CHAPTER III

MATERIALS AND METHODS

From the literature review and various studies mentioned, it is proposed that 1) total plasma LDH activity and LDH isoenzymes changes may be found in methomyl-exposed rats 2) These alterations can indicate the target organs of methomyl toxicity and 3) LDH isoenzymes is responsive as a marker of methomyl toxicity.

Therefore, the first purpose of this study is to assess and investigate the alterations of total plasma LDH activity and LDH isoenzymes in rats receiving various acute doses of methomyl and repeated administration of selected dose by using known validated methods (Helena Laboratories, 1995; Scandinavian Society for Clinical Chemistry and Clinical Physiology, 1974). The second purpose is to investigate the histopathological findings in rats treated with single and repeated dose of methomyl at various time points and explore the possible relationships between the effects of methomyl, total LDH activity, LDH isoenzymes, and hematological values as well as histopathologic findings. SGPT determination will be used in conjunction with LDH to rule out the hepatotoxicity.

Animals

A total of 240 male outbred Wistar rats (102.4 $\pm$ 6.3 g) were obtained from the National Laboratory Animal Center of Salaya Campus, Mahidol University. Rats were identified individually by marking with picric acid solution. Three rats were housed in each cage and provided with standard mice pellets from C.P. Ltd. (Thailand) (Appendix A) and tap water *ad libitum*. The animals were quarantined for at least a week prior to dosing to acclimatize them to the controlled environment

conditions (Appendix B). The body weight, food and water consumption of all rats were measured daily during acclimatization time and throughout the experiments.

All animal experiments were carried out at the Laboratory Animal Center, Department of Medical Sciences, National Institute of Health (NIH Thailand), Ministry of Public Health.

Chemicals

β-NADH, sodium pyruvate, N-acetyl-L-cysteine, TRIS, and EDTA were purchased from Sigma Chemical Co. (USA). Methomyl with a purity of >98% was obtained by courtersy from Du Pont (Thailand) Limited. The reagents for the assay of LDH isoenzymes were purchased from Helena Laboratories (USA). The SGPT Kit was purchased from Clinical Diagnostic Co.(Thailand). All other chemicals used were purchased from Sigma Chemical Co. (USA).

1. Determination of possible relationships between serum LDH activity and LDH isoenzyme changes and methomyl toxicity in rats.

The objectives of this study were: 1) To assess and investigate the alteration of total LDH activity and LDH isoenzymes alterations in rats treated with various single doses of methomyl and repeated doses. The single dose administration was performed to investigate the acute toxicity, whereas the repeated dose administration was to investigate the subacute toxicity that possibly occurred in the spraymen using methomyl repeatedly in the field. 2) To investigate the histopathologic findings in rats treated with single and repeated dose of methomyl at various time points and explore the posible relationships between the effects of methomyl, total LDH activity, LDH isoenzymes, and hematological values as well as histopathologic findings.

Experimental designs

1. Single dose administration of methomyl

After acclimatization, 96 rats were divided into 4 groups as shown in table 11. The control animals were dosed with distilled water. The other three treatment groups were administered with freshly prepared methomyl solution at doses 3, 5 and 7 mg/kg, respectively. Six of each group were anesthetized for blood collection and then sacrificed for tissue collection on day 1, 3, 5, and 7 post-methomyl exposure. The organs collected for histopathological examination included liver, heart, spleen and kidney .

Table 11. Single dose administration of methomyl in rats.

Group	Time after treatment (days)						
	1	2	3	4	5	6	7
Control	n=6		n=6		n=6		n=6
3 mg/kg methomyl	n=6		n=6		n=6		n=6
5 mg/kg methomyl	n=6		n=6		n=6		n=6
7 mg/kg methomyl	n=6		n=6		n=6		n=6

2. Repeated dose administration of methomyl

Forty-eight rats were divided into 2 groups, control group and treatment group with methomyl at a dose 5 mg/kg/day for 5 days (table 12). The control animals were dosed with distilled water. The sample collections were performed as in the single dose protocol.

Table 12. Repeated dose administration of methomyl in rats.

Group	Time after treatment (days)						
	1	2	3	4	5	6	7
Control	n=6		n=6		n=6		n=6
5 mg/kg/d methomyl (for 5 days)	n=6		n=6		n=6		n=6

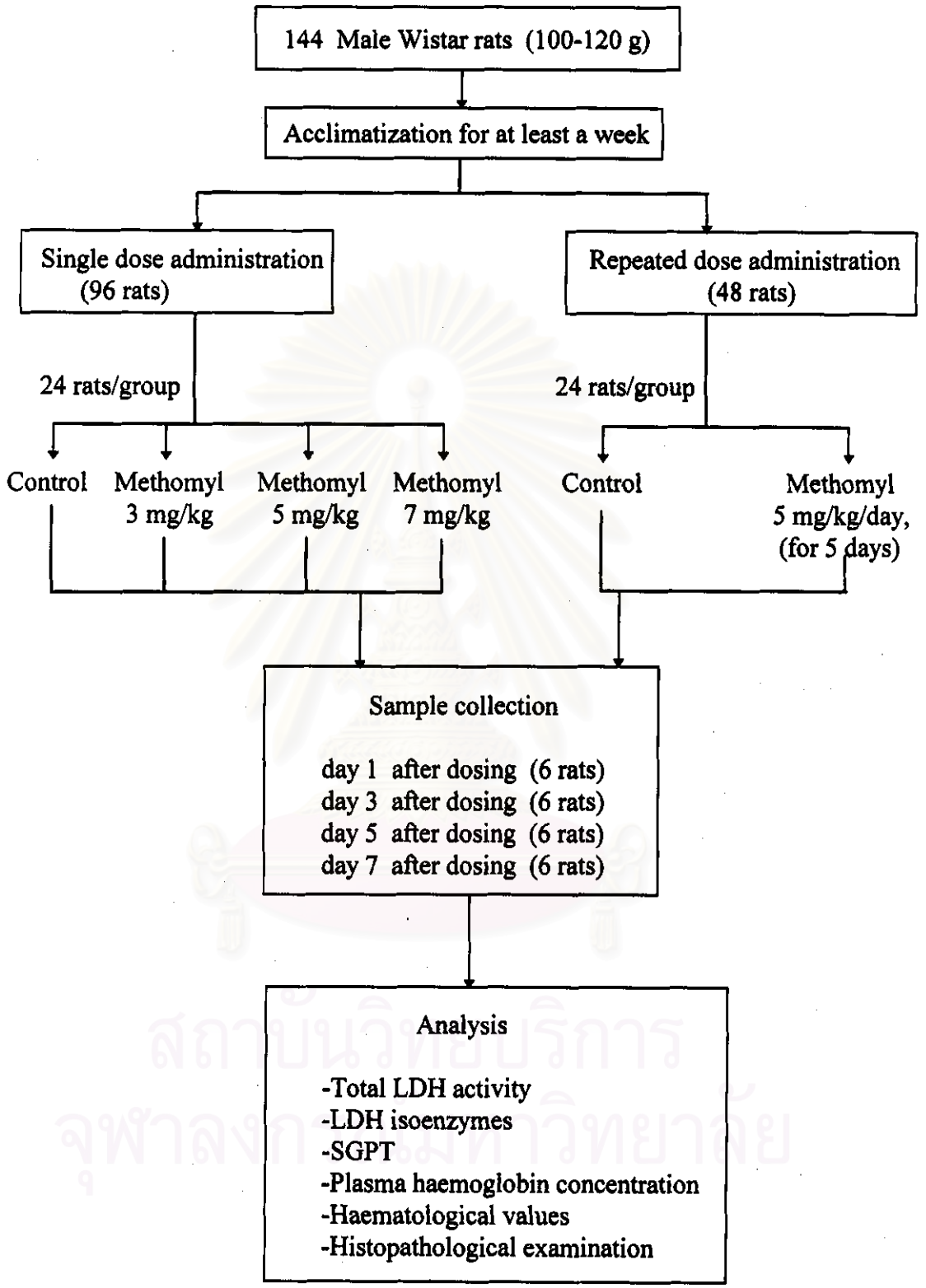


Figure 12. Experimental design for single dose and repeated dose administration of methomyl in rats.

Preparation of methomyl solution

Methomyl is well-dissolved in distilled water. The 0.12%, 0.20% and 0.28% water solutions of methomyl were prepared and administered to 3 mg/kg, 5 mg/kg, and 7 mg/kg-dose group, respectively.

1. Preparation of 0.28% methomyl solution. Weigh out 0.28g of methomyl and then dissolve in distilled water to 100 ml.

2. Preparation of 0.20% methomyl solution. Dilute 35.71 ml of 0.28% methomyl solution to 50 ml with distilled water.

3. Preparation of 0.12% methomyl solution. Dilute 21.43 ml of 0.28% methomyl solution to 50 ml with distilled water.

Dosing technique

The animals were weighed individually and then gavaged by a feeding needle attached to a 1-ml syringe. Methomyl was administered to animals at a constant dose volume (varying the dose concentration) (Chan and Hayes, 1989). The calculated dose volume of distilled water or methomyl solution for each rat was approximately 0.5 ml.

Blood collection technique

The blood samples were collected by jugular vein cannulation which was the most reliable method for blood collection in rats (Friedel, Diederichs, and Lindena, 1979). The PE-50 tube connected to 22-guage needle and 5-ml syringe was used for cannulation. The selected anticoagulant was heparin which did not affect the assay of activities of lactate dehydrogenase (LDH) and serum glutamate pyruvate transaminase (SGPT) enzymes (Moss and Henderson, 1994).

The animals were deeply anesthetized by pentobarbital sodium at a dose 50 mg/kg before collecting the blood (Cochetto and Bjornsson, 1983). Each blood sample was collected for 4 ml. One ml of the blood was transferred into a microtip for hematologic tests, 3 ml was transferred into a pyrex test tube. These samples were kept in ice-cold temperature and a little blood was smeared on two slides. The 3-ml blood samples were centrifuged as soon as possible at 2,000 g for 15 minutes (Kokusan, model H-108NA series). The plasma obtained was transferred into a microtip and then was kept in ice-cold temperature during waiting for enzyme assays.

Tissue collection technique

After collecting the blood, the anesthetized rats are operated for collecting organs including liver, spleen, heart, and kidney. These organs are removed and washed in saline solution. All changes in the size, color, or texture of these organs are recorded. They are weighed individually and then quickly fixed in 10% buffer formalin for further histopathological examination by an expert pathologist at the Department of Pathology, Faculty of Medicine, Chulalongkorn University. The evaluation of histopathological findings is graded from +1, +2, +3 and +4 according to the severity of lesions.

Determination of total LDH activity (Scandinavian Society for Clinical Chemistry and Clinical Physiology, 1974)

1. Principle

Pyruvate is reduced to lactate at pH 7.4 and 37°C in the presence of LDH. The progress of the accompanying oxidation of NADH to NAD^+ is monitored continuously by measuring the rate of absorbance decrease at 339 nm in a spectrophotometer.

2. Specimen

Heparinized plasma samples are obtained by centrifugation of the blood sample collected by jugular vein cannulation as mentioned above. These samples are analyzed as soon as possible (within 24 hours).

3. Materials

3.1 Instruments

1) Temperature controlled water bath
2) pH meter
3) Spectrophotometer (JASCO, model 7800)
4) Automatic pipette (2-ml, 200-μL, 50-μL)
5) 3-ml Polystyrene cuvet with 10-mm path length

3.2 Reagents

1) TRIS-EDTA buffer, pH 7.4 (37°C). Weigh out 6.7816 g of TRIS (56 mmol/L) and 2.0843 g of EDTA-disodium salt (5.6 mmol/L), dissolve in 900 ml of water, warm to 37°C, and carefully add HCl, 1 mol/L, to adjust the pH to 7.4 at 37°C. Make the solution up to 1 L. This reagent is stable for up to 6 wk when stored at 4°C in a tightly capped dark bottle.

2) β-NADH solution. Weigh out 10.8538 mg (170 μmol/L) of β-NADH (disodium salt) and dissolve in 90 ml of the TRIS-EDTA buffer, pH 7.4 (37°C). This reagent remains stable for at least 72 hours when stored in a tightly capped dark bottle at 4°C.

3) Pyruvate solution. Weigh out 154 mg (14 mmol/L) of sodium pyruvate and dissolve in 100 ml of water. This solution is stable for at least 20 days when stored at 4°C in a tightly capped bottle.

4. Procedure

4.1 Set up the spectrophotometer to the wavelength 339 nm with TRIS-EDTA buffer as reaction blank and warm the water bath. The absorbance is set up to record 2 times at every 30 seconds.

4.2 Pipet 2 ml of TRIS-EDTA buffer and 50 μL of plasma sample into a test tube. After mixing the contents, place the tube in the 37°C water bath for 15 minutes. This incubation permits a reduction by the NADH of any pyruvate and other oxo-acids present in the serum. At the end of the incubation period, add 200 μL of the pyruvate solution - which has been prewarmed to 37°C - mix rapidly, and transfer to a 3-ml polystyrene cuvet. Insert the cuvet in the spectrophotometer. Determine the initial reaction velocity by continuous recording (2 cycles, 30 second each) of the absorption at 339 nm.

The actual concentration of reaction components in the cuvet is as follows: TRIS buffer, 50 mmol/L (pH 7.4 at 37°C); EDTA, 5 mmol/L; pyruvate, 1.2 mmol/L; NADH, 150 μmol/L; volume fraction of serum = 0.023 (1:44).

5. Calculation

The LDH activity in international units at 37°C is obtained as follows:

$$U/L\ (\mu mol/min/L) = \frac{\Delta A/min}{6.3 \times 10^{-3}} \times \frac{2.250}{0.05} = \frac{\Delta A}{min} \times 7143$$

where 2.250 = total volume in cuvet, in milliliters

0.05 = volume of serum specimen, in milliliters

6.3×10^{-3} = micromolar absorption coefficient of NADH at 339 nm

ΔA/min = average absorbance change (decrease) per minute

6. Comments on precautions

6.1 Hemolysis samples must be ruled out by the determination of plasma hemoglobin concentration and the data is excluded.

6.2 Duplicated plasma samples are analyzed. Average total LDH activity is represented the enzyme activity of individual sample.

6.3 The calculation of the average absorbance change per minute (ΔA/min), using either the recorder tracing or the measured absorbance values. The linear portion of the rate curve or those consecutive individual values of ΔA/min is used.

Determination of LDH isoenzymes (Helena Laboratories, 1995)

1. Principle

The isoenzymes of LDH are separated according to their electrophoretic mobility on cellulose acetate in Tris-barbital-sodium barbital buffer. After separation, the isoenzymes can be detected colorimetrically. Using the Helena LD VIS ISOENZYME REAGENT, a tetrazolium salt is reduced with the formation of a colored formazan:

$$\text{NADH} + \text{Tetrazolium} \xrightleftharpoons{\text{Phenazine methosulfate}} \text{NAD} + \text{Formazan}$$

2. Specimen

Heparinized plasma is used. All samples are analyzed as soon as possible after collection (within 24 hours).

3. Materials

3.1 Instruments

1) Temperature controlled oven (Mammert)
2) Electrophoretic chamber (Zip Zone® Chamber, Helena Laboratories)
3) Power supply (300 V)
4) Scanning densitometer (Shimadzu, CS 930)
5) Cellulose acetate plate (TITAN® III PLATE, Helena Laboratories)
6) 8-sample Applicator (model Super Z, Helena Laboratories)
7) Blotters
8) Two pieces of glass (20 x 20 x 0.5 cm size)
9) Two pieces of glass slide
10) Water-proof marker pen

3.2 Reagents

1) Tris-barbital buffer (ELECTRA® HR BUFFER, Cat. No. 5805, Helena Laboratories). This commercial buffer is a Tris-barbital-sodium barbital buffer, pH 8.8 with an ionic strength of 0.029 when one package (18g) is diluted to 1750 ml with distilled water.

2) LDH isoenzyme reagent (LD VIS ISOENZYME REAGENT, Cat. No. 5909, Helena Laboratories). Reconstitute each vial of with 0.3 ml of distilled water. When reconstituted as directed, the concentrations of the reactive ingredients are 2.0 mM NAD, 68.5 mM lithium lactate, 0.9 mM MTT, 0.4 mM NBT, and $\geq$ 6.5 U Diaphorase.

3) LDH isoenzyme control (LD ISOENZYME CONTROL, Cat. No. 5919, Helena Laboratories). The control is used on each plate run to verify all phases of the procedure. The control is used as a marker for proper location of the isoenzyme bands and is quantitated for verification of quantitative results.

4. Procedure

4.1 Preparation of cellulose acetate plate (figure 13a).

1) Properly code the required number of plates by marking on the glossy, hard side with a water-proof marker pen. Place the mark in one corner of the plate so that it is always aligns with sample no.1. For each sample plate used, a substrate plate is also needed.

2) Soak the plates for 30 minutes in Tris-barbital-sodium barbital buffer. The plates should be wetted by slowly and uniformly lowering a rack of plates into the buffer. The same soaking buffer may be used repeatedly for approximately one week if stored tightly closed.

4.2 Preparation of LDH isoenzyme reagent. Reconstitute each vial of LDH isoenzyme reagent with 3.0 ml of distilled water.

4.3 Preparation of the electrophoretic chamber (figure 13b).

1) Pour approximately 100 ml of Tris-barbital-sodium barbital buffer into each of the outer sections of the chamber.

2) Wet two disposable wicks in the buffer and drape one over each support bridge being sure it makes contact with the buffer and that there are no air bubbles under the wick.

3) Cover the chamber to prevent buffer evaporation. Discard the buffer after use.

a b

Figure 13. Preparation of cellulose acetate plate (a) and electrophoretic chamber (b).

4.4 Sample preparation and application (figure 14)

1) Place a drop (approximately 10 μL) of sample into each well of the sample well plate using the microdispenser. Cover the samples with a glass slide if they are not used within 2 minutes (figure 14a).

2) Prime the applicator by depressing the tips into the sample wells 3-4 times. Apply this loading to a piece of blotter paper. Do not load the applicator again at this point, but proceed quickly to the next step. Priming the applicator makes the second loading more uniform (figure 14b).

3) Remove the wetted cellulose acetate plate from the buffer with the finger tips and blot once firmly. Place the plate in the aligning base, cellulose acetate side up, aligning the top of the plate with the black scribe line marker "cathode application". The identification mark should be aligned with sample no.1 (figure 14c).

4) Apply the sample to the plate by depressing the applicator tips into the sample well 3-4 times and promptly transfer the applicator to the aligning base. Press the button down and hold it 5 seconds (figure 14d).

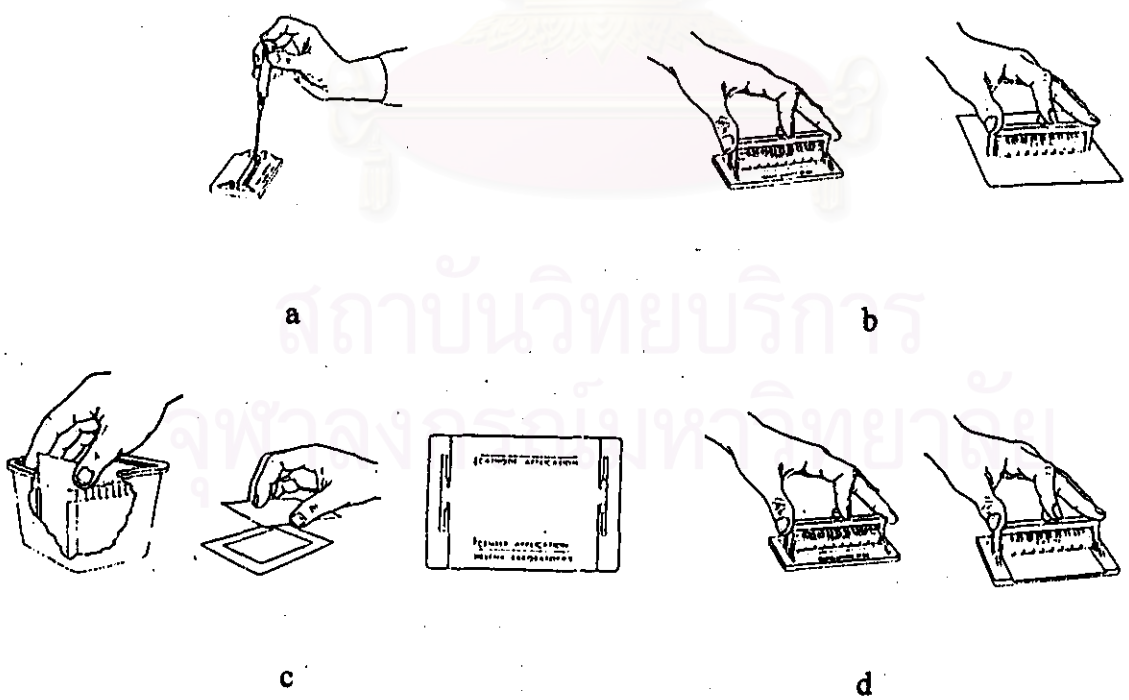


Figure 14. Sample preparation and application.

4.5 Electrophoresis of the sample plate (figure 15).

1) Quickly place the plate in the chamber, cellulose acetate side down. The application point should be close to the cathode (-). Place a glass slide on the plate to ensure contact with the wicks.

2) Allow the plates to lie in the chamber for 30 to 60 seconds before turning on the power. This allows the buffer to equilibrate in the plate.

3) Electrophorese the plates for 10 minutes at 300 volts.

4.6 Preparation of the substrate plate.

1) Immediately after starting the sample plats to electrophorese, remove the substrate plate from "the soaking buffer and blot once firmly.

2) Place the plate, cellulose acetate side up, on a clean glass development slide. Pipette 1.5 ml of the isoenzyme reagent (substrate) onto the cellulose acetate surface of the plate. Tilt the slide until the substrate covers the surface of the plate.

3) Allow the substrate to soak into the plate for the remaining period of electrophoresis. To ensure uniform distribution of the substrate, rotate the plate often during this period.

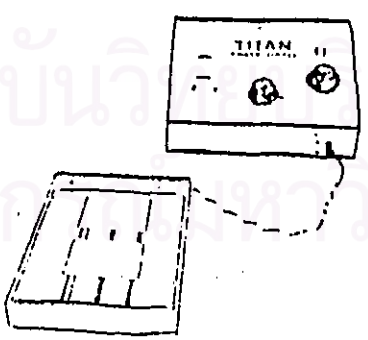


Figure 15. Electrophoresis of the sample plate.

4.7 Visualization of the isoenzyme bands (figure 16)

1) Remove the sample plate from the chamber at the end of the electrophoresis period and blot lightly.

2) Carefully layer the sample plate, cellulose acetate side down, onto the substrate plate. Place a blotter over the sandwiched plates. Remove the excess substrate from the sandwich by drawing the edge of the ALIGNING BASE across the plate several times. Blot away excess substrate (figure 16a).

3) After removing the excess substrate from the sandwiched plates, discard the blotter, but leave the plates on the DEVELOPMENT SLIDE.

4) Place the sandwiched plates on the glass slide in an incubator at 37°C (figure 16b).

5) Place another glass slide directly on the top of the sandwiched plates, and then place a preheated DEVELOPMENT WEIGHT on it.

6) Incubate the plates for 30 minutes at 37°C.

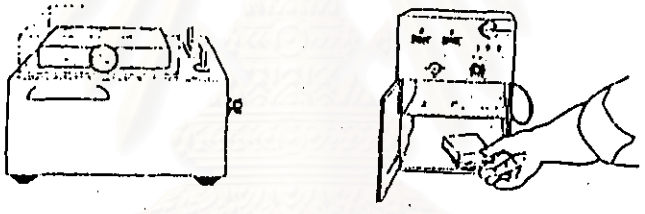
7) At the end of the incubation period, leave the sandwiched plate together and scan wet immediately by a scanning densitometer using 570 nm filter.

5. Calculation of each LDH isoenzyme.

Following electrophoresis, five zones of LDH activity can be demonstrated. The fastest zone is LDH-1 and the slowest zone is LDH-5. The remaining three zones have intermediate mobility. The LDH of normal plasma reflects the breakdown of numerous cells and all five components can be seen.

The relative concentration of the bands is the parameter determined. The scanning densitometer (Shimadzu, CS 930) with its accessories will automatically print the area under the curve of each band. If the data shows more than five bands, select the only five isoenzyme peaks by comparing the position of each band to the isoenzyme control which is concurrently scanned in the same plate, and then manually calculate the percent of each five isoenzymes selected. These calculated relative percents of the band of each isoenzyme are used as representative of each isoenzyme.

a



b

Figure 16. Visualization of isoenzyme bands.

6. Comments on precaution

6.1 Hemolysis sample must be avoided. Since erythrocytes contain 100-150 times more LDH than normal serum, the slightest hemolysis will contribute to gross error in assessment of LDH-1 and LDH-2 activity.

6.2 Do not freeze the plasma sample because LDH-5 is very unstable at freezing temperatures.

6.3 The sandwiched plate must be scanned at once after incubation because it will be dried or blurred and cannot be measured.

Haematologic tests

The determination of hematocrit, white cell count, and differential count were performed in this study (Shirley, 1982). The details of these tests are explained in Appendix C.

Determination of plasma haemoglobin

For this study, the assay of plasma hemoglobin was used for ruling out the hemolyzed sample which affected the analysis of enzyme activities (Fairbanks and Klee, 1994). The details of assay are explained in Appendix D.

Determination of glutamate pyruvate transaminase (GPT) activity

GPT is an enzyme used to indicate the toxicity of many organs, particularly liver. The plasma GPT determination performed in this study is to confirm the sample which has LDH-4 and LDH-5 elevation which may indicate hepatotoxicity (Clinical Diagnostics, 1996). The details of enzyme analysis are explained in Appendix E.

Data analysis

Results are expressed as mean $\pm$ S.E. For statistical comparisons of total LDH activity, each LDH isoenzymes and relative organ weight among control and treatment groups, the one-way or two-way ANOVA are used coupled with Duncan's multiple range test . A probability level (p-value) of <0.05 is regarded as significant.

2. Determination of splenotoxicity in methomyl exposed rats

From the previous study in rats (Kaplan and Sherman, 1977) and the total LDH activity and LDH-4 increases in serum of rats receiving methomyl in the first study, there were suggestions that the spleen may be a target organ of methomyl. The specific objective of this study was to investigate the splenotoxicity in methomyl-exposed rats by comparing the spleen weight and splenocyte viability of the treatment groups to the control groups. Erythrocyte cholinesterase activity and cholinesterase activity in the spleen were also determined to assess the relevance of the methomyl-induced splenotoxicity to anticholinesterase effect.

Experimental designs

After acclimatization, 96 rats were divided into 4 groups as shown in table 13. The control animals were dosed with distilled water. Two treatment groups were administered with freshly prepared methomyl solution at doses 6 and 8 mg/kg, respectively. The last group was pretreated orally with 60 mg/kg N-acetylcysteine (NAC) following an acute dose of 8 mg/kg of methomyl. Eight of each group were anesthetized for blood collection and then sacrificed for spleen collection on day 1, 3, and 5 after treatment.

Table 13. Administration protocol for determination of splenotoxicity in methomyl-exposed rats.

Group	Time after treatment (days)				
	1	2	3	4	5
Control	n=8		n=8		n=8
6 mg/kg methomyl	n=8		n=8		n=8
8 mg/kg methomyl	n=8		n=8		n=8
60 mg/kg NAC and 8 mg/kg methomyl	n=8		n=8		n=8

Preparation of methomyl solution

The 0.24% and 0.32% water solutions of methomyl were prepared and administered to 6 and 8 mg/kg-dose group, respectively.

1. Preparation of 0.32% methomyl solution. Weigh out 0.32g of methomyl and then dissolve in distilled water to 100 ml.

2. Preparation of 0.24% methomyl solution. Dilute 37.5 ml of 0.32% methomyl solution to 50 ml with distilled water.

Preparation of N-acetylcysteine solution

The 2% N-acetylcysteine (NAC) solution was prepared by weighing out 1 g of NAC and then dissolve in distilled water to 50 ml.

Dosing technique

The rats were weighed individually and dosed by gavage tube similar to those performed in the first study.

Blood collection technique

The 2-ml blood samples were collected by cardiac puncture. The 23-gauge needle was used. The blood sample was transferred into a microtip and kept in ice-cold temperature for determination of hemoglobin and erythrocyte cholinesterase activity.

Tissue collection technique

After collecting the blood, the anesthetized rats are operated for collecting the spleen. The spleen was removed, washed in saline solution, weighed and then quickly kept in ice-cold phosphate buffered saline (PBS) for determination of splenocyte viability and cholinesterase activity. The determination of splenocyte viability should be performed as soon as possible.

Determination of splenocyte viability (Updyke et al., 1991)

1. Preparation of single-cell splenic suspensions

1.1 Press the bottom of a test tube gently on the spleen to disperse the cells through a stainless-steel mesh screen which is located on a petri-dish.

1.2 Wash the cells on the mesh screen and petri-dish by PBS and then transfer the suspension into a test tube.

1.3 Centrifuge the suspension at 2,000g for 15 minutes and remove the supernatant.

1.4 Erythrocytes were removed by lysing with 2 ml of 0.15M Tris buffered ammonium chloride (pH 7.2) for 5 minutes and rapidly add 4 ml of PBS.

1.5 Centrifuge the suspension and remove the supernatant.

1.6 Lyse the erythrocytes and the cells once again (1.4-1.5).

1.7 The pellet (spleen cells) is dispersed in 4-ml PBS.

2. Determination of splenocyte viability

2.1 Pipet 20 µl of cell suspension into 0.2-ml trypan blue (1:10 dilution)

2.2 Mix and pipet the mixture into a hemocytometer.

2.3 Count the living cells and dead cells (blue-stained cells).

2.4 Calculate the percentage of splenocyte viability as follows:

$$\% \text{ Splenocyte viability} = \frac{\text{no. of living cells} * 100}{(\text{no. of living cells} + \text{no. of dead cells})}$$

Determination of erythrocyte cholinesterase activity (Ellman et al., 1961)

1. Principle

The acetylthiocholine is used as a substrate. The cholinesterase activity is continuously measured at 412 nm in a spectrophotometer by following the increase of yellow color preduced from thiocholine when it reacts with dithiobisnitrobenzoate (DTNB) ion.

acetylthiocholine $\xrightarrow{\text{cholinesterase}}$ thiocholine + acetate

thiocholine + dithiobisnitrobenzoate $\longrightarrow$ yellow color

2. Specimen

Whole blood is used in this assay and EDTA is used as an anticoagulant.

3. Materials

3.1 Instruments

1) pH meter
2) Spectrophotometer (JASCO, model 7800)
3) Automatic pipette (5-ml, 100-μL, 50-μL)
4) 3-ml Polystyrene cuvet with 10-mm path length

3.2 Reagents

1) 0.1 M Phosphate buffer (pH 8.0)
2) 0.075M Acetylthiocholine iodide (21.67 mg/ml)
3) 0.01 M Dithiobisnitrobenzoic acid (DTNB)

Dissolve 39.6 mg of DTNB in 10 ml pH 7.0 phosphate buffer (0.1 M) and then add 15 mg of sodium bicarbonate.

4) 0.1% quinidine sulfate (inhibitor of plasma cholinesterase)

4. Procedure

4.1 Set up the spectrophotometer to the wavelength 412 nm with mixture of 10 µl of 0.1% quinidine sulfate and 25 µl of DTNB reagent in 3-ml phosphate buffer as reaction blank. The absorbance is set up to record 7 times at every 1 minutes.

4.2 The blood cell suspension is prepared by dilute 10 µl of blood sample into 6 ml phosphate buffer (0.1M, pH 8.0).

4.3 Pipet 3 ml of blood cell suspention into a cuvet

4.4 Add 10 µl of 0.1% quinidine sulfate, 25 µl of DTNB reagent and 20 µl of substrate (0.075 M acetylthiocholine).

4.5 Mix rapidly and insert the cuvet in the spectrophotometer. Determine the initial reaction velocity by continuous recording of the absorption at 412 nm.

5. Calculation

The erythrocyte cholinesterase activity is obtained as follows:

$$\text{U (µmol/min) /g hemoglobin} = \frac{(4.41 \times 10^{-2})\ \Delta A/\text{min}}{\text{hemoglobin (g/L)}}$$

where $\Delta A/\text{min}$ = average absorbance change (decrease) per minute

Determination of cholinesterase activity in spleen (Ellman et al., 1961)

1. Principle

Simillar to the determination of erythrocyte cholinesterase activity.

2. Specimen

Spleen sample is kept in ice-cold PBS during waiting for enzyme assay.

3. Materials

3.1 Instruments

1) pH meter
2) Spectrophotometer (JASCO, model 7800)
3) Automatic pipette (5-ml, 100-μL, 50-μL)
4) 3-ml Polystyrene cuvet with 10-mm path length
5) Homogenizer
6) Refrigerated centrifuge

3.2 Reagents

1) 0.1 M Phosphate buffer (pH 8.0)
2) 0.075M Acetylthiocholine iodide (21.67 mg/ml)
3) 0.01 M Dithiobisnitrobenzoic acid (DTNB)

Dissolve 39.6 mg of DTNB in 10 ml pH 7.0 phosphate buffer (0.1 M) and then add 15 mg of sodium bicarbonate.

4. Procedure

4.1 Mince the spleen in 20-ml phosphate buffer (0.1 M, pH 8.0) and homogenized in a homogenizer.

4.2 Transfer into the centrifuge tube and centrifuge at 10,000 g, for 15 minutes (at 4 °C).

4.3 Remove the supernatant and resuspend with 20-ml phosphate buffer.

4.4 Recentrifuge, remove the supernatant and resuspend with 20-ml phosphate buffer.

4.5 Set up the spectrophotometer to the wavelength 412 nm with mixture of 100 μl of DTNB reagent in 3-ml phosphate buffer as reaction blank. The absorbance is set up to record 7 times at every 1 minutes.

4.6 Pipet 0.4 ml of the suspention into a cuvet and add 2.6 ml of phosphate buffer.

4.7 Add 100 µl of DTNB reagent and 20 µl of substrate (0.075 M acetylthiocholine).

4.8 Mix rapidly and insert the cuvet in the spectrophotometer. Determine the initial reaction velocity by continuous recording of the absorption at 412 nm.

5. Calculation

The cholinesterase activity of spleen is obtained as follows:

$$U\ (\mu mol/min)\ /g\ tissue = \frac{(5.74 \times 10^{-4})\ \Delta A/min}{C}$$

where $\Delta A/min$ = average absorbance change (decrease) per minute

C = original concentration of tissue (mg/ml)

Data analysis

Results are expressed as mean ± S.D. For statistical comparisons of spleen weight and splenocyte viability among control and treatment groups, the student's t-test was used.