Which reaction type had the best r^2 value, circle one: standard, inhibitor control, no GSH control **[1 point]**

18. Base on the results uploaded to Canvas, how well did our results match your predictions?
    a. Was there be a difference between males and females? If there was a difference, explain which sex had more activity and provide a potential reason as to why we might see this difference. If there is no difference, discuss likely reasons why this is the case. [2]

    b. Did pH have an effect on enzyme activity in either, or both sexes of mice? Discuss why you did or did not see a difference, and if there was a difference, which pH had the highest activity in male or female mice. [2]