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Indium as Internal Standard in Square Wave Anodic Stripping Analysis of Lead in Blood with Microelectrode Arrays

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The toxic effects of lead on humans, especially children have been well documented. ASV has been an important technique in the analysis of lead in blood of humans. This research has demonstrated that indium(III) can be used as an internal standard in the analysis of lead in blood samples with mercury film microelectrode arrays. Indium is a good choice because of a low endogenous blood concentration and because baseline separation of anodic stripping peaks among Cd, In, and Pb can be achieved under appropriate conditions. The concentration of sodium bromide strongly influences the resolution of these metals and the sensitivity of ASV to In(III) in solution. Square wave anodic stripping voltammetry together with screen-printed microelectrode arrays showed that the ratio of the anodic stripping peak currents of Pb and In varies linearly with the concentration of Pb in blood samples ranging from 1.2 to 30.0 μg/dL. The average intraassay precision (rsd) was 6.7%.

The toxic effects of lead on humans, especially children, have been well documented.[1-4] Elevated human blood lead concentrations are associated with damage to the kidney, the liver, and the gastrointestinal tract, as well as the central nervous system. In children, lower levels of lead can result in decreased intelligence, developmental disabilities, and behavioral disturbances. Childhood lead poisoning is one of the most common pediatric environmental health problems and is preventable. In response to studies that have identified harmful effects of lead in children at blood lead levels lower than previously thought, in 1991, the Centers for Disease Control (CDC) lowered the level of concern for blood lead concentration from 25 to 10 μg/dL and recommended universal screening for children. As a result, easy-to-use and sensitive devices for blood lead determination will be needed for the screening of a large number of children at risk of lead poisoning. Spurred by CDC funding, this has led to an effort to develop innovative technology for the measurement of lead in blood.

Current methods of blood lead analysis in screening programs include graphite furnace atomic absorption spectroscopy (GFAAS)[5] and anodic stripping voltammetry (ASV) at large-area Hg graphite electrodes.[6,7] GFAAS is accurate; however, the instrumentation is expensive and not portable. Commercially available ASV instruments lack the necessary precision at low blood lead levels (≤10 μg/dL) and are also not portable. These methods are mainly used in centralized analytical centers, not suitable for point-of-care testing programs.

The use of ASV with microelectrodes has generated great interest for the development of analytical systems in trace metal analysis because of its low detection limit. Electrochemical analyzers can be relatively inexpensive, easy-to-operate, rugged, and suitable for field use. Conventional ASV uses hanging mercury drop electrodes (HMDE) or mercury film electrodes (MFE). In the past decade, there have been many reports about the application of microelectrode arrays in ASV trace metal analysis.[8-11] Microelectrode arrays have proven to produce better signal/noise (S/N) ratios and larger currents compared to single microelectrodes. Carbon disk microelectrodes and screen-printed microelectrode arrays have been successfully used in this laboratory for low-level blood lead analysis in an effort to develop a low-cost, portable device for childhood lead poisoning screening programs.[12,13] In these studies, the determination of lead in blood has been based on square wave anodic stripping voltammetry (SWASV) using a single 10 μm diameter carbon microdisk electrode or a 287-element carbon microelectrode array. SWASV was used because of its advantages of excellent rejection of background currents[14,15] and applicability to solutions containing dissolved oxygen.[16,17] In our previous studies, the quantitative

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results were obtained by the standard addition method, which gave relatively good accuracy and precision for blood lead concentrations ranging from 2.0 to 99 μg/dL in the unstirred and undeaerated samples. However, the standard addition method would complicate the operation of any targeted portable blood lead analyzer and would require a higher level of operator skills.

In this work, internal standardization has been explored as the quantitation method in an effort to simplify the analytical procedure and improve analysis precision. Internal standards have been used in various analytical methods to compensate for variability and analyte losses. However, only a few ASV studies have utilized the internal standardization technique[18,19] so far. Pratt and Koch reported indium(III) as the internal standard for the determination of cadmium, copper, and lead at 10−25 ppb levels in water by using differential pulse ASV (DPASV) with a hanging mercury drop electrode. They used HMDE only and suggested that the use of indium as an internal standard at a mercury film electrode would probably result in severe interference because of the formation of intermetallic compounds between indium and other metals.[18]

However, this research shows that it is possible to use indium(III) as the internal standard for blood lead analysis with mercury film microelectrode arrays under appropriate conditions. The analysis of lead in blood poses a serious challenge to ASV technique mainly because of the electrode surface fouling problem by blood constituents. The use of an internal standard in blood lead analysis would simplify the analytical procedure and diminish the influence of uncertainties associated with the mass transport process and electrode surface response. Indium(III) was chosen as the internal standard in this research because of its low endogenous blood concentration and the feasibility of achieving anodic stripping peak separation between indium and cadmium, copper, and most importantly lead.

EXPERIMENTAL SECTION

Instruments and Chemicals. SWASV was performed on a computer-controlled BAS 100B electrochemical analyzer system with low-current module and C-2 cell stand (Bioanalytical Systems, West Lafayette, IN). Screen-printed carbon microelectrode array sensors were purchased from Enviromed (Ecossensors Limited, Enviromed, Sandy, Bedfordshire, U.K.). The microelectrode arrays were made by screen printing the carbon electrode material on a plastic substrate and by vapor depositing a uniform and conformal dielectric layer and then patterning the array by laser photoablation. The Ag/AgCl reference electrode and carbon counter electrode were also screen printed on the substrate. The microelectrode array used in this study had 280 elements, each being 20 μm in diameter and 160 μm apart in a 14 × 20 array (as shown previously in ref 13). The Ag/AgCl reference electrode and carbon counter electrode were not totally covered by the dielectric coating, with the area adjacent to the microarray being exposed. The screen printed Ag/AgCl reference electrode had an initial potential of −116 mV vs a regular Ag/AgCl reference electrode in 1 M NaBr and 1.2 M HCl solutions. All potentials are reported vs the screen-printed Ag/AgCl reference electrode.

Centrifugation of the acidified blood sample was accomplished in an Eppendorf Centrifuge 5414 (Brinkmann Instruments, Inc., Westbury, NY). Microcentrifuge tubes (1.5 mL) were from Fisher Scientific (Pittsburgh, PA). Nitric acid and hydrochloric acid were Fisher Optima grade. Mercury (Aldrich, Milwaukee, WI) was triple distilled. Lead ICP standard (1000 ppm) was purchased from G.F. Smith (Powell, OH); 1000 ppm indium AAS and 1000 mg/mL cadmium ICP standards were purchased from Alfa/Johnson Matthey (Ward Hill, MA). All solutions were prepared using type I reverse osmosis deionized water from a Milli-Q water purification system (Millipore, Bedford, MA).

To minimize extraneous Pb contamination in the analysis process, all sample preparation and electrochemical analysis were performed in a class 10 000 clean enclosure. All bottles and vials were acid cleaned, and microelectrode array sensors were cleaned as described before. All previously reported laboratory procedures were followed.[12,13]

SWASV Experiments. All analysis were performed under the following conditions. Electrochemical analysis was performed by placing 100 μL of sample solutions directly on the microelectrode array strips, which were placed horizontally on a support and connected to a BAS 100B system with subminiature crimp-type connectors. Care was taken to ensure that the solution drop covered the working electrode and the reference and counter electrodes. Experiments were conducted without either stirring or deaeration of the sample solution. Hg film deposition was accomplished by setting the potential at −1000 mV in a solution of 51 μM Hg^{2+} and 1.2 M HCl for 300 s. Electroanalysis was conducted by using Osteryoung square wave anodic stripping voltammetry with a deposition time of 120 s at −750 mV, square wave amplitude E_{sw} of 25 mV, step height ΔE of 5 mV, and square wave frequency of 100 Hz. The final potential of the anodic potential sweep was −250 mV. The small background current was taken into consideration by subtracting the background voltammogram obtained under the same conditions.

The multirun mode was used on the BAS 100B, with a rest time of 20 s between scans. Repeatable square wave stripping voltammograms on the same applied sample could be obtained after the first two scans for treated blood samples. Because of the small sample size (100 μL) and the evaporation of solvent at room temperature, the anodic stripping peaks tended to vary after eight or more repeated scans. So only data from the first five scans are used in this discussion. Following a set of repeated scans for a single treated blood sample, the Hg film was removed by setting the potential at +400 mV for 180 s after each blood sample analysis. Then the microelectrode arrays were ultrasonicated in 1.2 M HCl and H_2O for a few minutes. Each individual microarray was reused for sample analysis for no more than nine blood-derived solutions before being discarded.

Estimation of Hg Film Thickness and the Amount of Metals in Hg Film. Stripping coulometry was used to estimate the Hg film thickness on the microelectrode arrays.[20] After deposition of Hg film from 51 μM Hg^{2+} and 1.2 M HCl solution for 300 s, the potential was stepped from the rest potential of the electrodes to +400 mV for 300 s in the same solution, and the anodic stripping charge was measured. Background charge was measured in the same way without the deposition of Hg on the bare carbon microelectrode arrays. The anodic stripping charge was then converted to the amount of Hg plated on the microelectrode array by Faraday's law. The Hg film thickness was computed by assuming uniformity of the Hg film on the area of 280-element microarrays.

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As an approximation to the level of saturation of analytes in the Hg phase, the ratio of calculated amount of Pb and In from SWASV peak areas to that of Hg was used. The amount of each metal in the Hg film was calculated from the charge in the corresponding SWASV peak of each metal. The charge was determined by first converting the *x* axis from potential to time scale using the potential scan rate, which was obtained from the square wave step height and frequency, and then integrating the area under In and Pb peaks. The area under the peaks was assumed to be the stripping charge of the metal in Hg phase for the approximation, which, in turn, was converted to the amount of metal in Hg phase by Faraday's law. Assuming that the metals were completely stripped off the Hg film after each anodic stripping scan, the weight percentage of the metal in the Hg film was then calculated.

Procedures. Supporting Electrolyte Selection. Sodium salts of perchlorate, iodide, and bromide were explored as the supporting electrolyte for the SWASV analysis under conditions described above. Iodide solutions were not stable in the acidic conditions that were required for the blood sample treatment. Concentrations of 1.0, 2.0, 3.0, and 4.0 M $NaClO_4$ and 0.1, 0.5, 1, 1.5, and 2 M NaBr were studied separately as the supporting electrolyte for the SWASV of standard solutions containing both Pb and In. The solution without either ClO_4^- or Br^- was studied as well.

Selection of In(III) Concentration. The concentration of In was varied at 10, 50, 100, 500, and 1000 ppb while the concentration of Pb was kept at 10 ppb. At 10 ppb, the anodic stripping peak of In was very small compared to that of Pb peak, and the Pb peak was small compared to that of 1000 ppb In peak. In order to be to used as the internal standard for a wide range of the normally encountered blood Pb concentration, 100 ppb In was selected as the optimal concentration for concentrations of Pb between 2 and 100 ppb in standard solutions. Calibration curves of both standard solutions and treated blood samples were obtained with 100 ppb In as the internal standard.

Preparation of Blood Samples. Five blood samples were chosen from anonymous specimens in a childhood lead poisoning screening program in which our laboratory participates. The blood lead concentrations ranged from 1.2 to 30 mg/dL and were stored in Becton Dickinson (Rutherford, NJ) trace metal royal blue-top tubes containing EDTA anticoagulant. The Pb concentrations in the blood samples were determined on a Perkin-Elmer 3030 GFAAS,[12] whose accuracy was verified by participation in the American Association for Clinical Chemistry/College of American Pathologists Interlaboratory Comparison Program for Blood Lead. The blood samples were prepared as described before:[12] briefly, 100 μL of blood sample was diluted with 700 μL of H_2O and 200 μL of concentrated HCl and centrifuged at 15600*g* for 2 min. Then the supernate was diluted with supporting electrolyte having a final concentration of 100 ppb In, 1 M NaBr, 1.2 M HCl, and 46 μM Hg^{2+}. The total dilution factor for the blood sample was 1:20 after being corrected for the volume displacement of precipitated, lysed erythrocytes, which was assumed to be constant at 44% of total blood volume.

RESULTS AND DISCUSSION

In as Internal Standard. The electrochemical behavior of In(III)/In(0) (Hg) redox couple depends strongly on the composition and concentration of the supporting electrolytes.[21] Surface-active ligands, such as halides, are reported to be able to accelerate the electrode kinetics for this redox couple, even though the details of this effect are still unclear due to the difficulty in determining the complex equilibria of In(III) in such media. The anodic stripping peaks of In and Pb are within 100 mV of each other, so the baseline separation of both peaks is essential for quantitation. In order to obtain the appropriate conditions under which In(III) can be used as an internal standard in blood lead analysis, the effect of supporting electrolyte on the SWASV currents was studied. Pb and In anodic stripping peaks could be separated in both perchlorate and bromide solutions. The anodic stripping peak potentials of Pb and In were around −510 and −620 mV, respectively, in 1 M $NaClO_4$ solution, whereas the peaks of Pb and In were about −390 and −480 mV, respectively, in 1 M NaBr solutions. With $NaClO_4$ as the supporting electrolyte, the In peak was close to that of hydrogen evolution in acidic solutions, making it difficult to measure the peak current in some cases because of the sloping baseline. With NaBr as the supporting electrolyte, the deposition potential of the metals could be set at −750 mV, which was less negative than the hydrogen evolution potential. Therefore, NaBr was chosen as the supporting electrolyte.

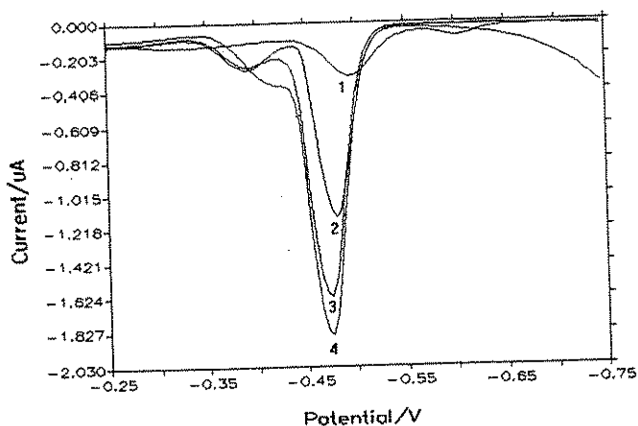

Figure 1. Square wave anodic stripping voltammograms of 10 ppb Pb and 150 ppb In in 1.2 M HCl and 51 mM Hg^{2+} solutions of different concentration of NaBr. SWASV parameters: t_d = 120 s; E_d = −750 mV, E_{sw} = 25 mV, ΔE = 5 mV, and f = 100 Hz. NaBr concentrations: (1) 0.0, (2) 1.0, (3) 1.5, and (4) 2.0 M.

Figure 1 shows the square wave voltammograms of 10 ppb Pb and 150 ppb In standard solutions containing different concentrations of NaBr as supporting electrolyte. Without bromide supporting electrolyte, the Pb and In peak potentials were the same as in solutions with perchlorate as supporting electrolyte. This is not surprising since ClO_4^- is not normally considered as a complexing agent. It is confirmed by spiking that the peaks at about −390 and −480 mV were Pb and In peaks, respectively, when bromide was added in the solutions as supporting electrolyte. From Figure 1 it can be seen that as the concentration of bromide was increased, the peaks of In shifted toward more positive potentials, and the In peak current increased, while the Pb peak current did not change appreciably. However, with the widening of In peak as bromide concentration became higher, the peaks of Pb and In gradually overlapped. This resulted in the unacceptable merge between these two peaks when the concentration of bromide was as high as 2 M. From these

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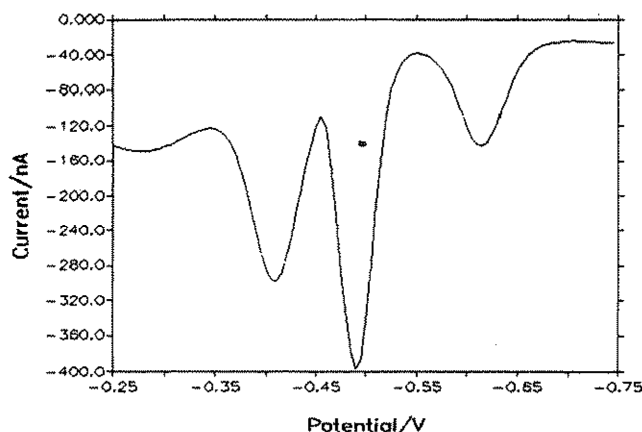

Figure 2. SWASV of 10 ppb Pb, 100 ppb In, and 10 ppb Cd in 1 M NaBr, 1.2 M HCl, and 51 mM Hg^{2+} solution. SWASV parameters are the same as in Figure 1.

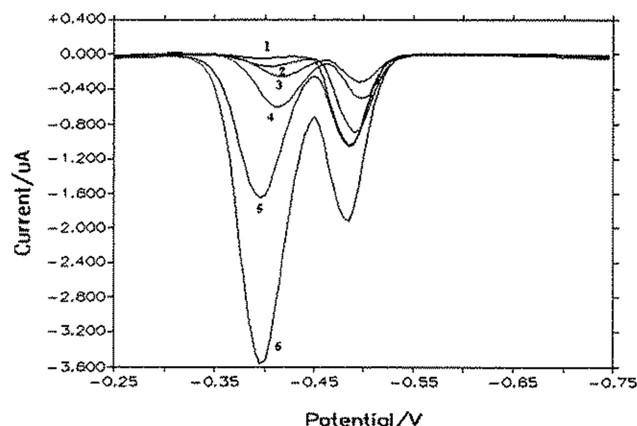

Figure 3. SWASVs of different concentrations of Pb in 51 mM Hg^{2+}, 1.2 M HCl, and 1 M NaBr with 100 ppb In as internal standard. SWASV parameters are the same as in Figure 1. Pb concentrations: (1) 2, (2) 5, (3) 10, (4) 20, (5) 50, (6) and 100 ppb.

experimental results it was decided that 1 M NaBr was chosen as the supporting electrolyte for the analysis of lead in blood.

The ASV peak potentials of Pb, In, and Cd are within a range of 200 mV and must be resolved during analysis. Under the chosen conditions, baseline separation of ASV peaks of Pb, In, and Cd could be achieved in 1 M NaBr solutions; see Figure 2. The anodic stripping peak at around −620 mV was for Cd and the other two peaks for Pb and In, respectively, as described above.

The square wave anodic stripping voltammograms of Pb and In standard solutions are shown in Figure 3. The Pb concentration was varied from 2 to 100 ppb in the experiments. These voltammograms were obtained with different microelectrode array sensors. The Pb peak current increased with the increase of Pb concentration in solutions, and the In peak height fluctuated for different sensors even though the concentration of In was constant at 100 ppb level. Using the ratio I_{Pb}/I_{In} as the measured response (where I_{Pb} and I_{In} denote the peak height of Pb and In measured against the baseline), the variation between samples and sensors was reduced. The calibration curve for Pb with 100 ppb In(III) as the internal standard is shown in Figure 4. Each point in Figure 4 was the average of three or four measurements on one sensor, with the relative standard deviation (rsd) between 2.0% for 100 ppb Pb solutions and 5.8% for 5 ppb Pb solutions (average of 4.1%). From Figure 4, it can be seen that the ratio I_{Pb}/I_{In} changes nonlinearly within the whole concentration range of Pb. The

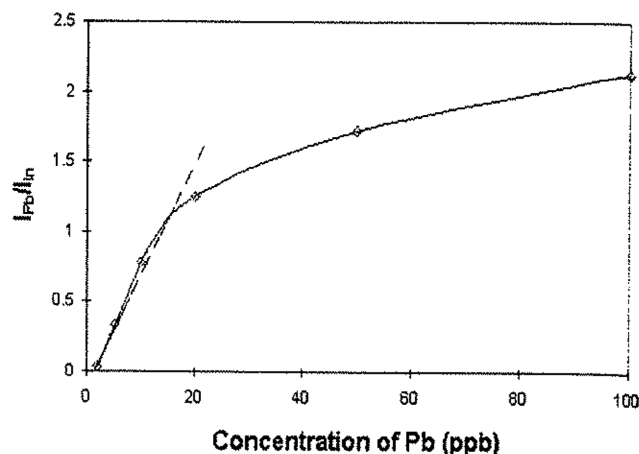

Figure 4. Calibration curve of Pb with 100 ppb In(III) as the internal standard in 1 M NaBr, 1.2 M HCl, and 51 mM Hg^{2+} solutions. The diamonds are experimental data. The dashed lines are drawn to show linear range of interest.

dashed line is drawn to show the approximation of a linear relationship in the range of interest corresponding to undiluted blood Pb concentrations of 0−30 μg/dL. However, a linear relationship between the ratio I_{Pb}/I_{In} and the logarithm of Pb concentration can be obtained by linear regression, with a slope of 1.27 and an intercept of −0.447. The correlation coefficient (r) of the linear regression is 0.996 and the standard error of estimate 0.0822. This transformed calibration curve (I_{Pb}/I_{In} vs log [Pb]) can be used for a wider range of concentration of Pb from 2 to 100 ppb.

Working with mercury film electrode, especially mercury film microelectrodes, care has to be taken that the solubility of metals in mercury is not exceeded. The solubility of indium in mercury is high, up to 68.3 wt %, compared to other metals. The solubility of lead in mercury phase is only 1.2 wt %.[22] The Hg film thickness on the 280-element microelectrode arrays, estimated by the coulometric method described above, ranged from 18 to 27 nm in this research. The charge under the In peak in the square wave anodic stripping voltammogram from the 100 ppb Pb and 100 ppb In solution, for example, shown in Figure 3 was estimated as 1.1×10^{-7} C. By assuming that the metals were completely stripped off the Hg phase after each anodic stripping scan, the concentration of In in the Hg film in the SWASV analysis was calculated as less than 0.5 wt %. In a similar manner, Pb concentrations in the mercury microelectrode arrays were estimated as less than 0.09, 0.32, 0.72, and 1.1 wt % for 10, 20, 50, and 100 ppb Pb solutions, respectively. Thus, the solubilities of these metals were not exceeded. The calculation also shows that more Pb was deposited into the mercury phase than In in the same period of time under the same experimental conditions. This means that the detection limit of Pb by ASV will be lower than that of In, because the preconcentration of Pb in the mercury phase is more efficient. This conclusion is in accordance with results reported before. The detection limit of Pb by DPASV is reported about 10 times lower than that of In.[22]

Indium(III) as Internal Standard in Blood Lead Analysis. Some examples of the square wave anodic stripping voltammograms of treated blood samples in 1 M NaBr, 1.2 M HCl, and 46

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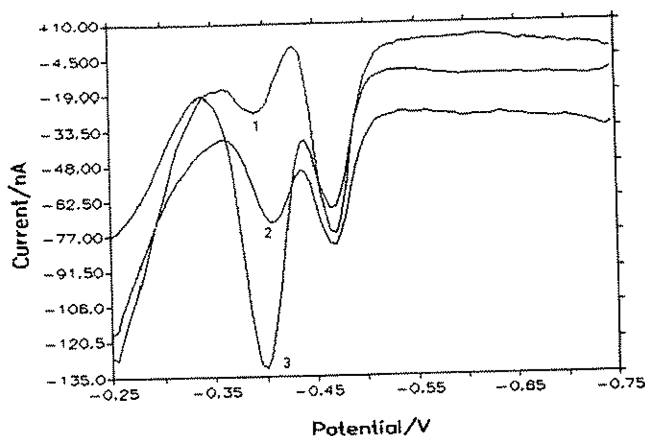

Figure 5. SWASVs of blood samples with 100 ppb In as internal standard in 1 M NaBr, 1.2 M HCl, and 46 mM Hg^{2+} solutions; 1:20 dilution factor for blood samples. SWASV parameters are the same as in Figure 1. Pb concentrations: (1) 50, (2) 105 and (3) 300 ppb Pb in original blood sample.

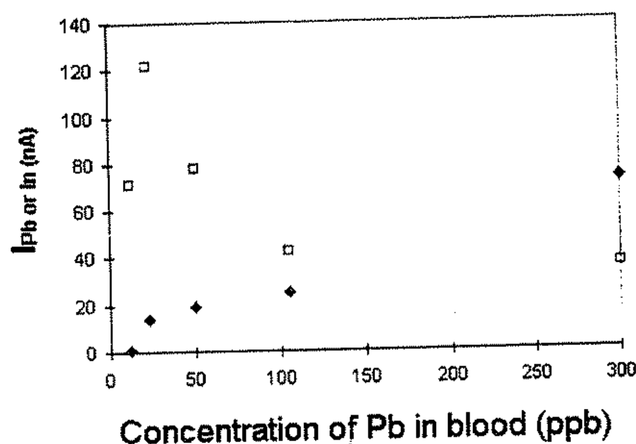

Figure 6. Variation of In and Pb peak currents of a typical treated blood sample on different electrode sensors in 1 M NaBr, 1.2 M HCl, and 46 mM Hg^{2+} solutions without using the normalization by internal standard. □ indicates In peak currents, and ◆ Pb peak currents.

μM Hg^{2+} solutions are shown in Figure 5. When 100 ppb In is used as the internal standard, accurate determination of blood Pb concentration of <1.2 μg/dL (0.6 ppb in diluted preparation) would be difficult because of the large difference between the peak currents of Pb and In. The peak at −390 and −480 mV were Pb and In peaks, respectively, in blood samples, which did not change significantly from those in standard solutions. However, the variation of the anodic stripping peak currents between different electrodes was more prominent for blood samples. A typical set of actual anodic stripping peak currents of both Pb and In of treated blood samples is shown in Figure 6. Even though the Pb peak currents seemed to respond, to some extent, linearly to the increase of Pb concentration, the In peak currents revealed the variation between different sensors and blood samples. This variation between samples could come from several factors, among which the variation in the Hg film preparation on each individual sensor and the uncertainty of the surface fouling propensity for each individual blood sample are probably most important.[13] This clearly demonstrates one of the advantages of using internal standard, that is, it can easily detect the variation of anodic stripping analysis and provide a viable process for the compensation of this variation.

When the measured response, the ratio of I_{Pb}/I_{In}, is plotted against the concentration of blood Pb, a linear calibration curve can be demonstrated. By linear regression of I_{Pb}/I_{In} vs blood [Pb], a slope of 0.006 74 and an intercept of −0.0752 are obtained. The correlation coefficient (r) of the linear regression is 0.999, and the standard error of estimate 0.0267. The data used in the calculation are the average of three or four measurements for each blood sample on one sensor. The rsd for the measured responses, I_{Pb}/I_{In}, range between 3.1% for original 10.5 μg/dL blood Pb sample and 9.8% for original 2.3 μg/dL blood Pb sample, with an average value of 6.7%. The precision when indium is used as an internal standard improves upon previous results, where an average value of relative standard deviation of 17.4% was obtained by using standard addition analysis.[13] The precision obtained from this research is well within the CDC requirement for innovative technology for blood lead analysis, which has called for the precision of ±1 μg/dL at 10 μg/dL (CDC program announcement no. 269, 1992).

Note that the blood samples were diluted 20 times in the sample preparation process. So, the highest original blood lead concentration of 30.0 μg/dL used in this research actually represents 1.5 μg/dL (15 ppb) Pb in the treated blood sample. Therefore, the whole range of the calibration curve of blood samples described above actually corresponds to the initial linear portion of the calibration curve of standard solutions shown in Figure 4. This explains why the calibration curve for blood samples is a straight line within the blood lead concentration range tested. Note that the intercept of the regression line is negative. However, the calibration curve for blood samples were obtained by calibrating against the actual Pb concentration of real blood samples, which were determined by GFAAS. This bias would not affect the analytical results for blood samples.

With the success of using internal standard in blood lead analysis, the whole analytical procedure could be simplified (e.g., combine all reagents on the sensor surface) and compatible with compact computerized instruments. Even though the use of internal standard has proven to be a viable concept for the targeted portable blood lead analyzer, the sample treatment method used in this research is not practical. Future work on semiselective coatings for the electrode are expected to enhance detection and reduce the number of sample preparation steps. The ratio of peak currents of Pb and In can be easily determined by computer software. This would be especially valuable for developing a point-of-care blood lead analyzer. Further research is being conducted in our laboratory toward the goal.

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