CHAPTER III

RESULTS

3.1 Methods for Metal Determination

Prior to the Pb binding proteins experiments , methods for metal determination were performed to check their sensitivity and limitation. In the determination of metals , two methods were commonly used (Man Amornsit and Amorn Petchsom , 1991) : the chemical method with Ferrozine for Fe determination and the physical methods with spectrometry. Inductively Coupled Plasma Spectrometry and Graphite Furnace Atomic Absorption Spectrophotometry were methods of choice. Although these methods were established as accurate , precise and acceptable methods of analysis for trace metals in plasma and erythrocytes (Whitehouse *et al.*, 1982 and Mori *et al.*, 1981) , they may be interfered by some factors such as proteins , buffers and other metals causing either a decrease or an increase in the absorbance signal of the sample. These effects must be identified and compensated if metal values are to be reliable. The experiments were thus performed by matching the major constituents of the sample ; serum , buffers and other metals ; in pure metal standard and compared their absorbance with pure standard. The results were shown in Figures 4-11.

3.1.1 Ferrozine Method (for Fe Determination)

For choosing the suitable buffer for Fe determination by Ferrozine , the concentration of Fe standards dissolved in various buffers were determined by Ferrozine method (2.2.1.3). The result is shown in Figure 4. Different buffers present different absorption values. The acetate buffer pH 4.15 gives the highest absorbance. This buffer gave the highest standard curve (Figure 4) but did not interfere the iron-determination system (Figure 5) , Pb (250-2,000 μg/l) hardly interferes this system. This point is very important since blood samples for Fe analysis (in the following experiments) are always contaminated with Pb , especially in the case of Pb-treated blood and Pb-toxic patient blood. Moreover, Pb binding was highest at pH 4.15, as compared to other pH's, the result is shown in Table 3.

3.1.2 Inductively Coupled Plasma Emission Spectrometer (ICPS)

Inductively Coupled Plasma Emission Spectrometry is a technique that has taken its place as a mainstay in the field of elemental analysis with wide dynamic range , μg/l detection limits , a lower degree from matrix interference and a minimum of sample pretreatment (Wolnik , 1988). Standard graph of Fe and Pb present in Figures 6 and 7 with detection range 0-1,000 μg/l. However, there may be some interference occured during analysis by ICPS: spectral

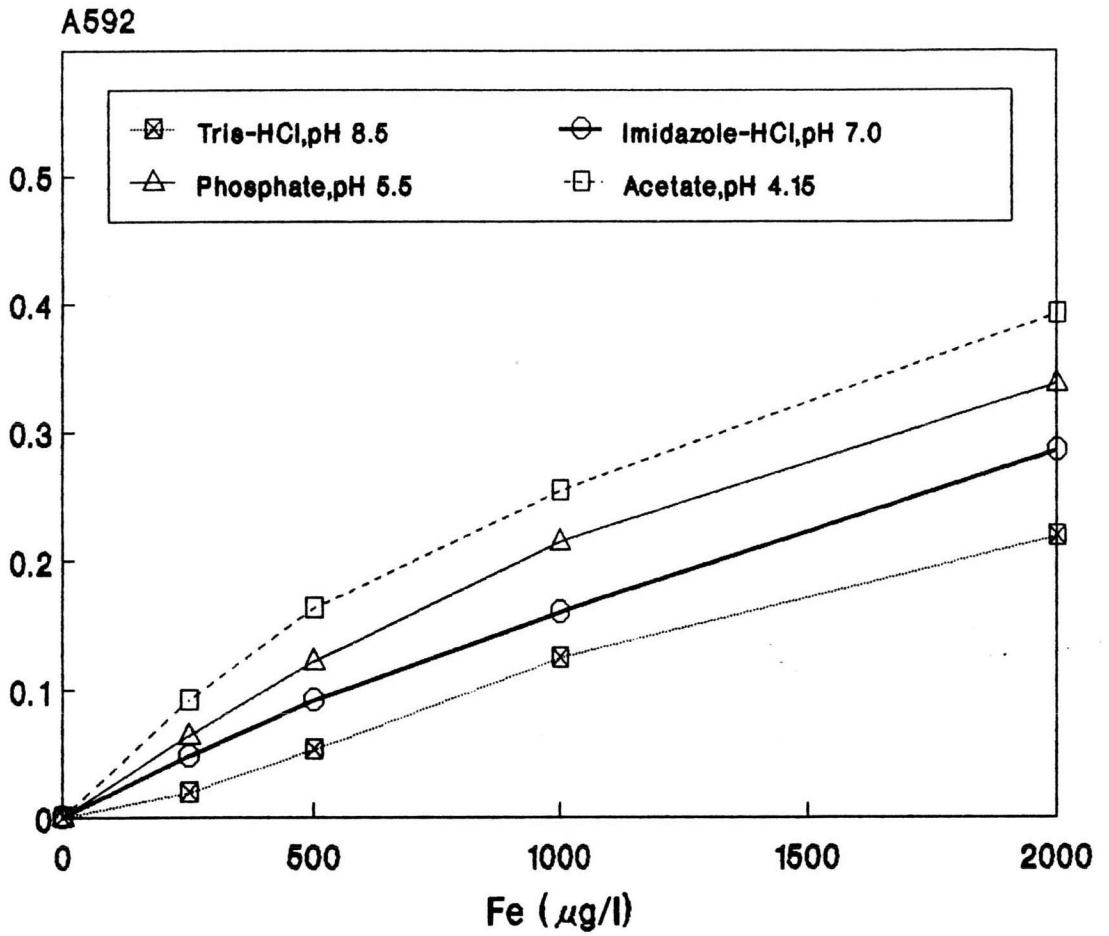


Figure 4 Effect of buffers on Fe determination by Ferrozine method

Fe standards were prepared in various buffers of 0.1 M as indicated in the Figure. Fe concentration was determined in each standard by Ferrozine method as described in 2.2.1.3.

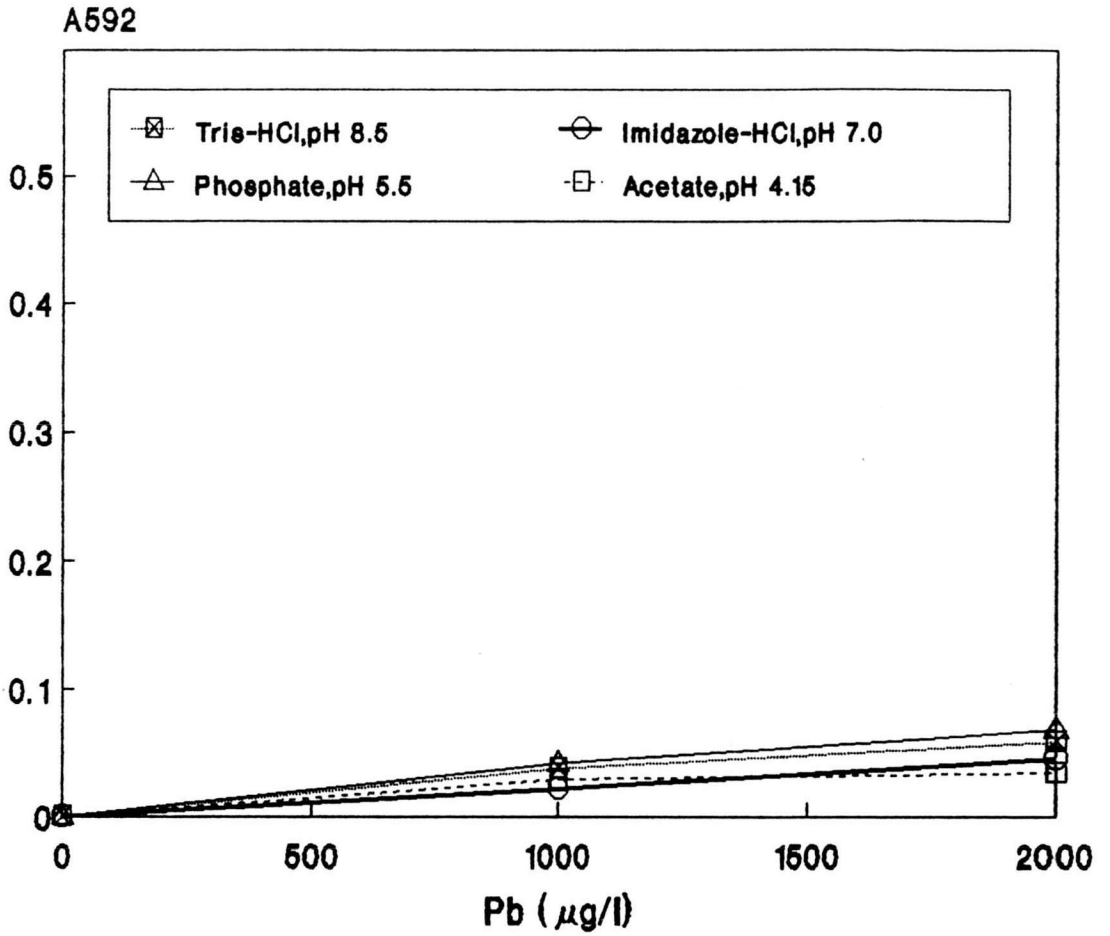


Figure 5 Specificity of Ferrozine method on Fe determination.

Pb standards (250 - 2,000 μg/l) were prepared in Fe-free buffers (0.1 M) of different pH as indicated. A_{592} were measured after Ferrozine reaction as mentioned in 2.2.1.3.

Buffers	Fe concentration (μg/l)		
	-Pb	+Pb	% decrease
acetate, pH 4.15	1350 ± 331	520 ± 87	61
phosphate, pH 5.5	1030 ± 321	600 ± 240	42
imidazole, pH 7.0	780 ± 275	700 ± 306	10
Tris-HCl, pH 8.5	400 ± 120	280 ± 99	30

Table 3 Effect of Pb on the concentration of protein bound iron at various pH's

Serum was incubated with or without Pb (1,800 μg/l) in 0.1 M of various buffer and then determined for protein-bound Fe with Ferrozine method as determined in 2.2.1.3

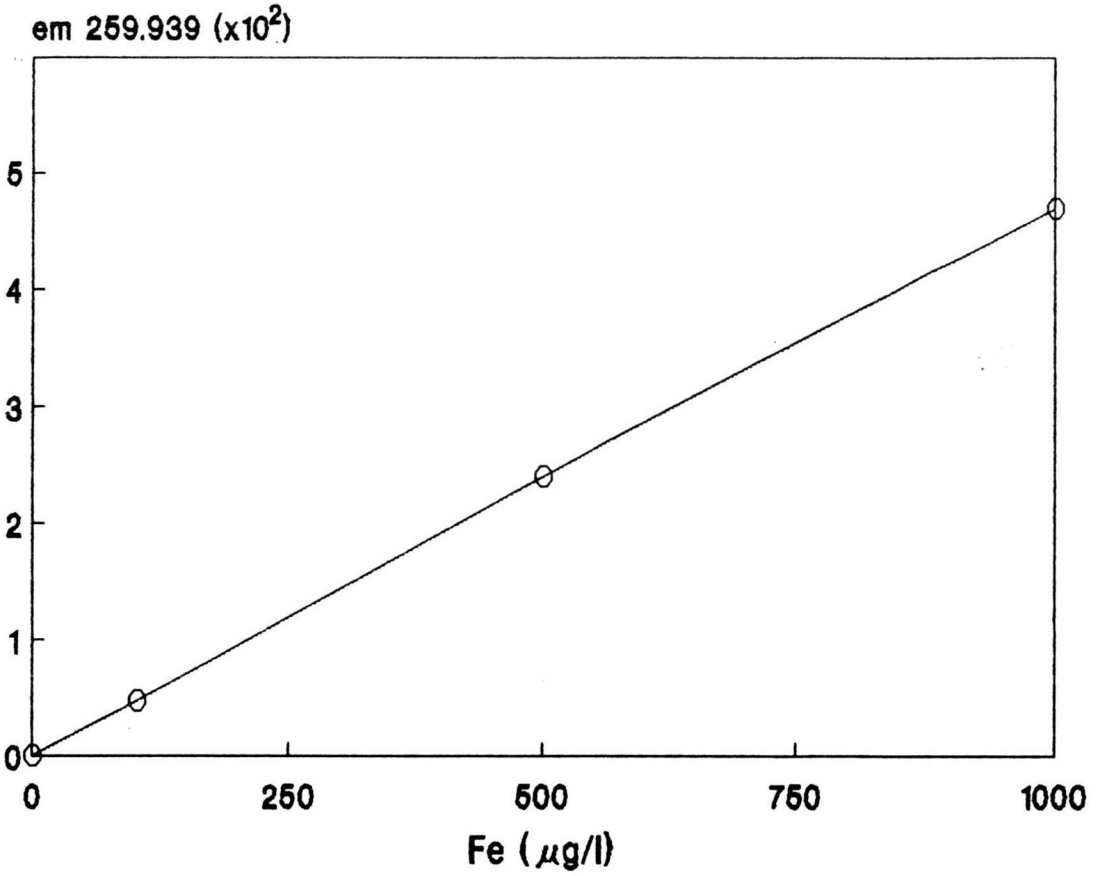


Figure 6 Standard Fe (µg/l) graph showing the relation between Pb concentrations and their emission (em) values determined by ICPS. The method is described in 2.2.1.2.

(em 259.939 = emission value at wavelength of 259.939 nm for Fe determination)

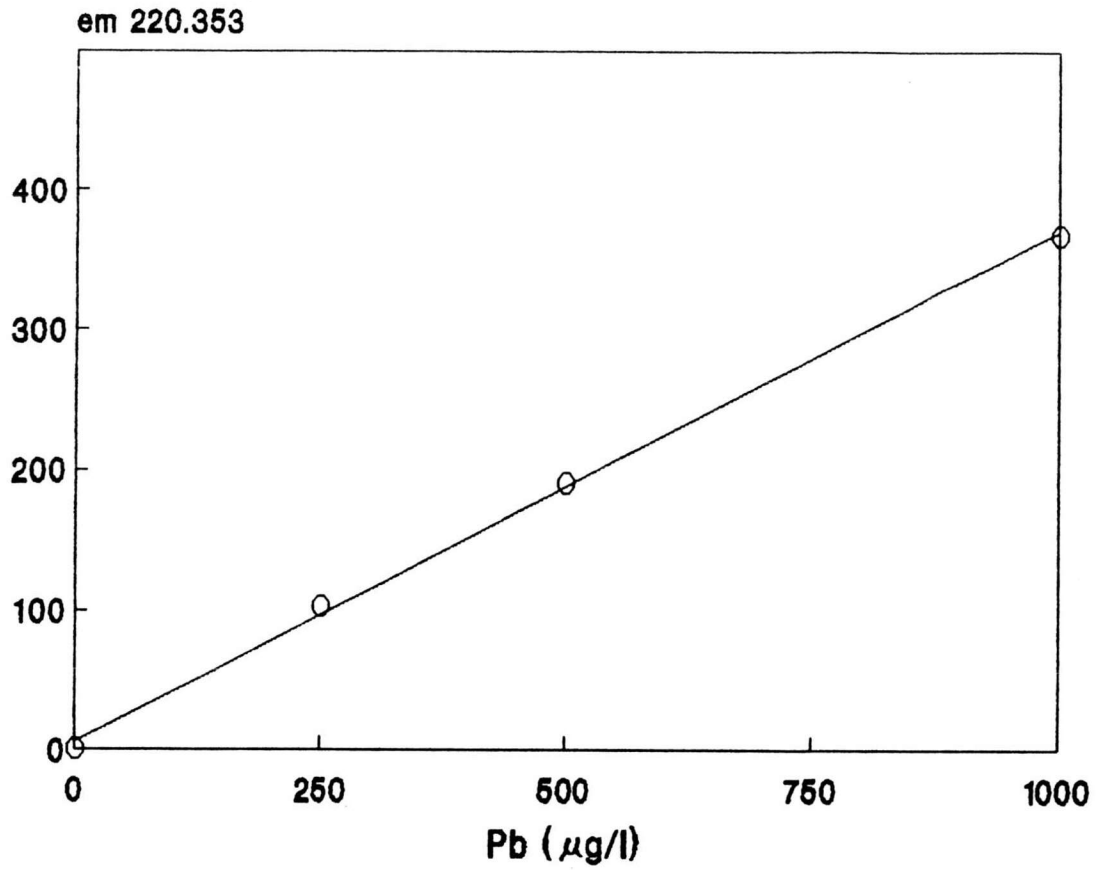


Figure 7 Standard Pb (µg/l) graph showing the relation between Pb concentrations and their emission (em) values determined by ICPS. The method is described in 2.2.1.2.

(em 220.353 = emission value at wavelength of 220.353 nm for Pb determination)

interference and matrix interference (Wolnik, 1988)

Spectral interference stems from spectra emitted by components of the sample solution other than the analyte or from shifts in background continuum caused by the introduction of sample aerosol. Spectral interference leads to errors in the measurement of analyte emission unless properly corrected (Wolnik, 1988).

Matrix interference effects are caused by components of the sample solution other than the analyte and result in increased analyte emission (Wolnik, 1988)

In this study , the experiments were usually performed on human serum in acetate buffer pH 4.15 which may interfere the metal determination. To check their interference on metal measurement by ICPS , serum diluted 30 folds or 0.1 M acetate buffer pH 4.15 were added to Fe and Pb standards and their emission were measured (3.3.1). The result indicates that serum or 0.1 M acetate buffer can not interfere the metal determination (data not shown).

Spectral interference of ICPS on Pb and Fe determination were also studied. The emission of 330 μg/l Pb and 420 μg/l Fe standards were measured at two wavelengths : 220.353 and 259.939 nm which claimed to be specific for Pb and Fe , respectively. The result in Table 4 indicates that ICPS is specific for Pb but is not for Fe determination. Fe emission could not be detected at 220.353 nm, the wavelength specific for Pb detection , but Pb emission could

Samples	emission value (em)	
	259.939 nm (Fe)	220.353 nm (Pb)
Fe	2,171.87	7.84
Pb	1,623.73	136.65
Fe+Pb	3,200.93	139.54

Table 4 Spectral interference of ICPS on metal determination

Fe (420 μg/l) and Pb (330 μg/l) standards were prepared in 0.1 M acetate buffer pH 4.15 and determined for metal concentration by ICPS at 259.939 nm and 220.353 nm, respectively.

be detected at both wavelengths. Detection of 330 μg/l Pb at 259.939 nm may lead to wrong interpretation *i.e.*, 324 μg/l Fe. Therefore, ICPS can not be used to analyse Fe in the sample containing Pb.

3.1.3 Graphite Furnace Atomic Absorption Spectrophotometer (F-AAS)

Atomic absorption method is most widely used for the determination of metals in biological materials (Slavin, 1988). It is inherently very specific and interferences that will reduce the accuracy below the level of the precision are few (Slavin, 1988). Since various experiments in this study were usually performed on human serum in acetate buffer of pH 4.15, the interfences, if exist, may arise from these two factors. Thus, it is necessary to check whether they interfere F-AAS on Fe and Pb determination. Pb standards were prepared in various solutions as described in Figure 8 and determined for its concentration by F-AAS (2.2.1.1) , 0.02 M HNO_3 which is suitable for analyzing metal concentration and is the most commonly used solvent in F-AAS (Man Amornsit and Amorn Petchsom, 1991) was utilized as the reference in this study. The standard graph of Pb in acid is a straight line while 0.1 M acetate buffer pH 4.15 interferes Pb determination, the higher the concentration of Pb - the more interference produced. However, this effect disappeared when the buffer

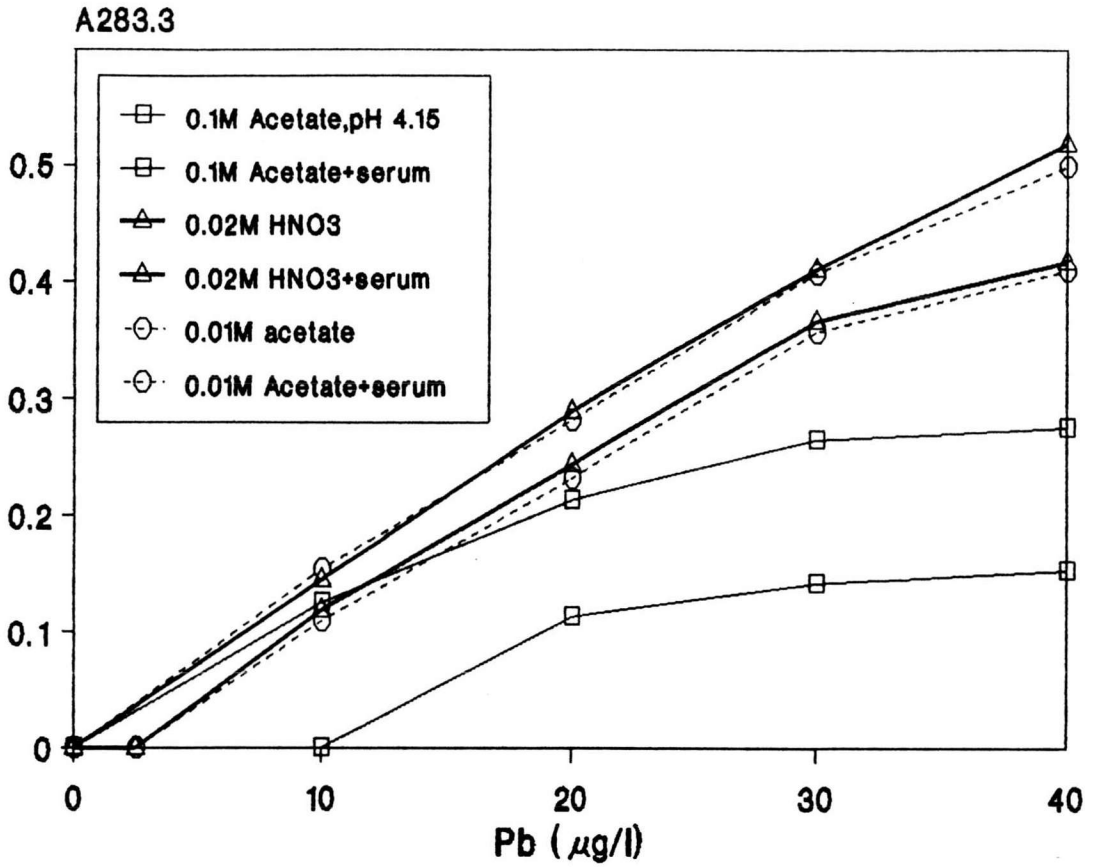


Figure 8 Effect of buffers and serum on Pb determination by F-AAS

Pb standards were prepared in various solutions with and without serum (30 fold dilution) as indicated in the Figure and determined for Pb concentration by F-AAS (2.2.1.1)

was diluted to 0.01 M.

Addition of serum in the amount used in several experiments (30 fold dilution), slightly reduces the absorbance of Pb in 0.02 M HNO_3 or 0.01 M acetate buffer (Figure 8). This interference effect of serum is more pronounced when Pb was dissolved in 0.1 M acetate buffer: about 50 % was observed. Thus, 0.01 M acetate buffer is the suitable buffer for Pb measurement in presence of serum with not more than 30 fold dilution.

The experiments on interferences of serum and buffer in Fe determination by F-AAS were also performed. Fe standards were prepared as described in Figure 9. The result showed that serum (30 fold dilution) and 0.01 M acetate buffer pH 4.15 did not interfere this system, so the measurement of Fe concentration can be done in the presence of 0.01 M acetate buffer and 30 fold-diluted serum by F-AAS.

Apart from checking the matrix interference, F-AAS was also checked for spectral interference created by Pb or Fe. The result in Figure 10 indicates that the absorption of Fe(up to 8,000 μg/l) at wavelength 283.3 nm which claimed to be specific for Pb, is very low. Figure 11 shows that Pb of up to 1,800 μg/l had little absorption at 248.3 nm, the specific wavelength for Fe.

From the above result, F-AAS is the suitable method for determining Fe and Pb concentration. F-AAS

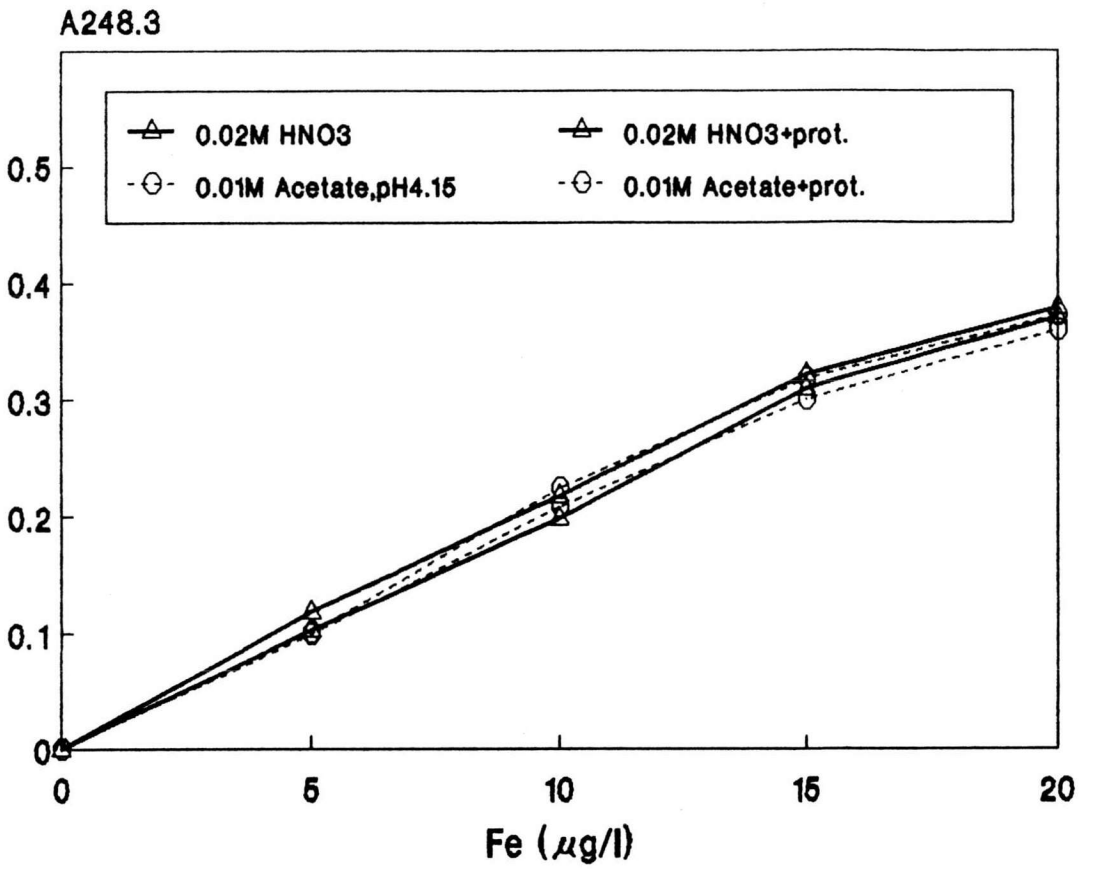


Figure 9 Standard curve of Fe, determined by F-AAS

Fe standards were prepared in different solutions with or without serum and determined for the concentration of Fe by F-AAS (2.2.1.1).

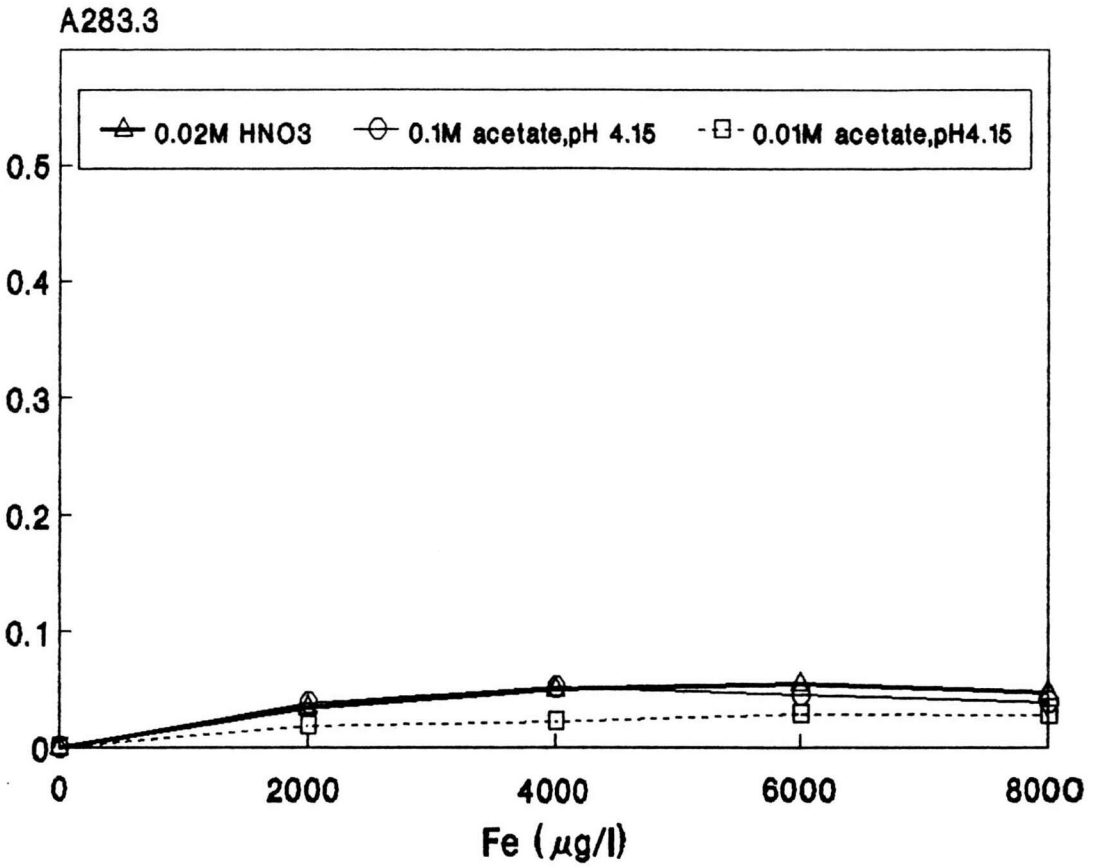


Figure 10 Spectral interference of Fe on Pb determination by F-AAS

Fe standards (2,000 - 8,000 µg/l) were prepared in 0.01 M acetate buffer pH 4.15 and Pb concentration determined by F-AAS.

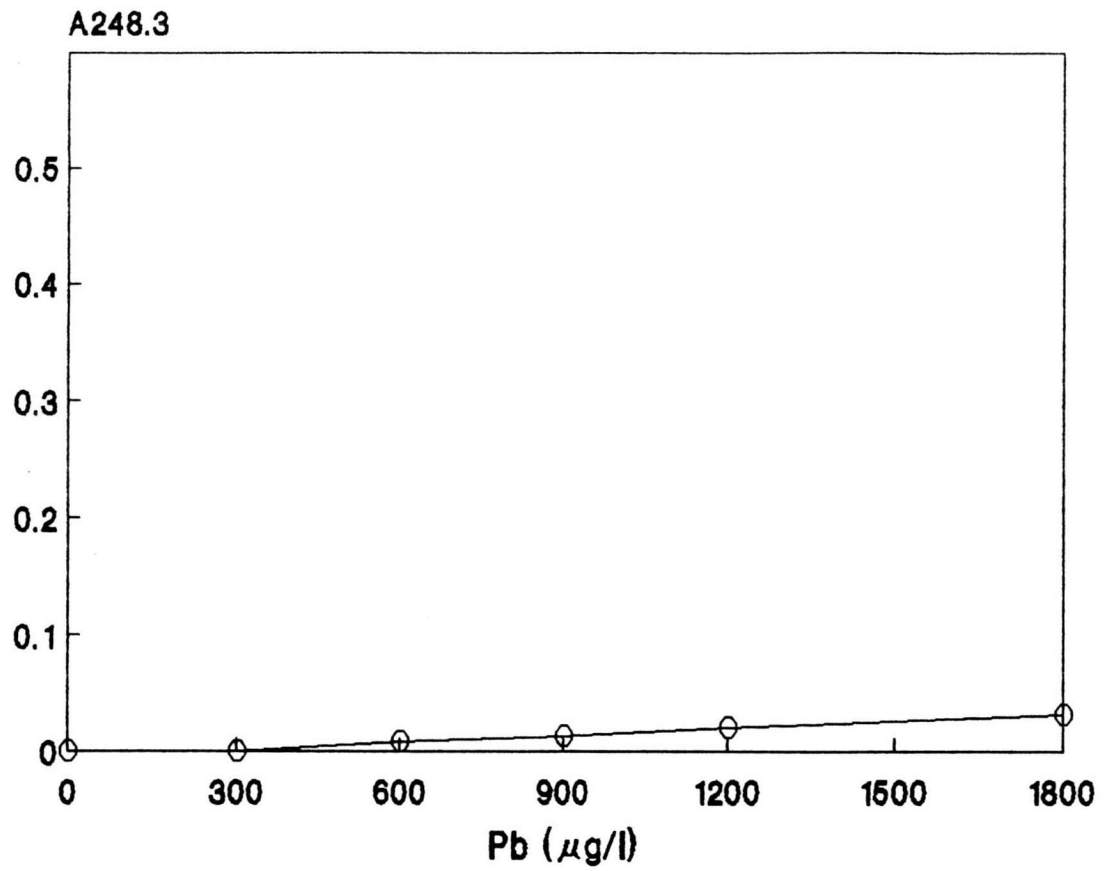


Figure 11 Spectral interference of Pb on Fe determination by F-AAS

Pb standards (300-1,800 µg/l) were prepared in 0.01 M acetate buffer pH 4.15 and Fe concentration determined by F-AAS (2.2.1.1)

is not only be less interfered by matrix and spectral interferences , but also requires small volume of sample solution and detects Fe and Pb concentrations in level of μg/l.

3.2 Distribution of Pb in Whole Blood

To study the kinetic of Pb uptake by blood cells, human heparinized blood was incubated with Pb at the concentration reported to cause anemia (600 μg/l and 1,800 μg/l) (Goyer, 1991). Pb was checked periodically for its localization in serum and blood cells by F-AAS as described in Figure 12.

At both concentrations of lead utilized, the metal was taken up rapidly by blood cells, reached saturation within 8 min, leaving Pb residues in the serum fraction (Figure 12). It was of great interest that after 8 min Pb concentration detected in the serum , in both assays did not reach zero but remained constant at 250 μg/l. This result suggests the presence of some factor(s) which can bind to Pb in plasma.

To prove the above statement , the blood cells was prepared and washed 3 times to remove plasma and preserved in phosphate buffer saline pH 7.4. The cells were then treated with Pb and its concentration determined as shown in Figure 13. The result indicates that at saturation , Pb uptake by isolated blood cells was only 30 % of that in

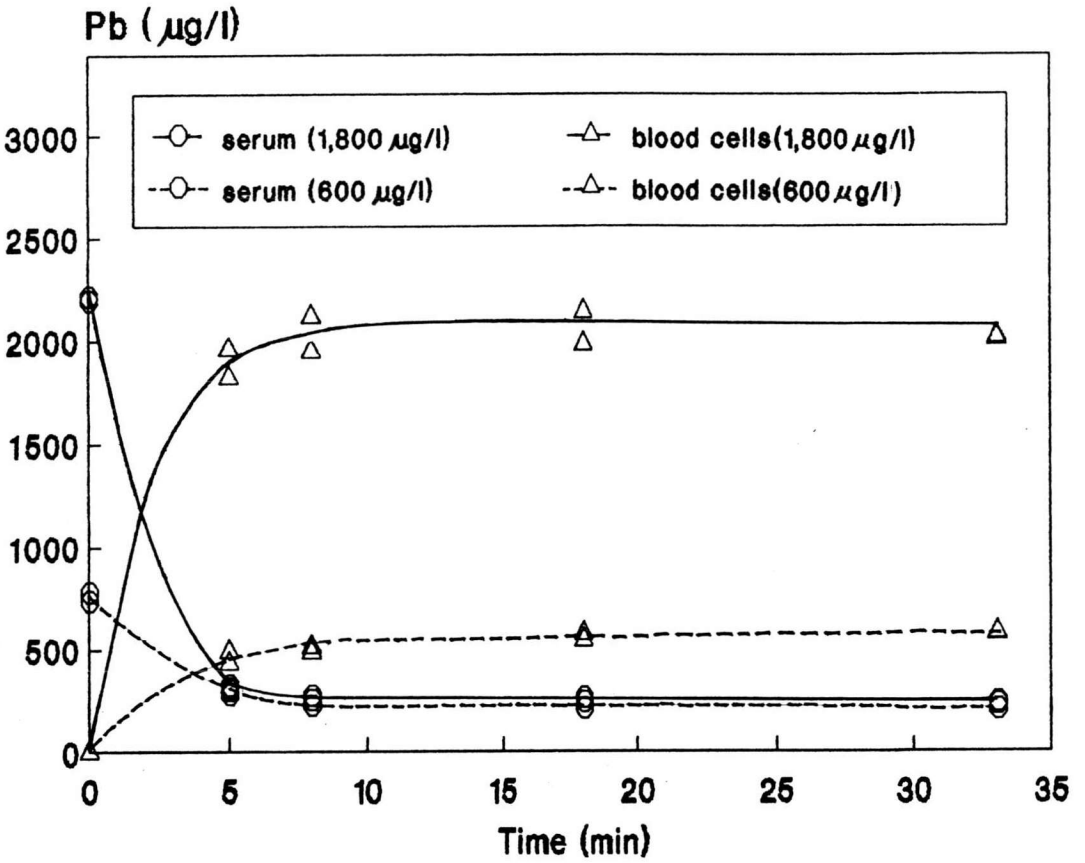


Figure 12 Pb uptake by blood cells

50 ml of human heparinized blood was incubated with 600 µg/l Pb and 1,800 µg/l Pb at 37°C. At indicated time, cells were separated from serum by centrifugation at 10,000 xg for 1 min. The concentration of Pb was determined in both fractions by F-AAS as described in 2.2.1.1.

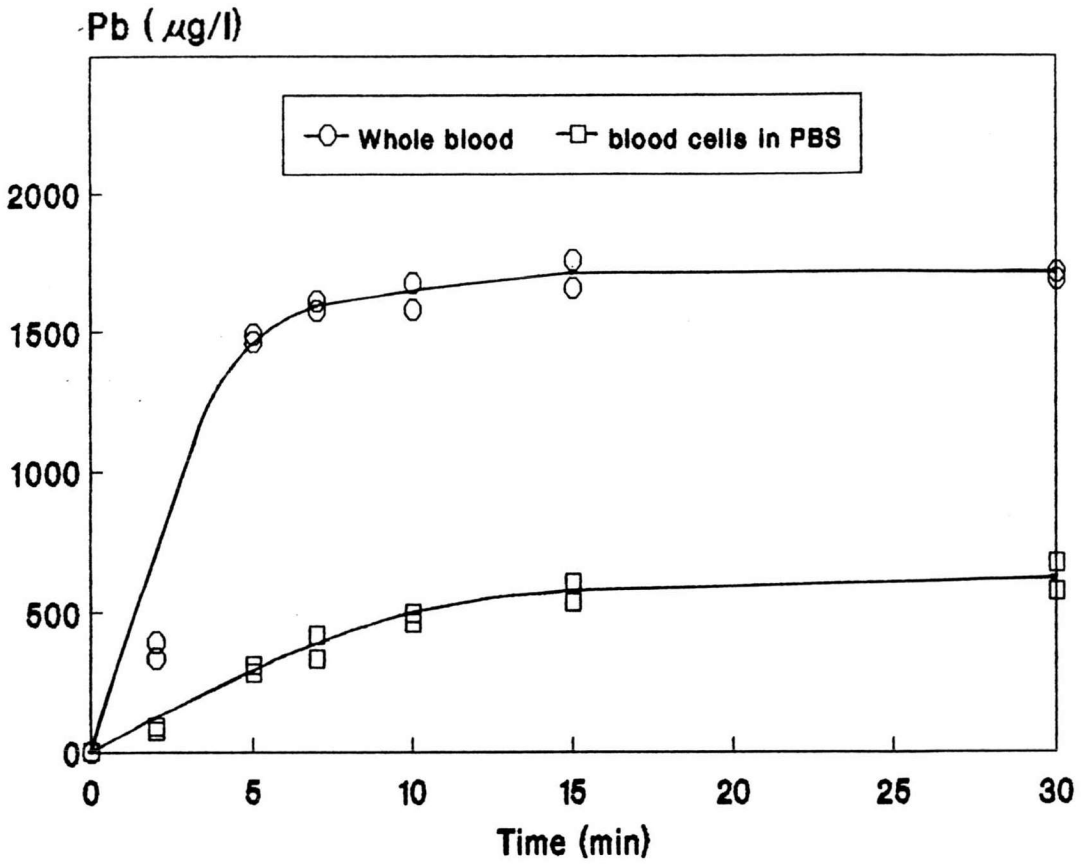


Figure 13 Effect of blood plasma on Pb uptake by blood cells

Blood cell fraction was prepared from heparinized whole blood by centrifugation at 10,000 xg for 1 min, washed 3 times and finally suspended in 0.155 M phosphate saline buffer (PBS). Whole blood fraction was the heparinized blood. Both fractions were adjusted to give the same amount of blood cells , as judged by A_{410} nm. Both of them were then incubated with 1,800 μg/l Pb at 37°C. At the indicated time, 1 ml of each solution was centrifuged at 10,000 xg for 1 min and Pb concentration was determined in packed cell fraction by F-AAS (2.2.1.1).

the presence of plasma (in whole blood fraction). Moreover, the uptake of Pb in the former case is slightly slower than that in the latter. This result strongly suggests the presence of factor(s) which can bind to Pb in plasma. This factor(s) may involve in the transport of Pb to blood cells.

3.3 Lead-binding Proteins

There are many proteins in serum, most of them are enzymes while others play important role in the transportation of essential metals into blood cells or other organs (Schultz , 1986). When Pb enters the blood stream , it is possible that the metal may bind to these metal-carrier proteins and transported into blood cells. Fe is one of the most important metals present in blood stream, it is an essential precursor for heme synthesis (Lehninger, 1982). Chisolm (1971) reported that Pb can cause iron deficiency which leads to anemia. This study proposes that if transferrin (Tf), Fe-carrier protein , is required for Fe-transport into the red cells, the competitive binding of Pb on Tf molecule will decrease Fe transport.

To prove this statement, choosing the appropriate buffer for studying Pb-binding is the first necessary step. Serum was treated at various pH's with or without Pb at the concentration reported to cause anemia (Goyer, 1991) and determined for Fe residues bound to serum proteins by Ferrozine method (2.2.1.3). The result shown in Table 3

indicates that Fe concentration in serum at various pH's are different , the highest was obtained at pH 4.15 in acetate buffer. The presence of Pb caused a decrease in serum bound Fe at every pH's , with the highest decrease (61 %) was also at pH 4.15. Acetate buffer pH 4.15 was thus chosen for the study of Pb-binding.

Furthermore , when serum was treated with Pb (600 and 1,800 μg/l) , the result in Table 5 indicates that the concentration of Pb bound to protein increased while bound Fe decreased. The higher the concentration of Pb used the lower the amount of Fe remained , this result suggests that Pb and Fe may bind to the same protein(s).

The experiment was also repeated with ICPS instead of F-AAS , the result (in Table 6) suggests similar finding.

3.4 Characterization of Pb-binding Proteins in Serum

3.4.1 Sephadex G-200 Column Chromatography

Since most of the human serum proteins have been well characterized by their molecular weight with Sephadex G-200 column chromatography (Killander, 1964) , this method was thus used to characterize Pb-binding protein in the following series of experiments.

Normal human serum was treated with Pb and directly applied to Sephadex G-200 column, after which the proteins and Pb concentrations were measured in each fraction by

Serum	Metal concentration (μg/l)	
	Fe	Pb
Normal	1503 ± 15	56 ± 17
Pb-treated (600 ug/l)	1112 ± 11	118 ± 24
Pb-treated (1,800 ug/l)	609 ± 188	248 ± 5

Table 5 The relationship between Pb binding and Fe released from serum protein(s), determined by F-AAS

One milliliter of serum was incubated with or without 600 μg/l and 1,800 μg/l Pb in 0.1 M acetate buffer pH 4.15. Free Pb was separated from serum proteins by passing through Sephadex G-25 column (1 x 20 cm) according to 2.2.1.1, protein pool was determined for Pb and Fe concentration by F-AAS as described in 2.2.1.1.

Serum	Metal concentration (μg/l)	
	Fe	Pb
Normal	1365 ± 98	67 ± 9
Pb-treated (1,800 ug/l)	819 ± 56	230 ± 19

Table 6 The relationship between Pb binding and Fe releasing on protein(s) in serum, determined by ICPS

One milliliter of serum was incubated for 30 min with or without 1,800 μg/l of Pb in 0.1 M acetate buffer pH 4.15. Free Pb was separated from serum proteins by passing through Sephadex G-25 column (1 x 20 cm) as shown in 2.2.2.1. Protein pool was determined for Pb and Fe concentration by ICPS (2.2.1.2).

spectrophotometer and ICPS, respectively. Figure 14 shows the chromatographic profile of the Pb-treated serum proteins. The proteins were resolved into 4 major peaks , while Pb was resolved into three. The second Pb peak (II) eluted from the column with the third protein peak suggests the binding of this metal to the protein. The major Pb peak (III) which eluted later with smaller molecular weight protein may be free Pb.

Standard transferrin (20 mg) was applied to the same column , the elution volume of transferrin as indicated by (▼) in Figure 14 , coincides with the Pb peak (II) in Pb-treated Sephadex G-200 profile. This suggests that one of the Pb-binding protein is transferrin.

Figure 15 shows the chromatographic profile when the experiment was repeated, using F-AAS for Pb determination. Although protein profile is still reproduced , Pb profile is not. High background is observed in this profile, which may be due to the high sensitivity of the F-AAS method. However, the Pb peak II in ICPS profile (Figure 14) is still observed.

It was speculated from previous experiment (3.3) that Pb caused a decrease in bound Fe concentration in serum by binding to the same serum protein molecules. To support with this speculation , normal serum was loaded onto Sephadex G-200 and determined for Fe concentration by F-AAS as shown in Figure 16. The protein profile in Figure 16

Figure 14 Sephadex G-200 column chromatography of Pb-treated serum

Two milliliters of serum was incubated with Pb (1,800 μg/l) for 30 min at pH 4.15 and then loaded onto Sephadex G-200 column (1.7 x 85 cm) equilibrated with 0.1 M of acetate buffer pH 4.15. The column was then eluted with about 250 ml of the same buffer at the flow rate of 20 ml/hr. The 2 ml fractions were collected, protein concentration was monitored by the absorbance of 280 nm Pb concentration was determined by ICPS according to 2.2.1.2.

(▼) indicates the eluted position of standard Tf.

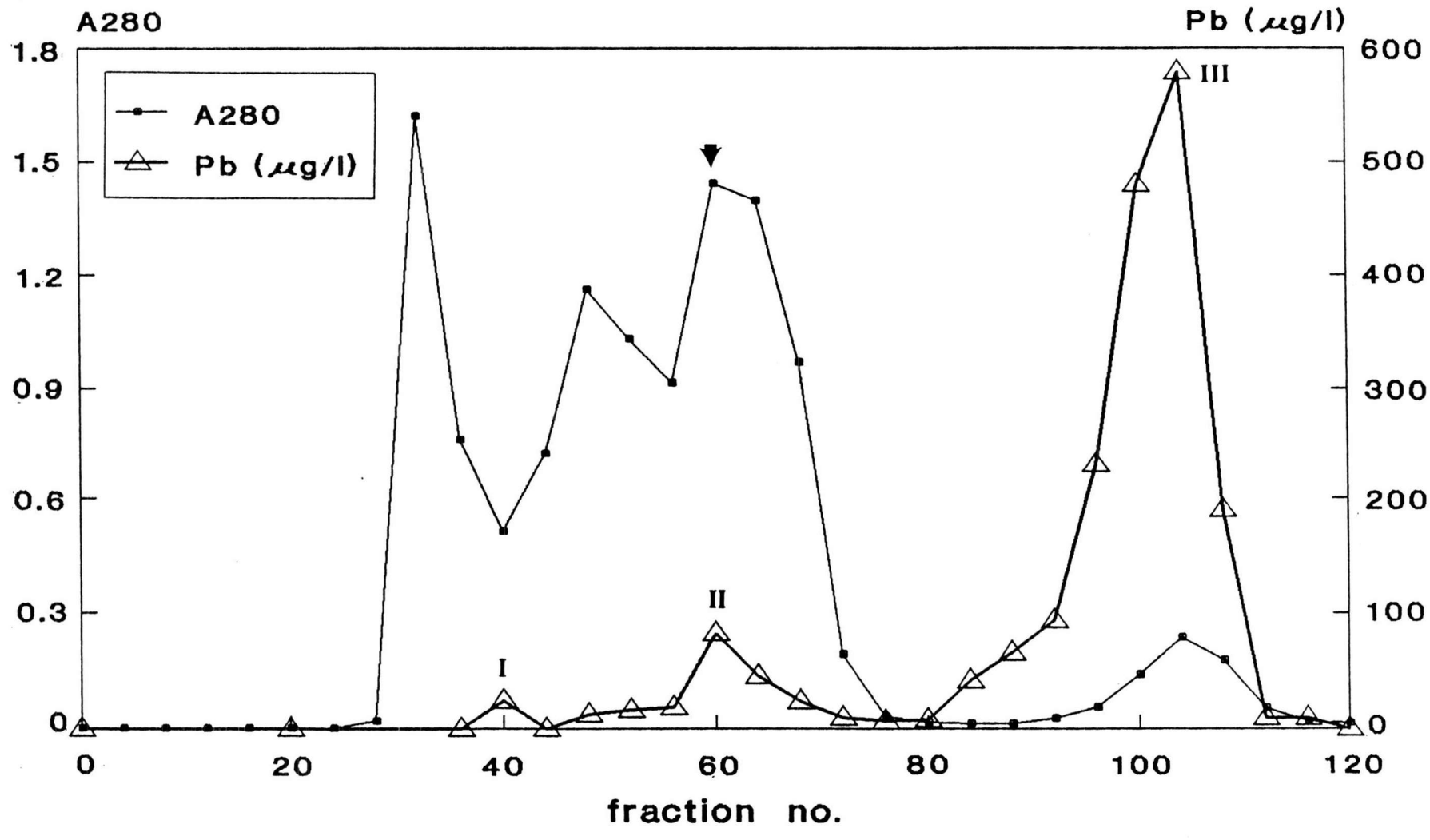

A280
Pb (μg/l)
A280
Pb (μg/l)
1.8
1.5
1.2
0.9
0.6
0.3
0
600
500
400
300
200
100
0
I
II
III
0
20
40
60
80
100
120
fraction no.

Figure 15 Sephadex G-200 column chromatography of Pb-treated serum

Two milliliters of serum was incubated with Pb (1,800 μg/l) at pH 4.15 for 30 min and then loaded onto Sephadex G-200 column (1.8 x 120 cm) equilibrated with 0.01 M acetate buffer pH 4.15. The column was then eluted with about 380 ml of the same buffer while maintaining the flow rate at 20 ml/hr The 2 ml fractions were collected, protein concentration was monitored by the absorbance of 280 nm. Pb concentration was determined by F-AAS according to 2.2.1.1.

(▼) indicates the eluted position of standard Tf.

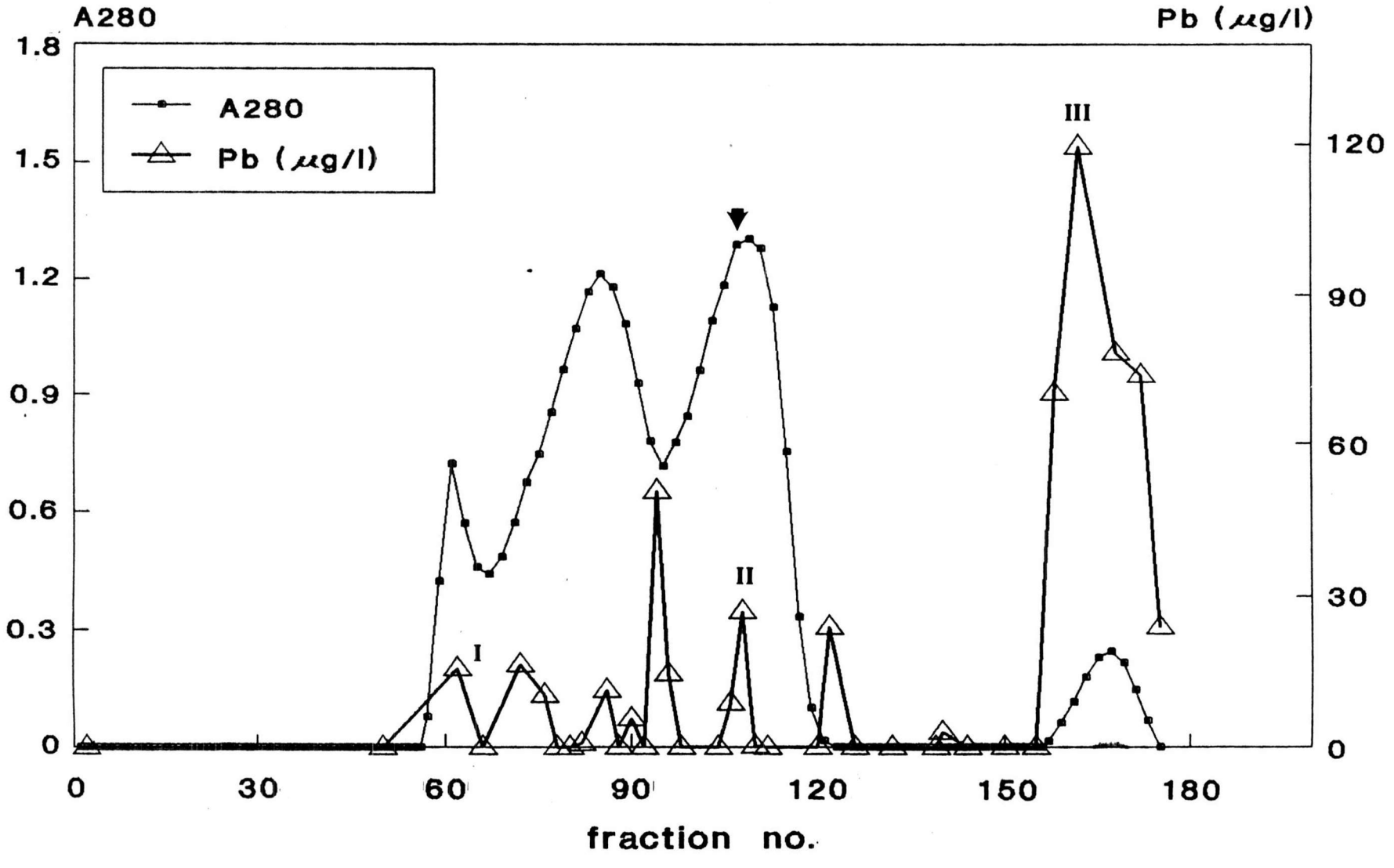
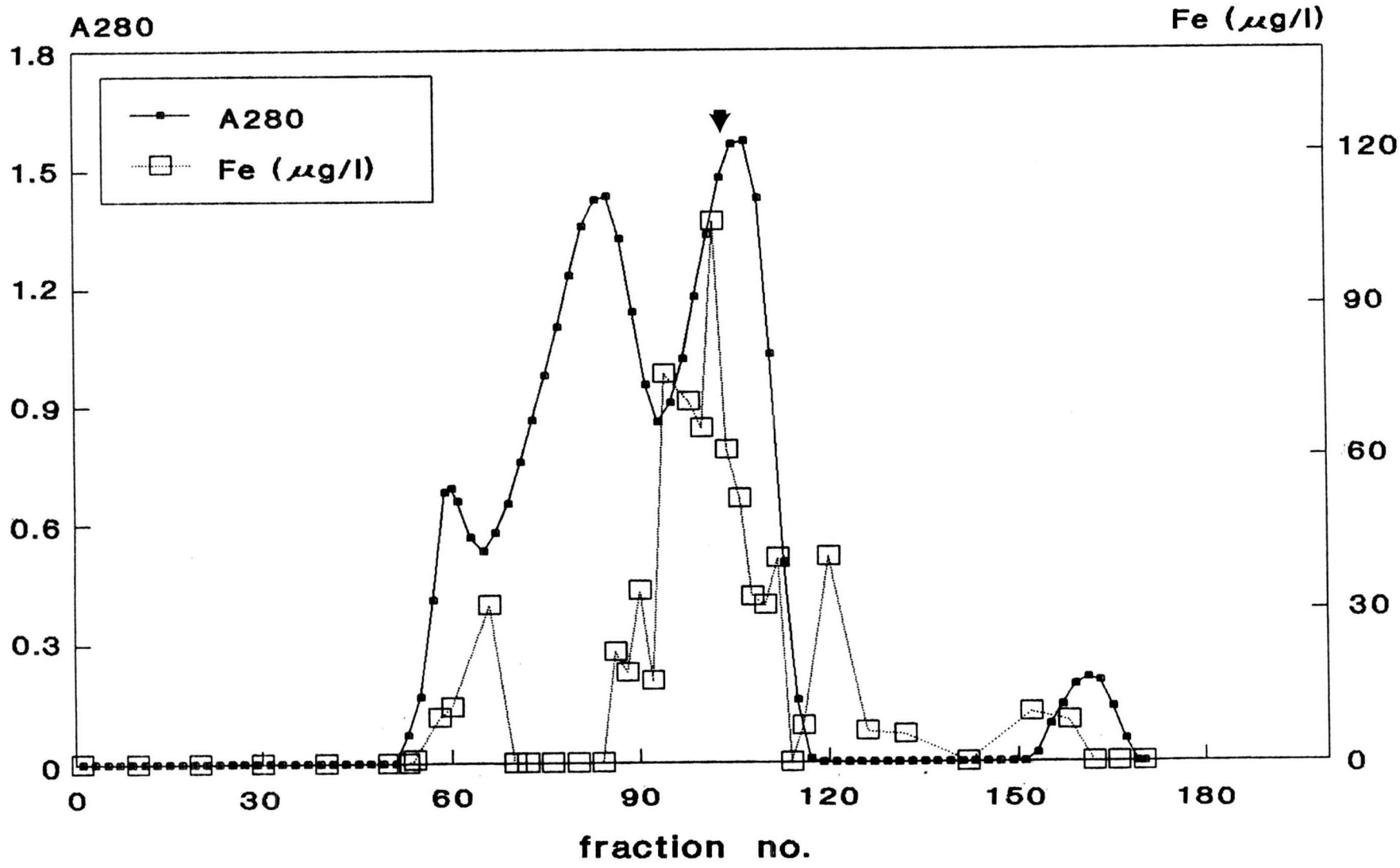

A280
Pb (μg/l)
A280
Pb (μg/l)
1.8
1.5
1.2
0.9
0.6
0.3
0
120
90
60
30
0
I
II
III
0
30
60
90
120
150
180
fraction no.

Figure 16 Sephadex G-200 column chromatography of normal human serum

Two milliliters of serum was loaded onto Sephadex G-200 column (1.8 x 120 cm) equilibrated with 0.01 M acetate buffer pH 4.15. The column was then eluted with about 380 ml of the same buffer while maintaining the flow rate at 20 ml/hr. The 2 ml fractions were collected, protein concentration was monitored by the absorbance of 280 nm. Fe concentration was determined by F-AAS according to 2.2.1.1.

(▼) indicates the eluted position of standard Tf.


A280
Fe (μg/l)
A280
Fe (μg/l)
1.8
1.5
1.2
0.9
0.6
0.3
0
120
90
60
30
0
0
30
60
90
120
150
180
fraction no.

is similar to that of the Pb-treated column and Fe peak appears at the same position as Pb-peak II in Pb-treated profile (Figure 15).

Although every experiments that have been performed lead to the same conclusion that Pb binds to Tf molecule , they were all *in vitro* experiments. To approach the physiological condition , Pb-binding proteins in serum of patient with Pb - toxicity were characterized by Sephadex G-200 chromatography and F-AAS was used to monitor Pb concentration. The result is depicted in Figure 17. Again , the protein and Pb profiles were similar to Pb-treated serum in Figure 14, except the Pb peak II is much lower in patient serum : about 12 μg/l compared to 100 μg/l in Pb-treated column. Pb peak III which was beleived to be free Pb is also smaller in patient serum , suggesting lower free Pb in patient serum. This result confirms that Pb can bind to the transferrin in physiological condition when it enters human blood stream.

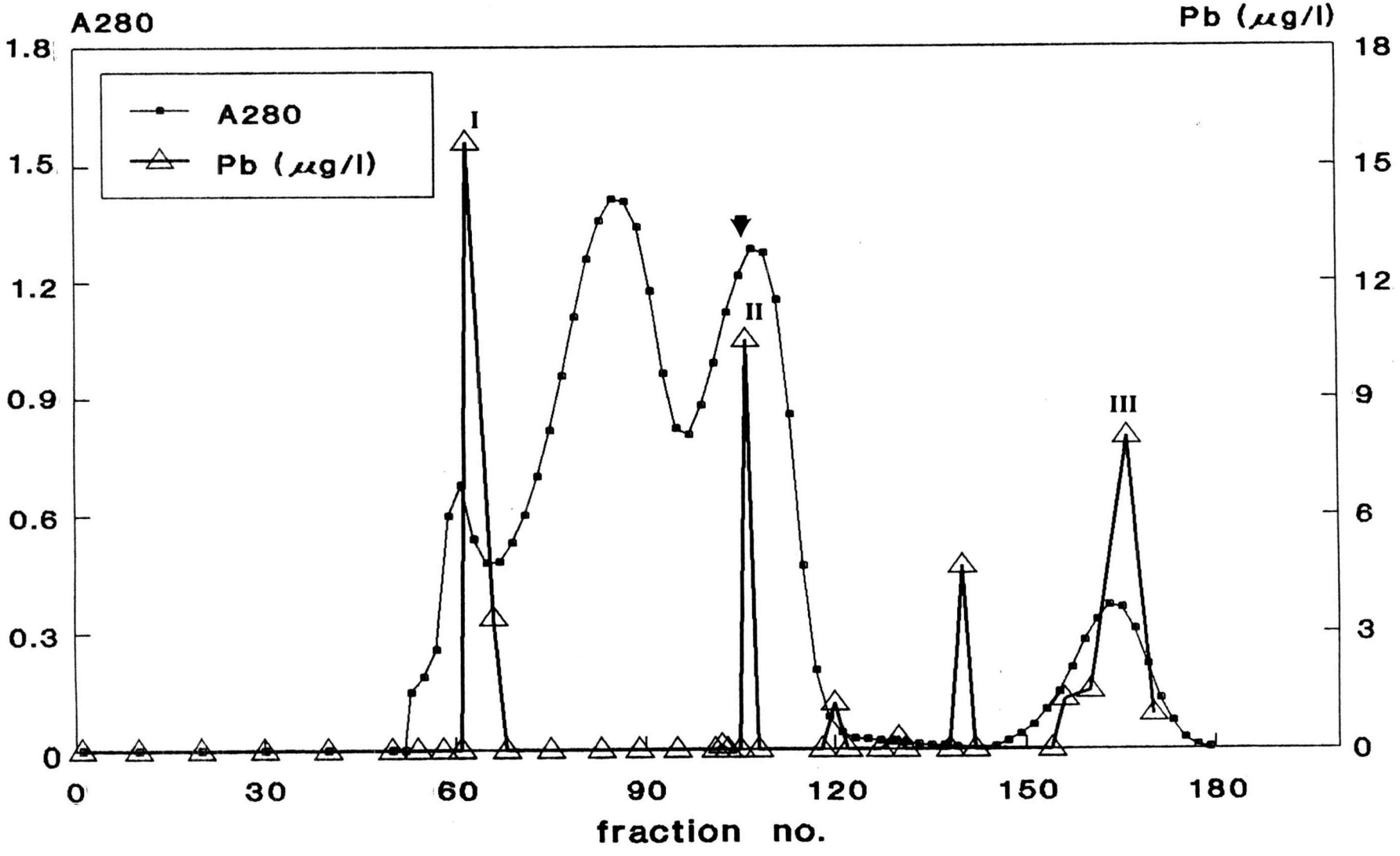
3.4.2 Molecular Weight Determination of Pb-binding Protein

Molecular weight of Pb-binding protein was determined by Sephadex G-200 gel filtration as described in 2.2.2.2 comparing with standard proteins. The result is shown in Figure 18. The molecular weight of Pb-binding protein is calculated to be 90,000 Da which is close to the reported molecular weight of transferrin, the Fe transported

Figure 17 Sephadex G-200 column chromatography of Pb-poisoned patient serum

Two milliliters of patient serum was loaded onto Sephadex G-200 column (1.8 x 120 cm) equilibrated with 0.01 M acetate buffer pH 4.15. The column was then eluted with about 380 ml of the same buffer while maintaining the flow rate at 20 ml/hr. The 2 ml fractions were collected, protein concentration was monitored by the absorbance of 280 nm. Pb concentration was determined by F-AAS according to 2.2.1.1

(▼) indicates the eluted position of standard Tf.


A280
Pb (μg/l)
A280
Pb (μg/l)
I
II
III
1.8
1.5
1.2
0.9
0.6
0.3
0
18
15
12
9
6
3
0
0
30
60
90
120
150
180
fraction no.

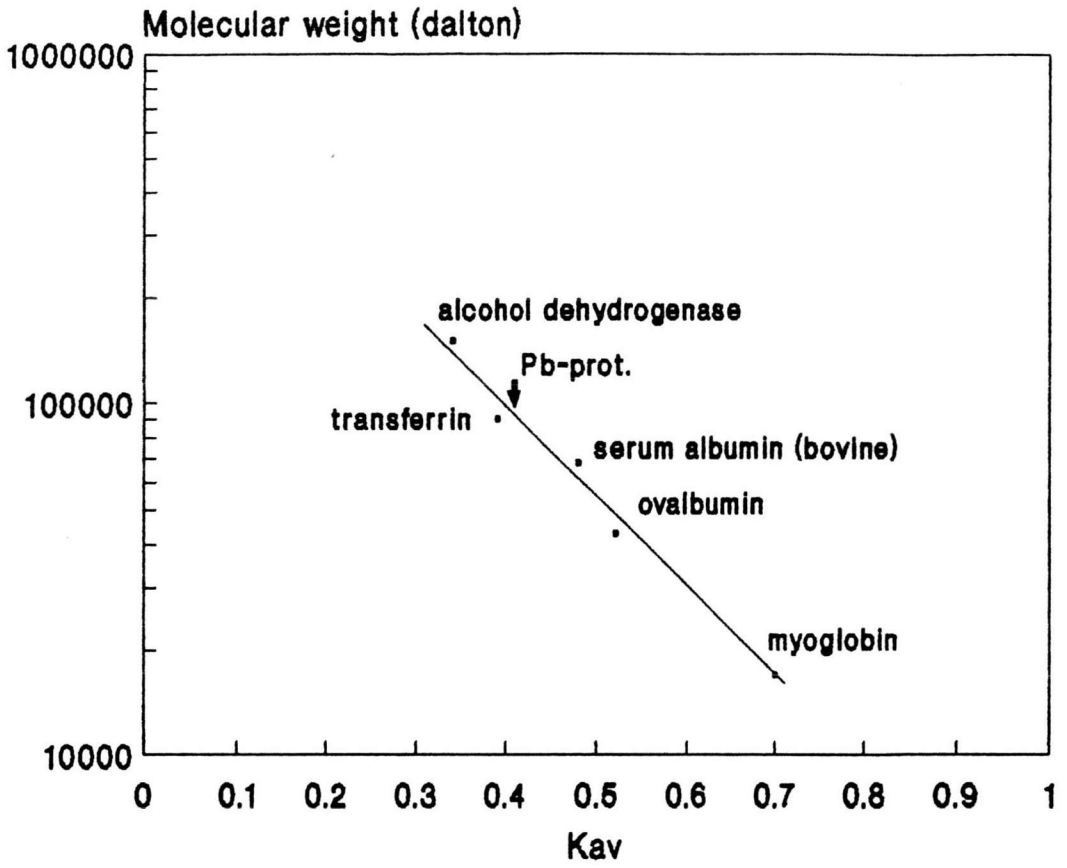


Figure 18 Molecular weight calibration graph for the determination of molecular weight on Sephadex G-200 gel filtration chromatography

Standard proteins were alcohol dehydrogenase (150,000 Da), human transferrin (90,000 Da), bovine serum albumin (68,000 Da), ovalbumin (43,000 Da) and myoglobin (17,000 Da).

protein in human serum (Kichi, 1990).

3.4.3 Polyacrylamide Gel Electrophoresis

Apart from molecular weight , charge has always been another important physical property to characterize proteins. Discontinuous Polyacrylamide Gel Electrophoresis at pH 8.3 (Disc-PAGE) was then used in this study to characterize Pb-binding protein.

Each fraction from Sephadex G-200 column (Pb peak II) was concentrated 10 folds by freeze drying before loading to Disc-PAGE as described in 2.2.3. From the protein pattern in Figure 19 , the upper band in serum fractions (lanes 2-7) can be identified as Tf by comparing to standard Tf (lanes 1 and 8). It shows that the Pb-bound fractions can not be completely separated from other serum proteins. However , fraction no. 60+61 (lane 4) which contains the highest Pb concentration appears to contain the richest amount of Tf. This result again suggests that the Pb-binding protein is transferrin.

To confirm the above result, fractions of Pb peak II in Pb-treated profile (Figure 15) , patient blood profile (Figure 17) and Fe peak in serum profile (Figure 16) were characterized with Disc-PAGE , the results are shown in Figures 20, 22 and 21, respectively. All protein patterns are similar and demonstrate the presence of Tf in Pb-rich fractions.

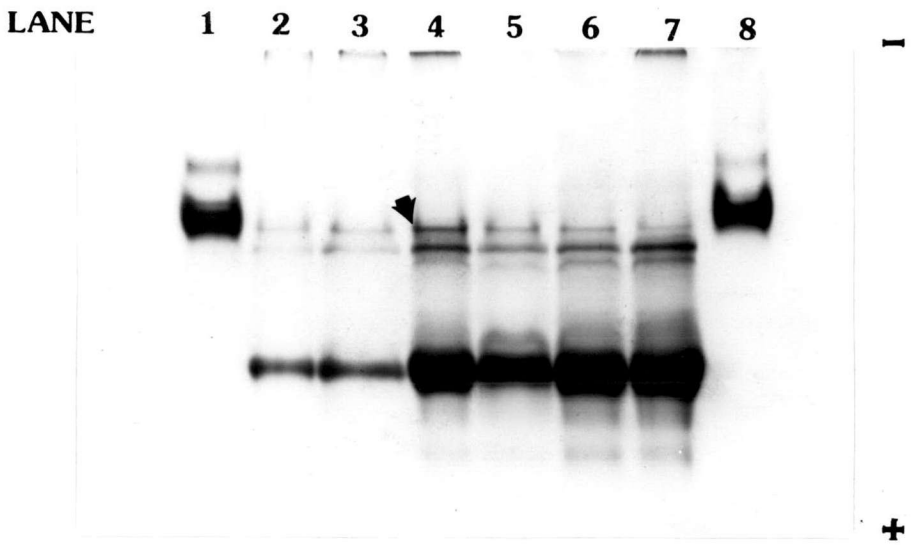


Figure 19 Protein patterns of Disc-PAGE of Pb-bound fraction eluted from Sephadex G-200 column (Figure 14)

Fractions from Pb-peak II from Sephadex G-200 column chromatography of Pb-treated serum was concentrated 10 folds by freeze drying before loading to the gel at equal amount of proteins (20 μg). Disc-PAGE was performed as shown in 2.2.3.

lane 1 transferrin standard
2 fraction no. 56+57
3 fraction no. 58+59
4 fraction no. 60+61
5 fraction no. 62+63
6 fraction no. 64+65
7 fraction no. 66+67
8 transferrin standard

(▼) indicates Tf major band.

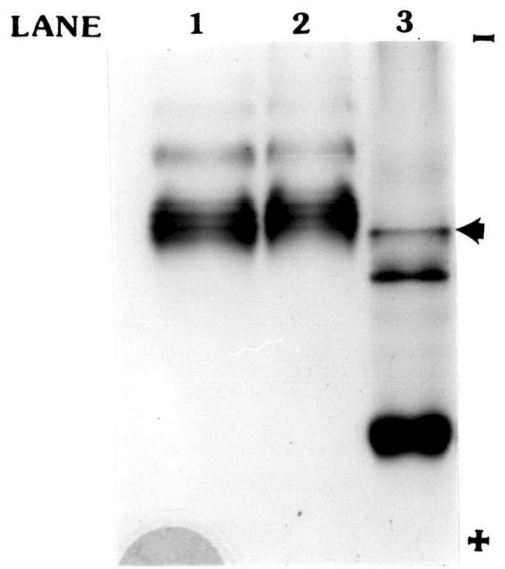


Figure 20 Protein pattern of Disc-PAGE of Pb-bound fraction eluted from Sephadex G-200 column (Figure 15)

Fraction from Pb-peak II from Sephadex G-200 column chromatography of Pb-treated serum was concentrated 10 folds by freeze drying before loading to the gel at equal amount of proteins (20 μg). Disc-PAGE was performed as shown in 2.2.3.

lane 1,2 transferrin standard

3 fraction no. 108 (Pb-peak II) from Sephadex G-200 column

(▼) indicates Tf major band.

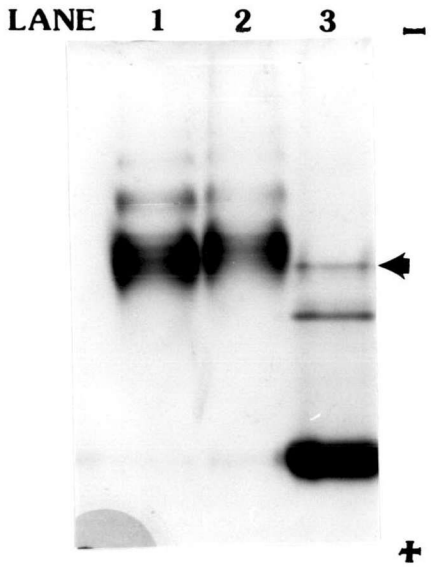


Figure 21 Protein pattern of Disc-PAGE of Fe-bound fraction eluted from Sephadex G-200 column

Fraction from Fe-peak from Sephadex G-200 column chromatography of normal serum was concentrated 10 folds by freeze drying before loading to the gel at equal amount of proteins (20 μg). Disc-PAGE was performed as shown in 2.2.3.

lane 1,2 transferrin standard

3 fraction no. 106 (Fe-peak) from Sephadex G-200 column

(▼) indicates Tf major band.

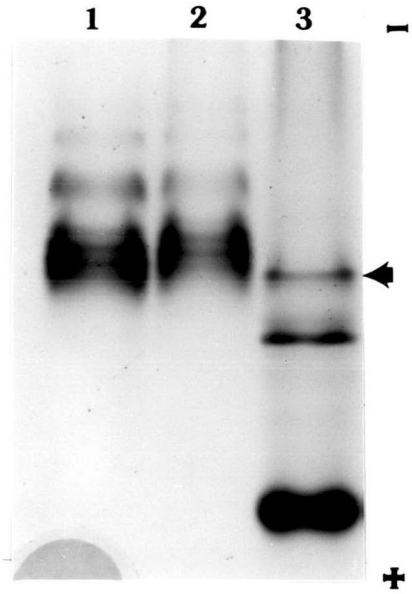


Figure 22 Protein pattern of Disc-PAGE of Pb-bound fraction eluted from Sephadex G-200 column (Figure 17)

Fraction from Pb-peak II from Sephadex G-200 column chromatography of Pb-poisoned patient serum was concentrated 10 folds by freeze drying before loading to the gel at equal amount of proteins (20 μg). Disc-PAGE was performed as shown in 2.2.3.

lane 1,2 transferrin standard

3 fraction no. 108 (Pb-peak II) from Sephadex G-200 column

(▼) indicates Tf major band.

3.5 Binding of Pb to Standard Transferrin (Tf)

3.5.1 Sephadex G-25 Column Chromatography

From previous experiments, serum was a source used for studying Pb-binding proteins. Those results indicate that Tf is one of the serum proteins which can bind to Pb. However, the system mentioned is not pure but highly contaminated with other serum proteins. To confirm the above results, purified Tf was used.

Standard Tf was treated with or without Fe and Pb , after which the free metals were removed by Sephadex G-25 column. The amount of protein and bound Pb were measured as presented in Table 7. It is shown that normal Tf is not saturated with Fe. The Fe:Tf mole ratio in normal sample is 0.77:1 , which is increased to 1.73:1 with a saturated amount of Fe , this ratio closes to that reported previously by Righetti (1990). When Tf saturated by Fe (no. 2) was treated with Pb in the concentration which can cause anemia (no. 4), the mole ratio decreases from 1.73:1 to 0.56:1 which is close to Pb-treated Tf (no. 3). Furthermore, the Pb:Tf mole ratios in both samples (no. 3 and 4) are equal and very low (0.03:1). The value is much lower than Fe:Tf ratio. Eventhough the Pb:Tf ratio cannot be calculated conclusively with low concentration of Pb utilized, this result suggests that Fe bound on Tf is not replaced by Pb at 1:1 ratio.

Table 7 The binding of Pb on Tf

Molecular weight of Tf = 90,000 Da

Atomic weight of Pb = 207.2 Da

Atomic weight of Fe = 55.9 Da

Three milliliters of standard transferrin solution (4 g/l) were divided into 3 fractions ; no. 1 was kept as control, no. 2 was treated with 0.1 ml of ferric ammonium sulfate (0.5 g/l) and no. 3 was treated with 1,800 µg/l Pb in 0.01 M acetate buffer pH 4.15 for 30 min at 37 °C. These fractions were separately applied to Sephadex G-25 (2.2.2.1). The protein eluted was concentrated with Freeze Dry System and determined for Fe and Pb concentration with F-AAS (2.2.1.1). A portion of concentrated fraction no. 2 was also treated with 1,800 µg/l Pb, loaded to Sephadex G-200 and measured for metal concentrations (2.2.1.1) and depicted as fraction no. 4.

Sample		Metal concentration					
		Fe			Pb		
no	description	μg/l	μM	mole ratio (Fe:Tf)	μg/l	μM	mole ratio (Pb:Tf)
1	Tf (control)	1907±194	34.11±3.47	0.77:1	29± 6	0.14±0.03	-
2	Tf+Fe	4292±353	76.78±6.32	1.73:1	21± 4	0.10±0.02	-
3	Tf+Pb	1249±206	22.34±3.69	0.53:1	321±45	1.51±1.30	0.03:1
4	Tf+Fe+Pb	1458±122	26.08±2.18	0.56:1	313±58	1.55±0.22	0.03:1

3.5.2 Isoelectric Focusing Polyacrylamide Gel Electrophoresis (IEF-PAGE)

Righetti (1990) reported that Fe can shift the mobility of Tf major band to more anodal side in IEF-PAGE. It is proposed in this study that if Pb and Fe bind to Tf in the same manner and with the same amount , the mobilities of Tf shifted by Pb or Fe will be the same. Tf was thus treated with Pb or Fe and applied to IEF-PAGE (pH 5-7). Their mobilities were compared to non-treated sample as shown in Figure 23. Three major bands of Tf are observed in non-treated sample (lane 1), with band II as the major band. The result corresponds well with that reported by Righetti (1990), stating that band I is Fe-free Tf, band II is monoferric Tf (Fe-Tf) and band III is Tf saturated with Fe (2Fe-Tf). Fe-saturated Tf (lane 2) in this experiment confirms Righetti's result with shifting of major band from II to III. The most striking observation is the similar shifting of Tf band in Pb-treated sample (lane 3). This result suggests that both Fe and Pb bindings to Tf molecule cause the change in net charge , resulting in mobility shift of Tf band. It further suggests that the net charge of Fe-Tf and Pb-Tf are close to each other or identical. This implies that it is very likely that the same amount of Pb or Fe is present on Tf molecule. However, this result does not correspond to F-AAS result (3.5.1) which shows that 1 mole of Pb can replace about 36 moles of Fe on Tf molecule.

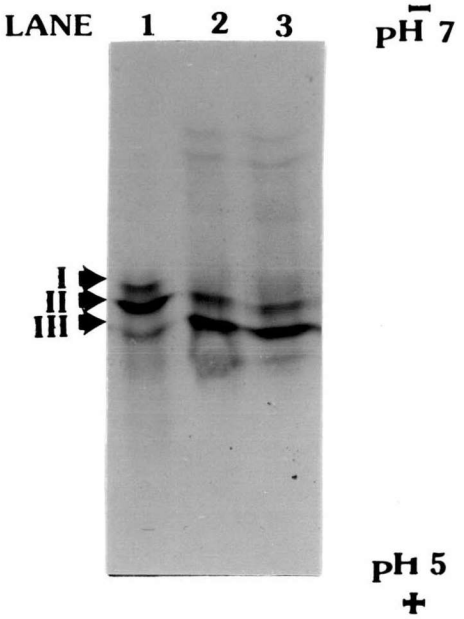


Figure 23 Effect of Pb and Fe on IEF-pattern of Tf

Ten microliters of 5 mg/ml standard Tf was incubated with and without 2 μl of 0.5 mg/ml ferric ammonium sulfate and 1.8 mg/ml Pb acetate. It was then applied to IEF-PAGE according to 2.2.4.

lane 1 standard Tf

2 standard Tf treated with Fe

3 standard Tf treated with Pb

(▼) indicates Tf major band.

3.6 Effect of Some Chelators on Protein-bound Pb

Pb is a heavy metal, it can be removed by some chelators such as EDTA, Dimercaprol and D-penicillamine (Chisolm, 1971). Chisolm (1971) reported that the anemia caused by lead poisoning is a reversible process, it can return to normal by the removal of Pb, using drug containing chelating agent.

In this experiment , EDTA , Dimercaprol and D-penicillamine were used for the removal of Pb from protein molecules. The result is presented in Table 8. $CaNa_2EDTA$, Dimercaprol and D-penicillamine reduces the concentration of Pb bound in serum protein by 88, 96 and 100 %, respectively.

Chelators	Pb concentration	
	μg/l	%
None	139 ± 17	100.00
$CaNa_2$ EDTA	16 ± 1	11.51
Dimercaprol	6 ± 2	4.32
D-Penicillamine	0	0

Table 8 Effect of some chelators on protein-bound Pb

One milliliter of serum was incubated with Pb (1,800 μg/l) in 0.01 M acetate buffer pH 4.15 at 37°C for 30 min, each was subjected to 5 μl of different chelators , 100 mg/l of $CaNa_2$EDTA, 70 mg/l of Dimercaprol and 1,000 mg/l of D-Penicillamine, at 37°C for 30 min. Each was then passed through Sephadex G-25 column and protein pool was determined for Pb concentration by F-AAS as described in 2.2.1.1.