CHAPTER III

Results

1. The study of ELISA technique

1.1 Conditions for Tests

The suitable conditions of the ELISA method for detection serum ferritin level were studied. The results of various conditions of the tests which were modified from Anderson and Kelly's ELISA technique were shown in Figures 10-15. Each condition of the test was repeated for 5 times.

The results were plotted between various quantity of standard ferritin in nanogram per millilitre (ng/ml) against the optical density (O.D.) of the test. The quantity of ferritin is on the horizontal axis and the optical density on vertical axis. In Figures 10-15, the downward vertical lines showed the variation of 2 S.D.. The upward 2 S.D. variation were omitted.

1.1.1 The Effect of Incubating Temperature

The sandwich reaction of ferritin with antiferritin and peroxidase conjugated antiferritin with ferritin were done at room temperature (RT) and at 37°C (water bath) while other conditions were kept according to the Anderson and Kelly's method.

Figure 10 showing the results of incubating temperature at 37°C (water bath) for the reaction of antiferritin with ferritin and peroxidase conjugated antiferritin with ferritin when compared to the incubating temperature at RT. It showed that the incubating temperature at 37°C (water bath) is more suitable because the slope was closed to 45 degrees (the theoretical slope should be 45 degrees). So, the incubating temperature of 37°C (water bath) was used for this study.

1.1.2 The Effect of Incubation Period on the Reaction of Ferritin with Antiferritin

The incubation periods of 15, 30 and 60 minutes for the reaction of antiferritin with ferritin were tested while other conditions were kept as the original Anderson and Kelly's method, except the temperature of incubation was 37°C (water bath) and the period for incubation ferritin with peroxidase conjugated antiferritin was 30 minutes at 37°C (water bath).

The results were compared in the Figure 11. All the curves showed similar characters, except the 15 minute incubation period showed less variation. So, in this study, the 15 minute incubation period for the reaction of ferritin with antiferritin was used. This period reduces one half of time required in the test.

1.1.3 The Effect of Incubation Period on the Reaction of Peroxidase Conjugated Antiferritin with Ferritin

The incubation periods of 15, 30 and 60 minutes for the reactions of ferritin with peroxidase conjugated antiferritin were tested while other conditions were kept as Anderson and Kelly's method, except the temperature of incubation was 37°C (water bath) and the incubation period of ferritin with antiferritin was 30 minutes at 37°C (water bath).

The results were compared in the Figure 12. All the curves also showed similar characters but the 60 minutes curve was slightly higher in optical density than the 15 and 30 minute ones, but it showed higher variation. The 15 and 30 minute curves was quite closed in variation. So, the 15 minute incubation period for this reaction was used.

1.1.4 The Comparison of Incubation Period between 15,15 minutes and 30,30 minutes for the Reaction of Antiferritin with Ferritin and Peroxidase Conjugated Antiferritin with Ferritin

The incubation periods of 15, 15 minutes for the reaction of antiferritin with ferritin and peroxidase conjugated antiferritin with ferritin were compared with 30,30 minute incubation period while other conditions were kept as the Anderson and Kelly's method, but the incubation temperature was 37°C (water bath).

Both curves were closely similar in both variation and character. These tests confirmed that the results of 15, 15 minute incubation period for the reaction of antiferritin with ferritin and peroxidase conjugated antiferritin with ferritin were comparable with 30,30 minutes, with considerable saving of working time.

1.1.5 The Optimal Dilution of Antiferritin for Coating

Figure 14 shows various dilutions of antiferritin for coating the polystyrene ELISA plate. The serial dilutions of 1:1000, 1:1500, 1:2000 and 1:2500 were tested. The results of 1:1000, 1:1500 and 1:2000 dilutions were nearly similar while the 1:2500 dilution curve was

deviate from all the others. However, the dilution of 1:2000 showed higher variation. In any case, when considered all factors including the economy, the 1:1500 dilution of antiferritin is the most suitable for coating.

1.1.6 The Optimal Volume of Peroxidase Conjugated Antiferritin for study

Figure 15 showing the results of the tests when the various volumes of peroxidase conjugated antiferritin were 40, 50 and 60 µl in 12 ml of conjugate diluent solution (NSS 9 ml, chicken serum 3 ml and added with 100 µl of 1.0 M.sodium citrate). All the curves were nearly similar variation but the curve of 40 µl started to drop at the level of 200 ng/ml. Considering the efficiency of the test and economy, the volume of 50 µl was used.

1.2 The Precision Test of Modified Anderson and Kelly's ELISA Technique

Figures 16 to 18 and Table 4 were the results of the precision test of modified Anderson and Kelly's ELISA technique. The study included both within assay and between assay using 3 levels of serum control, low, medium and high levels (n = 20). From table 4, the coefficient of variation (% CV) of within assay were 3.95, 3.67 and 12.52, and between assay were 4.96, 9.07 and 14.57,

in order of low, medium and high level. From this data, the precision of modified Anderson and Kelly's technique of low, medium level of serum control were acceptable for testing, but the variation of high level (about 430 ng/ml) was slightly too high and not suitable for test. In a case of high ferritin sample, the serum should be further diluted (the suitable of % CV should be less than 10 (97)).

1.3 The Accuracy of The Test

Figure 19 was the result of the comparison between modified Anderson and Kelly's ELISA technique and Radioimmuno Assay (RIA). The RIA Gammadab kit (Cat. no. CA-590 Clinical assays, Division of Travenol Laboratories, INC.) was used for RIA examination (98). Amounts of 30 random samples were examined , the coefficient correlation (r) = 0.987 and the correlation equation is: Y = .91 X + 3.8. This high coefficient correlation (r = 0.987) indicates that the result of modified Anderson and Kelly's ELISA technigue for serum ferritin determination was exactly equal to RIA technique.

1.4 The sensitivity of the test

The result of quantitative test for serum ferritin by the modified Anderson and Kelly's technique was shown in Figure 20. The sensitivity of this main method was

determined by the O.D. value in the standard curve. The least ferritin concentration which the O.D. value equals to the O.D. of mean + 2SD. of blank (no ferritin contained) is the sensitivity of the test (98,99,100), approximately 2 ng/ml can be detected by this method (Figure 21).

2. The Radial Immunodiffusion Technique(RID) and the Counter Immunoelectrophoresis Technique(CIEP)

The results of the RID and the CIEP technique were studied using variations of diluted standard ferritin as shown in Figures 22 and 24, respectively. The minimum amount of ferritin which can be detected by the RID technique is 10 µg/ml (9 µg/ml of ferritin produced no visible precipitin ring as shown in Figure 22). The standard curve of this method for quantitative detection of soluble antigen in agarose was shown in Figure 23.

In the case of the CIEP technique, 3 µg/ml was the smallest amount of ferritin which could be detected by this method for quanlitative detection of soluble antigen in agarose (Figure 24).

Note The sera which were concentrated by lyphogel were too sticky when the concentration was more than 3-4 folds. It is too sticky to be accurately pipetted and it caused irregularity in filling of agarose wells.

3. The serum ferritin level of all samples in each group

Serum ferritin levels of all serum samples in groups of lung cancer, non cancer lung disease, liver cancer, other cancers, and normal persons show in Figure 25. All data are tabulated in detail in Tables 5 - 10.

Table 5 shows the percentage of high serum ferritin samples from each group. It showed that all 13 males of metastatic lung cancer had high serum ferritin level. The percentage of high ferritin in the group of non-treated, non-metastatic lung cancer, inflammatory lung disease, non treated tuberculosis and pneumonitis in male were 64.5 %, (n=31), 52.3 % (n=21), and 46.3 % (n=28) and 42.8% (n=7) respectively. The results were non-significantly different when compared by chi-square test (X^2).

Table 6 shows mean and standard deviation of serum ferritin level in all samples of each group.

Table 7 shows the comparison of results of unpaired t-test.

Tables 8, 9 are the comparison by chi-square test between the non-treated, non-metastatic lung cancer with the during-treated, non-metastatic lung cancer(Table 8)

and between the untreated tuberculosis and the tuberculosis after treatment (table 9).

Table 10 shows the result of serum ferritin level in the non-treated, non-metastatic lung cancer in each cell type.

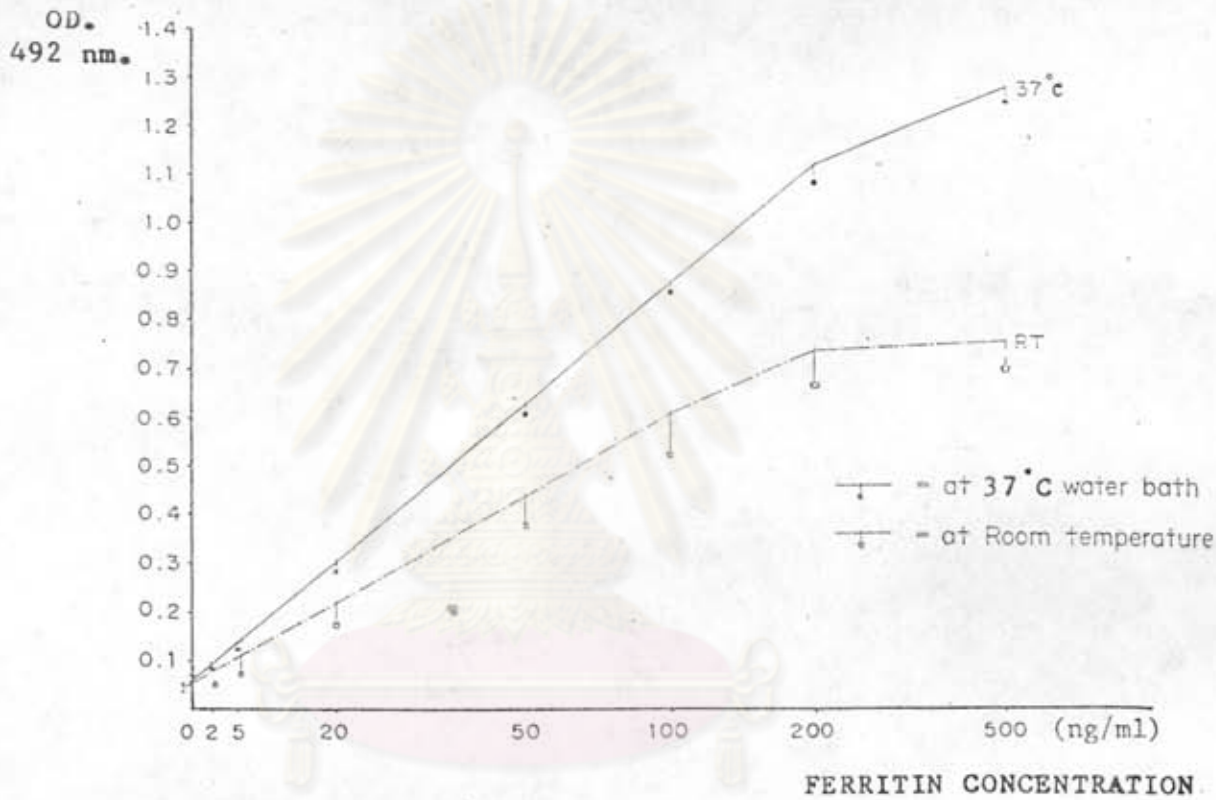


Figure 10 The comparison of incubating temperature for the reaction between ferritin with antiferritin and peroxidase conjugated antiferritin with ferritin on the ELISA technique.

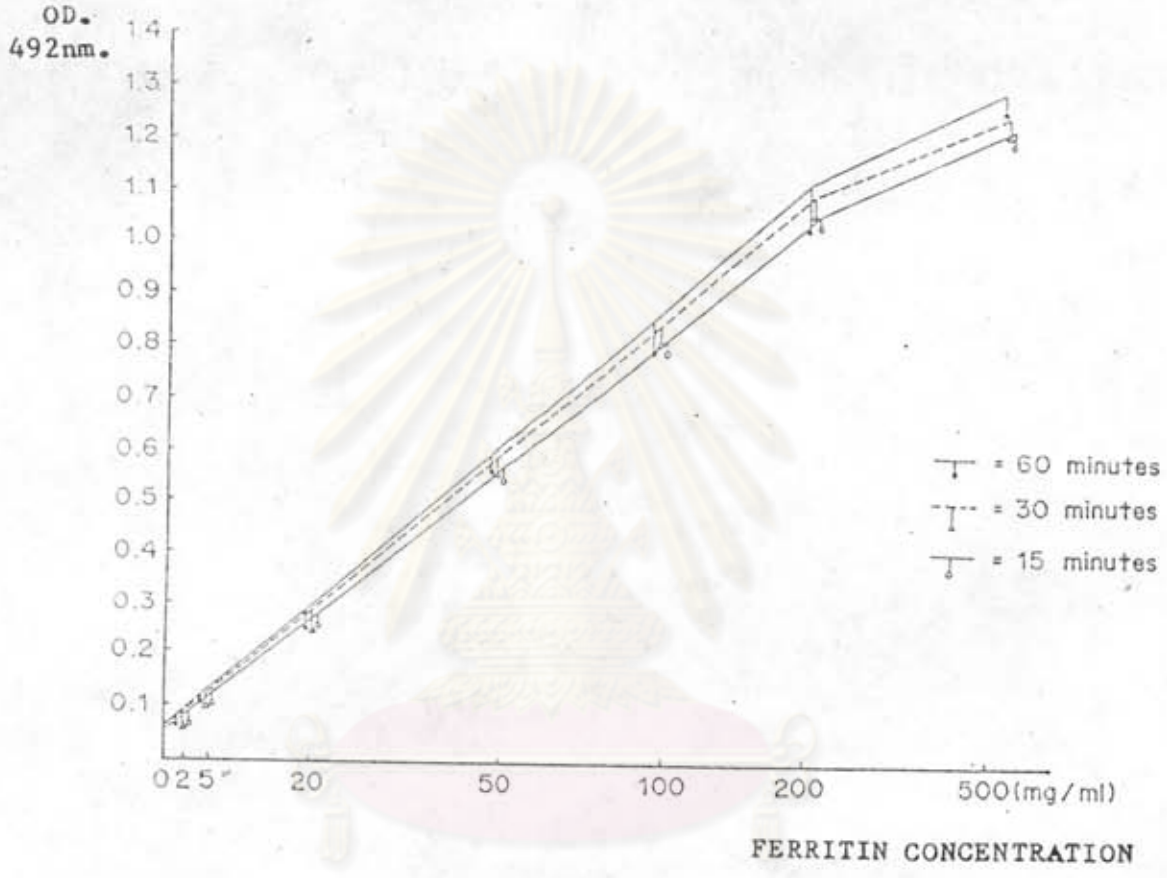


Figure 11 Incubation period for the reaction of ferritin with antiferritin at varing periods 15, 30 and 60 minutes for a comparison.

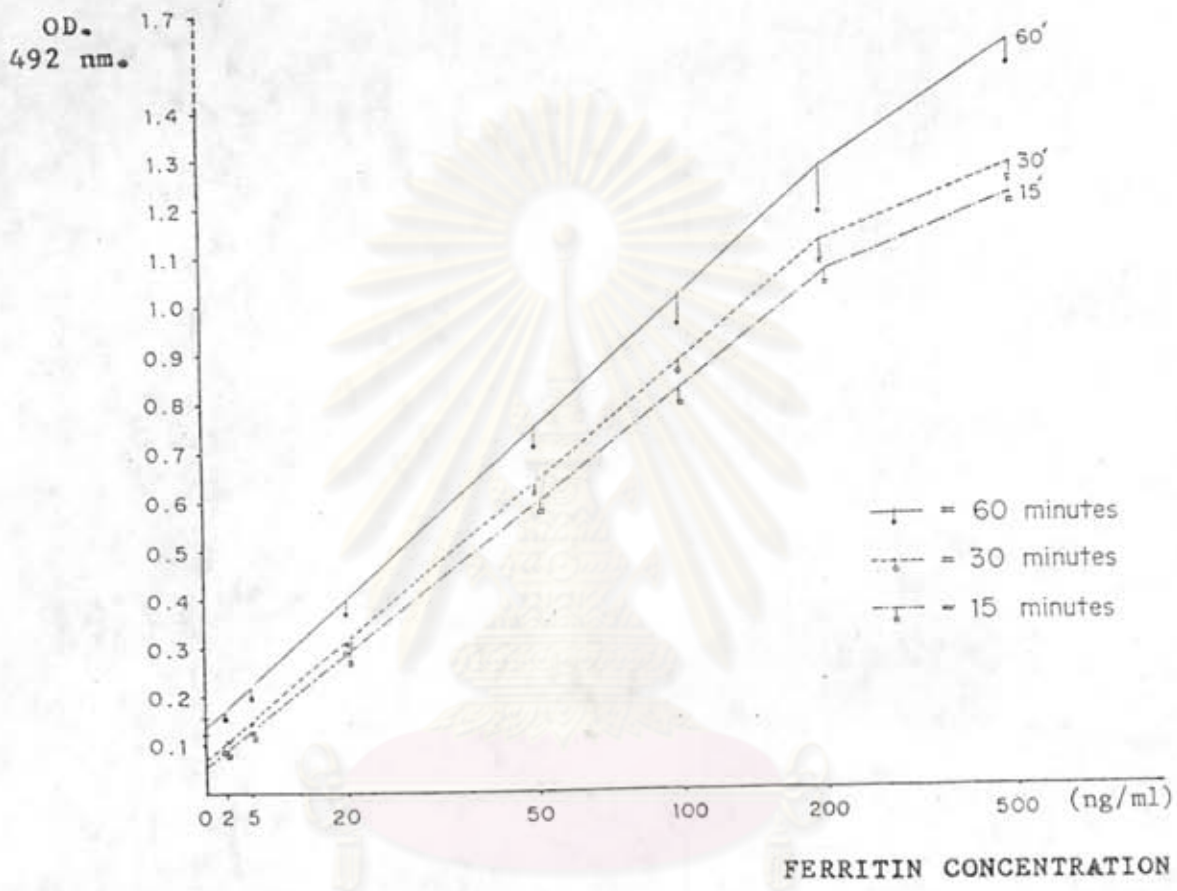


Figure 12 Incubation for the reaction of peroxidase conjugated antiferritin with ferritin at varing periods 15, 30 and 60 minutes for a comparison.

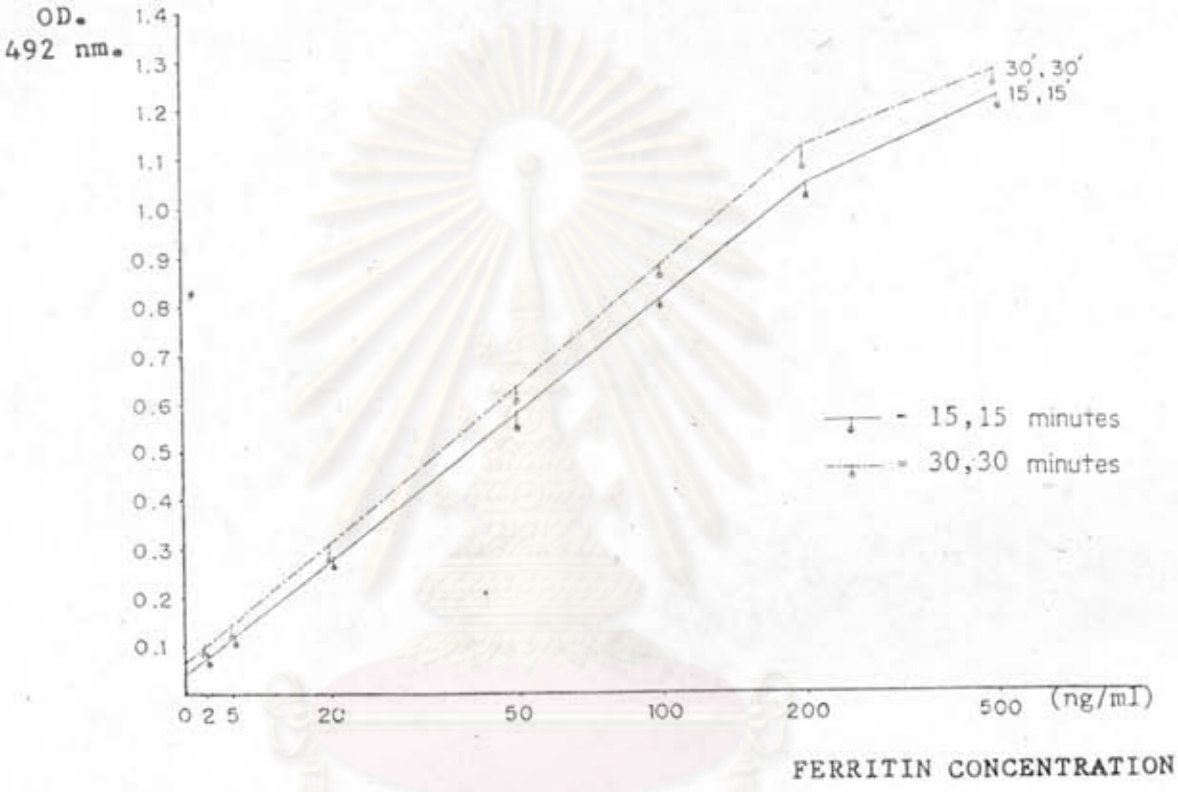


Figure 13 The comparison of incubation periods between 15, 15 minutes and 30, 30 minutes for the reaction of ferritin with antiferritin and peroxidase conjugated antiferritin with ferritin of the ELISA technique.

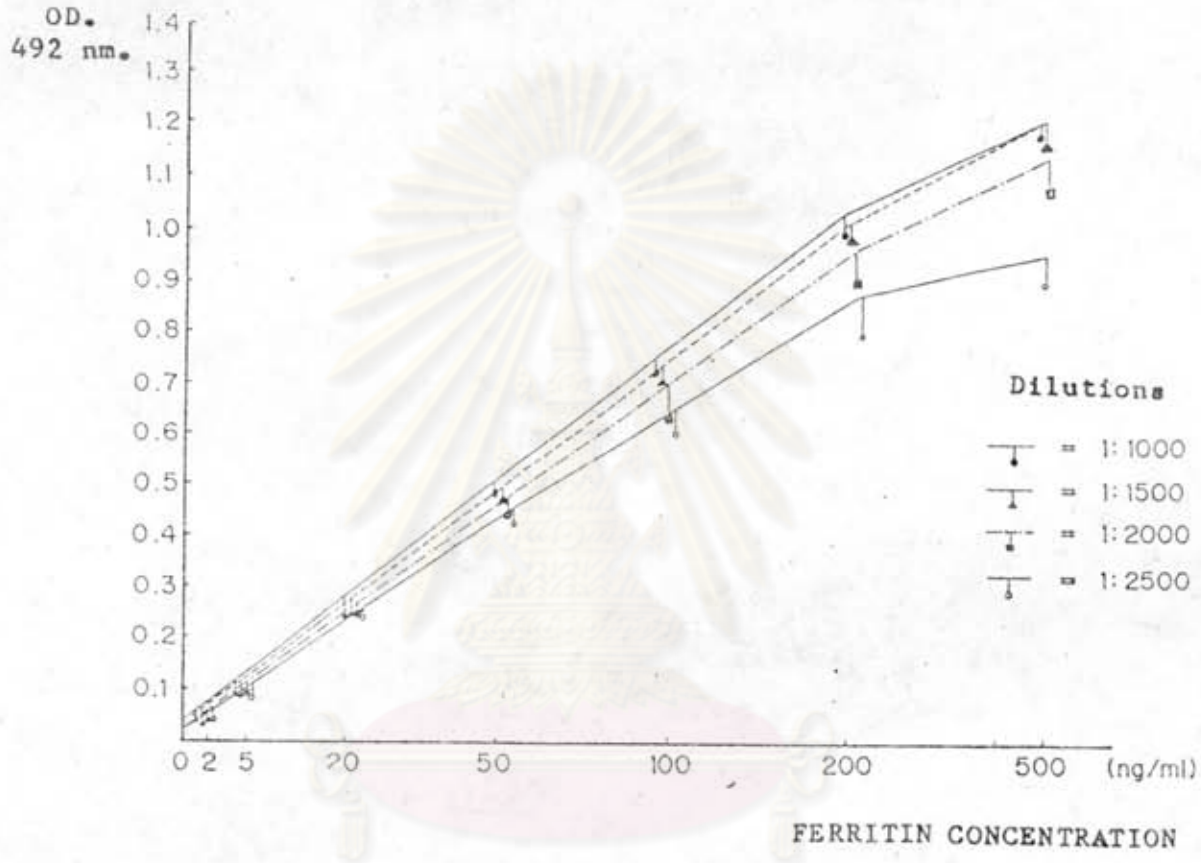


Figure 14 The comparison of the ELISA technique using dilutions of antiferritin (1:1000, 1:1500, 1:2000 and 1:2500 of antiferritin for coating plate).

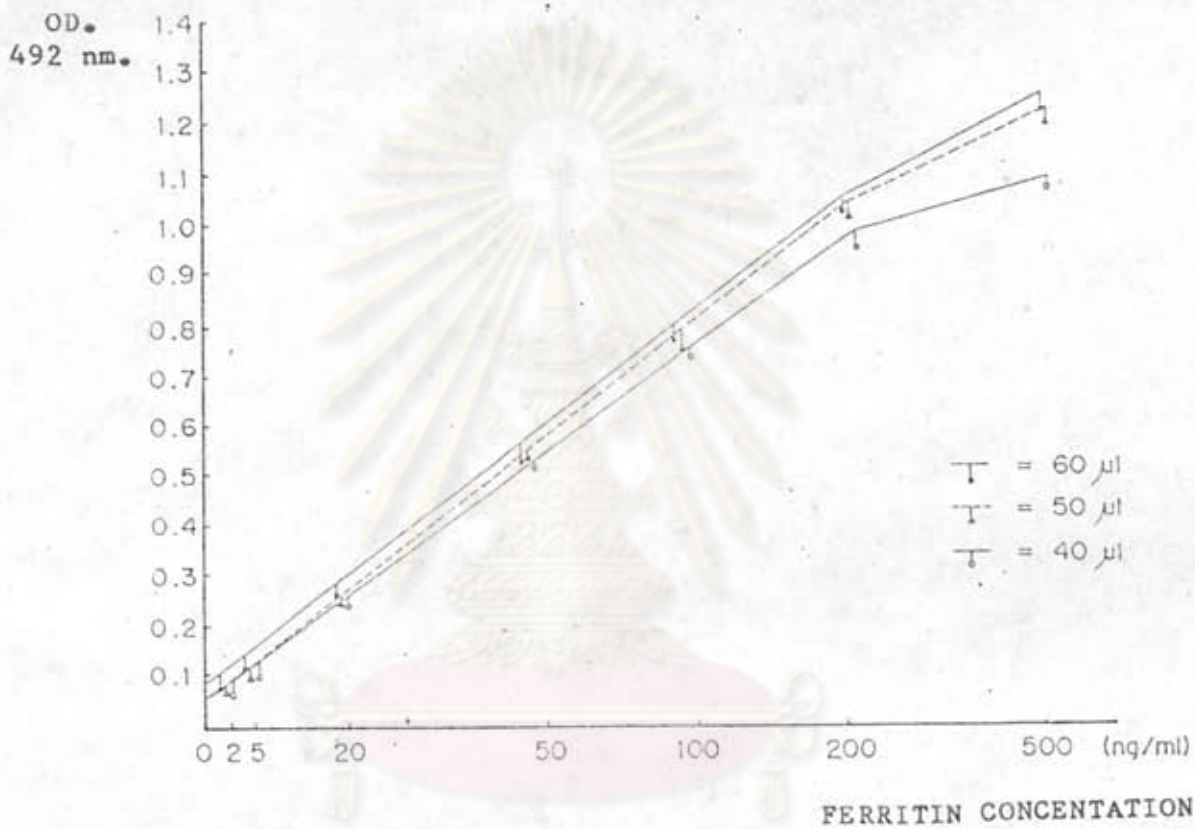


Figure 15 The comparison of the ELISA technique using various volume of peroxidase conjugated antiferritin. The volumes (40, 50 and 60 µl) were varied in 12 ml of conjugate diluent solution.

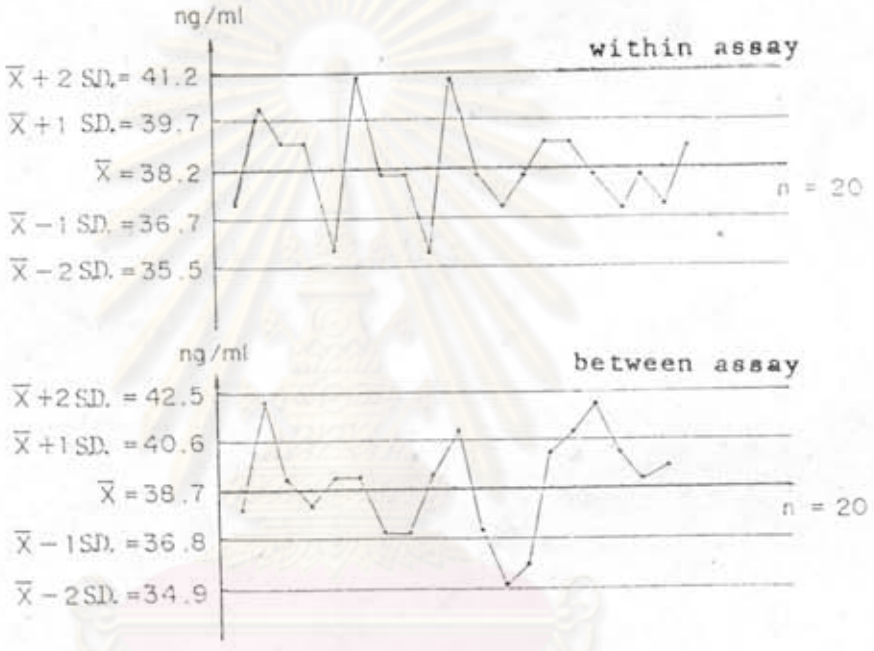


Figure 16 The precision of the ELISA technique, checking both within assay and between assay of low level of serum ferritin control (n=20).

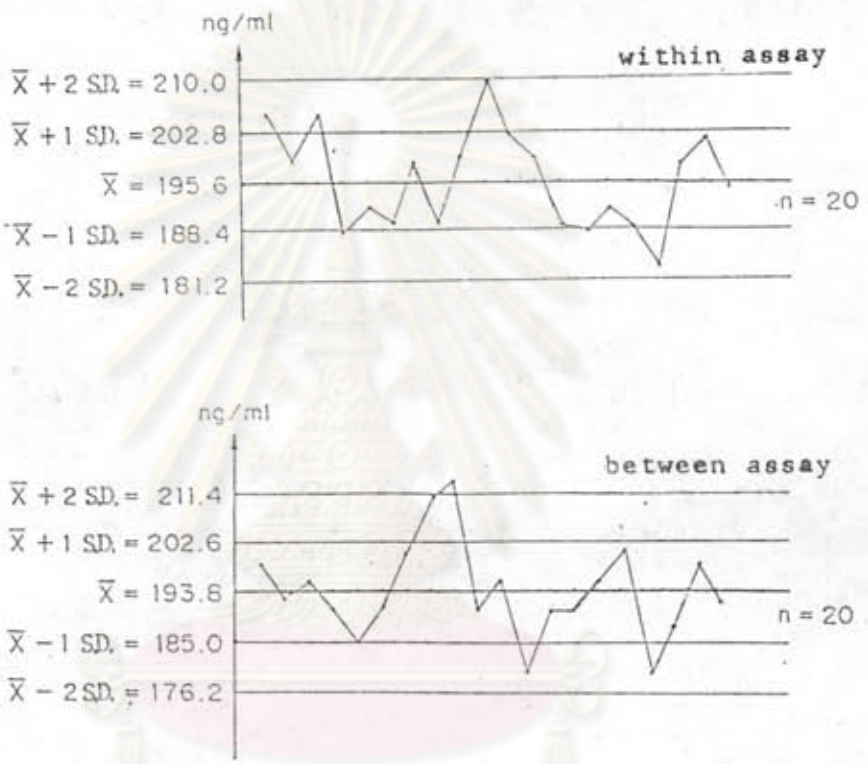


Figure 17 The precision of the ELISA technique, checking both within assay and between assay of medium level of serum ferritin control (n=20).

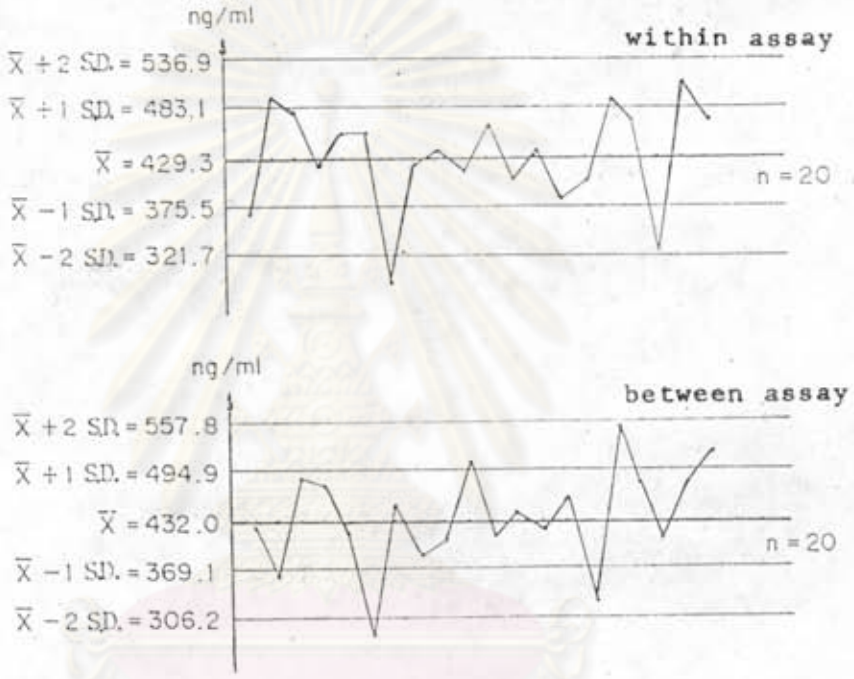


Figure 18 The precision of the ELISA technique, checking both within assay and between assay of high level of serum ferritin control (n=20).

Table 4 The precision study of within assay and between assay of ferritin level examination by ELISA method (modified Anderson and Kelly's technique), 3 level of samples (low, medium, and high) were checked, as shown in Figures 16-18.

Within assay of the precision test

	serum ferritin concentration(ng/ml)		
	$\bar{X}$	S.D.	% CV
Low value	38.2	1.5	3.95
medium value	195.6	7.2	3.67
high value	429.3	53.8	12.52

Between assay of the precision test

	serum ferritin concentration(ng/ml)		
	$\bar{X}$	S.D.	% CV
Low value	38.7	1.9	4.96
medium value	193.8	8.8	9.07
high value	432.0	62.9	14.57

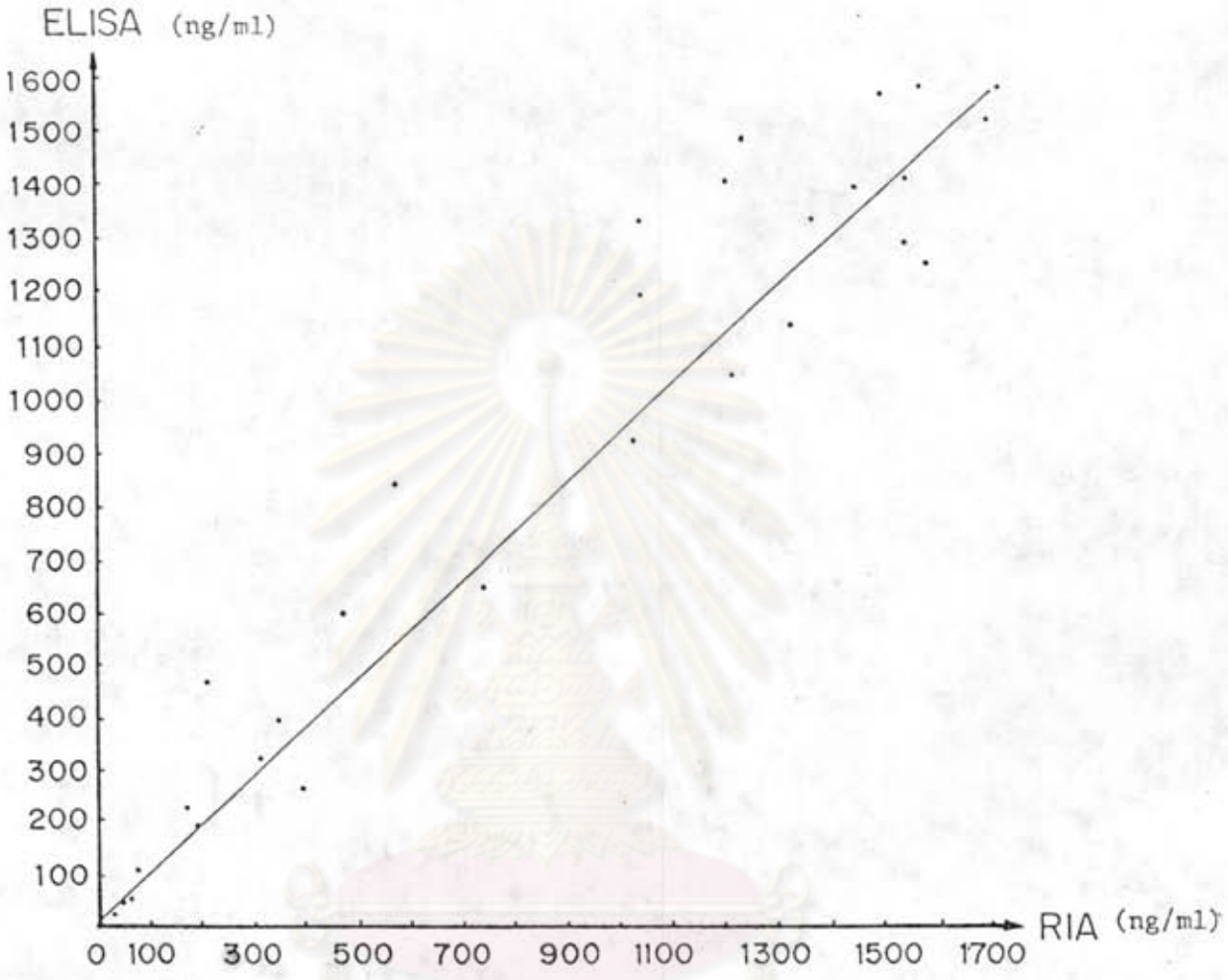


Figure 19 Correlation curve of modified Anderson and Kelly's ELISA technique and RIA technique (r = 0.987, y = 0.91x + 3.8)

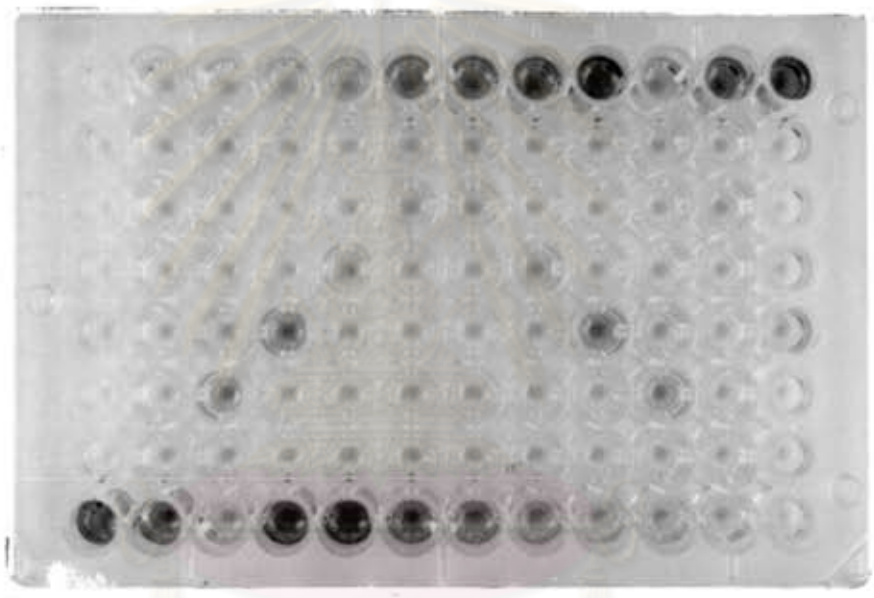


Figure 20 Photograph of ELISA technique result.
(the explanation of the procedure is in Figure 9)

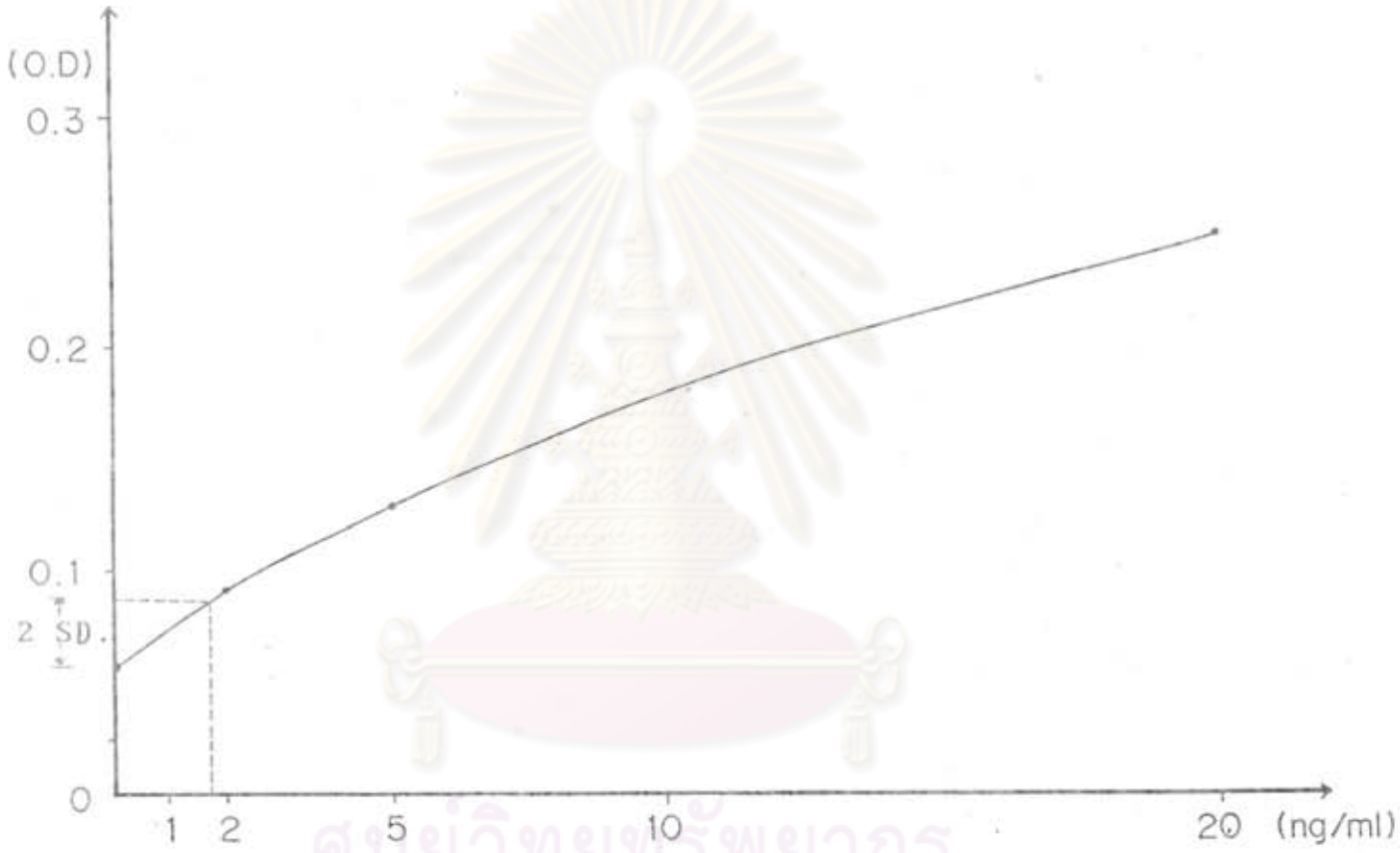
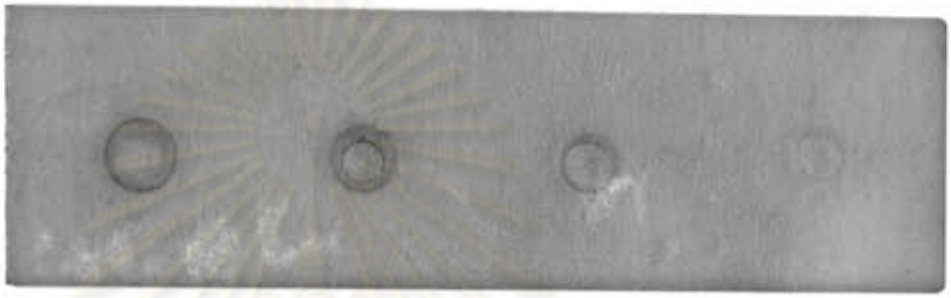


Figure 21 The sensitivity of modified Anderson and Kelly's ELISA technique, the smallest concentration of ferritin which can be detected is nearly 2 ng/ml.



A (20 µg/ml) B (15 µg/ml) C (10 µg/ml) D (9 µg/ml)

Figure 22 Photograph of Radioimmunodiffusion (RID) pattern in RID result. The precipitin ring was the reaction of diluted standard ferritin with antiferritin. The least amount of ferritin which can be detected was 10 µg/ml (The third (C) well, the precipitin ring of 9 µg/ml cannot be seen).

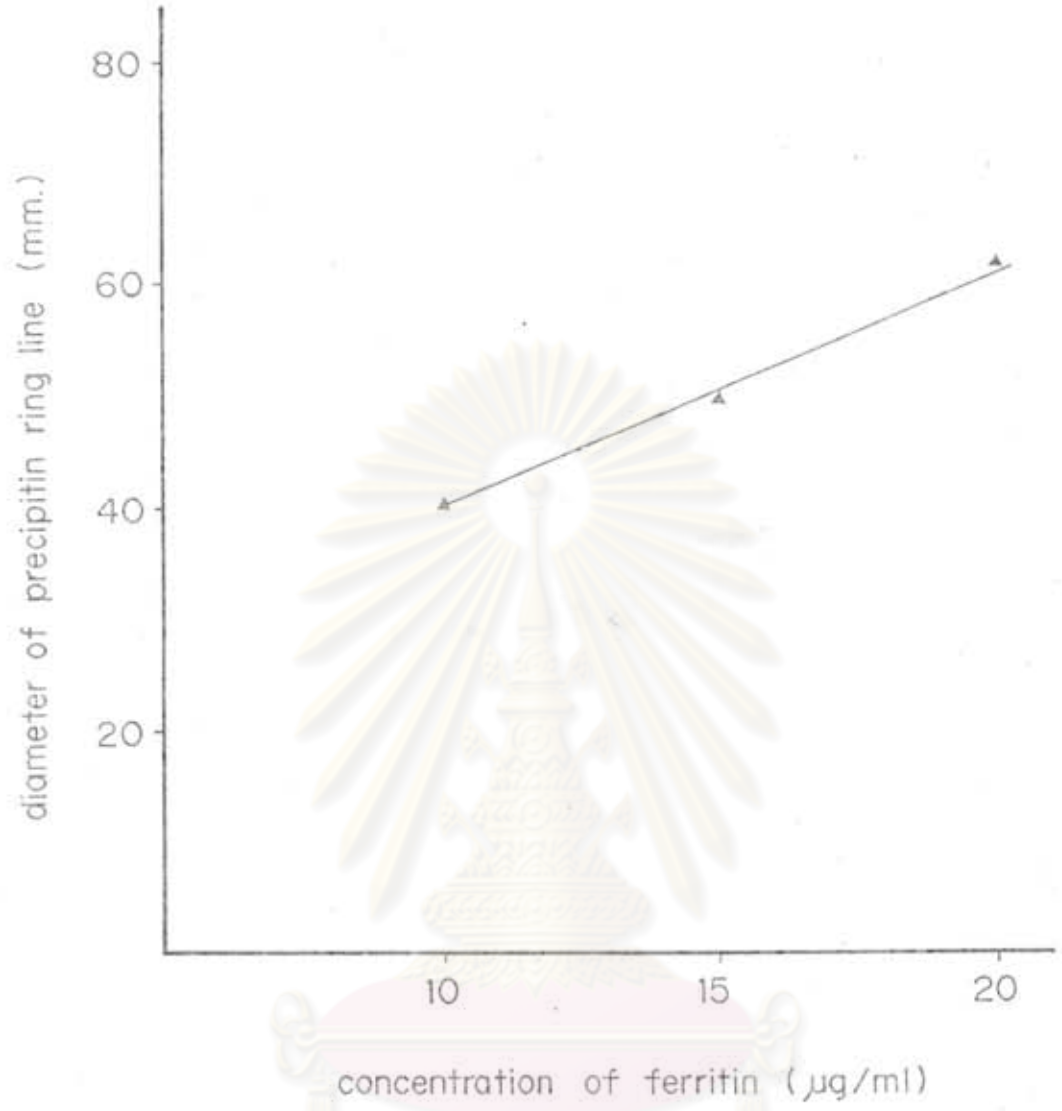
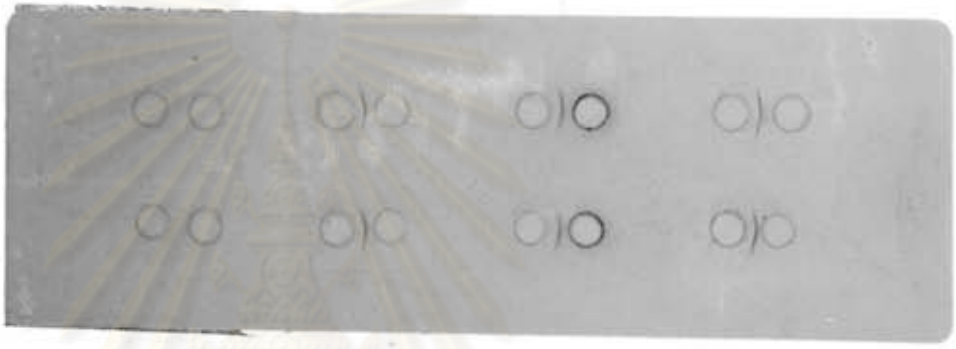


Figure 23 Standard curve of ferritin level of Radial Immunodiffusion technique (RID).



A (2 µg/ml) B (3 µg/ml) C (4 µg/ml) D (5 µg/ml)

Figure 24 Photograph of Counter Immunoclectrophoresis (CIEP) pattern. The precipitin lines were the reaction of diluted standard ferritin with antiferritin. The least amount of ferritin which can be detected was 3 µg/ml(3,000 ng/ml).

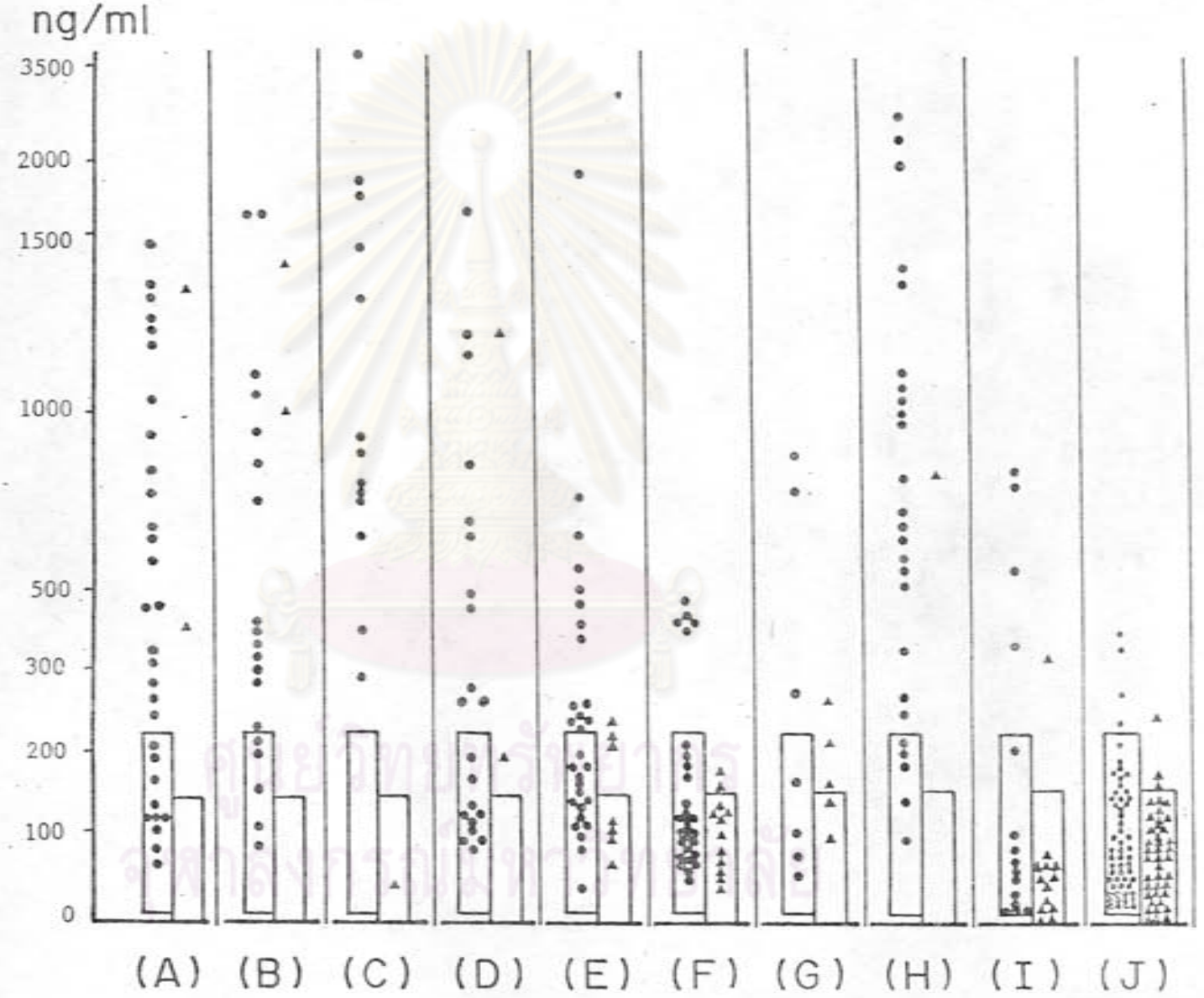


Figure 25 see illustration in next page.

Figure 25 The results of serum ferritin level of each sample group which were examined by modified Anderson and Kelly's method, the squares were the border of normal level which was calculated from $\bar{X}$ + 2S.D. of normal person group. (male = 221 ng/ml), female = 146 ng/ml)

Abbreviation:

(A) = non-treated, non-metastatic lung cancer

(B) = during-treatment, non-metastatic lung cancer

(C) = metastatic lung cancer

(D) = inflammatory lung disease

(E) = non treated tuberculosis.

(F) = tuberculosis after treated

(G) = pneumonitis

(H) = liver cancer (hepatocellular carcinoma)

(I) = other cancers such as cancers of colon, esophagus, stomach, breast, cervix and others.

(J) = normal persons.

table 5 Number and percent of disease groups with elevated serum ferritin, are divided into male and female.

Disease Group	No. of samples showing elevation[1]		% of samples showing elevation	
	Male	Female	Male	Female
non-treated, non-metastatic lung Ca	20/31[2]	2/3	64.5	66.7
non-metastatic lung Ca during-treatment	14/19	2/2	73.7	100.0
metastatic lung Ca	13/13	0/1	100.0	0.0
inflammatory lung disease	11/21	1/2	52.3	50.0
non treated tuberculosis	13/28	3/8	46.4	37.5
tuberculosis after treatment	5/28	2/12	17.8	16.7
Pneumonitis	3/7	3/5	42.8	60.0
liver Ca	20/25	1/1	80.0	100.0
other Ca	4/15	1/13	26.6	7.7
Normal	3/56	2/41	5.3	7.3

[1] male > 221 ng/ml, female > 146 ng/ml.

[2] the proportion of cases showing elevation per total.

Table 6 Value of mean ($\bar{X}$) and standard deviation (S.D.) of serum ferritin level in each disease group.

DISEASE	$\bar{X}$ (ng/ml)	S.D.
non-treated, non-metastatic lung Ca	566.6	449.9
during-treated, non-metastatic lung Ca	662.8	490.0
metastatic lung Ca	882.0	522.4
inflammatory lung disease	463.8	462.3
non treated tuberculosis	304.1	338.6
tuberculosis after treatment	122.8	116.1
Pneumonitis	261.0	289.0
liver Ca	886.6	758.3
normal	73.2	74.0

Table 7 Comparison results of unpaired t-test (one tailed) in each disease group by testing against the other selective one.

	t-test value	p value
non-treated, non-met* lung Ca vs non treated tuberculosis	2.52**	<0.05
non-treated, non-met lung Ca vs met lung Ca	1.91**	<0.05
non-treated, non-met lung Ca vs inflammatory lung disease	0.79	>0.05
non-treated, non-met lung Ca vs during-treated,non-met lung Ca	0.69	>0.05
During-treated, non-met lung Ca vs met lung Ca	1.20	>0.05
inflammatory lung disease vs non treated tuberculosis	1.33	>0.05
Non-treated tuberculosis vs tuberculosis after treated	2.72**	>0.05

*met = metastatic

** = the significantly different of 2 compared groups.

Table 8 X^2 comparison of non-metastatic lung Ca between non-treated group and during-treated group. (The number in brackets was the expected value).

	non-metastatic lung Ca.		
	non-treated	during-treated	
high	20 (21.1)	14 (12.9)	34
normal	10 (9.9)	5 (6.1)	16
	31	19	50

The tabulated X^2 for $\alpha = 0.05$ and df = 1 is 3.84 Since the calculated X^2 is 0.20, lower than the tabulated X^2, it concludes that the number of high serum ferritin of non-treated lung cancer was non-significantly differrent from the during treated lung cancer.

Table 9 X^2-test comparison of Tuberculosis disease patients between non-treated and after treated group. (The number in brackets was the expected value).

	Tuberculosis		
	non-treated	after-treated	
high	13 (9)	5 (9)	18
normal	15 (19)	23 (19)	38
	28	28	56

The tabulated X^2 for α= 0.05 and df = 1 is 3.84. Since the calculated X^2 is 4.02, greater than the tabulated X^2, it concluds that the number of high serum ferritin of non-treated tuberculosis was significantly different from tuberculosis after treated.

Table 10 Cell types of lung cancer showing mean($\bar{X}$), standard deviation (S.D.), range and percent of samples which showing elevation of serum ferritin (the data was shown only from males).

cell type	number	$\bar{X}$ (ng/ml)	S.D. (ng/ml)	range (ng/ml)	%of samples showing elevation
Squamous cell	14	736.8	528.1	35-1450	85.7
Adeno cell	6	390.6	327.4	70-860	50.0
Small cell	5	412.6	466.4	93-1200	40.0
Large cell	6	502.5	350.5	105-1020	83.3
All	31	566.6	449.9	35-1450	64.5