CHAPTER III

MATERIALS AND METHODS

The basic study is a retrospective study (case-control study), which analyses the concentration of chlorinated hydrocarbon insecticides in blood of Thai cancer patients compared with normal subjects.

1. Population

The studied population was adopted on cancer patients and healthy subjects who visited the National Cancer Institute, Medical Service Department, Public Health Ministry, during the period of June 1992 to December 1992.

In order to satisfy the objectives of this study, i.e., to investigate the carcinogenic risk of chlorinated hydrocarbon insecticides exposure, individuals visiting the institute and were occupationally exposed to other known carcinogens were excluded.

1.1 Selection of Samples

The study was conducted in twenty-three men and thirty-nine women whose age ranging from 26 to 67 years

with cancer of various organs newly diagnosed during the period of June 1992 to December 1992 at the National Cancer Institute, Medical Service Department, Public Health Ministry and had not yet been treated with any cancer medications. According to the physician's records a total of 62 cases were : 14 carcinomas of buccal cavity, 5 carcinomas of digestive system, 5 liver carcinomas, 6 nasopharyngeal carcinomas, 6 lung carcinomas, 10 breast carcinomas, 8 skin carcinomas, 4 lymphomas, 3 thyroid carcinomas and 1 mass of orbit.

1.2 Selection of Controls

The control group was recruited from the non-cancer subjects who visited the same institute during the same period for medical examination. They were matched with the cancer cases by sex, geographic area of residence and age (the age difference between the matched case and control is no more than 5 years).

2. Specimen Collection

Two ml of blood was taken from each subject for analysis of chlorinated hydrocarbon insecticides.The blood samples were stored frozen at -4 °C in glass containers with EDTA (ethylenediaminetetraacetic acid) as anticoagulant until analysis.

3. Collection of Data

The personal and medical data of the case and control groups were obtained by means of predesigned questionaire (form of questionaire is shown in Appendix D). Each subject was interviewed by the investigator and questionaire filled out. The completed questionaire provides the following informations : name and address, sex, age, marital status,incidence of cancer in the family, occupation, exposure to chemicals and organ with cancer. The validity of medical data (i.e.,the cancerous organ) was ascertained by checking with the physician's diagnosis report. The chlorinated hydrocarbon insecticide contents in the blood drawn from all subjects were quantitated by gas liquid chromatographic technique.

4. Determination of Chlorinated Hydrocarbon Insecticide in Blood by Gas Liquid Chromatography (35)

4.1 Principle

Chlorinated hydrocarbon pesticides in whole blood are liberated from blood structure by addition of 60 % H_2SO_4. Pesticides are then extracted from acidified blood by hexane-acetone (9:1) and analyzed by electron capture gas liquid chromatography (GLC) by comparison of peak heights or areas with those of suitable standard mixtures of chlorinated pesticides. If peak widths are 25 % of peak

heights,peak heights should be multiplied by 0.54 peak width or area measurements should be made.(36)

4.2 Apparatus

- gas liquid chromatography-electron capture detector : Varian model 3700
- recorder : Varian model 9176
- vortex mixer:Scientific industries model K-550-GE
- centrifuge : Kokusan Enshinki model H-103 N series
- 15 ml graduated concentrator tubes
- microsyringe 10 µl
- Pasteur pipet
- 20 ml round-bottom tubes with screw cap

4.3 Chemicals and reagents

4.3.1 The chemicals obtained from J.T.Baker Chemical Co. U.S.A. were hexane (A.R.grade) and petroleum ether (A.R.grade).

4.3.2 Acetone (A.R. grade) obtained from Mallinckrodt, Inc. France.

4.3.3 Conc. sulphuric acid (A.R.grade) obtained from E.Merck Germany.

4.3.4 The following stock standard solutions were obtained from FAO/WHO ; aldrin, chlordane, dieldrin, p,p'-DDD(para,para'-dichlorodiphenyl dichloroethane), o,p'-DDE (ortho,para-dichloro diphenyldichloroethylene), p,p'-DDE(para,para-dichlorodiphenyldichloroethylene), o,p'-DDT

(1, 1, 1- trichloro -2-(ortho-chlorophenyl)-2-(para-chlorophenyl) ethane,endrin, heptachlor, heptachlor epoxide, lindane as concentration 1 g/l in petroleum ether.

4.3.5 The working standards were 1 mg/l of aldrin, chlordane dieldrin, p,p'-DDD, o,p'-DDE, o,p'-DDT, endrin, heptachlor, heptachlor epoxide and lindane. They were prepared by diluting the 1 g/l stock solution 1000-fold. In the case of p,p'-DDE, the standard concentration was 0.2 mg/l which was prepared by diluting from 1g/l stock solution 5000-fold.

4.4 Operating system

4.4.1 Column : 10% DC-200 on 100/120 mesh Chromosorb W-HMDS 1.8 m x 2 mm

: 6% QF-1 on 80/100 mesh Chromosorb W-HMDS 1.8 m x 2 mm

: temperature 160 °C

4.4.2 Detector : eletron-capture detector

: temperature 290 °C

4.4.3 Injector port : temperature 230 °C

4.4.4 Gas flow rate : N_2 30 ml/min

4.4.5 Sensitivity : $256x10^{-11}$

4.4.6 Chart flow rate : 0.25 cm/min

4.5 Extraction procedure

4.5.1 Take a 2 ml sample of whole blood into a 20 ml tube.

4.5.2 Add a 1.5 ml of 60 % by weight of sulphuric acid (60 g H_2SO_4 / 40 g water) and mix 3-4 seconds on Vortex mixer. Add a second 1.5 ml of 60 % sulphuric acid and mix as before.

4.5.3 Finally, add a 2 ml of sulphuric acid and mix for 10 seconds (5 ml of acid total).

4.5.4 Cool 2-3 minutes, then add 3 ml of hexane/ acetone (9:1)

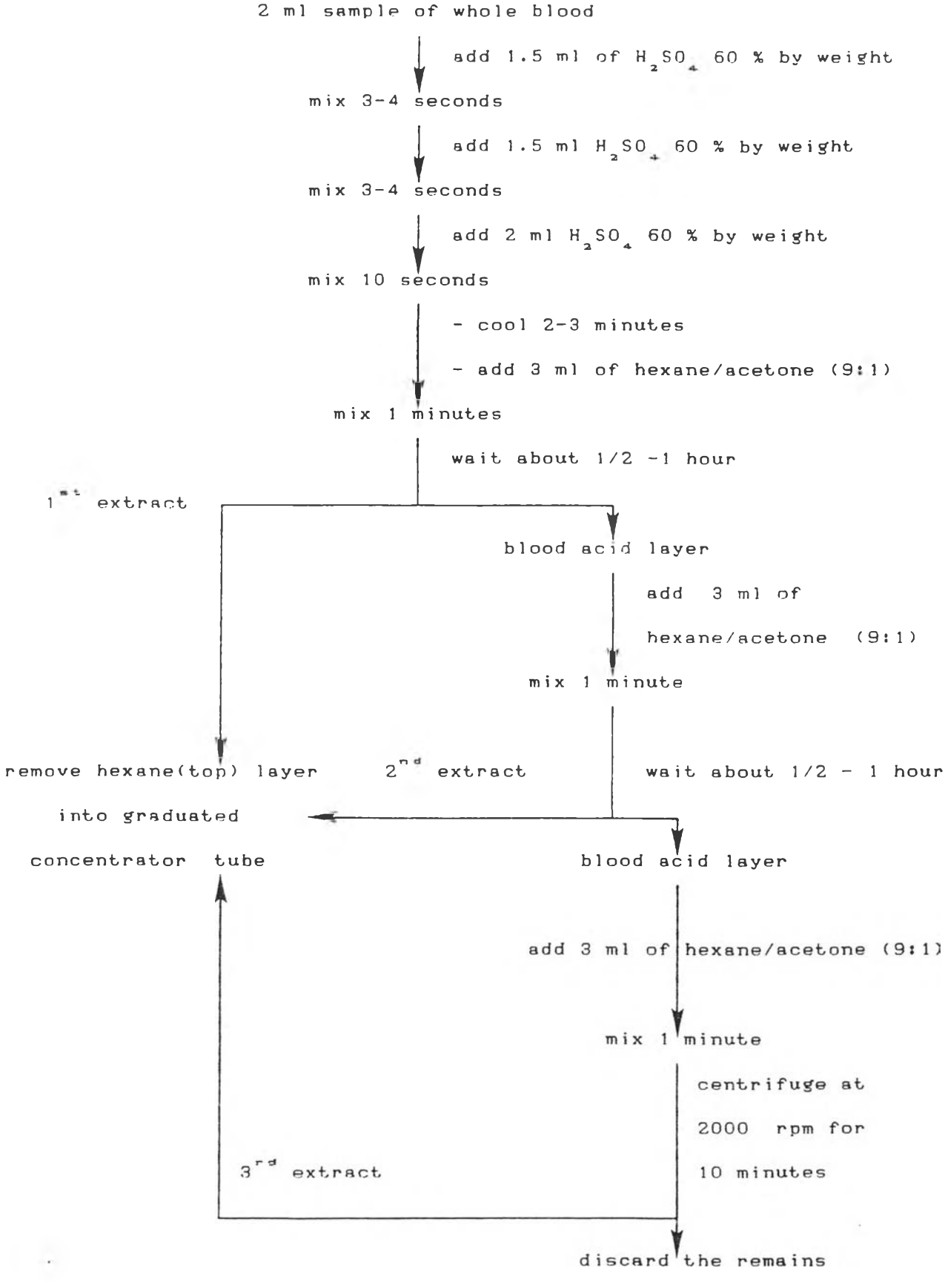
4.5.5 Cap the tube and mix on Vortex mixer for 1 minute.

4.5.6 The tube was left standing until the hexane-acetone layer seperate from blood-acid mixture (about 1/2-1 hour),then remove the hexane(top) layer with Pasteur pipet and place the extract in a 15 ml graduated concentrator tube. Stopper tube and mix.

4.5.7 Complete all 3 extractions during 1 working day, since decomposition of the pesticides occure when they are allowed to be in contact with the blood-acid mixture for an excessive length of time.(For the last extraction, after mixing on Vortex mixer, the mixture was transferred to centrifuge tube and centrifuge for 10 minutes at 2000 rpm before remove the hexane layer.)

4.5.8 Add sodium sulfate to sample container to make moisture-free extact.

4.5.9 Reduce extract volume by passing gentle stream of clean, dry air over the surface of extract to volume of 0.2 ml or 0.5 ml in some cases.

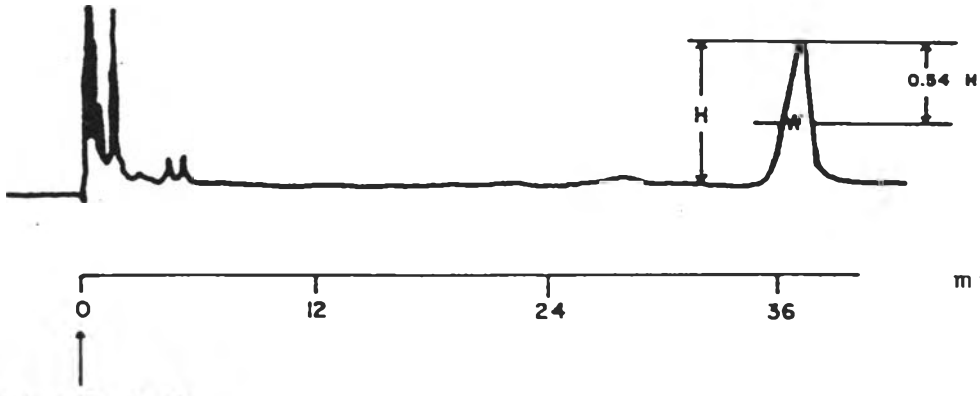


FLOW CHART OF EXTRACTION PROCEDURE

4.6 Qualitative and quantitative determinations of chlorinated hydrocarbon insecticides from chromatograms

The identity of chlorinated hydrocarbon insecticides in the blood extract was achieved by comparing the retention time of the peaks in the chromatogram of blood extract with that of known standards. Two different columns were employed in order to confirm the identity of the insecticides. When the identity of the insecticides is known, their quantity can be determined by comparing their peak heights or areas with that of the standards as described in section 4.1. Most blood sample contain chlorinated hydrocarbon insecticides in the range 1-400 ppb. Dilute stock insecticides to obtain standard in proper range. An example of the qualitative and quantitative measurements of chlorinated hydrocarbon insecticides from chromatograms is shown in figure 3.

Sample chromatogram



Inject 2 µl of sample

retention time = 36.8 minutes

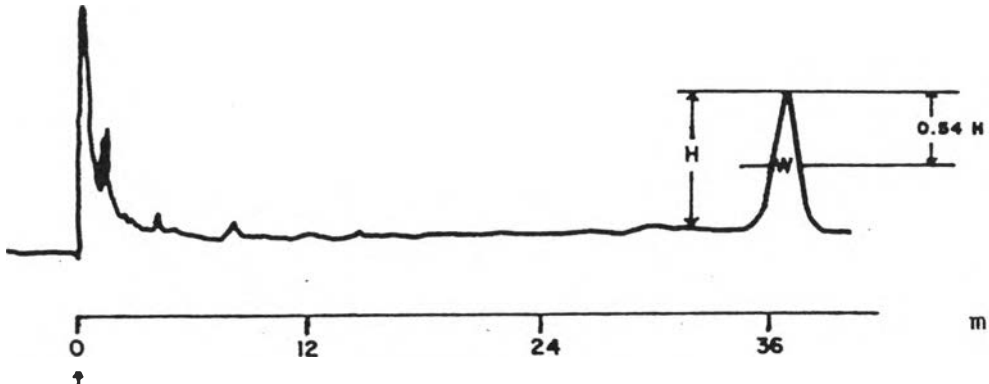
peak height = 1.9 cm

width (at 0.54 of height) = 0.35 cm

peak area = 1.9 x 0.35

= 0.665 cm^2

Standard chromatogram



Inject 0.5 ul of
1 ng/µl of pp'-DDE

retention time = 36.8 minutes

peak height = 1.8 cm

width (at 0.54 of height) = 0.35 cm

peak area = 1.8 x 0.35

= 0.630 cm^2

Figure 3. Example of chromatograms in qualitative and quantitative measurement of chlorinated hydrocarbon insecticide

Therefore, by comparison of the retention times, the insecticide in sample chromatogram is p,p'-DDE.

$$\text{The amount of p,p'-DDE in 2 } \mu\text{l sample (all=0.2 ml)} = \frac{0.665}{0.630} \times 0.5$$

$$= 0.528 \text{ ng}$$

$$\text{The concentration of p,p'-DDE in blood} = \frac{0.528 \times 0.2 \times 1000}{2 \times 2}$$

$$= 26.4 \text{ ng/ml(ppb)}$$

4.7 Recovery of chlorinated hydrocarbon insecticides from blood

Recovery experiments were performed in order to determine whether the chlorinated hydrocarbon insecticides in blood sample were satisfactorily recovered by the adopted extraction method. The organochlorine standards were added to control blood to give concentrations of 5 and 50 ppb. The insecticides were then extracted and quantitated as described in section 4.5 and 4.6 respectively. The results of recovery experiments are shown in table 2.

Table 2. The results of recovery experiments

Compound	Blood fortified to 50 ppb		Blood fortified to 5 ppb	
	amount recovered	% recovery	amount recovered	% recovery
Aldrin	46.15	92.30	5.00	100.00
	55.77	111.54	4.35	87.00
Chlordane	57.44	114.89	4.90	98.00
	52.13	104.25	5.20	104.00
Dieldrin	43.21	86.42	5.13	102.60
	43.21	86.42	5.13	102.60
Heptachlor	43.93	87.87	5.65	113.00
	45.56	91.12	5.65	113.00
Heptachlor epoxide	44.12	88.24	5.83	116.60
	55.88	111.76	4.86	97.20

Table 2. The results of recovery experiments (continued)

Compound	Blood fortified to 50 ppb		Blood fortified to 5 ppb	
	amount recovered	% recovery	amount recovered	% recovery
Lindane	46.92	93.83	5.54	110.80
	49.60	99.20	5.56	111.20
o,p'- DDE	41.67	83.33	5.00	100.00
	58.33	116.67	5.00	100.00
p,p'- DDD	48.69	97.38	-	-
	46.43	92.86	-	-
p,p'- DDE	55.65	111.30	-	-
	43.42	83.10	-	-
n	18		14	
$\bar{X}$	48.78		5.20	
SD	5.62		0.40	
% CV	11.52		7.69	

5. Pilot study

Pilot study was performed with 22 cancer patients and 22 normal subjects. They were interviewed by the investigator and the questionaires filled out to collect personal and medical data. The blood sample was taken from each subject. The extraction of chlorinated hydrocarbon insecticides from blood sample was conducted as follow :

5.1 Take a 2 ml sample of whole blood into a 50 ml seperatory funnel

5.2 Add 10 ml of hexane and shake vigorously for 10 minutes

5.3 The seperatory funnel was left standing for 2-3 minutes to seperate the hexane layer.

5.4 Keep the hexane (top) layer and transferred into 50 ml glass beaker.

5.5 Repeat step 5.2-5.4 three times.

5.6 Pool the hexane extract and then add sodium sulfate to make it moisture-free.

5.7 Reduce extract volume to 0.2 or 0.5 ml by passing gentle stream of clean, dry air over the surface of the extract in graduated concentrator tube.

The identity and quantity of the chlorinated hydrocarbon insecticides in the extract were determined according to section 4.6.The result is shown in table 3.

Table 3. The results of pilot study

	mean of chlorinated hydrocarbon(total DDT) Conc^{n} $\bar{X}$ (ppb)	SD
Sample (n=22)	17	21.3
Control (n=22)	6	8.9

Mean difference = 17-6 ppb

= 11 ppb

S_p^2 = 266.45

6. Sample Size

In this study, populations of cancer pateints and normal subjects are equal. Sample size determination can be computed from the formula (37).

$$n = \frac{(Z_\alpha + Z_\beta)^2 \times 2 \times S_p^2}{D^2}$$

n = sample size

Z_α = critical values of the significant level of α (type I error)

Z_β = critical values of the significant level of β (type II error)

S_p^2 = pool variance

D = mean difference between two groups

From pilot study

Z_α = 1.96 when α = 0.05

Z_β = 1.64 when β = 0.10

D = mean difference of DDT which is 11 ppb

S_p^2 = 266.45

So $$n = \frac{(1.96 + 1.64)^2 \times 2 \times 266.45}{(11)^2}$$

$$= 57$$

Thus,the number of case and control subject in this study each must be at least 57. The sample method employed is the purposive sampling.

7. Data analysis

Unpaired Student's t-test was employed to determine the statistical significance of the difference in blood chlorinated hydrocarbon insecticides content between the case and control groups. In the case of determination of the difference of that among more than two groups in various factor, ANOVA (Analysis of variance) was employed. This and other data analysis were carried out with SPSS/PC+ (Statistical Package for the Social Sciences Personal Computer Plus).