EXPERIMENT 1:

PHASE 1 REACTIONS

ETHOXYRESORUFIN-O-DEETHYLASE (EROD) ACTIVITY AND DETERMINATION OF COTININE

A) General Information:

Living creatures are exposed to many drugs and chemicals due to increasing industrialization in recent years. Among these compounds that enter the organism, those with lipophilic properties are easily absorbed, but their excretion is limited. These lipophilic chemicals are transformed into hydrophilic ones, which must be removed from the organism, that is, biotransformation takes place via xenobiotic metabolizing enzymes. These enzymes can are generally divided into two main groups, namely, Phase 1 and Phase 2 enzymes. While Phase 1 enzymes are involved in oxidation, reduction and hydrolysis reactions, Phase 2 enzymes also play a role in conjugation reactions. In these reactions, where cytochrome P450 dependent enzyme system usually plays a role, the xenobiotics become more polar and are eliminated from the body.

The cytochrome p450-dependent enzymes is found in many organs and tissues both in the liver and outside the liver. These enzymes are involved in the oxidative, peroxidative and reductive metabolism of endogenous substances such as steroids, bile acids, fatty acids, prostaglandins, leukotrienes, vitamins A and D, biogenic amines, retinoids, lipid hydroperoxidases and phytoalexins. Many of these enzymes also metabolize a large number of xenobiotics, including drugs, synthetic chemicals, environmental pollutants, pesticides, aromatic amines, and polycyclic aromatic hydrocarbons. sddddThis enzyme system converts the chemical substances into effective or ineffective, which can lead to changes in the therapeutic activities of the drugs or to the development of specific toxic effects such as carcinogenicity and mutagenicity. For this reason, changes in this system can lead to changes in the activities and / or toxicity of xenobiotics. The most important component of this enzyme system, P450, has numerous forms, and each of them encoded and synthesized by a different gene. The liver is the organ that plays the most important role in the metabolism of xenobiotics.

The most important reactions in Phase 1 reactions are the oxidation reactions. 90-95% of xenobiotics are metabolized in this way. Although metabolism usually results in inactivation of the parent substance, some metabolites may be more toxic than the parent substance, ie, metabolic activation. The enzyme systems that catalyze oxidation reactions are as follows:

a) Microsomal Enzyme System

i. Monooxygenases (CYPs) containing cytochrome P450
ii. Flavin containing monooxygenases (FMO)

b) Non-microsomal Enzyme System

i. Cytosolic enzymes (alcohol dehydrogenase, aldehyde dehydrogenase)
ii. Mitochondrial enzymes (MAO, DAO)

A significant proportion of the Phase 1 reactions are catalyzed by microsomal enzymes. These enzymes add small molecules such as OH- or O- to their substrates. Phase 1 enzymes (CYPs) in the xenobiotic metabolizing enzyme system are responsible for the metabolism of many xenobiotics, including drugs.

7-ethoxyresorufin O-deethylase (EROD) activity, one of the Phase 1 enzymes, is measured as an indicator of CYP1A1 activity. CYP1A1 is responsible for the activation of many chemicals. PAHs (benzo(a)pyrene ; BaP) present in cigarette smoke and aflatoxins are some of these chemicals.

Nicotine, which enters the organism with cigarette smoke, also rapidly transforms to cotinine, which is the metabolite of nicotine. This is why cotinine is measured in biological fluids to support the association of EROD activity with cigarette smoking.

1) Ethoxyresorufin-o-deethylase (EROD) activity

Solvents, materials and devices used in the experiment:

1. Resorufin solution

2. 1 mM 7-ethoxy resorufin solution

3. 12 mg / ml albumin solution

4. 0.1 M pH: 7.8 Tris HCl buffer solution

5. NADPH-generating system

6. Spectrofluorimeter

7. Methyl alcohol

8. Automatic pipettes

9. Shaking water bath

10. Centrifuge

11. Vortex

12. Experimental animal-rat / Mouse

Preparation of tissues and microsomal fractions

The liver tissue removed from the experimental animals is washed with pure water and cold water to remove its blood. The tissue is then taken up on an absorbent paper to provide drying. The weight of the tissue is measured to a precision of 0.001 grams. All these operations are carried out in ice cubes at + 4 ° C.

Rat liver tissues are cut with scissors to separate small pieces. The disrupted tissue is placed in the glass homogenizer and added with 1.15% of the KCl solution as 5 mL per gram of tissue (3.5 mL per gram of tissue if it was mouse). The homogenizer is used to homogenize the tissues. The homogenates are centrifuged for 20 minutes at 10,000 x g. The pellet, which is tissue fragment that collapse at 10,000 x g, contains the mitochondria and cell nuclei. In

the supernatant (liquid above the sediment), there are endoplasmic reticulum fragments and soluble cytoplasm. The resulting supernatant is then centrifuged for 1.5 hours at 30,000 rpm (108,000 x g) in a Beckman refrigerated ultracentrifuge. The pellet obtained after centrifugation contains microsomes. The pellets are homogenized by the addition of 20% glycerol, so as to be 0.5 mL for 1 g of tissue. A portion of the resulting microsomal fraction is taken for protein determination and the remaining microsomal fractions are stored at -80 ° C freezer for the calculation of enzyme activities.

Protein quantification method

The protein content of the prepared microsome is determined by the method of Lowry [Lowry et al., 1951]. 2.5 ml Reagent D is added onto 1/100 (100 µl microsomal solution completed with 10 ml distilled water) and 1/200 (50 µl microsomal solution completed with 10 ml distilled water) diluted microsomes. After 10 minutes, 0.25 mL of Folin solution is added to all tubes as quickly as possible. After each addition, the mixture is vortexed, shaken at 37 ° C for 30 minutes in a water bath, and read at spectrophotometer against a blank at 660 nm. Protein concentration are calculated using the standard curve (0.05 mg / mL, 0.10 mg / mL, 0.015 mg / mL and 0.2 mg / mL bovine serum albumin, BSA). A sample standard curve graph is shown below.

Preparation of Reactive D:

****** Reagent A: 0.1 ml of 2% $CuSO_4.5H_2O$,

Reagent B: 0.1 ml of 2% potassium sodium tartrate,

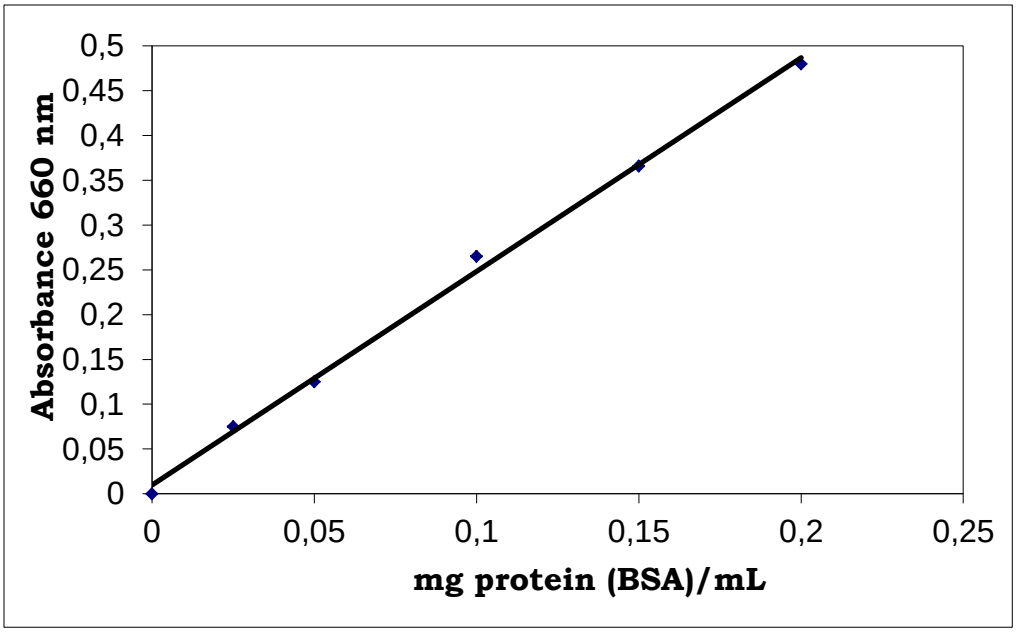
Reagent C: 10 ml of a solution of 2% Na_2CO_3 in 0.1 N NaOH

Reagent D solution is freshly prepared just before use.

****** Folin solution: 2 N Folin reactive, diluted 1: 1 with distilled water.

Tube number	Elements of the reaction medium	Volume (ml)	Added solution (2.5 ml)	Added solution (0.250 ml)
1	Distilled water	0.5	Reactive D	Folin solution
2	Distillde water	0.5	Reactive D	Folin solution
3	0.1 mg/ml BSA*	0.5	Reactive D	Folin solution
4	0.1 mg/ml BSA	0.5	Reactive D	Folin solution
5	0.1 mg/ml BSA	0.5	Reactive D	Folin solution
6	0.1 mg/ml BSA	0.5	Reactive D	Folin solution
7	1/100 dil. Liver microsome solution	0.5	Reactive D	Folin solution
8	1/1001 dil. Liver microsome solution	0.5	Reactive D	Folin solution
9	1/2001 dil. Liver microsome solution	0.5	Reactive D	Folin solution
10	1/200 dil. Liver microsome solution	0.5	Reactive D	Folin solution

* BSA: Bovine serum albumin



Determination of EROD activity

Once the protein is assayed for the microsomes, 7-ethoxyresorufin O-deethylase (EROD) activity is determined. 7- ethoxyresorufin O-deethylase is the enzyme that provides 7-ethoxyresorufin to resorufine conversion. The activity of this enzyme is determined by a method based on the spectrofluorimetric measurement of the amount of resorufin formed. NADPH-generating system is used as cofactor.

Resorufin solution was used as the standard for the measurement of EROD enzyme activity. Different FI values corresponding to the different amounts of resorufin that are measured with spectrofluorimeter by the standards added to the reaction medium at the four different concentrations are also plotted in the standard curve.

In the reaction medium, in a total volume of 1.0 ml, 0.2 mg of microsomal protein, 1 µM 7-ethoxyresorufin as substrate, pH 7.8 Tris HCl buffer; 12 mg/ml albumin, as the cofactor (NADPH generating system) 2.5 mM glucose-6-phosphate, 2.5 mM $NADP^+$, 1 U / 0.5 mL glucose-6-phosphate dehydrogenase, 2.5 mM $MgCl_2$, 0.4 mM pH 7.8 potassium phosphate buffer. The cofactor is prepared just before it is used.

Elements of the reaction medium	Stock solutions	Additional volume
7- ethoxyresorufin	1 mM	0,100 mL
pH 7.8 Tris HCl buffer	100 mM	0,550 mL
Albumin	1.2 mg/mL	0,100 mL
Microsomal protein	0.2 mg/mL	0,100 mL
$NADP^+$	10 mM	0,025 mL
$MgCl_2$	100 mM	0,025 mL

Glucose-6-phosphate dehydrogenase	1000 Unit (U)/0,2 mL	0,0002 mL
Potassium phosphate buffer pH: 7,8	400 mM	0,0748 mL
Glucose-6-phosphate	100 mM	0,025 mL

Experimental procedure

The reaction is initiated by adding NADPH generating system to the tubes containing the reaction medium elements except the NADPH generating system. The tube mouths are left open for 5 minutes at 37 ° C in a shaking water bath. The reaction is then stopped by the addition of 3 ml of methanol. The tubes are placed in ice cubes and the denatured protein is precipitated by centrifugation at 4000 rpm for 20 minutes. 3 ml of the solution is taken in another tube and the FI values of the solution are measured spectrofluorimetrically (Excitation: 538 nm, Emission: 587 nm). After reading the FI values, the EROD activity is calculated against the resorufin standards.

2) Determination of cotinine in urine

Urinary cotinine values is determined by barbituric acid (BA) spectrophotometric assay. This method is based on the principle that cotinine and barbituric acid form a colored complex. The absorbance of the resulted colored product is measured spectrophotometrically.

Solvents, materials and devices used in the experiment:

1. 4 M sodium acetate buffer solution pH: 4.7

2. 10% potassium cyanide

3. 10% Chloramin-T

4. 1% solution of barbituric acid (in acetone : water - 1: 1)

5. Urine sample

6. Spectrophotometer

Experimental procedure:

-0.2 ml of 4 M sodium acetate buffer pH: 4.7, 0.1 ml of 10% potassium cyanide, 0.1 ml of 10% Chloramin-T, 0.5 ml of 1% barbituric acid solution are added respectively to 0.5 ml of urine.

- Wait 20 minutes.

- Measured at 506 nm in a spectrophotometer.

Absorbances that are read at spectrophotometer are calculated according to the absorbance values of standard solutions prepared at different concentrations and the amount of cotinine in the urine is found.