CHAPTER 2

MATERIALS AND MEDTHODS

2.1 Apparatus and Chemicals

2.1.1 Apparatus

1. Autopipette size 100-1000 ul
2. Autopipette size 2-20 ul
3. Centrifuge
4. Centrifuge tube, Kontron
5. Feed tube
6. Freezer - 70° C, Forma- Scientific
7. Freezer -20 °C, Sharp
8. Homogenizer, Glas- Col
9. High performance liquid chromatography system, Varian HPLC system, contained Varian pump 9012 Q, Aldrich guard column, reverse phase C18 u bondapack column (30 cm x 4.6 mm) Water, UV detector, Varian ProStar model 310 and Polychrome/ Polyview solfware
10. Ice generator, NT 609 New ton
11. Light microscope, Olympus provis
12. Materials for surgery such as tissue forceps, scissors, artery clamp
13. Spectrofluorometer, Jasco FP-777
14. Spectrophotometer, Beckman Du 650
15. Ultracentrifuge, Kontron
16. Vortex mixture, VM-300
17. Weighting balance, Oertling NA 164

2.1.2 Chemicals

1. Acectaminophen, Fluca
2. 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB), Fluca
3. 7-Hydroxy coumarin, Sigma
4. Acetronitrile HPLC grade, Fisher Scientific
5. B- Nicotinamide adenine dinucleotide phosphate, Sigma
6. B- Nicotinamide adenine dinucleotide, Sigma
7. BCA protein assay kit, Sigma
8. Bovine serum albumin, Sigma
9. Coumarin, Sigma
10. Dextromethorphan, Merck
11. Disodium hydrogen phosphate anhydrous, Ajax Finechem
12. Disulfiram, T.O. Pharma.Co.LTD
13. Ethylenediaminetetraaceticacid (EDTA), Fluca
14. Formaldehyde, Chiang Mai Winner
15. Glucose -6- phosphate, Sigma
16. Glycerol, Merck
17. Hydrocholric acid, Merck
18. Magnesium chloride, May and baker
19. Metaphosphoric acid, Merck
20. Methanol HPLC grade, Fisher Scientific
21. *N*-acetylcysteine, Fluca
22. *p*- nitrophenol, Fluca
23. Perchloric acid, J.F. baker
24. Potassium chloride, Merck
25. Reduced glutathione, Sigma
26. Sodium chloride, Merck
27. Sodium citrate dihydrate, Merck
28. Sodium dihydrogen phosphate dihydrate ,Seelza-hannover
29. Sodium hydroxide, Merck

30. Sucrose, Fisher Chemical

31. Trichloroacetic acid, Merck

2.2. Preparation of Solutions

See in Appendix A

2.3 Experimental animal

Male and female Wistar rats, 200 – 250 g were purchased from the National Laboratory Animal Center, Salaya Mahidol University, Nakom Pathom, Thailand. The animals were kept in animal room where the temperature was maintained at 24 ±1 °C and approximately 45 percent relative humidity. They were exposed to 12 hours light dark cycle. They had free access to food and water and acclimatized for at least one week before starting the experiments.

2.4 Experimental protocol

In this experiment, Wistar rats were randomly allocated into 6 groups by random sampling as number of table (see in appendix B). Each group consists of 36 male and 36 female. All animals were fasted 12 hours before the experiment started.

The first group, the control group, received 0.9% normal saline (I.P) and 0.5% methyl cellulose vehicle (P.O).

The second group, the positive control, received 4.25 g/kg acetaminophen (P.O) and 0.9% normal saline (I.P)

The third group received 4.25 g/kg acetaminophen (P.O) after 6 hours of started treatment by giving 140 mg/kg NAC (I.P) every 4 hours for 7 times.

The fourth group received 4.25 g/kg acetaminophen (P.O) after 6 hours of started treatment by giving 140 mg/kg NAC (I.P) every 4 hours for7 times and 100 mg/kg disulfiram

The fifth group received 4.25 g/kg acetaminophen (P.O) after 6 hours started treatment by giving 140 mg/kg NAC (I.P.) every 4 hours for 7 times and 200 mg/kg disulfiram

The sixth group received 4.25 g/kg acetaminophen (P.O) after 6 hours started treatment by giving 140 mg/kg NAC every 4 hours for 7 times and 400 mg/kg disulfiram

The Wistar rats were fed 12 hours after received acetaminophen.

Wistar rats were injected with pentobarbital and sacrificed at 6, 12, 24, 48, 72 and 168 hours after received acetaminophen respectively. Each group of experiment is shown in Table 1.

2.5. Collection and storage of samples

Blood sample was collected into 5 – ml tube which contained 200 µl 8% (w/v) EDTA , 3 ml of blood was shared and stored at 4 C for glutathione determination in blood and 2 ml of blood was stored at – 20 °C for determined acetaminophen concentration determination. Clotted blood was collected into 5 – ml tube which uncontained 8% EDTA and centrifuged for 10 minutes at 1,400 × g. Serum was separated and stored at 4 °C for AST and ALT. Livers were washed weighted and cut at least 5 pieces in each lobe and were fixed in 10% formalin to study of histology and were stored at – 70 °C for catalytic activity and reduced glutathione determination.

2.6. Determination of mortality rate

The mortality rate was recorded after acetaminophen administation in each group. Results are expressed and calculated the percentage of mortality rate each time.

2.7. Determination of serum alamine aminotransferase (ALT)

ALT level was determined with a semiautomatic kit assay from Sirivej hospital. The ALT level was expressed as IU/L.

2.8. Determination of serum aspartate aminotransferase (AST) level.

AST level was determined by a semiautomatic kit assay from by Sirivej hospital. The AST level was expressed as IU/L.

Table 1 The experimental animals were shown in 6 groups.

Group	Treatment	0	4	6*	8	12*	16	20	24*	28	48*	72*	168*
1	Control group Methyl cellulose	MC	NS	-	NS	NS	NS	NS	NS	NS	-	-	-
2	Ac 4.25 g/kg	Ac	NS	-	NS	NS	NS	NS	NS	NS	-	-	-
3	Ac 4.25 g/kg + NAC 7x 140mg/kg	Ac	NA C	-	NA C	NA C	NA C	NA C	NA C	NA C	-	-	-
4	Ac 4.25 g/kg + NAC 7x 140 mg/kg + DS 100mg/kg	Ac	NA C+ DS	-	NA C	NA C	NA C	NA C	NA C	NA C	-	-	-
5	Ac 4.25 g/kg + NAC 7x 140 mg/kg + DS 200mg/kg	Ac	NA C + DS	-	NA C	NA C	NA C	NA C	NA C	NA C	-	-	-
6	Ac 4.25 g/kg + NAC 7x 140 mg/kg + DS400 mg/kg	Ac	NA C + DS	-	NA C	NA C	NA C	NA C	NA C	NA C	-	-	-

When Ac = acetaminophen

DS= disulfiram

NAC = N-acetylcysteine

*= time for killed rats

2.9 Determination of reduced glutathione level in liver

Level of reduced glutathione in liver was measured by modified method of Meng et al. (94)

1. The frozen liver tissue was weighted in 20 mg.
2. The liver tissue was homogenized in 2.0 ml cold tricloroacetic acid (4% w/v) and 2.0 ml phosphate buffer pH 7.4.
3. The sample was kept at 4 °C at least 1 hour and then the sample was centrifuged at 1200 ×g and 4 °C for 15 minutes.
4. The assay mixture contained 0.1 ml supernatant, 2.7 ml phosphate buffer pH 7.4 and 0.2 ml DTNB solution.
5. The yellow color was developed and read immediately at 412 nm by a spectrophotometer.
6. The level of reduced glutathione in the liver was determined by comparing its absorption with standard curve. Data was expressed as ng/ mg tissue

2.10 Determination of glutathione level in blood

Levels of glutathione in blood were measured by the method of Leelarungrayab et al. (95)

1. 0.4 ml of whole blood and 1.6 ml distilled water were added in precipitation agent.
2. The sample was centrifuged at 2000 rpm for 5 minutes.
3. The assay mixture contained 0.2 ml supernatant, 1.0 ml phosphate buffer pH 7.4, and 0.25 ml DTNB solution.
4. The yellow color was developed and read within 5 minutes at 412 nm by spectrometer.
5. The level of glutathione in blood was determined by comparing its absorption with standard curve. Data was expressed as ng/ml blood.

2.11 Preparation of microsomal enzyme

Hepatic microsomes were prepared by method of Potter et al. (28)

1. Frozen livers were removed on ice within 30 minutes.
2. 3 g of liver were cut by scissors and pooled in 9 ml Tris-KCl buffer 4° C, pH 7.4.
3. Liver tissues were homogenized with a motor- driven glass-Teflon homogenizer.
4. The homogenate was centrifuged at 9,000 × g for 20 minutes and the supernatant was decanted and recentrifuged at 105,000 × g for 1 hour.
5. The microsomal pellet was kept in Tris buffer pH 7.4 at -70^{o}C

2.12 Protein assay

Protein contents were determined by bicinchonic acid (BCA) protein assay kit.

1. Microsomal proteins were diluted by phosphate buffer pH 7.4.
2. The different concentrations albumin standards (200, 400, 600, 800 and 1000 ug/ml) were prepared in different concentration.
3. BCA working reagent was prepared by mix 50 ml reagent A and 2 ml reagent B. The BCA reagent was mixed working in light green color.
4. 2 ml of the BCA working reagent was added with 0.1 ml of BSA protein standard, blank, and unknown samples. Vortex was gently mixed and incubated at 60° C for 15 minutes.
5. The tube was allowed cool in room temperature and transfered it into a cuvette.
6. The absorbance of the solution was measuered at 562 nm.
7. Protein concentration was determined by comparison of the absorbance of standard curve. The data was expressed as ug/ml.

2.13 Determination of catalytic activity CYP 2E1

The catalytic activity of CYP 2E1 was determined by measuring p-nitrophenol hydroxylation as described by Koop et al. (96)

1. Reaction mixture contained 1.68 ml phosphate buffer pH 6.8, 40 ul 100 mM p-nitrophenol and 150 ul microsomal suspension.

2. Reaction mixture was perincubated at 37 ° C for 5 minutes and started by adding 100 ul 10 mM NADPH.

3. After 10 minutes, the reactions were terminated by adding 0.5 ml 0.5M TCA.

4. Protein was precipitated by centrifuged at 2500 ×g for 15 minutes.

5. 1.0 ml of the supernatant was mixed with 0.1 ml 10 N NaOH for the measurement of 4-nitrocatechol at 511 nm by spectrophotometer.

6. The level of 4-nitrocatechol was determined by comparing its absorption with standard curve. Data was expressed as nmol/mg/min

2.14 Determination of catalytic activity CYP2A6

The catalytic activity of CYP2A6 was determined by measuring coumarin 7-hydroxylation as described by Draper et al. (97).

1. Reaction mixture contained 1.5 ml phosphate buffer pH 7.4, 100 ul 3mM $MgCl_2$, 100 ul 1mM EDTA, 100 ul 1 mM NADP, 100 ul 5 mM glucose-6-phosphate, 100 ul 50 uM coumsrin and 150 ul microsomal suspension.

2. The reaction mixture was preincubated at 37°C for 5 minutes and started by adding 100 ul 10 mM NADPH.

3. After 10 minutes, the reactions were terminated by the addition of 125 ul 15% trichloroacetic acid and 2 ml dichloromethane.

4. The tubes were vigorously mixed on a vortex. 1 ml aliquot of organic phase was removed and added with 2 ml 0.01 N NaOH containing 1mM NaCl.

5. After further mixing, the concentration of 7-hydroxy coumarin in the alkaline phase was determined by spectrofluorometer at wave length excitation 371 nm and wave length emission 454 nm.

6. The level of 7-hydroxy coumarin was determined by comparing its absorption with standard curve. Datas were expressed as pmol/ng/min

2.15 Determination of catalytic activity of CYP2D6

The catalytic activity of CYP2D6 was determined by modified method of Dong et al. (15)

1. Reaction mixture contained 150 ul microsomal suspension and 400 ul 1 M erythromycin and preincubated at 37°C for 15 minutes.
2. 200 ul dextromethorphan was added in the reaction mixture and incubated at 37°C for 5 minutes.
3. 100 ul NADPH was added to start the reaction.
4. After 10 minutes, the reactions were terminated by the addition of 25 ul 70% perchloric acid.
5. Protein was precipitated by centrifuge at 12,000 × g for 10 minutes and supernatant was determined final amount of dextromethorphan.

Chromatographic condition

Mobile phase :

methanol: acetronitrile: phosphate buffer pH3.5 in the ratio 20: 25: 55 by volumn, at flow rate 1.0 ml/ min and injection volumn is 20 ul. The detection wave length is 280 nm.

Standard curve

Standard curves were prepared by using standard solution contained 25,50,100,200,400,600 and 800 ul/ml of dextromethorphan. Erytromycin was used internal standard.

6. An approximately 1:1 stoichiometry was observed for the formation of dexthorphan.

Then calculation of amount dextrophan form

mole dextromethorphan = mole dexthorphan

$mole_{start}$-$mole_{final}$ = mole dexthorphan

when $mole_{start}$ = mole of dextromethorphan before added NADPH in to reaction mixture

$mole_{final}$ = mole of dextromethorphan after the reaction were stopped

2.16 Determination of acetaminophen concentration

2.16.1. Preparation of sample

Serum was added with methanol in ratio 1:1 (v/v) and centrifuged to remove plasma protein at 12,000 × g for 10 minutes.

2.16.2. Chromatographic conditions

Mobile phase : methanol : water in the ratio 35 : 65 (v/v)

Flow rate : 1.0 ml/ min

Detector wave length: 254 nm

Volumn of injection : 20 ul

2.16.3. Standard curve

Standard curve was prepared using standard solution containing 100, 200, 300, 400, 500,600 ug/ml of acetaminophen.

2.17. Calculation of half-life and clearance

From $C = C_0 . e^{-ke.t}$

$\log C = \log C_0 + \log e^{-ke.t}$

$\log C = \log C_0 - k_e.t \log e$

$\log C = \log C_0 - (k_e/2.303)t$

1. Plot data of acetaminophen concentration by taking logalithm with time see in Figure 2.1

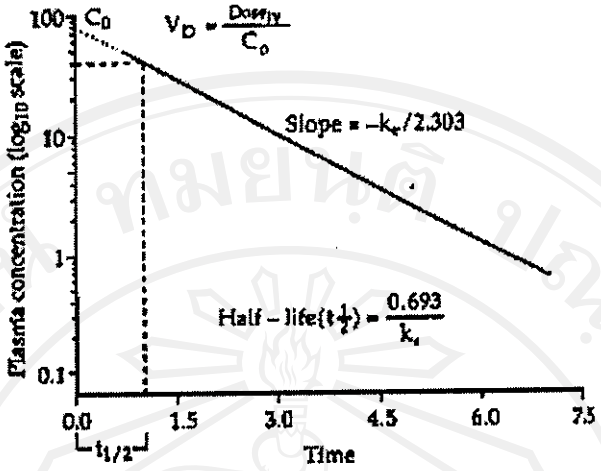

Figure 9 Calculation of pharmacokinetic of drug from curve between drug concentration (log scale) and time (98)

2. Calculation half-life ($t_{1/2}$) of acetaminophen from equation

$$t_{1/2} = 0.693/ k_e$$

3. Calculation clearance (CL) of acetaminophen from equation

$$CL = [\ 0.693 \times (\text{volumn of distribution})] / t_{1/2}$$

2.19 Histopathological studies

1. Livers representative sections were taken from the midportion of each lobe and were stored in 10 % formalin. Fixed sections were embedded in paraffin, sectioned into 4 ul slices, and stained with heamatoxylin and eosin.
2. Livers were examined by an expert pathologist who did not aware sample assignment in each experimental group and blinded manner by light microscopy for evidence of hepatocellular necrosis. The hepatic necrosis score (HNS) were shown in table 2 and calculated percentage of necrosis by using score ≥ 2 in all lobes sampling.

Table 2 The hepatic necrosis score (99)

Scale	Charactter
0	No evidence of cellular necrosis
1	Necrosis limited to the region surrounding the central vein
2	Necrosis extending beyond the central vein but not to the portal triads
3	Necrosis extending to the portal triad in some region
4	Severe generalized necrosis

2.19 Statistical analysis

Datas were presented as mean + standard deviation. Two ways analysis of variance (ANOVA) followed Turkey's multiple comparison test were used to contrast group. The level of significance was P less than 0.05. In cases where the variance analysis precluded utilization of ANOVA for statistical comparisons, use was made of the Student 't test. SPSS program was used.