CHAPTER 2

EXPERIMENTAL

2.1 Materials and apparatus

1. Peristaltic pump (FIA Lab, USA)
2. Peristaltic pump (Alitea, USA)
3. Six-port selection valve (Pittman, USA)
4. Six-port injection valve (Metrohm, Germany)
5. Spectronic 21 (Spectronic Instrument, USA)
6. Perspex for making bead retention cell
7. Two-way pinch valve (Cole-Parmer, USA)
8. Solenoid valve (Guardian Electric, USA)
9. Flow through cell 1 cm, 80 µl (HELLMA, Germany)
10. Computer softwares (LabVIEW®, USA, Origin Northampton, USA)
11. Computer
12. Bone ALP ELISA kit 96 well (Metra BAP EIA kit, Cat. No. 8012, Quidel, USA)

2.2 Reagents

1. Tris (hydroxymethyl) aminomethane: Tris-HCl (Sigma, USA)
2. Hydrochloric acid: 37% HCl (Merck, Germany)
3. Magnesium Chloride: $MgCl_26H_2O$ (Carlo Erba, Italy)
4. Sodium Chloride: NaCl (AJAX, Australia)
5. Sodium hydroxide: NaOH (Merck, Germany)
6. Triethanolamine: $N(CH_2CH_2OH)_3$ (BDH, United Kingdom)

7. Zinc chloride: $ZnCl_2$ (Carlo Erba, Italy)

8. p-Nitrophenyl phosphate (Sigma, USA)

9. Glacial acetic acid: 99.8% (wv^{-1}) CH_3COOH (Merck, Germany)

10. Calcium chloride: $CaCl_2$ (Carlo Erba, Italy)

11. Wheat germ lectin beads: (Lot. No. 103K4119, Product No. L1394, bead size 200-300 μm diameter, Sigma, USA)

12. Bovine serum albumin (Sigma, USA)

13. Fetal bovine serum (Lot. No. CN J0094, Cat. No. CH 30160.03, HyClone Laboratories, Inc., USA)

14. Bone alkaline phosphatase (Lot. No. 4-1-8 Human bone, Cat. No. 124A0001, Calzyme Laboratories, Inc., USA)

15. Liver alkaline phosphatase (Lot. No. 2 Human liver, Cat. No. 178A0001, Calzyme Laboratories, Inc., USA)

2.3 Preparation of standard solutions and reagents

2.3.1 Acetate buffer solution pH 4.5, 50 mM

Acetate buffer solution was prepared by dissolving 0.89 g $CaCl_2$, 7.90 g NaCl in 800 ml distilled water. Then 3 ml CH_3COOH was added before adjusting to pH 4.5 with NaOH. Finally, the volume was made up to 1000 ml with distilled water.

2.3.2 Tris-HCl buffer pH 9.5, 100 mM

Tris-HCl buffer solution was prepared by dissolving 12.1 g Tris (hydroxymethyl) aminomethane, 2.03 g $MgCl_2$ $6H_2O$ and 5.85 g NaCl in 800 ml

distilled water. Then HCl 4.1 ml was added before adjusting to pH 9.5 with NaOH, Finally, the volume was made up to 1000 ml with distilled water.

2.3.3 Diluent pH 7.6, 0.1 M

Triethanolamine 1.86 g was dissolved in distilled water. Then 0.41 ml HCl, 0.1 ml $MgCl_2$ solution (0.1 M) and 0.1 ml $ZnCl_2$ (0.1 M; prepared freshly) was added. The pH was adjusted to 7.6 with NaOH and then the volume was made up to 100 ml with distilled water.

2.3.4 Stock standard bone alkaline phosphatase solution, 20000 U/L

Bone ALP (activity 0.5 U/mg solid) 0.04 g was dissolved in the diluent to the final volume of 1 ml. The stock standard solution was stored at -20^{o}C.

2.3.5 Stock standard liver alkaline phosphatase solution, 20000 U/L

Liver ALP (activity 2 U/mg solid) 0.01 g was dissolved in the diluent to the final volume of 1 ml. The stock standard solution was stored at -20^{o}C.

Note: Unit (U) is defined as 1 U liberates 1 μmol of p-nitrophenol per minute at 37 ^{o}C, pH 10.15 in Tris buffer.

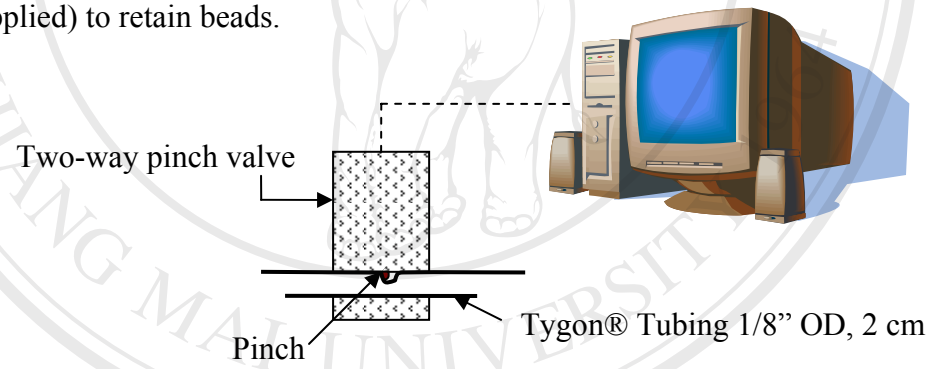
2.3.6 p-nitrophenol phosphate solution, 5 mM

pNPP solution was prepared by diluting 0.5 M pNPP stock solution with Tris-HCl buffer solution to obtain 5 mM. The stock substrate solution (0.5 M) was stored at -20^{o}C for 3 days (see APPENDIX A for its stability over time)

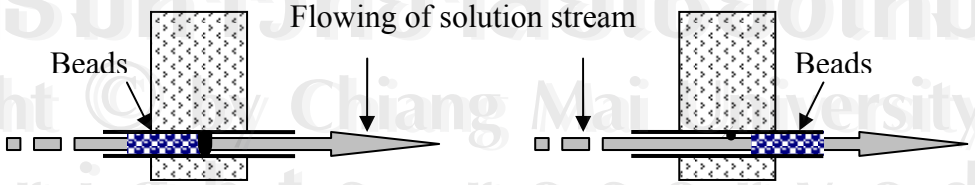
2.4 Bead retention cell

In this research, a specialized flow cell was designed to trap the wheat germ coated beads while the solution was flowed through the system. Two different designed were studied. The first one is based on retention of beads in the tygon® tubing itself by pinching the tube close with a solenoid valve. Another one is a laboratory made Perspex glass cell.

For the first design (Figure 2.1), a solenoid valve was used to control the retaintion and rejection of the beads. This was done by using the computer program (LabVIEW® software) (see APPENDIX B).The pinch valve was opened (without applied voltage) to let beads flow into the tubing. The pinch valve was closed (voltage was applied) to retain beads.



(a) The system for retention of beads in the Tygon® tubing using a 2-way pinch valve

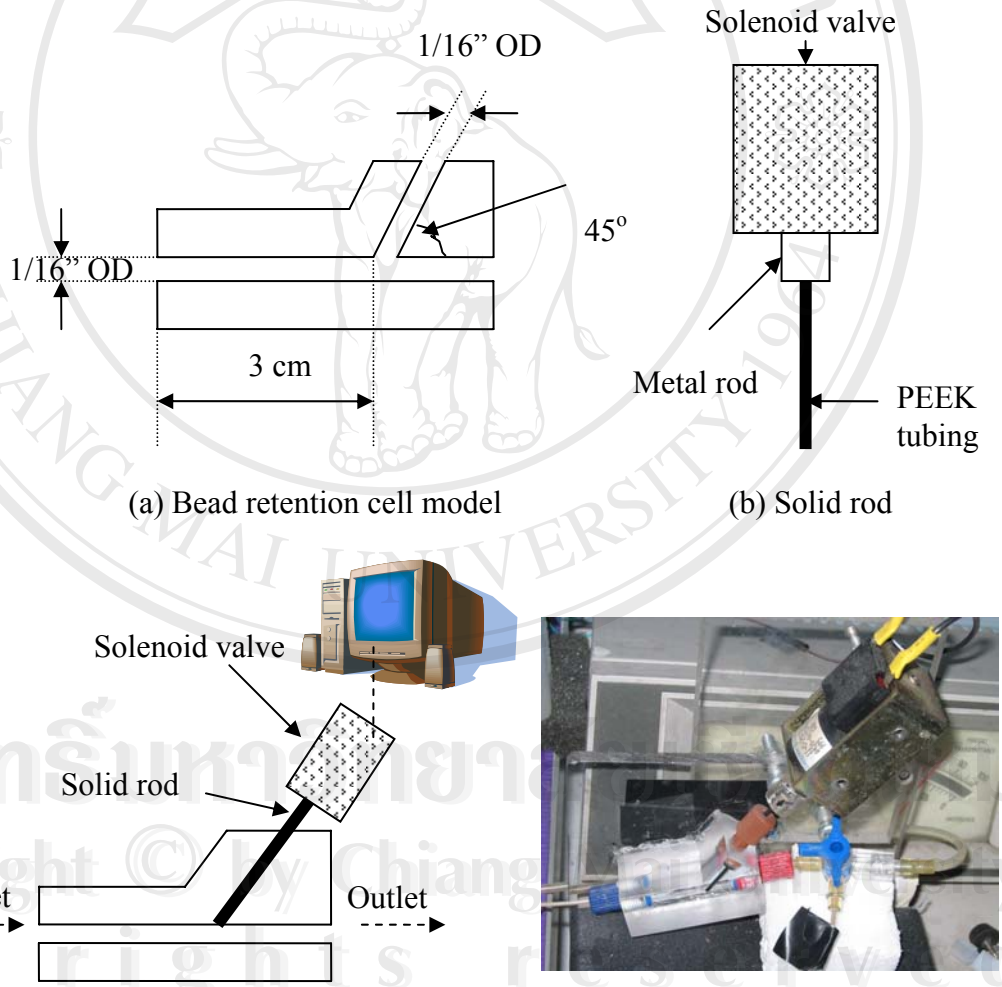


(b) Pinch valve was closed (beads were retained in the cell while solution flowed through)

(c) Pinch valve was opened (beads were rejected from the cell with a flowing of solution stream)

Figure 2.1 A schematic diagram of the bead retention cell

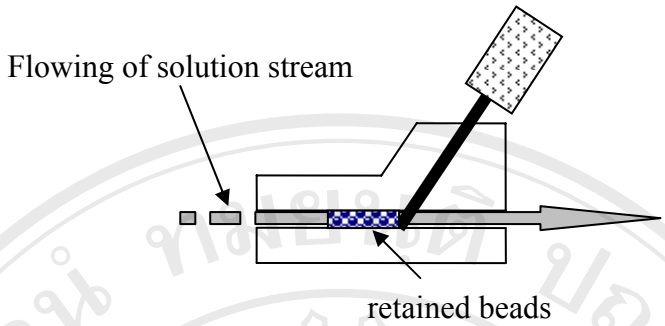
The second bead retention cell design, the channel was created in the Perspex glass (Figure 2.2 a). Solenoid-solid rod, composed of a PEEK tubing connected to the metal rod of solenoid valve as shown in Figure 2.2 b, was inserted 45^{o} to the flow path to retain beads. After analysis, the beads were rejected by pulling back the solid rod (with the applied voltage). Then beads were pushed out with a flowing stream of solution. The movement of solid rod can be controlled by applying the voltage to a solenoid valve using the LabVIEW® solftware (see APPENDIX B)



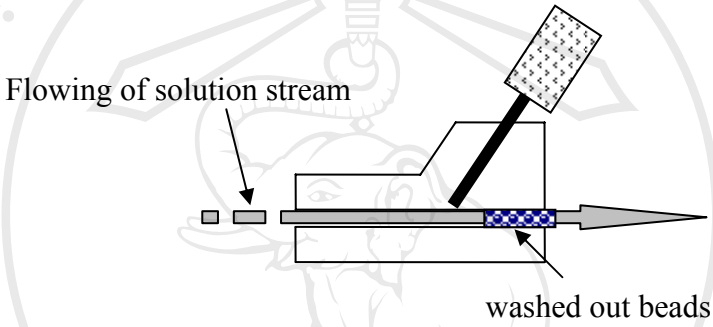
(a) Bead retention cell model (b) Solid rod

(c) Composition of bead retention cell made from Perspex

Figure 2.2 A schematic diagram of the bead retention cell made from Perspex glass



(a) Beads are retained in the cell while the solution is flowing through



(b) Beads are rejected from the cell with a flowing of solution stream

Figure 2.3 A schematic diagram of beads retention and beads rejection by a solenoid valve

2.5 Manifold and operation step

The bead-injection (BI) technique is the combination of using special functionalized beads with a flowing stream of solution in the flow injection (FI) system. The peristaltic pumps were used to deliver beads and buffer solutions. Wheat germ lectin coated on beads were introduced and retained in the bead retention cell while solution was flowing through the small space between cell surface and solid rod. Samples and substrate were consequently introduced by means of a six-port injection valve into the FI system. Specific lectin (wheat germ) on the bead surfaces

interacted with bone ALP in the sample solution that was passed through the flow cell. Bone ALP was detected by p-nitrophenyl phosphate. The detectable species, p-nitrophenol can be detected at 405 nm (see Figure 2.4). when it passed into the flow through cell with 1 cm path length which was placed in the spectronic 21. The absorbance was converted to voltage and recorded with a computer software (LabVIEW®, USA) installed in a personal computer (see APPENDIX B). These data were transferred and integrated with Origin (Northampton, USA) and Microsoft Excel (Microsoft, Corp., USA). The Manifold used in the work is shown in Figure 2.5.

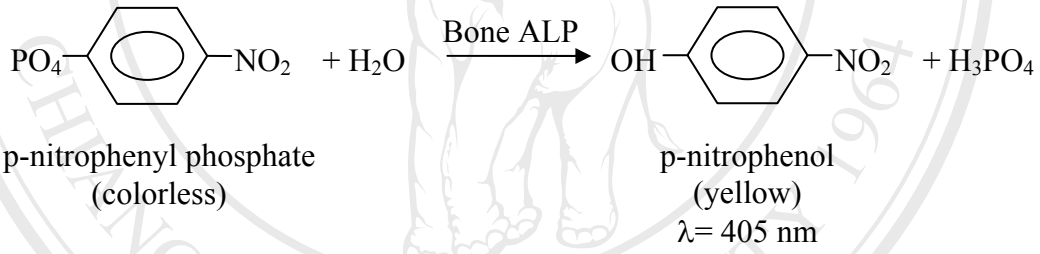


Figure 2.4 The enzyme-substrate reaction of bone alkaline phosphatase with p-nitrophenyl phosphate

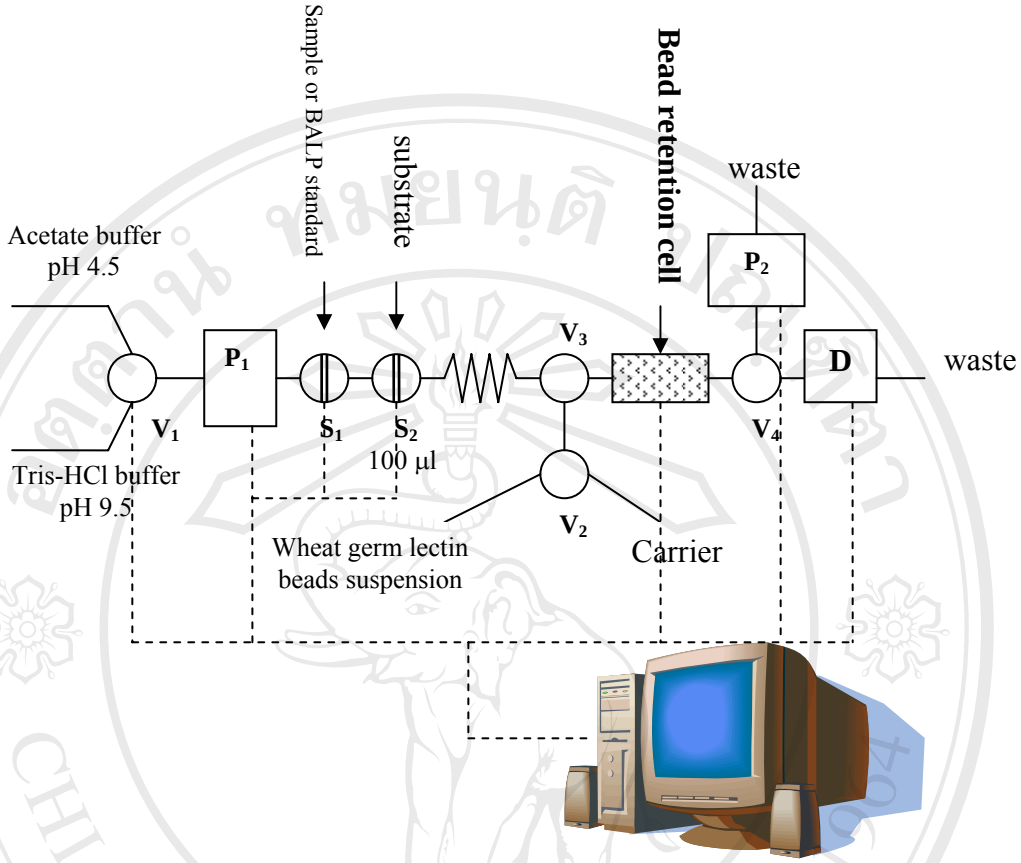


Figure 2.5 The FI-BI system for the determination of bone alkaline phosphatase, S_1 and S_2 are the six-port injection valves; V_1 is a six-port selection valve; V_2, V_3 and V_4 are three way valve; P_1 and P_2 are the peristaltic pumps and D is a spectronic 21.

Operation steps of the system are as follows; first, wheat germ lectin beads were loaded into the flow cell with a peristaltic pump (P_2) through valves V_2, V_3 and V_4 out to waste. Then DI water was pumped in to pack beads in the bead retention cell by switching valve V_2 to the carrier After that, the pump (P_2) was stopped. Then valve V_3 and V_4 were switched to introduce the acetate buffer solution pH 4.5 to the system by a peristaltic pump (P_1). Serum sample was injected into the acetate buffer solution

stream via an injection valve (S_1) and passed through the bead retention cell. Then the pump (P_1) was stopped to allow incubation of bone ALP with wheat germ lectin coated beads for a desired period of time. After that, the Tris- HCl buffer solution pH 9.5 was pumped through the system by switching valve V_1 to remove unbound species. p-Nirophenyl phosphate was injected into the buffer solution stream by means of an injection valve (S_2) with a 100 µl loop and passed through the bead retention cell and out through the flow cell. Then the pump was stopped for incubation. Finally, the enzyme-substrate product, p-nitrophenol was pushed to the flow through cell and was detected at 405 nm by pumping the buffer solution through the Spectronic 21 (see APPENDIX C).

2.6 Optimization

Various parameters were optimized. These include the bead loading time, sample volume, flow rate (P_1) and processing time (condition time, washing time and incubation time).

All experiments in this section employ fetal bovine serum as a sample of bone ALP to mimic matrices of real serum samples to be standard in the future.

2.6.1 Sample volume

The sample volume was investigated for acceptable sensitivity. Various volume were studied by changing the size of sample loop in the range of 50, 100, 200 and 300 µl.

2.6.2 Flow rate

Flowing of solution at high flow rate through the packed bead retention cell may result in high back pressure. On the other hand, a low flow rate has a draw back of a long analysis time. Thus, the suitable flow rate was studied. It was varied from 1.0 to 2.5 ml/min.

2.6.3 Bead loading time

In this work, the suitable bead volume was investigated by studying the bead loading time. The homogeneous bead suspension was loaded through P_2 pump with flow rate of 3.0 ml/min and retained in the system. The bead loading time was varied from 5s to 20s.

2.6.4 Processing time

The operation steps for measuring bone ALP include the bead conditioning step, incubation steps and washing step. The optimum time of each step was studied and chosen based on the acceptable sensitivity and analytical time.

2.6.4.1 wheat germ lectin beads conditioning time

The optimum condition of wheat germ lectin coated bead is weak acidity (pH 4.5). Therefore, its suspension was prepared in the acetate buffer solution. However, interaction bone ALP and wheat germ is the best at pH 4.5. So, beads need to be conditioned before trapping bone ALP. Because Tris-HCl buffer (pH 9.5) was used in the last step of analysis that the FI system was in weak basic condition. The flowing time of the acetate buffer was investigated in the range of 1, 2 and 3 min.

2.6.4.2 Incubation time of wheat germ lection with bone ALP

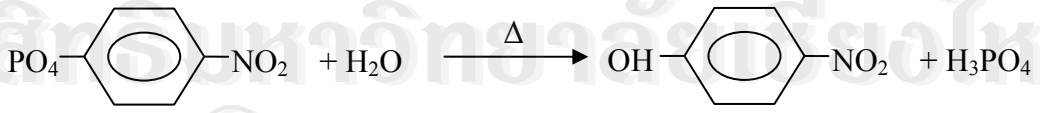
The longer incubation time allows the higher interaction of wheat germ lectin with bone ALP. The optimum incubation time was investigated by stopping the flowing stream of acetate buffer at 3, 4, 5 and 10 min.

2.6.4.3 Washing time

After incubation of wheat germ with bone ALP, Tris-HCl buffer pH 9.5 was flowed though the system to remove unbound species and to adjust the condition of the system for the enzyme-substrate reaction. Thus, the flowing time of the Tris-HCl buffer was studied in the range of 1, 2, 3 and 6 min.

2.6.4.4 Incubation time of bone ALP with p-nitrophenyl phosphatase

The longer the enzyme-substrate incubation time, the more products is produced yielding higher signal. However, the long incubation slows down the analysis and the product may be degraded or substrate may be auto-oxidized (Figure 2.6). The optimization of incubation time was investigated from 1 to 10 min.



p-nitrophenyl phosphate (colorless)

p-nitrophenol (yellow) λ= 405 nm

Figure 2.6 p-nitrophenol obtained from auto-oxidation of p-nitrophenyl phosphate

2.7 Within-run precision

The FI-BI system was operated under the optimum condition and a fetal bovine serum was used repeatedly 10 times with in one day. The percentage of relative standard deviation (%RSD) was estimated.

2.8 Between-run precision

The FI-BI system was operated under the optimum condition to determine bone ALP in a fetal bovine serum sample repeatedly every day for 10 days. The percentage of relative standard deviation (%RSD) was estimated.

2.9 Calibration curve

The optimum condition of the FI-BI system was used to obtain the calibration graph. The bone ALP activities were investigated in the range of 10-10,000 U/L to cover amount of bone ALP found in normal person and bone patients. The detection limit and working range were determined from this curve.

2.10 Cross-reactivity with liver ALP

Even though wheat germ lectin is 90% specific toward Bone ALP[17], the liver ALP which is another important isoenzyme of ALP in human serum, may have cross-reactivity with the wheat germ lectin. The cross-reactivity was investigated by spiking liver ALP activities into 6% BSA in the range of 50-1000 U/L. Spiked liver ALP samples were measured by a simple FI-BI system then compared the analytical signal with the calibration curve of bone ALP activity. Percentage of cross-reactivity was calculated by equation 2.1.

$$\text{Cross-reactivity} = \frac{[(\text{calculated activity from calibration curve}) \times 100]}{(\text{Activity of spiked liver ALP})} \quad (2.1)$$

2.11 Testing of accuracy

Accuracy of the method was investigated by spiking various bone ALP activities into normal serum sample as follows; 100, 500 and 1,000 U/L. The accuracy was calculated as the percentage of difference between the calculated activities of spiked bone ALP with the measured amount of bone ALP found by using the FI-BI system.

2.12 Dilution of serum sample

The limited amount of sample affects the study of serum. Thus a suitable sample dilution is required for an effective use of sample. In this work, 6% BSA was used as a diluent (pH 7.6) to dilute serum sample 1/2, 1/3, 1/5 and 1/10 times.

2.13 Application of developing FI-BI technique for real serum samples

The FI-BI technique was used to estimate bone ALP in real samples of normal persons and patients of osteoporosis. The results from different sample groups were compared. The possibility of using this method for screening and monitoring was evaluated.

2.14 Comparison of the FI-BI system with ELISA kit

ELISA kit as commercial method is an immunoassay in a microtiter strip format utilizing a monoclonal anti-Bone ALP coated on the strip to capture bone ALP in the sample. The enzyme activity of captured bone ALP is detected with p-nitrophenyl phosphate. Sample volume used was 20 µl. ELISA kit requires 3-h for incubation, 3 times washing step and 30 min for developing detectable species. Enzyme-substrate product was monitored at 405 nm. A comparison of the FI-BI assay and ELISA results for 15 normal serum samples and 9 abnormal serum samples (osteoporosis patient) were carried out.