

TED ANKARA COLLEGE FOUNDATION
PRIVATE HIGH SCHOOL

Comparison of destabilizing activities of a well-known $\alpha\beta$ -fibril destabilizer
rifampicin with methylene blue for its usage in Alzheimer's disease treatment

BIOLOGY EXTENDED ESSAY

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Word Count: 3987

ABSTRACT

The aim of this experiment was to determine whether methylene blue has a destabilizing effect on $\alpha\beta$ -fibrils or not for the usage in Alzheimer's disease treatment. So that this extended essay includes the comparison of the destabilizing effect of methylene blue with rifampicin which is a well-known $\alpha\beta$ -fibril destabilizer.

My research question was "Does the destabilizing effect of methylene blue on A β -fibril formation in vitro sufficient for its usage in the Alzheimer's disease's treatment compared to Rifampicin?"

My hypothesis was "Methylene blue can destabilize A β -fibril formation which is a symptom of Alzheimer's disease in vitro as much as Rifampicin."

In order to answer the research question and test the hypothesis, Ellman's spectrophotometric method and thioflavin-t fluorometric method was used. Ellman's spectrophotometric method was to calculate the IC₅₀ value of methylene blue which will be used in the second part. Second part was the comparison of the fluorescence concentrations at the end of the 24th hour. Destabilizers at their IC₅₀ values were put into bathtubs and the bathtubs were put into bacteriological incubator. At the end of the 24th hour tubes were put into fluorometer device and the fluorescence concentrations were measured. As fluorescence concentration is directly proportional with $\alpha\beta$ -fibril formation this measurement gave the necessary data about the destabilizing activity of methylene blue and rifampicin.

At the end of the 24th hour fluorescence concentrations in the tubes that contain methylene blue and rifampicin were close to each but t-test results showed that there is difference between them. After the calculation of percentage effects of the destabilizer, the difference was more observable: the effect of methylene blue was more compared to rifampicin. This gives hope about the treatment of Alzheimer's disease but also brings new questions to investigate.

Word count:287

Table of Contents

Introduction	1
Hypothesis	4
Method, Development & Planning	5
Material List	8
Method	9
Data Collection and Processing	11
Conclusion and Evaluation	19
Appendices	
Appendix 1	22
Appendix 2	23
Appendix 3	24
Appendix 4	26
Appendix 5	29
Bibliography	30

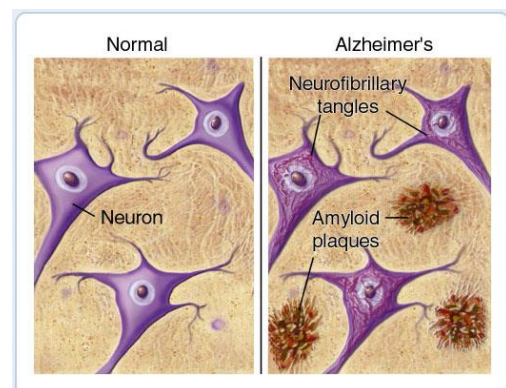
INTRODUCTION

4 years ago my grandfather diagnosed with Alzheimer's disease. He comes and stays with us with regular time intervals for his doctor controls. We have a dog for two years now but he forgets it and in his every visit he is surprised because of it. Also there are some notes everywhere on post-its like "Don't forget to extinguish the furnace!" or "Don't forget to close the cover of the refrigerator!". So I did a research about Alzheimer Disease; I learned that there are some symptomatic treatment options and some drug mechanism tests are being done.

To start with I got a complete definition; Alzheimer's disease (AD) is a progressive neurodegenerative disease that occurs at hippocampus and cortex regions of the brain, associated with irreversible loss of neurons leading to deterioration in cognitive functions. It is characterized by memory loss; personality or behavioral disorders; difficulties in speaking, direction finding, decision making, thinking and interpreting.¹

As a summary of the work and research I had done, it can be said that Alzheimer's disease is thought to occur at brain with three major modifications;

- Loss of nerve cells,
- Intercellular protein deposits consist of extremely phosphorylated tau proteins which stabilize microtubules,
- Extracellular protein deposits which are called amyloid plaques which I will be mentioning as A β -fibril formation in further parts.²



I learnt that although Alzheimer's disease is one of the most studied diseases in recent years there is no effective treatment yet. There are some symptomatic treatment options and the most common way is the usage of cholinesterase inhibitors by maintaining acetylcholine levels since Alzheimer's disease pathogenesis has been linked to a deficiency in the brain neurotransmitter acetylcholine.

My research showed me that cholinesterases are widely distributed enzymes divided into two groups according to their substrate specificities and kinetic behaviors towards to their inhibitors.³ Butrylcholinesterase hydrolyze butrylcholine. Acetylcholinesterase hydrolyzes

¹ Nussbaum R.L., Ellis C.E. Alzheimer's Disease and Parkinson's Disease. *N Engl J Med.*, 348, 1356-1364, (2003)

² Robinson S.R., Bishop G.M., A β as bioflocculant: implications for the amyloid hypothesis of Alzheimer's Disease. *Neurobiol Aging*, 23,1051-1072, (2002)

³ Maurstad G., Prass M., Serpell L.C., Sikorski P. Dehydration stability of amyloid fibrils studied by AFM. *Eur Biophys J.* 38(8): 1135-40, (2009)

acetylcholine to acetic acid and choline. Hydrolysis of acetylcholine is a necessary reaction to allow a neuron to return to its resting state after activation.

Experiments and research which have carried out until today exposed that new effective cholinesterase inhibitors are needed for Alzheimer's Disease treatment since the treatment effect of those in use is modest and, there is evidence of wide variability in the outcomes reported. Moreover, there is no known A β -fibril destabilizing agent in use for Alzheimer's Disease treatment yet.

According to the information that I collected during my research, cholinesterase inhibitors are being used for Alzheimer's disease treatment and I learnt that Methylene blue is one of the substances that can inhibit acetylcholinesterase. Methylene Blue is a dye for a number of different staining procedures in Biology recently shown to inhibit cholinesterases⁴ and my project aims to determine the inhibitory activity of methylene blue on cholinesterase and to investigate the destabilizing effect of this inhibitor on A β -fibril formation in vitro⁵. In case that my study demonstrates that methylene blue may inhibit the A β -fibril formation in vitro, methylene blue may be presented as a promising novel agent in Alzheimer's disease treatment with dual effects as acetylcholinesterase inhibitor and an A β -fibril destabilizer. When the action mechanism of Alzheimer's disease is thought, Methylene blue can inhibit the loss of nerve cells and also destabilize the extracellular protein deposits.

Currently, there are more than 100 clinical trials being conducted for the treatment of Alzheimer's disease and there is a thought that methylene blue can inhibit the acetylcholinesterase and A β -fibril formation in vitro so that it can be a new treatment option. I will try to support the idea that Methylene Blue can be used as an option in the treatment process of Alzheimer's disease by a comparison of Methylene Blue with Rifampicin.

Rifampicin is a semisynthetic compound derived from *Amycolatopsis rifamycinica* that is used in the treatment of a number of bacteria.⁶ Also it is a known A β -fibril destabilizer that is used in Alzheimer's disease's treatment. So I thought that comparing the destabilizing activity of Methylene Blue with Rifampicin will give an idea about the possibility of using Methylene Blue in Alzheimer's disease's treatment.

⁴Kucukkilinc Tuylu, T., Ozer, I. (2007), Multi-site inhibition of human plasma cholinesterase by cationic phenoxazine and phenothiazine dyes. Archives in Biochemistry and Biophysics, Volume 461, Issue 2, Pages 294-298.

⁵ In vitro studies are studies in experimental biology that are conducted using components of an organism that have been isolated from their usual biological context in order to permit a more detailed or more convenient analysis than can be done with whole organisms.

"In Vitro." *Wikipedia, The Free Encyclopedia*

http://en.wikipedia.org/wiki/In_vitro

⁶Sensi P, Margalith P, Timbal MT (1959). "Rifomycin, a new antibiotic—preliminary report". *Farmaco Ed Sci* 14: 146–147.

Therefore, my research question is “Does the destabilizing effect of methylene blue on A β -fibril formation in vitro sufficient for its usage in the Alzheimer’s disease’s treatment compared to rifampicin?”

HYPOTHESIS

As I search, there is no definite cure or treatment for Alzheimer's disease so I believe that the main targets in the treatment process of those patients is to improve the life standards and maximize the operational performance by correcting their cognitive functioning, mood and behavior.

As I mentioned in introduction, for symptomatic treatment of Alzheimer's Disease, generally cholinesterase inhibitors are preferred to maintain acetylcholine level.

The cholinesterase inhibitors donepezil hydrochloride, galantamine hydrobromide, and rivastigmine tartrate are the current mainstays of symptomatic treatment for patients with Alzheimer's Disease. In clinical trials for all three agents, beneficial effects on standard measures of cognitive and global function have been observed in patients with mild to moderate Alzheimer's Disease. Although none of the cholinesterase inhibitors has been approved for treatment of patients in advanced stages of Alzheimer's Disease, all three agents have had beneficial cognitive effects among patients with less severe forms of the disease.⁷

Methylene blue is one of the substances which can inhibit cholinesterase activity like the ones I have mentioned in the upper paragraph⁸ and this means that it may be used in symptomatic treatment of Alzheimer's Disease but there is still no information about the effect of Methylene Blue on that A β -fibril formation. My project aims to show that A β -fibril formation which is another symptom of Alzheimer's Disease may be inhibited by methylene blue in vitro. The comparison will be made with a well-known A β -fibril destabilizer, Rifampicin.

Since Methylene Blue can inhibit acetylcholinesterase activity it is thought that it may also inhibit A β -fibril formation⁹. Therefore it can be hypothesized that methylene blue can destabilize the A β -fibril formation in vitro as much as rifampicin.

⁷ Jay M. Ellis, DO, Director, Neuroscience Research of the Berkshires, 100 Wendell Ave, Pittsfield

<http://www.jaoa.org/content/105/3/145.abstract>

⁸ Kucukkilinc Tuylu, T., Ozer, I. (2007), Multi-site inhibition of human plasma cholinesterase by cationic phenoxazine and phenothiazine dyes. Archives in Biochemistry and Biophysics, Volume 461, Issue 2, Pages 294-298.

⁹ Kucukkilinc Tuylu, T., Ozer, I. (2007), Multi-site inhibition of human plasma cholinesterase by cationic phenoxazine and phenothiazine dyes. Archives in Biochemistry and Biophysics, Volume 461, Issue 2, Pages 294-298.

METHOD, DEVELOPMENT & PLANNING

After deciding to work on Alzheimer's Disease I talked to my grandfather's doctors and they guided me to the Pharmacy Faculty of Hacettepe since the laboratory opportunities in there are the best. So I send an e-mail to the head of the department of biochemistry Gülberk Uçar which includes all the necessary information about me and the extended essay. I got a response telling that they can help me. So I went to a meeting to meet and talk



Figure 1: Laboratory of Faculty of Pharmacy of Hacettepe University

the details with Gülberk Uçar, I learnt that I can use the laboratory and an expert pharmacist Tuba Tüylü Küçükkılınç can help me about my project.

Then we had meetings with her and when I mentioned her about my project, she told me that thioflavin-T fluorometric method could be used for the comparison of the destabilizing activities of Methylene Blue and Rifampicin. After this I learnt that IC_{50} values of the substances must be used for this kind of comparison as a procedure. IC_{50} value is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half.¹⁰ So I decided to use a standard method to find the IC_{50} value of Methylene blue and my research showed that the Ellman's spectrophotometric method is the most suitable way for me as methylene blue is a dye and this method gives the most accurate results on these kinds of substances

Methylene blue, rifampicin, acetylcholinesterase, acetylthiocholine, 3-(N-morpholino) propanesulfonic acid (MOPS) and 5,5'-ditiobis-(2-nitrobenzoic acid) (DTNB) are obtained by marketing from the medical firm Sigma.

As I learnt from her, the spectrophotometric method, which I will utilize in my experiment, is usually used for the experiments that include dyes such as Methylene Blue. The main principle of Ellman's spectrophotometric method is to determine the change in the absorbance which is directly proportional with color change. So that, to provide a color

¹⁰ "IC50." *Wikipedia, The Free Encyclopedia*
<http://en.wikipedia.org/wiki/IC50>

change in the bathtubs, Acetylthiocholine reacts with human acetylcholinesterase with the presence of 5,5'-dithio-bis-(2-nitrobenzoic acid), DTNB. DTNB is a substance that gives yellow color when it reacts with thiol so that human acetylcholinesterase will be made to react with acetylthiocholine instead of acetylcholine. This means that the absorbance will increase with the increasing reaction rate. The reaction must occur also in the presence of 3-(N-morpholino)propanesulfonic acid, MOPS, too to keep the pH constant.

There is more than one way to determine the inhibitory activity of Methylene Blue but spectrophotometric method is the more common one because of the easiness of its usage. Also it gives the data in a very short time and it is claimed to be that the data collected by this method is the most accurate one. Moreover, a peltier-equipped Shimadzu 1601 PC Spectrophotometer, keeps the internal temperature constant at desired value during the measurement.



Figure 2: Peltier-equipped Shimadzu 1601 PC Spectrophotometer



Figure 3: Inside of Peltier-equipped Shimadzu 1601 PC Spectrophotometer

As the standard procedure, Methylene Blue activity is observed by using three different concentrations and a control group. While adjusting the three different concentrations of Methylene Blue criteria is to take three values that are not close to each other to get a more accurate best line for the graph. Then the collected data will be used to draw a graph and the equation of the best line of the graph will be used to find IC_{50} value of Methylene Blue. IC_{50} value of Rifampicin is $100\mu M$.¹¹

¹¹ Kucukkilinc Tuylu, T., Ozer, I. (2007), Multi-site inhibition of human plasma cholinesterase by cationic phenoxazine and phenothiazine dyes. Archives in Biochemistry and Biophysics, Volume 461, Issue 2, Pages 294-298.

After these, thioflavin-T fluorometric method will be used to compare the destabilizing activities of Rifampicin and Methylene Blue. The main principle of thioflavin-T fluorometric method is to measure the fluorescence at a certain moment so that it can be used at experiments which include fluorescence change. Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation.¹² As Methylene blue or Rifampicin destabilizes A β -fibril formation fluorescence value will decrease because of the decreasing light emission of the plaques. To make the reaction occur NaCl (sodium chloride) will put into the test tubes and to stabilize the pH at high levels MKB (mono basic potassium phosphate, KH₂PO₄) will be added.



Figure 4: Fluorometer device



Figure 5: Inside of fluorometer device

Making the measurement at 0th, 2nd, 6th and 24th hours will give the opportunity to calculate and compare the fluorescence changes in three test groups. (Control group, Rifampicin group, Methylene Blue group) Destabilization of A β -fibrils cause a fluorescence change so that the thioflavin-T fluorometric method is preferred. Measuring the fluorescence change at 0th, 2nd, 6th and 24th hours will give the needed data to draw a second graph to compare the effects of

Rifampicin and Methylene Blue. Also there must be a control group which does not contain any inhibitor to see the inhibitor activity. Between the two measurements, bathtubs must be incubated at 37^oC which is the natural human body temperature.¹³

Furthermore, the primary reason why I have chosen these two methods is that these methods will provide me quantitative data that I need to compare Methylene blue with Rifampicin. Although there are more ways to get the needed data, I preferred these two methods because making the measurements with these devices are easy to learn and use. At the same time these two methods are two of the methods that give the most accurate data as I learnt from the experts at the laboratory.

¹² "Fluorescence." *Wikipedia, The Free Encyclopedia*
<http://en.wikipedia.org/wiki/Fluorescence>

¹³ Normal temperature is about 37^oC or 98.6^oF.
<http://www.healthcare-online.org/Normal-Body-Temperature.html>

MATERIAL LIST

10000 μL	3-(N-morpholino)propanesulfonic acid, (MOPS)
10500 μL	H_2O
2000 μL	5,5'-dithio-bis-(2-nitrobenzoic acid), (DTNB)
2000 μl	Acetylthiocholine, (ATC)
200 μL	Human acetylcholinesterase, (AChE) (Sigma)
25 μL	25 μM Methylene Blue, (MB) (Sigma)
150 μL	5 μM Methylene Blue, (MB) (Sigma)
10 μL	0.125 μM Methylene Blue, (MB) (Sigma)
3000 μL	$\text{A}\beta$ -fibril (Sigma)
7500 μL	100 μM Mono basic potassium phosphate – MKB, (KH_2PO_4)
1500 μL	1000 μM Sodium chloride, (NaCl)
50 μL	10 μM Rifampicin (Sigma)
$\times 25$	Bathtubs ($1 \times 1 \times 2 \text{cm}^3$)
$\times 1$	1000 μL micropipette
$\times 1$	200 μL micropipette
$\times 1$	20 μL micropipette

Refrigerator

Bacteriological incubator

Peltier-equipped Shimadzu 1601 PC
Spectrophotometer

Fluorometer device



Figure 6: Materials that are used in the experiment (inside the refrigerator)

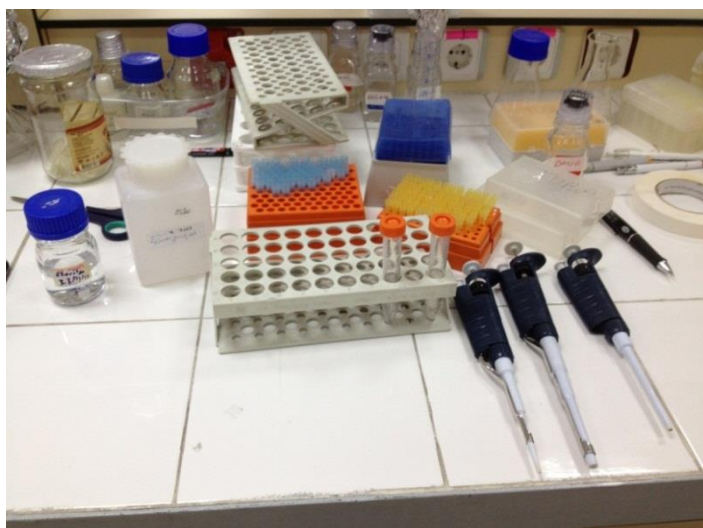


Figure 7: Materials that are used in the experiment

METHOD

A) CALCULATION OF IC₅₀ VALUE OF METHYLENE BLUE (ELLMAN'S SPECTROPHOTOMETRIC METHOD)^{14,15}

1) To create a control group; put the substances below into 5 bathtubs.

- 500 µL MOPS,
- 390 µL water,
- 100 µL ATC
- 100 µL DTNB
- 10 µL AChE

2) Put the substances below into other 15 bathtubs.

- 500 µL MOPS,
- 370 µL water,
- 100 µL ATC
- 100 µL DTNB
- 10 µL AChE

3) -Add 5µL 25µM methylene blue into 5 of those 20. (to have 125nM finally)

-Add 15µL 5µM methylene blue into 5 of those 20. (to have 75nM finally)

-Add 2µL 0.125µM methylene blue 5 of those 20. (to have 0.25nM finally)

4) Measure the absorbance values with a Peltier-equipped Shimadzu 1601 PC Spectrophotometer.

5) Draw a graph by using the measured absorbance values and calculate IC₅₀ value by using the equation of the best line of the graph.

¹⁴ See Appendix 3

¹⁵ See Appendix 4

**B) MEASURING FLOURESCENCES OF THE SOLUTIONS CONTAINING
DIFFERENT DESTABLISERS****(THIOFLAVIN-T FLUOROMETRIC METHOD)¹⁶**

1) To create a control group; put the substances below into 5 bathtubs.

-200 μL A β -fibril

-190 μL water,

-500 μL 100 μM KP

-100 μL 1000 μM NaCl

2) Put the substances below into other 10 bathtubs.

-200 μL A β -fibril

-190 μL water,

-500 μL 100 μM KP

-100 μL 1000 μM NaCl

3) - Add 10 μL 10 μM Rifampicin into 5 of those 10. (to have 100nM finally)

- Add 14 μL 5 μM Methylene blue 5 of those 10. (to have 70nM finally)

4) Measure the fluorescence values with the fluorometer device.

5) Record the data (initial fluorescence concentration)

5) Put the bathtubs into the bacteriological incubator and adjust the temperature to 37 $^{\circ}\text{C}$.

6) Measure the fluorescence values at 2 $^{\text{nd}}$, 6 $^{\text{th}}$ and 24 $^{\text{th}}$ hours with the fluorometer device.

7) Record the data.

¹⁶ See Appendix 5

DATA COLLECTION AND PROCESSING

	TYPE OF DESTABLIZER	FLUORESCENCE CONCENTRATION($\pm 0.001\mu\text{M}$)														
		NO DESTABLIZER (CONTROL GROUP)					RIFAMPICIN					METHYLENE BLUE				
		TRIAL	1	2	3	4	5	1	2	3	4	5	1	2	3	4
TIME (HOUR)	0	0.135	0.136	0.136	0.135	0.133	0.168	0.165	0.166	0.166	0.165	0.161	0.159	0.158	0.159	0.158
	2	0.824	0.822	0.821	0.821	0.822	0.160	0.160	0.159	0.160	0.161	0.162	0.163	0.162	0.163	0.160
	6	0.937	0.935	0.935	0.936	0.937	0.032	0.034	0.033	0.032	0.034	0.039	0.041	0.041	0.040	0.039
	24	0.932	0.936	0.935	0.933	0.934	0.048	0.045	0.046	0.045	0.046	0.040	0.041	0.038	0.038	0.038

Table 1: Table 1 shows the fluorescence concentrations at the beginning and at the end of the 2nd, 6th and 24th hours in 5 trials which are measured to check that the destabilizers are working.

After these measurements to observe the change more clearly average fluorescence concentrations are calculated by using the method below:

$$f_{c_{average}} = \frac{f_{c_1} + f_{c_2} + f_{c_3} + f_{c_4} + f_{c_5}}{5}$$

Where;

f_c is fluorescence concentration

x in f_{c_x} is trial number

Example calculation:

(Average fluorescence concentration calculation for

time=2 hours and

destabilizer= methylene blue)

$$f_{c_{average}} = \frac{f_{c_1} + f_{c_2} + f_{c_3} + f_{c_4} + f_{c_5}}{5} = \frac{0.162 + 0.163 + 0.162 + 0.163 + 0.160}{5} = 0.162$$

		AVERAGE FLUORESCENCE CONCENTRATION ($\pm 0.001\mu\text{M}$)		
		NO DESTABLIZER (CONTROL GROUP)	RIFAMPICIN	METHYLENE BLUE
TIME (HOUR)	0	0.135	0.166	0.159
	2	0.822	0.160	0.162
	6	0.936	0.033	0.040
	24	0.934	0.046	0.039

Table 2: Table 2 shows the average fluorescence concentrations at the beginning and at the ends of 2nd, 6th and 24th hours.

TYPE OF DESTABILIZER	TRIAL	FLUORESCENCE CONCENTRATION ($\pm 0.001\mu\text{M}$)	TOTAL TIME (hour)	TYPE OF FIBRIL	VOLUME OF FIBRIL ($\pm 0.05\mu\text{L}$)	VOLUME OF KP ($\pm 0.05\mu\text{L}$)	CONCENTRATION OF KP (μM)	VOLUME OF NaCl ($\pm 0.05\mu\text{L}$)	CONCENTRATION OF NaCl (μM)	VOLUME OF H ₂ O ($\pm 0.05\mu\text{L}$)	TYPE OF WATER	FINAL CONCENTRATION OF DESTABILIZER (nM)	WAITING TEMPERATURE ($\pm 0.05^\circ\text{C}$)
Methylene blue	1	0.040	24	A β -fibril (mark: Sigma)	200.00	500.00	100	100.00	1000	190.00	Pure water	70 (IC ₅₀ value)	37.00
	2	0.038											
	3	0.038											
	4	0.041											
	5	0.038											
	AVERAGE	0.039											
Rifampicin	1	0.048	24	A β -fibril (mark: Sigma)	200.00	500.00	100	100.00	1000	190.00	Pure water	100 (IC ₅₀ value)	37.00
	2	0.046											
	3	0.045											
	4	0.046											
	5	0.045											
	AVERAGE	0.046											
No destabilizer (Control group)	1	0.932	24	A β -fibril (mark: Sigma)	200.00	500.00	100	100.00	1000	190.00	Pure water	-	37.00
	2	0.934											
	3	0.935											
	4	0.933											
	5	0.936											
	AVERAGE	0.934											

Table 3: Table 3 shows the fluorescence concentrations in the bathtubs which contain different destabilizers (Rifampicin and Methylene Blue) at their IC₅₀ values when Sigma marked 200 μL A β -fibril, 500 μL 100 μM KP, 100 μL 1000 μM NaCl, 190 μL pure water are used and the waiting temperature is constant at 37 $^\circ\text{C}$.

FLOURESCENCE CONCENTRATION (μM)			
	INHIBITOR TYPE		
TRIAL	- (CONTROL GROUP)	RIFAMPICIN	METHYLENE BLUE
1	0.932	0.048	0.040
2	0.936	0.045	0.041
3	0.935	0.046	0.038
4	0.933	0.045	0.038
5	0.934	0.046	0.038
MEAN OF THE FLOURESCENCE	0.934	0.046	0.039
MODE	-	0.045	0.039
MEDIAN	0.934	0.046	0.039
RANGE	0.004	0.003	0.003
VARIANCE	0.000	0.000	0.000
SD	0.002	0.001	0.001
SE	0.001	0.001	0.001
t	2.776	2.776	2.776
%95CI (SE X T(0,05,df))	0.002	0.002	0.002
%95CI (EXEL)	0.001	0.001	0.001

Table 4: Table 4 shows the fluorescence values in the bathtubs after 24 hours with the mean, mode, median, range, variance, standard deviation, standard error, t and 95% confidence values of the collected data.

t-Test: Two Sample for Means

	<i>Variable 1</i>	<i>Variable 2</i>
Average	0.046	0.039
Variance	0.0000015	0.000002
Observation	5	5
Pearson Correlation	0.144337567	
Estimated Average Difference	0	
Df	4	
t Stat	9.036961141	
P(T<=t) one-tailed	0.000415322	
t Critical one-tailed	2.131846782	
P(T<=t) two-tailed	0.000830645	
t Critical two-tailed	2.776445105	

Table 5: t-test: two sample for means of the fluorescence concentrations in the bathtubs that contain one of methylene blue or rifampicin at the end of 24th hour.

P value (written in bold) is smaller than 0.05 and this means that there is a difference between two groups.

To see the difference; with a simple calculation percentage effect of destabilizer on fluorescence concentration can be found:

$$ed = \frac{|\Delta fc|}{fc_{initial}} \times 100 = \frac{|fc_{final} - fc_{initial}|}{fc_{initial}} \times 100$$

Where;

ed is percentage effect of destabilizer

fc is fluorescence concentration

Example calculation:

(for methylene blue)

$$ed = \frac{\Delta fc}{fc_{initial}} \times 100 = \frac{fc_{final} - fc_{initial}}{fc_{initial}} \times 100$$

$$ed = \frac{0.039 - 0.159}{0.159} \times 100$$

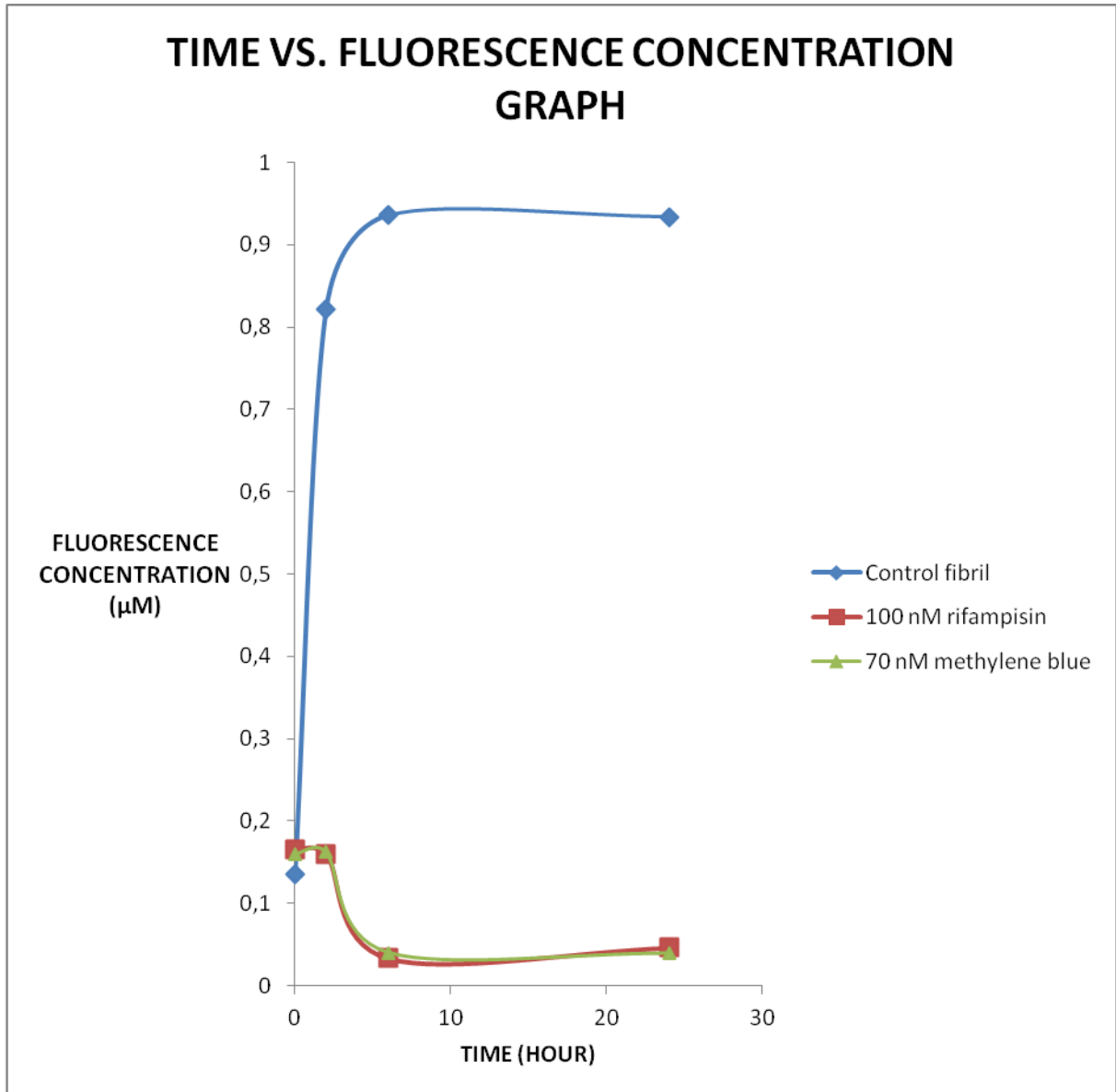
$$ed = \frac{-0.12}{0.159} \times 100$$

$$ed = -75.4716981$$

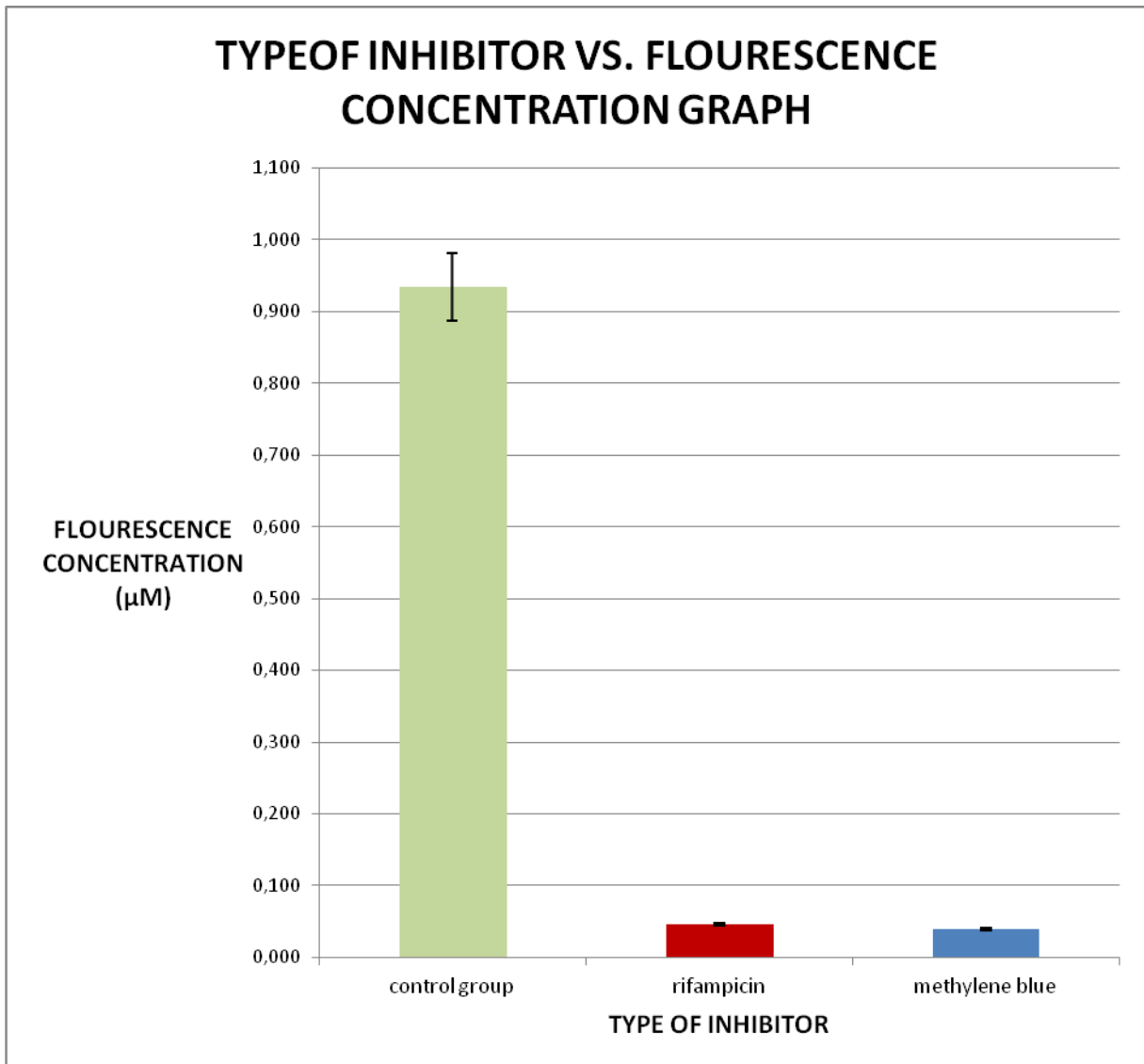
$$\approx -75.472\%$$

TYPE OF DESTABLIZER	PERCENTAGE EFFECT OF DESTABILIZER ON FLUORESCENCE CONCENTRATION (%)
NO DESTABLIZER (CONTROL GROUP)	+591.852
RIFAMPICIN	-72.289
METHYLENE BLUE	-75.472

Table 6: Percentage effect of the destabilizer at the end of the 24th hour where “-“ means decrease in fluorescence concentration and “+” means increase in fluorescence concentration.



Graph 1: Graph 1 shows the change in the fluorescence concentrations in the tube due to time and destabilizer effect where the blue line is for control group (which includes no destabilizer), red line is for rifampicin and green line is for methylene blue.



Graph 2: Graph 2 shows the average fluorescence concentrations in the bathtubs that contains rifampicin or methylene blue or none of them (control group) at the end of the 24th hour.

CONCLUSION AND EVALUATION

Alzheimer's disease (AD) is a progressive neurodegenerative disease that occurs at hippocampus and cortex regions of the brain, associated with irreversible loss of neurons leading to deterioration in cognitive functions.¹⁷ One of the major modifications that Alzheimer's disease causes is extracellular protein deposits ($\alpha\beta$ -fibril formation).

As there is no known $A\beta$ -fibril destabilizer agent that can be used for Alzheimer's disease treatment yet, this experiment was about testing the destabilizing activity of Methylene blue for its usage in Alzheimer's disease's treatment in the way that it can destabilize $A\beta$ -fibril formation and also inhibit acetylcholinesterase.

In this experiment my aim was to show that Methylene blue can destabilize $A\beta$ -fibril formation like Rifampicin (a well-known $A\beta$ -fibril destabilizer which cannot be used in Alzheimer's disease treatment because of several reasons). To prove my hypothesis I used Ellman's Spectrophotometric method and thioflavin-T fluorometric method. Ellman's spectrophotometric method was to find the IC_{50} value of Methylene blue and thioflavin-T method was to measure the fluorescence concentration change which is directly related with $A\beta$ -fibril presence.

200 μ L $A\beta$ -fibril, 190 μ L water, 500 μ L 100 μ M KP and 100 μ L 1000 μ M NaCl were put into bathtubs and rifampicin or methylene blue is added to tubes. The fluorescence concentration was measured by a fluorometer device and data is recorded. Measurements were made at the beginning and at the ends of the 2nd, 6th and 24th hours to check if the destabilizers are working.

Table 1 contains the results that are obtained in trials and table 2 shows the calculated average values to see the pattern easily. In the control group which does not include any of the destabilizers the fluorescence concentration increases from the beginning to the end of the 6th hour. At the beginning fluorescence concentration is 0.135 μ M; it becomes 0.822 μ M at the end of 2nd hour and 0.936 μ M at the end of 6th hour. This shows that the $\alpha\beta$ -fibril formation occurs. Also in the first two hours, the fluorescence concentration increases 0.687 μ M which means that the formation rate is high in the first two hours when there is no destabilizer. On the other hand fluorescence concentrations in the tubes that contain one of the two destabilizers (methylene blue or rifampicin) decrease with time. Fluorescence concentration in the rifampicin containing tube is 0.166 μ M at the beginning. It becomes 0.160 μ M and 0.033 μ M as time passes. Fluorescence concentration in methylene blue containing tube increases 3 μ M in the first two hours but then it decreases to 0.040 μ M. This shows that both of the destabilizers, did destabilize the formation of $\alpha\beta$ -fibril but the small increase in the methylene blue containing tube means that the methylene blue becomes

¹⁷ Nussbaum R.L., Ellis C.E. Alzheimer's Disease and Parkinson's Disease. *N Engl J Med.*, 348, 1356-1364, (2003)

more active with the time unlike rifampicin. At the end of the 24th hour, fluorescence concentration in the control group is almost same with the value at the end of the 6th hour (0.934 μ M). This proves the presence of A β -fibril. The increase in the fluorescence concentration in rifampicin containing tube shows that the destabilizer becomes less effective after a time between the 6th and 12th hour. On the other hand it can be seen that the fluorescence concentration in methylene blue containing tubes are decreased between the 6th and 12th hours; it becomes 0.039 μ M. This means that the effect of methylene blue is still continuing.

Graph 1 was for visualizing the data obtained and given in table 2. In the graph it can be seen that the fluorescence concentration in control group bath tubes is increasing rapidly whereas the fluorescence concentrations of the other tubes which include rifampicin or methylene blue is decreasing. (Where increase in the fluorescence means increase in emission of fibrils and increase in emission of fibril means increase in volume of fibril whereas the decrease means the opposites.) Moreover, it is observable that the fluorescence concentration values in the tubes containing methylene blue or rifampicin are close to each other.

In graph 2 the fluorescence concentrations at the end of the 24th hour is compared. In graph 2 it can be seen that the fluorescence concentrations in the tubes that contain rifampicin and in the ones that include methylene blue are really close to each other. This shows that the destabilizing activity of methylene blue and rifampicin are close to each other. On the other hand it is not possible to make a detailed comparison of the destabilizing activity of rifampicin and methylene blue by just looking at the average results at the end of the 24th hour.

Table 3 shows the fluorescence concentrations in the bathtubs at the end of the 24th hour with the details of the trials. The fluorescence concentrations in the tubes that contain rifampicin are 0.048 μ M, 0.046 μ M, 0.045 μ M, 0.046 μ M, 0.045 μ M whereas the concentration in tubes containing methylene blue are 0.040, 0.038, 0.038, 0.041, 0.038 μ M. To compare the destabilizing activity of rifampicin and methylene blue collected data is analyzed by making a t-test. Although they look similar in the previous graphs and tables, p value is 0.000830645 which is smaller than 0.05 giving the result that they differ from each other.

To decide which was more effective at the end of the 24th hour percentage effects of the destabilizers are calculated. Percentage effect of methylene blue is -75.472%. This means that methylene blue decreased the formation of $\alpha\beta$ -fibril by 75.472% with respect to the beginning. Percentage effect of rifampicin is -72.289%. These percentages show that methylene blue was more effective at the end of the 24th hour and this shows that methylene blue is a good destabilizer.

These results give a positive thought and hope for the treatment of Alzheimer's disease.

On the other hand there were some errors:

- 1) First of all, to check if the experiment is working or not the bathtubs were taken out from the Bacteriological incubator at the end of the 2nd and 6th hours in which the temperature is stabilized at 37^oC. So that the change in the temperature might have affected the results.
- 2) Secondly, when Table 2 is observed carefully, it can be seen that the fluorescence concentration at the end of 2nd and 6th hour in bathtubs that contain rifampicin is less than the ones contain methylene blue. This means that Rifampicin destabilized more in the first 6 hours. But when we look at the results that is collected at the end of 24th hour fluorescence concentration in the tubes that contain methylene blue is less. This means that rifampicin is more active in the first two hours and methylene blue is more active after a point after 6th hour. (Rifampicin is no longer affective after a point after the 6th hour.)
- 3) When using spectrophotometer or fluorometer, transparency and cleaning of the tubes are the factors that affect the results. Although same tubes are used and the conditions were same for all trials, tubes were put into devices with gloves and this might have caused a decrease in transparency a little.

For further investigation on this subject and further applications of this experiment there are a few things that can be done to improve the experiment.

- 1) Six trials for each destabilizer should be made. In this way, 6th tube will be taken out to check if the experiment is working and that tubes will not be used for getting results.
- 2) 24th hour is not the best time to compare the destabilizing activity as the effect of rifampicin decreases earlier. Getting the results at the end of the 8th or 9th hour will be better to see the fluorescence concentrations when the effects of the destabilizer are at maximum level.
- 3) For not touching the tubes before the measurements to be sure that there is no decrease in transparency, tubes can be filled in the spectrophotometer/fluorometer after cleaning them carefully.

As a similar experiment has not performed before there is no quantitative data to compare the results. Further studies will give the opportunity to compare the results and to comment on them. Although this results give hope about the treatment of Alzheimer's disease in the way that methylene blue can destabilize the $\alpha\beta$ -fibril formation, later studies on this should test the availability of methylene blue in treatment of Alzheimer's disease in humans by looking for the side effects and trying to reduce them.

APPENDIX 1

UNITS

PREFIX	SYMBOL	10^n	EXAMPLE
yotta	Y	10^{24}	1YM= 10^{24} M
zetta	Z	10^{21}	1ZM= 10^{21} M
exa	E	10^{18}	1EM= 10^{18} M
peta	P	10^{15}	1PM= 10^{15} M
tera	T	10^{12}	1TM= 10^{12} M
giga	G	10^9	1GM= 10^9 M
mega	M	10^6	1MM= 10^6 M
kilo	k	10^3	1kM= 10^3 M
hector	h	10^2	1hM= 10^2 M
deca	da	10^1	1daM= 10^1 M
		10^0	1M
deci	d	10^{-1}	1dM= 10^{-1} M
centi	c	10^{-2}	1cM= 10^{-2} M
milli	m	10^{-3}	1mM= 10^{-3} M
micro	μ	10^{-6}	1μM=10^{-6}M
nano	n	10^{-9}	1nM=10^{-9}M
pico	p	10^{-12}	1pM= 10^{-12} M
femto	f	10^{-15}	1fM= 10^{-15} M
atto	a	10^{-18}	1aM= 10^{-18} M
zepto	z	10^{-21}	1zM= 10^{-21} M
yocto	y	10^{-24}	1yM= 10^{-24} M

Table 7: units

APPENDIX 2

ABBREVIATIONS & SYMBOLS

AD: Alzheimer's disease

MOPS: 3-(N-morpholino) propanesulfonic acid

DTNB: 5-5'-dithio-bis-(2-nitrobenzoic acid)/ also known as Ellman's reagent

ATC: Acetylthiocholine

AChE: Acetylcholinesterase

MB: Methylene blue

KP: Potassium phosphate

NaCl: Sodium chloride

ThT: Thioflavin-t

μM : micro molar

nM: nano molar

μL : micro liter

cm^3 : cubic centimeter

APPENDIX 3:

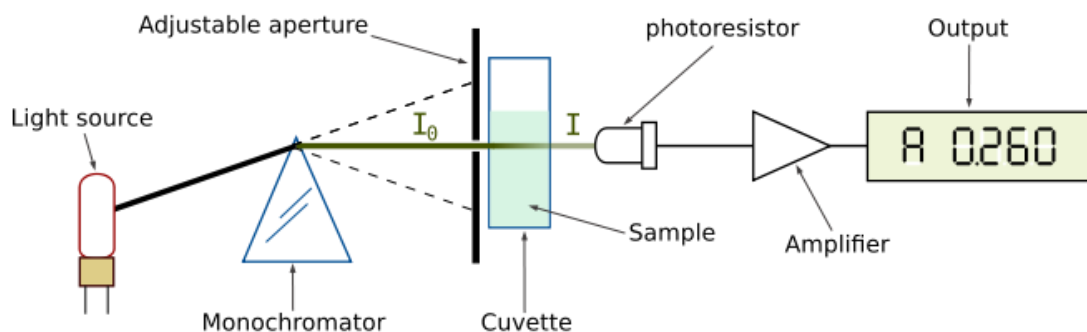
SPECTROPHOTOMETRIC METHOD & ABSORBANCE

Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength.¹⁸

Spectrophotometry involves the use of a spectrophotometer. A spectrophotometer is a photometer that can measure intensity as a function of the light source wavelength. Important features of spectrophotometers are spectral bandwidth and linear range of absorption or reflectance measurement.¹⁹

The sequence of events in a modern spectrophotometer is as follows:

1. The light source is imaged upon the sample
2. A fraction of the light is transmitted or reflected from the sample
3. The light from the sample is imaged upon the entrance slit of the monochromator
4. The monochromator separates the wavelengths of light and focuses each of them onto the photodetector sequentially.



¹⁸ Allen, D., Cooksey, C., & Tsai, B. (2010, October 5). Spectrophotometry. Retrieved from <http://www.nist.gov/pml/div685/grp03/spectrophotometry.cfm>

¹⁹ "Spectrophotometry." *Wikipedia, The Free Encyclopedia*
<http://en.wikipedia.org/wiki/Spectrophotometry>

As DTNB (Ellman's reagent) is used in the experiment, special name of the method is Ellman's spectrophotometric method.

In spectroscopy, the absorbance of a material is a logarithmic ratio of the radiation falling upon a material, to the radiation transmitted through a material.^{20 21}

Absorbance is the fraction of radiation absorbed at a specific wavelength.²² It can be calculated by Beer-Lambert Law.

Beer-Lambert Law relates the absorption of light to the properties of the material through which the light is travelling.²³

$$A = \epsilon \times b \times c$$

Where;

A is absorbance,

ε is the molar absorptivity

b is the path length of the sample - the path length of the bathtub in which the sample is contained.

c is the concentration of the compound in solution

In the experiment;

ε is stabilized at $14.2 \text{ mM}^{-1}\text{cm}^{-1}$ by the spectrophotometer.

b is stabilized by using the bathtubs that are identical (**b** =1cm)

So that the absorbance depends on **c** .

AChE reacts with ACT and produces thiol which DTNB reacts with to produce yellow color.

The inhibitor (in this case MB) inhibits the reaction of AChE with ATC, so that the production of yellow color is decreases; absorbance decreases. (**c** in this case is the concentration of the substance with yellow color which is produced when DTNB reacted with thiol)

²⁰ Mehta, A. UV-Visible Spectroscopy- Derivation of Beer-Lambert Law

²¹ "Dictionary — Definition of absorptance". Websters-online-dictionary.org. <http://www.websters-online-dictionary.org/ab/absorptance.html>. Retrieved 2011-11-21.

²² "Absorbance." *Wikipedia, The Free Encyclopedia*

<http://en.wikipedia.org/wiki/Absorbance>

²³ "Beer Lambert Law." *Wikipedia, The Free Encyclopedia*

http://en.wikipedia.org/wiki/Beer%E2%80%93Lambert_law

APPENDIX 4: CALCULATION OF IC₅₀ VALUE

The half maximal inhibitory concentration (IC₅₀) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process by half.²⁴

In this experiment IC₅₀ value of MB refers to the concentration which MB can inhibit the reaction of AChE by 50%.

To calculate the IC₅₀ value three concentrations of MB were put into the bathtubs that contain MOPS, water, ATC, DTNB and AChE.

Final concentration of MB is calculated by using the formula below:

$$M_1V_1 = M_2V_2$$

Where;

M₁ is the concentration in the first solution,

V₁ is the volume of the first solution,

M₂ is the concentration in the final solution,

V₂ is the volume of the final solution.

5 of the tubes contain 5μL 25μM MB,

$$25 \times 5 = M_2 \times 1000$$

$$M_2 = \frac{25 \times 5}{1000} = \frac{125}{1000} = 0.125 \mu M = 125 nM$$

5 of the tubes contain 15μL 5μM MB,

$$25 \times 5 = M_2 \times 1000$$

$$M_2 = \frac{5 \times 15}{1000} = \frac{75}{1000} = 0.075 \mu M = 75 nM$$

²⁴ "IC50." *Wikipedia, The Free Encyclopedia*
<http://en.wikipedia.org/wiki/IC50>

5 of the tubes contain 2µL 0.125µM MB.

$$M_2 = \frac{0.125 \times 2}{1000} = \frac{0.25}{1000} = 0.00025\mu M = 0.25nM$$

Then the bathtubs were put into spectrophotometer and the change in absorbance is measured. As absorbance is directly proportional with the color change, absorbance also gives the activity of AChE. The concentration where the activity is 50% gives the IC₅₀ value of MB.

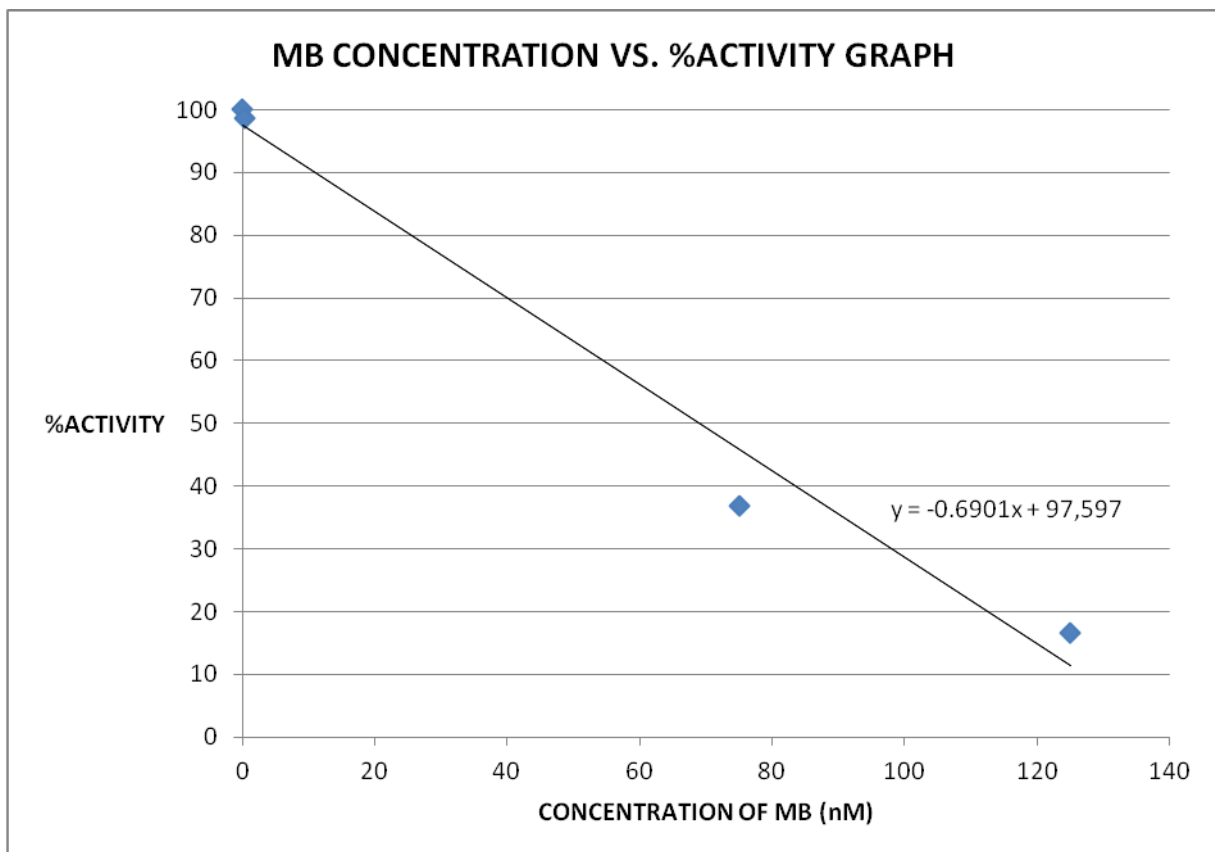
CONCENTRATION OF MB (nM)	ACTIVITY (ABSORBANCE)				CONTROL GROUP
	TRIAL 1	TRIAL 2	TRIAL 3	MEAN	
125	0.0215	0.0226	0.0215	0.0219	0.1312
75	0.0185	0.0164	0.0153	0.0167	0.0453
0.25	0.0283	0.0228	0.0270	0.0260	0.0287

Table 8: Shows the absorbance values in the test tubes. Control groups are the ones which do not include inhibitor and the absorbance in the control groups change because of the decrease in the enzyme activity due to time.

Percentage activity is calculated by using the formula below:

CONCENTRATION OF MB (nM)	%ACTIVITY
125	16.7%
75	36.9%
0.25	98.6%

Table 9: Shows the calculated %activity of MB in different concentrations.



Graph 3: Graph 3 shows the %activity of methylene blue when it is not used or used in different concentrations (0.125nM, 75nM, 125nM).

As the wanted activity of MB is 50%, 50 is put instead of y in the equation of the best line.

$$y = -0.6901x + 97.597$$

$$50 = -0.6901x + 97.597$$

$$-0.6901x = 50 - 97.597$$

$$-0.6901x = -47.597$$

$$0.6901x = 47.597$$

$$x = 68.9711636$$

As the IC_{50} value of MB is found to be 68.97.. which is close to 70nM, 70 nM MB will be used in the second part of the experiment.

APPENDIX 5: THIOFLAVIN-T FLUOROMETRIC METHOD

Fluorometry is an analytical technique for identifying and characterizing minute amounts of a substance by excitation of the substance with a beam of ultraviolet light and detection and measurement of the characteristic wavelength of fluorescent light emitted.²⁵

Fluorometry has been firmly established as method for the sensitive, specific and economic detection of trace quantities of substances. Generally, a fluorometric method has the potential of being 10—100 fold more sensitive than a colorimetric procedure, since dyes have a molar absorptivity of 10^5 .¹ By coupling a fluorometric read-out with a catalytic or a non-stoichiometric chemical process even greater enhanced sensitivity may be achieved. The greatly improved fluorometric instrumentation becoming commercially available for automated analyses has further contributed to the increasing utility of fluorometry in such diverse applications as environmental pollution, clinical chemistry, biology and metallurgy. Criteria for the choice of a set of reaction conditions for use in fluorometry include: rapid rates, fluorescent product stability, lack of internal quenching, and a highly fluorescent product.²⁶

$\alpha\beta$ -fibrils are obtained commercially which means that are solved in 0.02% ammonia solution. It is known that when these $\alpha\beta$ -fibrils are mixed with NaCl, MKB, and H₂O; and made to stay in a bacteriological incubator at 37⁰C a typical fluorescence is observed²⁷. So that putting NaCl, MKB and H₂O is the standard procedure to measure the fluorescence concentration in the tubes that contain $\alpha\beta$ -fibrils.

²⁵ <http://medical-dictionary.thefreedictionary.com/fluorometry>

²⁶ http://www.seminaronly.com/Chemical_Engineering/Fluorometric_Analytical_Methods.php

²⁷ Kucukkilinc Tuylu, T., Ozer, I. (2007), Multi-site inhibition of human plasma cholinesterase by cationic phenoxazine and phenothiazine dyes. Archives in Biochemistry and Biophysics, Volume 461, Issue 2, Pages 294-298.

BIBLIOGRAPHY

1. "Absorbance." *Wikipedia, The Free Encyclopedia*
<http://en.wikipedia.org/wiki/Absorbance>
2. Allen, D., Cooksey, C., & Tsai, B. (2010, October 5). Spectrophotometry. Retrieved from <http://www.nist.gov/pml/div685/grp03/spectrophotometry.cfm>
3. "Dictionary — Definition of absorbance".
<http://www.websters-online-dictionary.org/ab/absorptance.html>. Retrieved 2011-11-21.
4. "Fluorescence." *Wikipedia, The Free Encyclopedia*
<http://en.wikipedia.org/wiki/Fluorescence>
5. "IC50." *Wikipedia, The Free Encyclopedia*
<http://en.wikipedia.org/wiki/IC50>
6. "In Vitro." *Wikipedia, The Free Encyclopedia*
http://en.wikipedia.org/wiki/In_vitro
7. Jay M. Ellis, DO, Director, Neuroscience Research of the Berkshires, 100 Wendell Ave, Pittsfield
8. Kucukkilinc Tuylu, T., Ozer, I. (2007), Multi-site inhibition of human plasma cholinesterase by cationic phenoxazine and phenothiazine dyes. Archives in Biochemistry and Biophysics, Volume 461, Issue 2, Pages 294-298.
9. Maurstad G., Prass M., Serpell L.C., Sikorski P. Dehydration stability of amyloid fibrils studied by AFM. *Eur Biophys J.* 38(8): 1135-40, (2009)
10. Nussbaum R.L., Ellis C.E. Alzheimer's Disease and Parkinson's Disease. *N Engl J Med.*, 348, 1356-1364, (2003)
11. Robinson S.R., Bishop G.M., A β as bioflocculant: implications for the amyloid hypothesis of Alzheimer's Disease. *Neurobiol Aging*, 23,1051-1072, (2002)

12. Sensi P, Margalith P, Timbal MT (1959). "Rifomycin, a new antibiotic—preliminary report". *Farmaco Ed Sci* 14: 146–147.
13. "Spectrophotometry." *Wikipedia, The Free Encyclopedia*
http://en.wikipedia.org/wiki/Beer%E2%80%93Lambert_law
14. Healthcare
<http://www.healthcare-online.org/Normal-Body-Temperature.html>
15. Medical Dictionary
<http://medical-dictionary.thefreedictionary.com/fluorometry>
16. "Beer Lambert Law." *Wikipedia, The Free Encyclopedia*
http://en.wikipedia.org/wiki/Beer%E2%80%93Lambert_law