CHAPTER 3

RESULTS AND DISCUSSION

3.1 Bead retention cell designs

The bead retention cell was utilized to trap the beads while the solution was flowed through the system. This cell has multi-purposes of accommodation of chemical reaction and retention of the analyte. A few designs were tried in this study.

The two-way pinch valve bead retention cell (Figure 2.1) could trapped the beads by pinching the Tygon® tubing. This is a simple design but it was found that the Tygon® tubing could be degraded from pinching pressure after repeatedly uses for a period of 20 days. This led to a high back pressure due to bead retention in the tubing.

The flow injection (FI)-microcolumn system was also used for bone ALP assay. Wheat germ lectin coated beads were packed into a micro column shown in Figure 3.1.

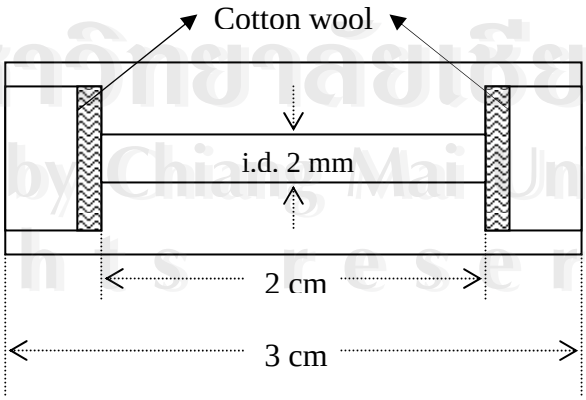


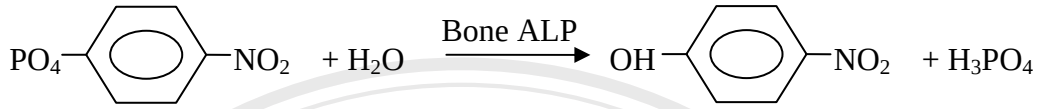
Figure 3.1 A micro column

After analysis, the beads could not be discarded due to the closed ends column. Therefore, they must be regenerated prior to the next assay. The bead regeneration was required for breaking the bone ALP-wheat germ lectin bonding. In this work, the bead regeneration used a long time (> 1 hr). In addition, the wheat germ coated beads performance was decreased. From these problems, bead injection system was used to replace beads with fresh ones to save time and avoid a decrease performance.

In bead injection technique, the bead retention cell made from a Perspex (Figure 2.3) could trap the beads by a solid rod connected to a solenoid valve while solution was flowed through the small space between the cell surface and the solid rod. The back pressure problem was not found using this bead retention cell. The automatic operation of this bead retention cell was made possible by controlling it with a LabView® computer program (see APPENDIX B). Thus, the bead retention cell made from the Perspex was chosen for the FI-BI system for determination of bone alkaline phosphatase in human serum sample.

3.2 Manifold and operation steps

Wheat germ lectin coated beads were flowed and retained in the bead retention cell made from the Perspex. Specific lectin on the bead surfaces interacted and separated bone ALP isoform from human serum sample. The unbound species were passed through the bead retention cell. The bone ALP was measured by using the enzyme-substrate reaction. Bone ALP changes p-nitrophenyl phosphate to p-nitrophenol that can be detected spectrophotometrically at 405 nm as shown below.



p-nitrophenyl phosphate
(colorless)

p-nitrophenol
(yellow)
λ = 405

In this research, the flowing stream of solution was used to carry beads though the system. There was no need to regenerate the bead surfaces because they were discarded after each use and replaced by fresh ones. This helps to reduce the risk of contamination, denaturation, fouling system and analysis time.

The FI-BI system for determination of bone ALP was controlled by a computer (Figure 2.5). This system can reduce the operation time of some tedious steps such as washing and incubation steps. In addition, the FI-BI is a closed system which has less contamination from the environment. It also helps to minimize the direct contact between the operator and blood sample.

3.3 Optimization

3.3.1 Sample volume

In the FI technique, the dispersion can be decreased by increasing sample volume but the excess sample volume gives rise to peak broadening that led to lower sensitivity of the measurement. The effect of sample volume was studied in the range of 50, 100, 200 and 300 μl. The peak height was recorded and the results are shown in the Figure 3.2.

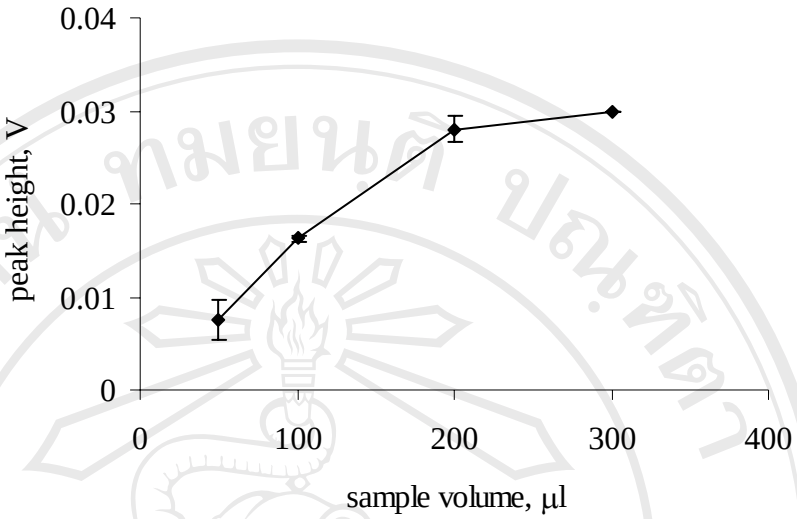


Figure 3.2 Effect of the sample volume on analytical signal

From the results, a low sensitivity was observed when a low sample volume was used. This was due to a low amount of bone ALP available to interact with wheat germ coated beads. On the other hand, the rise in analytical sensitivity was found when sample volume was increased. When sample volume was higher than 200 µl, the sensitivity was only slightly increased due to excess sample volume for the available amount of bead surfaces. In addition, a high sample volume was applied to cover beads zone in the bead retention cell and reduce the dilution on both trials of sample zone. In this work, the sample volume of 200 µl was selected for the further work to save sample while obtaining the best result.

3.3.2 Flow rate

The change in flow rate affects peak shape of the FI gram due to various degrees of dispersion. In this research, the flow rate of the buffers was investigated as follows: 1.0, 1.5, 2.0 and 2.5 ml/min. The results are shown in the Figure 3.3.

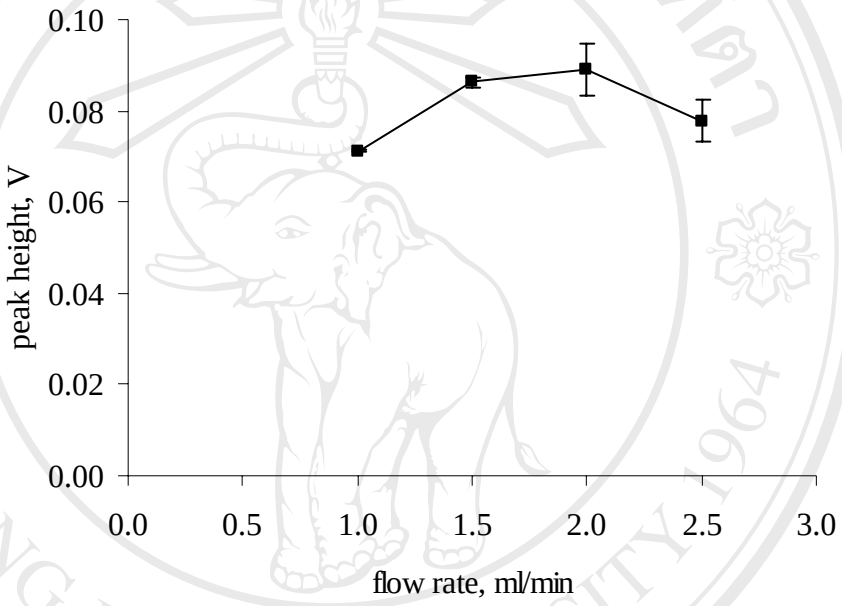


Figure 3.3 Effect of the buffer flow rate (P_1) on the analytical signal

These results indicated that the flow rate of 1.0 ml/min gave a low peak height, which may be resulted from the high dispersion effect of analytical zone in a slow flowing stream. Similarly, the decrease in peak height was also observed at the high flow rate of 2.5 ml/min which may be due to ineffective mixing. For future experiments, the flow rate of 1.8 ml/min was selected because it resulted in the stable range of reasonable sensitivity.

3.3.3 Bead loading time

The suitable bead volume was investigated by studying the bead loading time. The homogeneous of bead suspension was loaded with flow rate (P_2) 3.0 ml/min and retained in the system (Figure 2.4). The bead loading time was varied as follows; 5s, 10s and 20s which corresponds to the bead volumes of 15 μl, 30μl and 60 μl respectively. The increase of bead loading time led to increase sensitivity as shown in Figure 3.4. To save beads, the bead loading time selected was 10s at the flow rate (P_2) 3.0 ml/min using 30 μl bead suspension prepared from 0.25 ml of beads in 4 ml acetate buffer pH 4.5. Higher beads loading time was not used because sufficient sensitivity was obtained at 10s bead loading time.

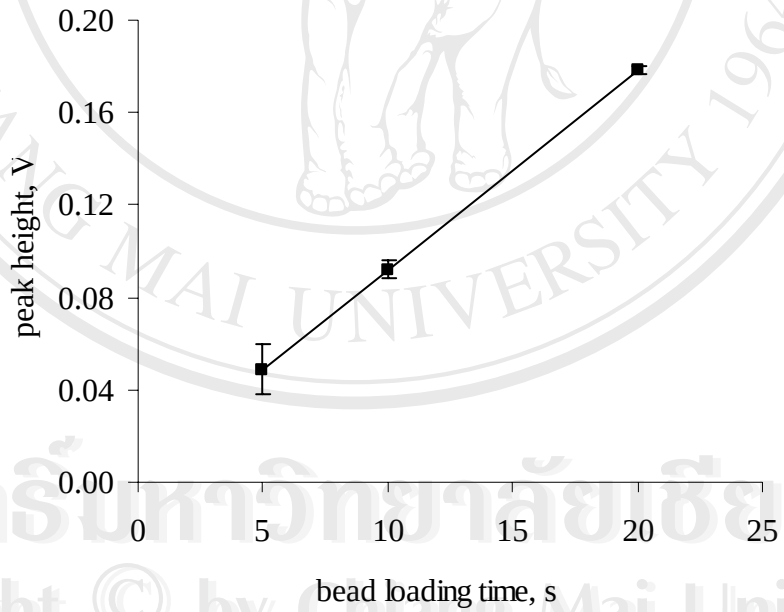


Figure 3.4 Effect of the bead loading time on the analytical signal

3.3.4 Processing time

3.3.4.1 Wheat germ lectin beads conditioning time

A high performance of bone ALP binding with wheat germ lectin coated beads was operated in weak acidic condition. Although, bead suspension was prepared in the acetate buffer solution but it needs to be conditioned. Because Tris-HCl buffer solution (pH 9.5) was used in the last step of each analysis, thus prior to the next assay the system condition have to be changed to weak acidity. The flowing time was investigated in the range of 1, 2 and 3 min at the flow rate (P_1) 1.8 ml/min. which corresponds to the volume of 1.8 ml, 3.6 and 5.4 ml, respectively. The results are shown in Figure 3.5.

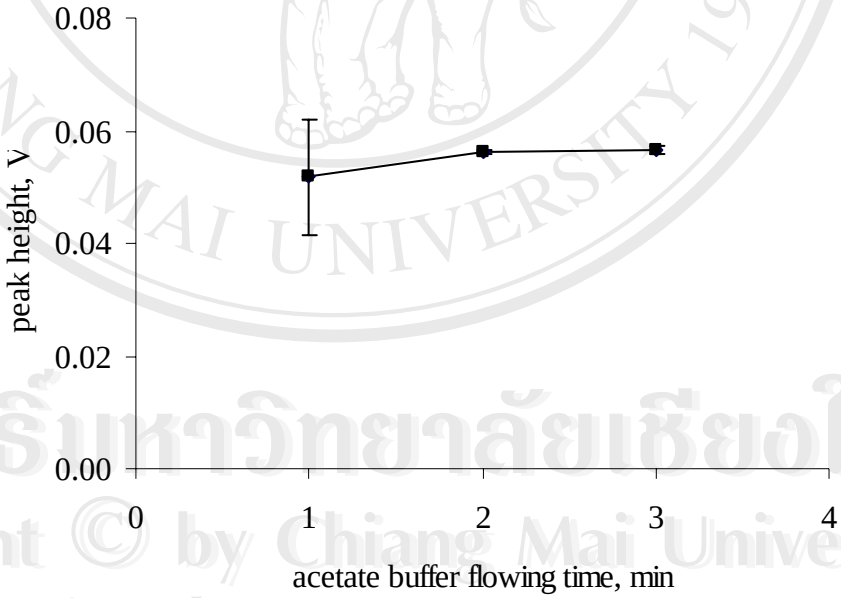


Figure 3.5 Effect of wheat germ lectin beads conditioning time on the analytical signal

The analytical signals were not significantly different in the flowing time range of 1, 2 and 3 min. However, the standard deviation of flowing time 1 min is large which may be due to the inefficient acetate buffer volume for wheat germ lectin beads conditioning. Hence, the acetate buffer flowing time at 2 min was chosen for further experiments.

3.3.4.2 Incubation time of wheat germ lectin with bone ALP

After the wheat germ lectin beads conditioning, Bone ALP was introduced to the system and interacted with wheat germ lectin coated on beads. The degree of interaction depends on incubation time. The longer incubation time allows the higher interaction and the better analytical sensitivity. In this experiment, the optimum incubation time was investigated by stopping the flowing stream of acetate buffer at 3, 4, 5 and 10 min. Effect of incubation time is presented in Figure 3.6. It was found that the range of incubation time at 3 and 4 mins are not significantly different in term of analytical signal. At longer incubation time, peak height was increased and remained steady in the range of 5 and 6 min. To compromise the analytical time and analytical sensitivity, incubation time of wheat germ lectin with bone ALP at 5 min was used for further experiments.

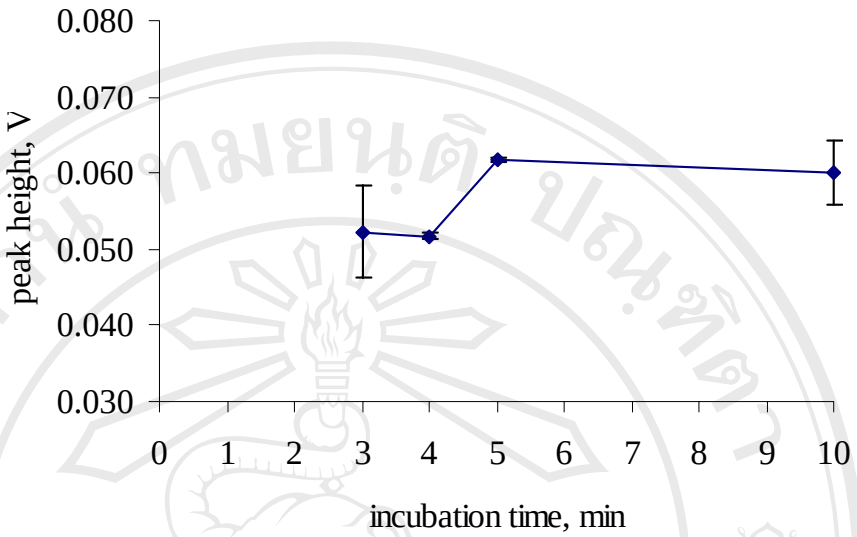


Figure 3.6 Effect of incubation time of wheat germ lectin with bone ALP on the analytical signal

3.3.4.3 Washing time

After incubation of wheat germ lectin with bone ALP, Tris-HCl buffer pH 9.5 was flowed trough the system to remove unbound species and to adjust the condition of the system for the enzyme-substrate reaction. In the washing step, baseline signal was changed due to unbound species removed though the flow cell of spectronic 21. When unbound species were completely removed, the baseline turned to the original baseline signal. The flowing time of the Tris-HCl buffer was investigated as follows; 1, 2, 3 and 6 min. The results are presented in Figure 3.7. It was found that the signals were not significantly different. However, the washing profiles shown in Figure 3.8 indicate that baseline signal of the flowing time 1 and 2 min did not turn to original baseline signal due to some remaining unbound species in the system. For the flowing time of 3 and 6 min, unbound species could be completely

removed from the system. Hence, the flowing time of Tris-HCl buffer for 3 min was selected for the further work.

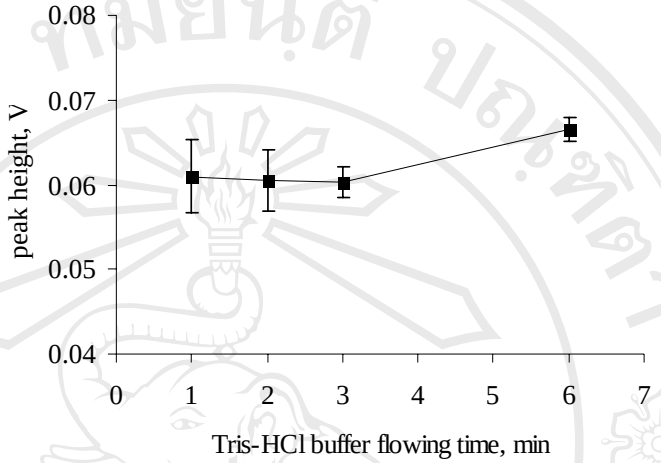


Figure 3.7 Effect of the Tris-HCl buffer pH 9.5 flowing time on the analytical signal

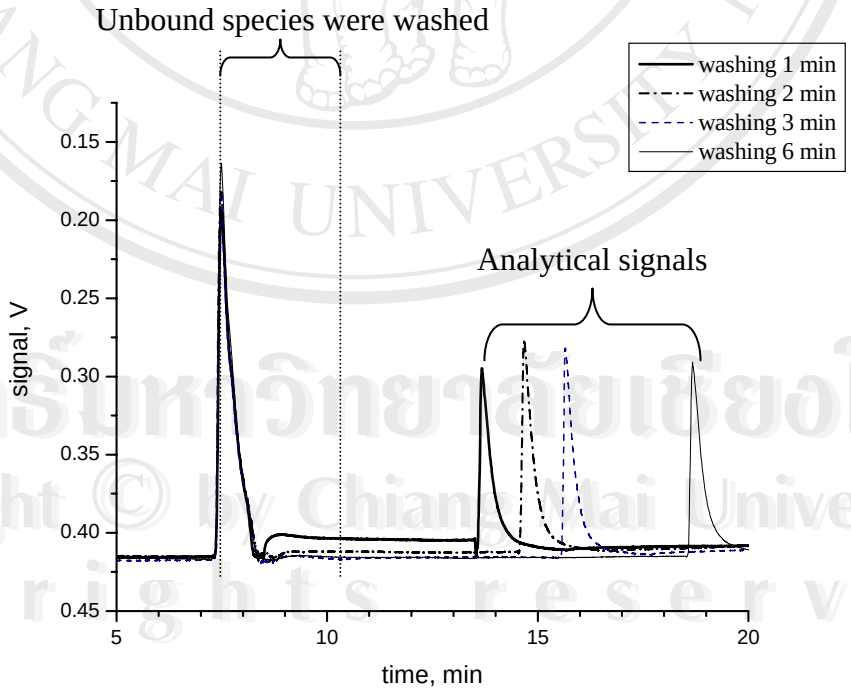


Figure 3.8 The washing profile at various flowing times of the Tris-HCl buffer pH 9.5

3.3.4.4 Incubation time of bone ALP with p-nitrophenyl phosphate

The longer the enzyme-substrate incubation time is, the more products is produced yielding in higher signal. However, long incubation slows down the analysis and the product may be degraded before detection process takes place. This incubation time was investigated as follows; 1, 3, 5 and 10 min. It was observed that the rise in incubation time led to the increase of peak height as shown in Figure 3.9.

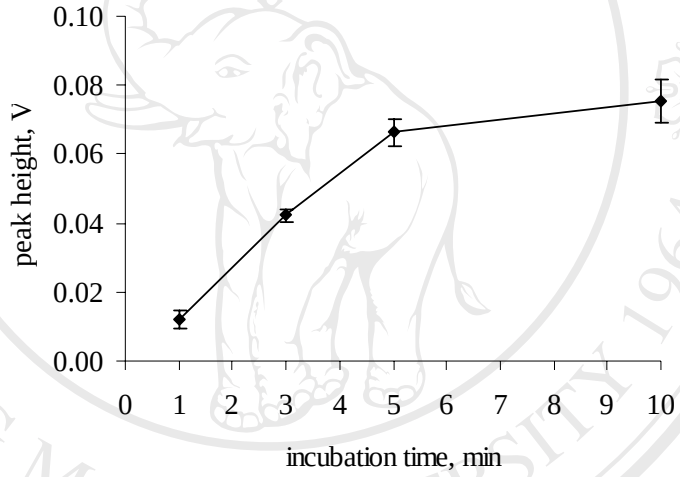


Figure 3.9 Effect of incubation time of bone ALP with p-nitrophenyl phosphate on the analytical signal

For this work, incubation time of 5 min was chosen based on acceptable sensitivity and analytical time.

Total processing time (Section 3.3.4), for determination of bone ALP in human serum samples by using FI-BI system is summarized in the Table 3.1.

Table 3.1 Operation time for determination of bone ALP by using FI-BI system

Processing step	Time (min)
1. Operation of bead injection system	2
2. Conditioning of wheat germ lectin beads	2
3. Incubation of wheat germ lectin with bone ALP	5
4. Washing step and conditioning of bone ALP condition	3 x 2 times
5. Incubation of bone ALP with p-nitrophenyl phosphate	5 x 2 times
Total	25

After Incubation of bone ALP with p-nitrophenyl phosphate, p-nitrophenol as the enzyme-substrate product was introduced to the detector. Two repeated enzyme-substrate incubation and detection was done for each sample. Therefore, the total analytical time of determination of bone ALP was about 25 min.

3.4 Optimum condition of the FI-BI system

From the optimization study discussed in Section 3.3, the conditions of the FI-BI system used in this work are summarized in Table 3.2.

Table 3.2 The optimum condition of the FI-BI system for determination of bone ALP in human serum samples

Conditions	
Bead Injection	
- Bead suspension	0.25 ml beads in 4 ml acetate buffer pH 4.5
- Flow rate (P_2)	3.0 ml/min
- Bead loading time	10 sec (about 30 µl of bead volume)
Flow injection	
- Flow rate (P_1)	1.8 ml/min
- Sample volume	200 µl
- Substrate volume	100 µl
- Detected at	405 nm

3.5 Within-run precision

Within-run precision or Repeatability[30] was determined by assaying bone ALP in a single fetal bovine serum that was assayed repeatedly 10 times within a day. The percentage of relative standard deviation (%RSD) was 6.1, see detail in Table 3.3.

Table 3.3 Within-run precision of the FI-BI system obtained from 10 injections of a fetal bovine serum in one day

No. of Run	Peak height, V
1	0.060
2	0.070
3	0.065
4	0.066
5	0.062
6	0.063
7	0.073
8	0.069
9	0.067
10	0.064
Average	**0.066**
SD	**0.004**
%RSD	**6.1**

3.6 Between-run precision

Between-run precision or Reproducibility[30] was determined by 10 runs bone ALP assay of a fetal bovine serum in different days (1 run per day). The percentage of relative standard deviation (%RSD) was 4.8, see detail in Table 3.4.

Table 3.4 Between-run precision of the FI-BI system obtained from 10 runs of a fetal bovine serum in 10 days

Date	Peak height
1	0.060
2	0.062
3	0.067
4	0.062
5	0.062
6	0.060
7	0.067
8	0.067
9	0.062
10	0.067
Average	**0.063**
SD	**0.003**
%RSD	**4.8**

3.7 Calibration curve

The bone ALP activities were investigated in the range of 1-10,000 U/L to cover amount of bone ALP normally found in normal person and bone patients. Analytical signals of bone ALP activities profile are shown in Figure 3.10. The corresponded calibration curve is presented in Figure 3.11.

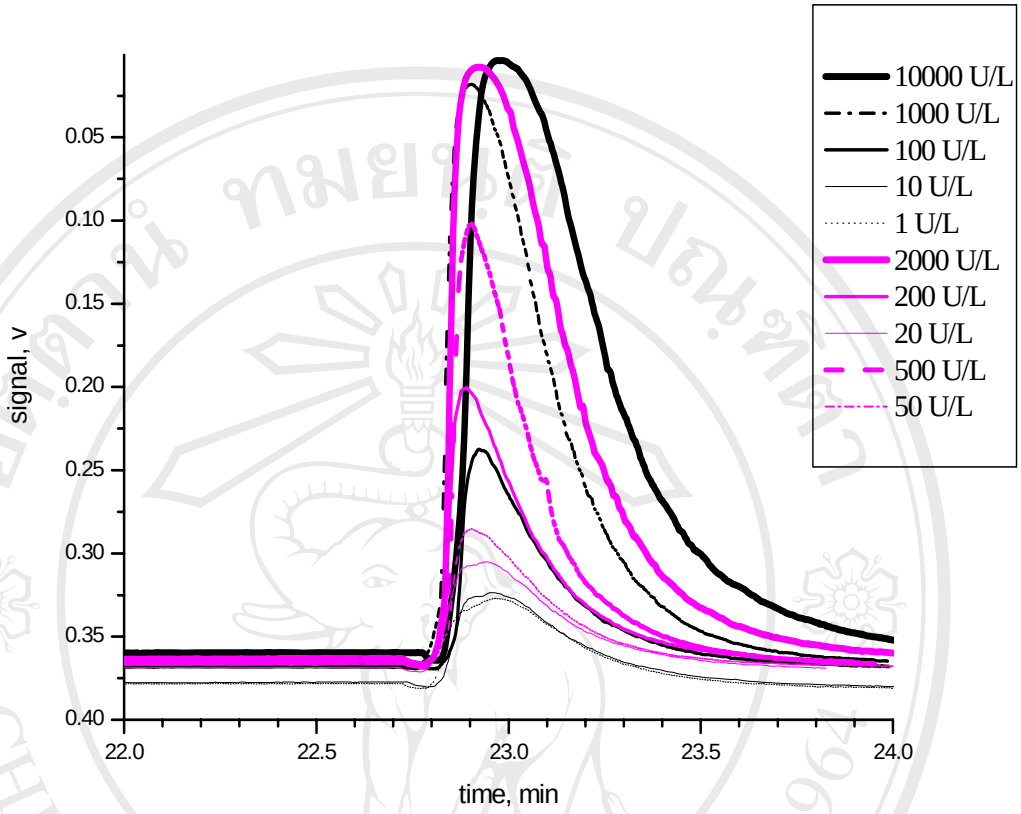


Figure 3.10 Analytical signal profile of standard bone ALP of various concentrations

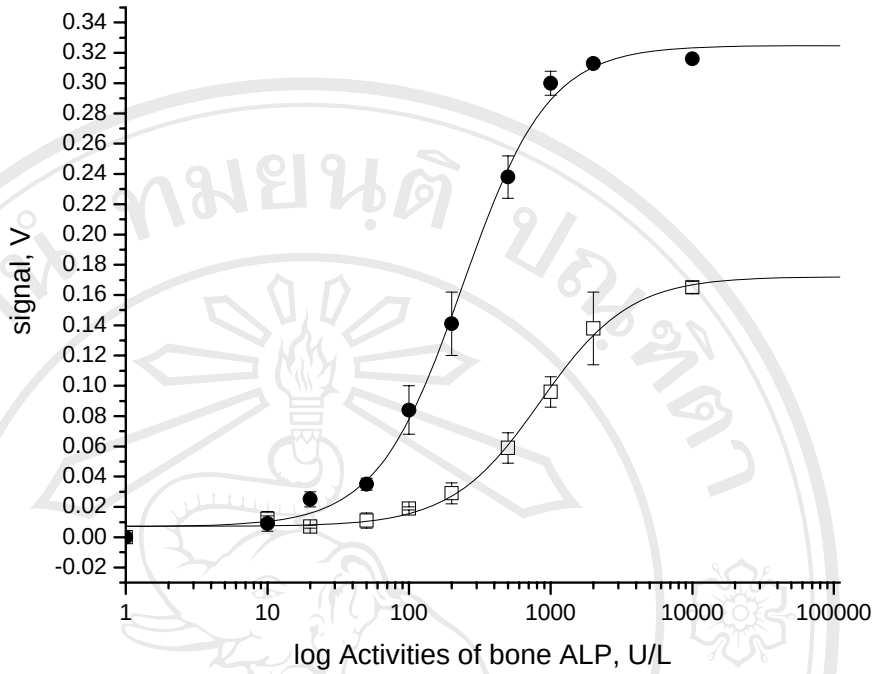


Figure 3.11 Calibration curve of the bone ALP activities

(□ = peak area, ● = peak height)

For comparison, the peak height and peak area are shown here. Calibration curve is the sigmoidal (logistic) fit obtained from the computer software (Origin version 7.0). When signal was recorded as peak height, sensitivity was higher than when it was recorded as peak area. Therefore, peak height was chosen for further use. This calibration curve is demonstrated in sigmoidal equation as follows;

$$Y = [(A_1-A_2) / (1+(X/X_0)^p)]+A_2 \quad ; R^2 = 0.9992 \qquad (3.1)$$

Where Y is analytical signal as peak height in volts, X is activities of bone ALP in U/L, A_1 is initial Y value = 0.01, A_2 is final Y value = 0.32, X_0 is X value at Y equal to half of the limit A_1 and A_2 = 238.40 and P is power =1.44.

From this calibration curve, the lowest detectable activity of bone ALP was found to be 6.3 U/L and the working range was 50-1000 U/L. The lowest detectable activities were determined as the lowest distinguishable analytical signal ± 2SD with 95% confidence from blank signal while the highest detectable activities were determined similarly on another end of the calibration curve. This range can very well covered the bone ALP level reported to be found in normal human in the range of 35-95 U/L[21] and at various evaluated level in patients with bone diseases such as osteoporosis.

3.8 Cross-reactivity with liver ALP

Even though wheat germ lectin is 90% specific toward Bone ALP[17], the liver ALP may have cross-reactivity with the wheat germ lectin that may interfere bone ALP assay. Because bone and liver ALP are existed in tissue-nonspecific ALP gene locus, they have similar structures. In this work, various liver ALP activities were spiked into 6% BSA. The results are presented in Table 3.5.

Table 3.5 Cross-reactivity of liver ALP with bone ALP assay

Activities of spiked liver ALP (U/L)	**Calculated activity from calibration curve (U/L)**	**Cross-reactivity (%)**
50	Not detected	Not detected
100	Not detected	Not detected
200	7±4	3±2
500	34±3	7±1
1000	67±9	7±1

From the results, it was found that the liver ALP activities have very minute cross-reactivity with the bone ALP assay. Wheat germ lectin is 90% specific toward bone ALP.

3.9 Testing of accuracy

Accuracy is the nearness of a measurement to the accepted true value. The accuracy can be expressed as a range, about the true value, in which measurement occur[31]. In this research, the accuracy of working range of calibration curve was expressed as the percentage of difference. The percentage of difference was investigated by spiking various bone ALP activity in normal serum and calculated from the percentage of difference between expected values with found values. The percentage of difference was calculated by using equation 3.2. The results are shown in Table 3.6.

% Difference = [(found value – expected value)/(expected value)] x 100 (3.2)

Table 3.6 Percent difference of spiked bone ALP in normal serum sample

Sample	Obtained bone ALP, U/L	Expected bone ALP, U/L	% Difference
Normal serum	16		
Normal serum + 20 U/L std. bone ALP	43±5	36	19±16
Normal serum +100 U/L std. bone ALP	94±7	116	19±6
Normal serum + 500 U/L std. bone ALP	379±16	516	27±3
Normal serum + 1000 U/L std. bone ALP	687±79	1016	32±8

The results from Table 3.6 indicate that accuracy of working range of calibration curve (50-1,000 U/L) was decreased when bone ALP activity spiked higher than 100 U/L. Therefore, bone ALP activity as near or upper 100 U/L required dilution.

3.10 Dilution of serum sample

In serum samples, matrices affect bone ALP assay. A suitable sample dilution is required to obtain the bone ALP activity in the working range of the calibration curve. The changes in percentage of difference when various dilution ratios of serum sample were used are shown in Table 3.7**.**

From the results, high percentage of difference was observed in the bone ALP activity higher than 100 U/L lead to low accuracy of this proposed method at higher

activity. The working range was about 0-100 U/L. Dilution of serum sample is necessary for the serum sample with of bone ALP activity near or higher than 100 U/L. The dilution ratio 1/2 was required. After dilution ratio 1/2 was used, if bone ALP activity was still higher than 100 U/L, higher dilution ratios (1/4, 1/6) must be applied. In this case, it means that bone ALP activity in serum sample was very high.

Table 3.7 Testing of accuracy of spiked bone ALP in normal serum samples.

Sample	Expected (U/L)	Found (dilution factor) (U/L)	% Difference
Normal sample 1	10	10	
Normal sample 1 +100 U/L std. BALP	110	141±5	28±5
		153±31 (1:2)	39±28
		219±10 (1:3)	99±9
Normal sample 1 +500 U/L std. BALP	510	314±56	38±11
		499±43 (1:3)	6±3
		767±74 (1:5)	50±14
		735±95 (1:10)	44±19
Normal sample 1 +1000 U/L std. BALP	1010	286±36	72±4
		397±15 (1:3)	61±1
		625±19 (1:5)	38±2
		920±79 (1:10)	9±8

Table 3.7 (continued)

Sample	Expected (U/L)	Found (dilution factor) (U/L)	% Difference
Normal sample 2	0	0	
Normal sample 2 +100 U/L std. BALP	100	62±10	38±10
		92±13 (1:2)	9±11
		98±33 (1:3)	23±3
Normal sample 2+500 U/L std. BALP	500	320±21	36±4
		433±12 (1:3)	13±2
		377±9 (1:5)	25±2
		446±156 (1:10)	22±15
Normal sample 2+1000 U/L std. BALP	1000	484±19	52±2
		969±50 (1:3)	4±4
		463±26 (1:5)	54±3
		456±18(1:10)	54±2

3.11 Determination of bone ALP in serum samples using the system

The FI-BI system was developed for determination of bone ALP in serum samples of normal persons and patients (osteoporosis). Normal serum samples were obtained from male (n=5, age 25-43 years) and female (n=10, age 23-33 years) subjects. Abnormal serum samples were obtained from osteoporosis patients (n = 9, age over 60 years). The determination of bone ALP in real samples by using FI-BI

method compared to a commercial ELISA kit[31] gave regression equation of y = 1.76x – 3.83 with a correlation coefficient (r) of 0.74 at the significance level 0.001 (p= 0.001), where y = FI-BI system and x = commercial ELISA kit as shown in Figure 3.12. Methods are not significantly different as they showed a good correlation.

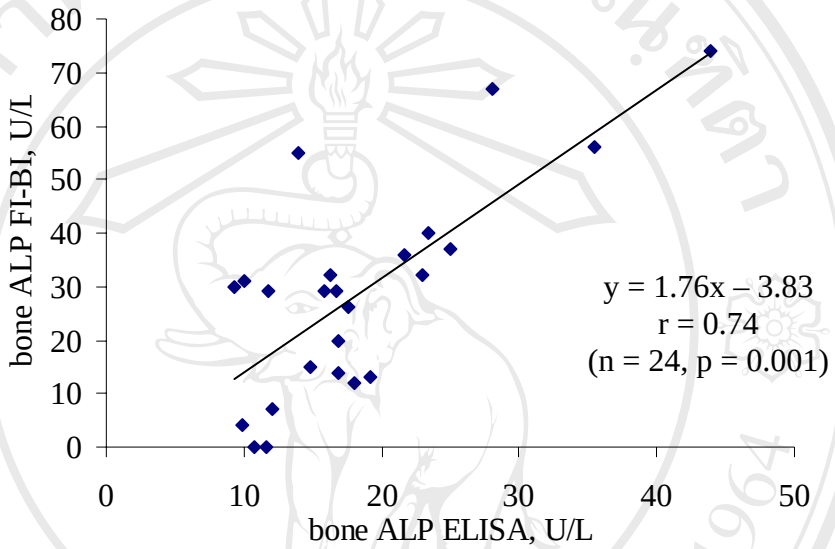


Figure 3.12 The method comparison between FI-BI and commercial ELISA kit

From Figure 3.12, it was found that slope of linear line was not equal 1. This might be because wheat germ lectin has less specific interaction to bone ALP than antibody used in ELISA kit. By average bone ALP activity obtained from the FI-BI assay has 2 times to bone ALP activity found when using a commercial ELISA kit. According to the ELISA kit manufacture, its cross reactivity with liver ALP is 3-8% while this work found that wheat germ lectin has cross reactivity to liver ALP about 3-7%. However, number of samples used to evaluate the cross reactivity of wheat

germ lectin in this work may be too small. It is possible that wheat germ lectin may have some cross reactivity with other isoforms of ALP more than the ELISA kit does.

Approximately 95% of the total ALP activity in human serum is obtained from bone and liver which occur in a ratio of 1:1, which means that the ratio of bone ALP: total ALP is 1:2. In bone patients, bone ALP activity was increased, led to increase in the ratio of bone ALP: total ALP. The ratio of bone ALP: total ALP in normal serum samples and abnormal serum samples were investigated and compared as presented in Figure 3.13. It was found that the ratio of bone ALP: total ALP in normal serum samples and abnormal serum samples obtained from the FI-BI assay are greater than that found when using a commercial ELISA kit. This means that the FI-BI technique can better distinguish normal from patient groups.

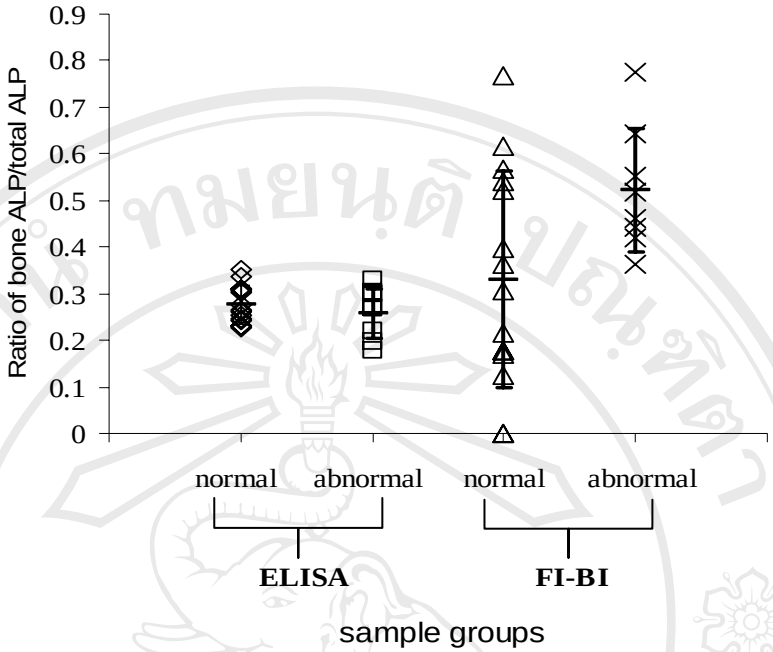


Figure 3.13 The ratio of bone ALP: total ALP in normal serum samples and abnormal serum samples were obtained from the FI-BI assay and commercial ELISA kit (◇ = normal serum samples obtained from ELISA, □ = abnormal serum samples obtained from ELISA, △ = normal serum samples obtained from FI-BI and × = abnormal serum samples obtained from FI-BI)

Averages bone ALP activities found in normal and abnormal groups using the FI-BI technique are shown in Figure 3.14 (see detailed results in APPENDIX F). It demonstrates the significant difference of average bone ALP activity in normal group and abnormal group.

Comparison of bone ALP activities in normal group and abnormal group from the FI-BI assay and commercial ELISA kit demonstrated that the level of bone ALP in

two group samples are significantly different. p-value (see APPENDIX E) of 1.0×10^{-4} (significantly level 0.0001, 99.99% confidence level) was found with the FI-BI assay and p-value of 6.4×10^{-3} (significantly level 0.01, 99.9% confidence level) was found with ELISA. The results are presented in Figure 3.15.

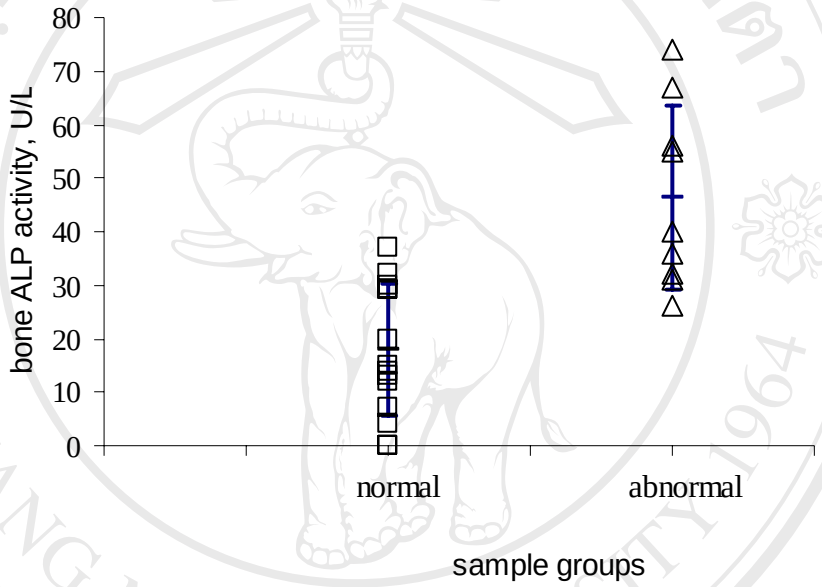


Figure 3.14 Distribution of bone ALP activity in normal serum group and abnormal serum group (osteoporosis) from the FI-BI assay (□ = normal serum samples, △ = abnormal serum samples)

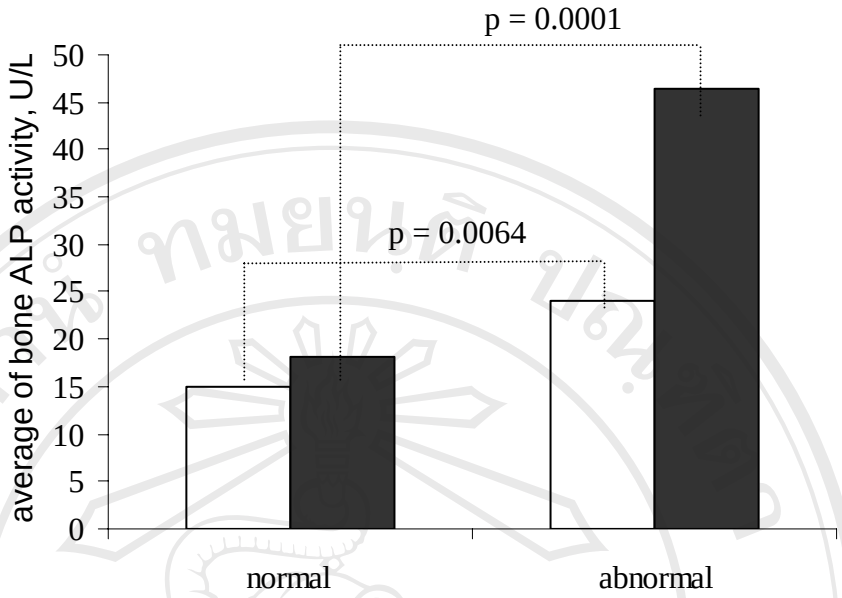


Figure 3.15 Comparison of bone ALP activities in normal group and abnormal group from the FI-BI assay and ELISA (□ = ELISA, ■ = FI-BI)