



The Association of Heavy Metal of Blood and Serum in the Alzheimer's Diseases

Ji-Yeon Lee^{1,2}, Jae-Hoon Kim¹, Dal-Woong Choi², Dong-Woo Lee³, Jun-Hyun Park³,
Hye-Jung Yoon⁴, Hee-Soo Pyo⁵, Ho-Jang Kwon⁶ and Kyung-Su Park¹

¹Advanced Analysis Center, Korea Institute of Science and Technology, Seoul 136-791

²Department of Environmental Health, College of Health Sciences, Korea University, Seoul 136-703

³Department of Psychiatry, Sanggye Paik Hospital, College of Medicine, INJE University, Seoul 139-707

⁴National Institute of Food And Drug Safety Evaluation, Cheongwon 363-700

⁵Molecular Recognition Research Center, Korea Institute of Science and Technology, Seoul 136-791

⁶Department of Preventive Medicine, Dankook University College of Medicine, Cheonan 330-714, Korea

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This study has attempted to establish an analysis method through validation against heavy metals in the body (Pb, Cd and Hg) using ICP-MS and Gold amalgamation and find out the relevance between heavy metal and Alzheimer's disease after analyzing the distribution of heavy metal concentration (Pb, Cd and Hg) and correlations between a control group and Alzheimer's disease group. In this study, Pb and Cd levels in the blood and serum were validation using ICP-MS. For analysis of Hg levels in the blood and serum, the gold amalgamation-based 'Direct Mercury Analyzer' has been used. According to an analysis on the heavy metal concentration (Pb, Cd and Hg concentration) in the blood, Cd concentration was high in the Alzheimer's disease group. In the serum, on the contrary, Pb and Hg were high in the Alzheimer's disease group. For analysis of correlations between heavy metal levels in the blood and serum and Alzheimer's disease, t-test has been performed. Even though correlations were observed between the blood lead levels and Alzheimer's disease, they were statistically insignificant because the concentration was higher in a control group. No significance was found in Cd and Hg. In the serum, on the other hand, no statistical significance was found between the heavy metal (Pb, Cd and Hg) and Alzheimer's disease. In this study, no statistical significance was observed between heavy metal and decrease in cognitive intelligence. However, it appears that a further study needs to be performed because the results of the conventional studies were inconsistent.

Key words: Alzheimer's diseases, Heavy metal, Association, Biomonitoring

INTRODUCTION

With industrial advancement, a demand for the measurement of environmental pollution-related hazardous substances to the body has been on the rise. It's been known that environmental pollutants such as heavy metal cause a problem in immune system functions or in various physiological functions. They can also disease susceptibility including cancer. Because people can be exposed to heavy metals in diverse routes, it is desirable to estimate the exact exposure levels and evaluate risk using biological indexes

in order to figure out the exposure to heavy metals (Kim, 2002; Lee *et al.*, 2004; NCCLS, 1997; Moyer, 1999; AACC, 1996; Haddad *et al.*, 1998; Ash and Komaromy-Hiller, 1997; Shaw *et al.*, 2001).

With the aging of population, Alzheimer's disease prevalence has dramatically increased by age. Specifically, it stayed at 7~10% among those aged 65~74, 18~20% among the aged 75~84 and 35~40% among those aged 85 or older (Rubin *et al.*, 2001). It appears that the number of the patients with Alzheimer's disease would further increase.

In Korea, about 8.4% of the aged population (65 or older) is suffering from Alzheimer's disease. According to a study on the number of the patients with Alzheimer's disease in urban and rural areas, it is forecasted that it would increase up to 730,000 in 2020 and 1.95 million in 2050. The heavy metal poisoning was included in 'non-occupational sentinel

Correspondence to: Kyung Su Park, Korea Institute of Science and Technology, Hwarangno 14-gil 5, Seongbuk-gu, Seoul 136-791, Korea
E-mail: pks6475@kist.re.kr

and environmental diseases' designated by the US Center for Disease Control and Prevention to achieve the national health goal for year 2000 (US CDC, 2000). The US National Institute of Environmental Health Sciences (NIEHS) also mentioned lead poisoning and mercury poisoning as leading heavy metal-related environmental diseases (US NIEHS, 2004). In Korea, the Ministry of Environment pointed out Pb, Hg and Cd as hazardous materials.

In foreign countries, correlations between heavy metal and Alzheimer's disease have already been discussed and analyzed. In Korea, however, it's been hard to find a study related to the said topic. Instead, there have been studies on evaluation of the exposure to heavy metal in the blood or urine. According to foreign references, there has been no consistency in correlations between heavy metal and Alzheimer's disease.

In terms of a trace metal analysis method on biological specimens, Graphite Furnace Atomic Absorption Spectrometry (GFAAS) has been used for a long time. However, this method is partially limited due to high LOD, long analysis time, poor reproducibility and difficult operations. Therefore, Inductively Coupled Plasma/Mass Spectrometry (ICP/MS) through which multiple items can be analyzed at the same time with low LOD has become more popular as an alternative of the GFAAS. Recently, there have been a lot of studies on the analysis of blood and biological indexes using ICP/MS. Since 2000, ICP/MS has been used as a monitoring analysis method in the U.S. and Europe (NCCLS, 1997; Moyer, 1999; AACC, 1996; Haddad *et al.*, 1998; Ash and Komaromy-Hiller, 1997).

Direct Mercury Analyzer (DMA), one of the mercury analysis methods, is the apparatus to measure mercury in liquid (or solid) specimen in a fast, precise and convenient way. Under this method, mercury can be analyzed without sample preparation (acid digestion). Therefore, the time for sample preparation can be saved. The mercury which is oxidized in the solid or soluble sample by the heat which is precisely adjusted in the furnace is thermally and chemically degraded after drying process (USEPA, 2007).

This study has attempted to establish an analysis method after conducting a validation on heavy metals (Pb, Cd and Hg) in the blood and serum of the patients with Alzheimer's disease using ICP-MS and Gold amalgamation and figure out correlations between heavy metal and Alzheimer's disease after analyzing the distribution of heavy metal (Pb, Cd and Hg) concentration and the correlations between a control group and Alzheimer's disease group in Korea.

MATERIALS AND METHODS

Sampling. For this study, blood and serum were sampled from a control group (130 people) and Alzheimer's disease group (80 patients) in a hospital in Seoul. Because the general public in which concentration would be low are included in the samples, in particular, the effect of pollution

which can occur during sampling and inspection on the results of analysis is relatively more important. To minimize the possibility of this kind of pollution, therefore, blood was sampled using disposable stainless steel needles after disinfecting the part in which blood was collected with 70% isopropyl alcohol. Then, the blood was sampled in Serum Separation Tube (SST, 5 ml each) for the analysis of serum. The Heparin tube was vertically stirred 4 to 6 times to make anticoagulant well mixed with blood. The serum was centrifuged at 2500 rpm for five (5) minutes, and the collected samples were kept at -70°C until they were used in the test. Right before the analysis, they were stored at -24°C and then at 4°C . After that, the final test was performed.

Reagents. The standard solution of each target material (Pb and Cd) used in this study is Perkin-Elmer (New York, USA)'s multi-element calibration standard 3 (10 mg/kg). In case of the standard solution of Hg, Kinto Chemical (Tokyo, Japan)'s solution (1,000 mg/l) was used.

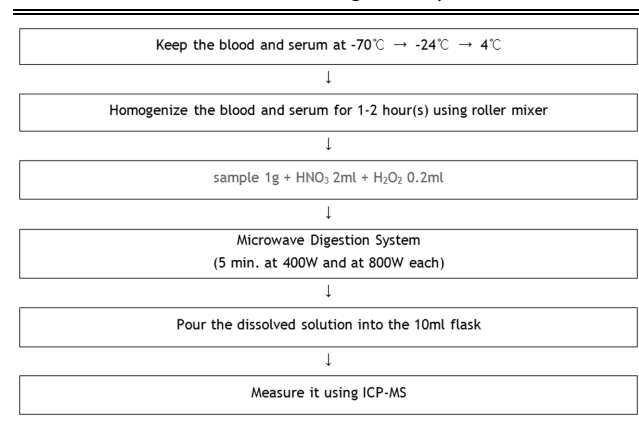
In terms of distilled water used in sample preparation and analysis, the triple distilled water of Mili-Q system (Mili-pore, MA; USA) was used. For nitric acid (67% v/v) and hydrogen peroxide (31% v/v) which would be used in the Microwave Digestion System, DONGWOO FINE-CHEM's semiconductor level was used.

For the accurate validation on the analysis method, a certified reference material (Lyphochel[®] Whole Blood Metals Control Level 1 manufactured by Biorad (California, USA) was used.

Sample preparation. The blood and serum of a control group and Alzheimer's disease group were kept at -70°C . Right before the analysis, they were moved and stored at -24°C and later at 4°C . Before the test, a roller mixer was used for 1~2 hour(s) for homogenization and prevention of coagulation.

To analyze Pb and Cd in the homogenized blood and serum with ICP-MS, 1 ml of specimen was put into a

Table 1. Scheme of microwave digestion system and ICP-MS



Teflon vessel using the microwave digestion system. Then, the lid was closed after adding 2 ml of nitric acid and 0.2 ml of hydrogen peroxide. Then, after inserting the vessel into the safety shield, it was dissolved at 400 W for five (5) minutes and at 800 W for another five minutes (Table 1).

Determination. As a microwave digestion system used for pre-treatment of Pb and Cd in this study, the system

Table 2. Instrumental parameters for ICP-MS

Descriptions	Conditions
R.F. generator	Free-running type, 40 MHz
R.F. power	1500 W
Plasma gas flow rate	18.0 l/min.
Auxiliary gas flow rate	1.50 l/min.
Nebulizer gas flow rate	1.00 l/min.
Lens Voltage	8.5 V
Nebulizer	Concentric type
Spray chamber	Cychronic type
Torch	Demountable
Interface cones	Platinum
Quadrupole chamber	1×10^{-6} torr
Dwell time	600 ms
Pb/Mass	208, 206
Cd/Mass	114, 112

Table 3. Instrumental parameters for Mercury Analyzer (Gold amalgamation)0

Descriptions	Conditions
Wavelength	253.65 nm
Interface filter	254 nm
Detector	Silicon UV photodetector
Catalyst	Platinum
Drying	Blood 300 °C, 120 sec
	Serum 300 °C, 60 sec
Decomposition	Blood 850 °C, 280 sec
	Serum 850 °C, 180 sec
Purge	60 sec
Amalgam record	12 sec
	30 sec

manufactured by Milestone (Soriso, Italy) has been used. Regarding Inductively Coupled Plasma - Mass Spectrometry (ICP-MS), the system manufactured by Perkin-Elmer (New York, USA) was used for measurement. Regarding the gold-Amalgamated Direct Mercury Analyzer (DMA)-80, the system manufactured by Milestone (Soriso, Italy) was used for analysis of Hg. The analysis conditions for each system were stated in the tables below (Tables 2, 3).

Method validation. In this study, validation was performed through of analysis equipment between ICP-MS for analysis of blood and serum. For method validation, validation parameters were selected, and a validation test was conducted. The validation parameters include linearity, sensitivity, selectivity, precision, accuracy and Limit Of Detection (LOD) & Limit Of Quantification (LOQ). If there is a possibility of matrix effect, the difference of slope between two calibration curves by adding standard reagent. In this study, the analysis method was validated using spiked sample and certified reference materials (KATS No.2008-242).

The LOD and LOQ were calculated using the sigma method (LOD: 3σ , LOQ: 6σ) (BS EN 15673, 2009). The LOD and LOQ which considered dilution factor were calculated as follows; MDL = dilution factor \times LOD/MQL = dilution factor \times LOQ.

RESULTS

Validation of analysis methods of blood lead and cadmium. In this study, validation by analysis method of blood lead and cadmium by ICP-MS.

In terms of the validation of blood lead level analysis, first of all, MDL was 0.55 $\mu\text{g}/\text{d}$ while MQL was 1.10 $\mu\text{g}/\text{d}$ in ICP-MS. In terms of calibration curve, good linearity was observed (0.9993). Precision which was calculated through %RSD was 1.23% while recovery was 90.7%.

In terms of validation of blood cadmium analysis, MDL and MQL were 0.07 $\mu\text{g}/\text{kg}$ and 0.13 $\mu\text{g}/\text{kg}$ respectively in ICP-MS. In calibration curve, good linearity was observed (0.9998). Precision which was calculated through %RSD

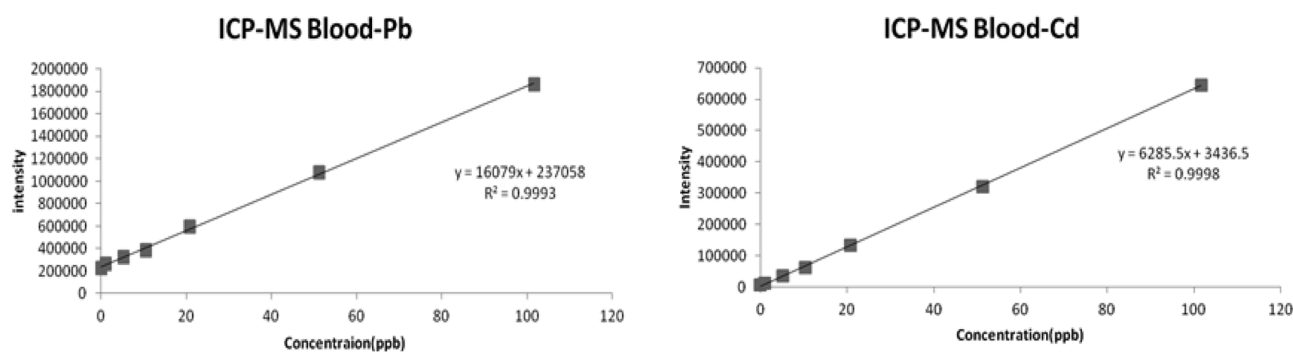


Fig. 1. Calibration curve of Pb and Cd in Blood by ICP-MS.

Table 4. Method validation of Pb and Cd in blood by ICP-MS

Blood	Pb	Cd
MDL	0.55 (µg/dl)	0.07 (µg/kg)
MQL	1.10 (µg/dl)	0.13 (µg/kg)
Linear equation	$y = 16079x + 237058$	$y = 6285.5x + 3436.5$
R ²	0.9993	0.9998
Precision (%)	1.23	1.32
Recovery (%)	90.7	83.8

Table 5. Method validation of Pb and Cd in serum by ICP-MS

Serum	Pb	Cd
MDL	0.10 (µg/dl)	0.07 (µg/kg)
MQL	0.21 (µg/dl)	0.14 (µg/kg)
Linear equation	$y = 16159x + 42293$	$y = 6089.5x + 231.7$
R ²	0.9975	0.9998
Precision (%)	1.82	2.40
Recovery (%)	90.7	87.3

was 1.32% while recovery was 83.8% (Fig. 1. and Table 4).

Validation of analysis methods of serum lead and cadmium.

First of all, in terms of the validation of serum lead level analysis, MDL was 0.10 µg/dl while MQL was 0.21 µg/dl in ICP-MS. In terms of calibration curve, good linearity was observed (0.9975). Precision which was calculated through %RSD was 1.82% while recovery was 90.7%.

In terms of validation of serum cadmium analysis, MDL and MQL were 0.07 µg/kg and 0.14 µg/kg respectively in ICP-MS. In calibration curve, good linearity was observed (0.9998). Precision which was calculated through %RSD was 2.40% while recovery was 87.3% (Fig. 2. and Table 5).

Validation of analysis methods of blood and serum mercury.

For validation of the analysis method on mercury levels in the blood and serum, Gold amalgamation has been used. Because this method does not require pre-treatment process, it can reduce analyzing time and eliminate the possibility of pollution or loss caused by the pre-treatment.

In blood, LOD and LOQ were 0.62 ng/g and 1.24 ng/g

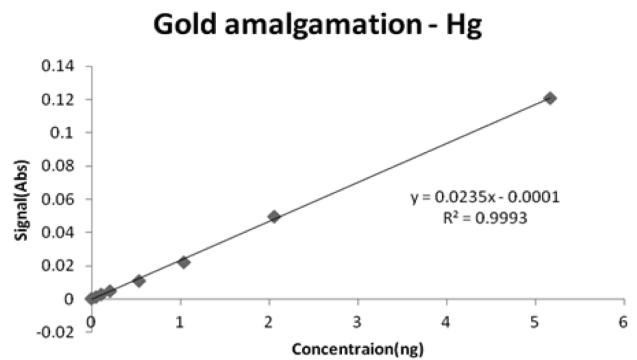


Fig. 3. Calibration curve of Hg by gold amalgamation.

Table 6. Method Validation of Hg in Blood and Serum by Gold amalgamation

Hg	Gold amalgamation	
	Blood	Serum
LOD (µg/kg)	0.62	0.22
LOQ (µg/kg)	1.24	0.44
Linear equation	$y = 0.0235x - 0.0001$	
R ²	0.9993	
Precision (%)	3.82	6.84
Recovery (%)	82.8	-

respectively. Good linearity was observed with 0.9993. Precision which was calculated through %RSD was 3.82% while recovery was 82.8%. In serum, LOD and LOQ were 0.22 ng/g and 0.44 ng/g respectively. Good linearity was observed with 0.9993. Precision which was calculated through %RSD was 6.84%. As a result, validation has been verified (Fig. 3 and Table 6).

Results of the analysis of Certified Reference Material (CRM).

Using the certified ICP-MS and Gold amalgamation, the certified reference material (CRM) was analyzed. In terms of CRM, Biorad (California, USA)'s Lyphochel Whole Blood Metals Control Level 1 was used.

According to the analysis on the CRM, lead, cadmium and mercury were 9.01 µg/dl, 4.96 µg/kg and 5.67 ng/g

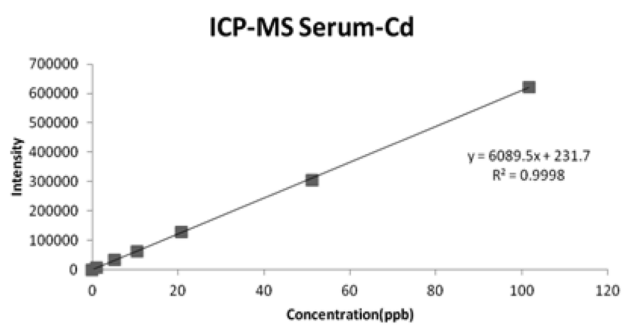
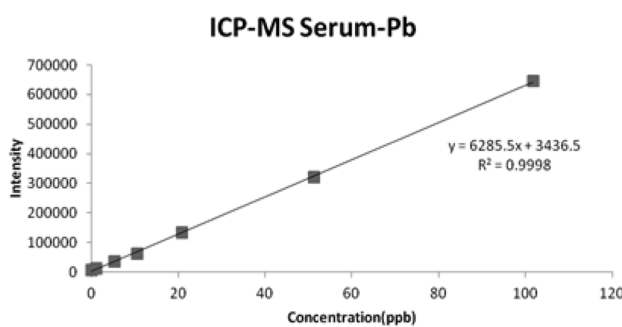


Fig. 2. Calibration curve of Pb and Cd in serum by ICP-MS.

Table 7. Result of certified reference material in blood

	Pb ($\mu\text{g}/\text{dl}$)	Cd ($\mu\text{g}/\text{kg}$)	Hg ($\mu\text{g}/\text{kg}$)
Institute	Biorad whole blood metals control Level-1		
Result	9.01	4.96	5.67
Reference value	9.58 ± 1.91	5.13 ± 1.02	6.85 ± 1.37

respectively. All materials were within the scope of the reference value (Table 7).

Analysis of heavy metal levels in the blood and serum among the patients with Alzheimer's diseases. Using the certified ICP-MS and Gold amalgamation, the blood and serum lead, cadmium and mercury levels among elderly people were analyzed.

The exposure to heavy metal was investigated against a control group (130 people) and Alzheimer's disease group (80 patients). According to the investigation, a control group was $2.19 \mu\text{g}/\text{dl}$ while Alzheimer's disease group was $1.90 \mu\text{g}/\text{dl}$ in terms of the blood lead levels. In terms of blood cadmium levels, a control group and Alzheimer's disease group were $1.06 \mu\text{g}/\text{kg}$ and $1.12 \mu\text{g}/\text{kg}$ respectively. In terms of blood mercury levels, on the contrary, a control group and Alzheimer's disease group were $3.23 \mu\text{g}/\text{g}$ and $2.93 \mu\text{g}/\text{g}$ respectively.

In terms of serum lead levels, a control group was $0.17 \mu\text{g}/\text{dl}$ while Alzheimer's disease group was $0.20 \mu\text{g}/\text{dl}$. In terms of serum cadmium levels, both control group and Alzheimer's disease group were $0.07 \mu\text{g}/\text{kg}$ (LOD) or below. In terms of serum mercury levels, a control group and Alzheimer's disease group were $1.43 \mu\text{g}/\text{g}$ and $1.45 \mu\text{g}/\text{g}$ respectively.

In terms of blood lead and mercury levels, a control group was higher than Alzheimer's disease group. In terms of blood cadmium levels, on the contrary, Alzheimer's disease group was higher than a control group. In case of serum, however, serum cadmium levels were LOD or below. In terms of serum lead and mercury levels, Alzheimer's disease group was higher than a control group (Tables 8, 9).

Analysis of correlations between heavy metal and Alzheimer's disease.

Based on an analysis on the exposure to heavy metal against a control group (130 people) and Alzheimer's disease group (80 patients), the correlations between heavy metal and Alzheimer's disease were investigated. To analyze the correlations, t-test was performed with the hypothesis that if the p-value was ≤ 0.05 , the sample results were deemed statistically significant.

According to the analysis of correlations between blood heavy metal levels and Alzheimer's disease, lead, cadmium and mercury were 0.035, 0.43 and 0.27 respectively in terms of p-value. In case of serum, lead, cadmium and mercury were 0.45, 0.24 and 0.9 respectively in terms of p-value.

To analyze correlations between blood and serum heavy metal levels and Alzheimer's disease, t-test was performed. Even though correlations were observed between the blood lead levels and Alzheimer's disease (p-value = 0.035), they were not statistically significant because a control group was higher than Alzheimer's group in terms of concentration levels. No statistical significance was found in both cadmium and mercury. In case of serum, no statistical significance was observed between heavy metals (lead, cadmium and mercury) and Alzheimer's disease (Table 10).

Table 8. Comparison of blood heavy metal level between normal group and Alzheimer disease group

	Blood	Average	Geomean	Stedv	Median	95 percentile	Min	Max	Count (ea)
Pb*	Control group	2.19	1.99	0.94	1.99	3.96	0.48	4.96	130
	Patient group	1.90	1.68	0.97	1.79	3.73	0.38	5.92	80
Cd**	Control group	1.06	0.98	0.46	0.97	1.99	0.32	2.59	130
	Patient group	1.12	0.98	0.58	1.06	2.15	0.12	3.61	80
Hg**	Control group	3.23	2.79	2.10	2.70	6.94	0.85	13.8	130
	Patient group	2.93	2.55	1.79	2.46	6.73	0.88	9.95	80

* $\mu\text{g}/\text{dl}$, ** $\mu\text{g}/\text{kg}$, control group: the normal aged, patient group: the aged with Alzheimer's disease.

Table 9. Comparison of serum heavy metal level between normal group and Alzheimer disease group

	Serum	Average	Geomean	Stedv	Median	95 percentile	Min	Max	Count (ea)
Pb*	Control group	0.17	0.14	0.12	0.13	0.39	0.041	0.69	130
	Patient group	0.20	0.14	0.27	0.13	0.54	0.036	2.12	80
Cd**	Control group	< ND	-	< ND	< ND	< ND	0	0.13	130
	Patient group	< ND	-	< ND	< ND	< ND	0	0.16	80
Hg**	Control group	1.43	1.27	0.89	1.295	2.54	0.30	8.44	130
	Patient group	1.45	1.31	0.77	1.29	2.45	0.48	4.91	80

* $\mu\text{g}/\text{dl}$, ** $\mu\text{g}/\text{kg}$, control group: the normal aged, patient group: the aged with Alzheimer's disease.

Table 10. Correlations of heavy metal level in blood and serum

Matrix	Element	p-value
Blood	Pb	0.035
	Cd	0.43
	Hg	0.27
Serum	Pb	0.45
	Cd	0.24
	Hg	0.9

DISCUSSION

In this study, validation by analysis method was tested using ICP-MS and Gold amalgamation in order to analyze heavy metals using the blood and serum of patients with Alzheimer's disease and find out correlations between heavy metals and Alzheimer's disease. In addition, lead, cadmium and mercury were compared between a control group and Alzheimer's disease group.

For analysis of lead and cadmium levels in the blood and serum, ICP-MS was obtained in terms of good linearity, precision, MDL, MQL and recovery. Therefore, it was applied to the samples.

To analyze mercury levels in the blood and serum, the Gold-amalgamated Direct Mercury Analyzer was used. As a result, good linearity, precision, recovery and LOD were obtained.

According to the analysis of lead, cadmium and mercury levels in blood. In terms of cadmium concentration levels, Alzheimer's disease group was higher than a control group.

In terms of the serum cadmium levels, both control group and patient group were 0.07 µg/kg (LOD) or below. In terms of lead and mercury levels in the serum, a control group was higher than Alzheimer's disease group.

For analysis of correlations between heavy metals in the blood and serum and Alzheimer's disease, t-test has been performed. Even though correlations were observed between the blood lead levels and Alzheimer's disease (p-value = 0.035), they were statistically insignificant because the concentration was higher in a control group. No significance was found in Cd and Hg. In the serum, on the other hand, no statistical significance was found between the heavy metal (Pb, Cd and Hg) and Alzheimer's disease.

In this study, no statistical significance was observed between heavy metal and decrease in cognitive intelligence. However, it appears that a further study needs to be performed.

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