# Direct fluorescence detection of $Pb^{2+}$ and $Cd^{2+}$ by high-performance liquid chromatography using 1-(4-aminobenzyl)ethylenediamine-*N,N,N'*,*N'*-tetraacetate as a pre-column derivatizing agent

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**Abstract**

A highly sensitive HPLC with direct fluorescent detection ($\lambda_{ex}$ = 235 nm, $\lambda_{em}$ = 355 nm) was developed for $Pb^{2+}$ and $Cd^{2+}$ complexes with an aromatic polyaminocarboxylate, 1-(4-aminobenzyl)ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetate as a pre-column derivatizing agent. A reversed phase partition column pretreated by a cationic surfactant was employed. Although this ligand forms thermodynamically stable complexes with various metal ions, only peaks of $Pb^{2+}$ and $Cd^{2+}$ were detected with the ligand-centered emission in the HPLC due to the emissive activity and kinetic stability in the dissociation reaction. The detection limits obtained were $1.5 \times 10^{-8}$ and $3.3 \times 10^{-9}$ mol $l^{-1}$ for $Pb^{2+}$ and $Cd^{2+}$, respectively.

*Keywords:* Lead ion; Cadmium ion; Aromatic polyaminocarboxylate; Dissociation kinetics; Pre-column derivatization; fluorescent detection

## 1. Introduction

It is well known that $Pb^{2+}$ and $Cd^{2+}$ have accumulative toxicity for the human body. The allowable concentrations are 3 and 10 μg $l^{-1}$ for $Cd^{2+}$ and $Pb^{2+}$ set by the World Health Organization (WHO) for drinking water quality. Accordingly, the Japanese government reduced the allowable concentration values for $Pb^{2+}$ and $Cd^{2+}$ for water pollutants from 50 ppb to 10 ppb in 2003. Therefore, there is a pressing need for the development of a simple and sensitive conventional method to measure lead and cadmium ion in environmental water and tap water. There are many quantitative testing methods for toxic heavy metal ions in matrix samples; however, because of the high setting up costs and high running costs of sophisticated instrumental methods, atomic absorbance spectrometry (AAS) and inductively coupled plasma mass spectrometry (ICP-MS) [1,2], small local governments and developing countries cannot realistically make practical use of them. An easier, simpler, more durable and more inexpensive technique is required. Although high-performance liquid chromatography (HPLC) [3-5] and ion chromatography (IC) [6] could be excellent candidates in terms of their simplicity, toughness and low-energy consumption, in most chemical systems in studies employing HPLC and IC, their sensitivity and selectivity has been shown not to satisfy the required concentration levels for toxic heavy metal ions. While there are several methods satisfying the criterion [5-8], most of them require complicated pre-treatment, such as pre-concentration processes, which spoils the advantage of simplicity in using HPLC.

In the detection mode of pre-column complexing HPLC methods, sensitivity for certain metal ions is high, comparable to that of sophisticated methods reported so far [9-14]. In those methods, called kinetically differentiation modes, a derivatizing agent (ligand) was intentionally not added in the eluent, so that a strong dissociative driving force was generated due to the absence of the ligand from the band of the metal complex, which resulted in a kind of concentration jump. Consequently, only kinetically inert metal complexes through the elution are selectively detectable. Moreover, in this technique, ultra trace analysis is accessible due to the substantially silent baseline noise in the absence of light-absorbing ligands. A complexing system, however, which is suitable for the sensitive detection of relatively soft heavy metal ions including $Pb^{2+}$ and $Cd^{2+}$ by the pre-column derivatizing technique, has not yet been developed. The fact that $Pb^{2+}$ and $Cd^{2+}$ tend to generally form labile complexes on ligand-exchange reaction as judged by their large water exchange rates ($k_{H2O,Pb} = 1 \times 10^{10}$ $s^{-1}$ and $k_{H2O,Cd} = 2 \times 10^{8}$ $s^{-1}$) [15], actually makes the chemical system design difficult.

The aim of this study is to develop a simple pre-column complexing HPLC method for ng $ml^{-1}$ level of toxic heavy metal ions. Furthermore, we employed fluorescence detection with respect to the selectivity and sensitivity. When the system is applied to practical samples, there is an advantage of using the fluorescence detection over the UV detection in terms of interference from organic compounds. However, it is generally known that complexes with heavy metal ions tend to quench the ligand-centered emission by what is known as the heavy atom effect. Although many studies about chromatographic methods for heavy metal ions with UV/vis detection have been reported so far [6], there is no work about a pre-column complexing HPLC technique with direct fluorescence

detection for heavy metal ions, to our knowledge. Both the emissive characteristics and the kinetic inactiveness in the dissociation reaction of complexes with heavy metal ions is necessary to develop such a pre-column HPLC technique.

We employed an aromatic polyaminocarboxylate, 1-(4-Aminobenzyl)ethylenediamine-*N*,*N*,*N* ',*N* '- tetraacetic acid (abbreviated as ABEDTA or L) as a candidate ligand. It is likely that, at least, the thermodynamic stability of $Pb^{2+}$, $Cd^{2+}$ and $Hg^{2+}$-abedta complexes are large judging from the stability constants of methyl-EDTA complexes ($K_{\text{Pb-medta}} = 10^{18.97}$ and $K_{\text{Cd-medta}} = 10^{18.83}$, $K_{\text{Hg-medta}} = 10^{22.81}$) [16], for which the chemical structure of the binding sites is an analogue of ABEDTA. It is unknown whether the ABEDTA complexes with those metal ions are kinetically stable since such hard ligands including oxygen and nitrogen atoms are not generally suite to bind to relatively soft metal ions with regard to the hard and soft acid and base (HSAB) principle. This ligand is originally employed as a bifunctional chelating reagent (Meares reagent) for labeling of biopolymers [17-19]. There are, however, no studies in which this ligand has been used as a derivatizing reagent for a separation system, except for a report about capillary electrophoresis (CE) for lanthanide ions with UV detection [20].

## 2. Experimental

### *2.1. Apparatus*

Fluorescent spectra were measured using a Shimadzu model RF-1500 spectrofluorimeter (1 cm cell length, bandpass 10 nm). The used HPLC setup consisted of a Model LC10-AD pump unit, a Model RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan) or a Model L-4200 UV/vis spectrophotometric detector (Hitachi, Tokyo, Japan), and a Rheodyne Model 7725 sample injection valve with a 100 μL loop. The analytical reversed-phase partition column used was a Mightysil RP-18 from Kanto Chemical (Tokyo, Japan) (150 mmL × 4.6 mm I. D. packed with 5 μm particle size), with the silica packing fully end-capped. The absorption and fluorescent spectra in batch solutions were measured using an UV 2400 PC spectrophotometer and a RF-1500 spectrophotofluorometer (Shimadzu, Kyoto, Japan), respectively.

### *2.2. Chemicals*

The reagent, ABEDTA (>90% purity), was purchased from Dojindo Lab (Kumamoto, Japan), and dissolved in ultra pure water. A pH buffer, *N*-2-Hydroxyethylpipeazine-*N*'-2-ethansulfonic acid (HEPES) (99.0 % purity, Dojindo Lab, Kumamoto, Japan ), was dissolved in ultra pure water, and then the solution's pH was adjusted by concentrated sodium hydride. Tetra-*n*-butylannmonium bromide, TBABr (analytical grade, Tokyo Kasei Kogyo, Tokyo, Japan), *n*-hexadecyltrimethyl ammonium bromide (CTAB) (analytical grade, Kishida Chemical, Osaka, Japan) and sodium sulfate (Wako Pure Chemical Industries, Osaka Japan) were dissolved in pure water. Standard solutions of metal ions were prepared by dissolving the chloride salts (99.9 % purity, Wako chemical Industries, Osaka, Japan) in pure water with a few drops of concentrated hydrochloric acid to adjust the pH value to 2.

### *2.3. HPLC procedure*

The modified procedure by Bedsworth et al. [21,22] was employed to coat a column with cationic detergent; 30 % (w/w) methanol solution containing $1 \times 10^{-3}$ mol $l^{-1}$ of CTAB was 0.5 ml / min for 10-16 hours. After the coating, the column was washed with pure water for 120 minutes with a flow rate gradually increasing from 0.1 to 0.5 ml /min.

The eluent solution, 15.0 % (w/w) methanol-water, containing $1 \times 10^{-3}$ mol $l^{-1}$ of HEPES-NaOH (pH 7.0) and $1 \times 10^{-3}$ mol $l^{-1}$ of $Na_2SO_4$, was prepared for typical HPLC conditions using the coated column. The flow rate was set at 1.5 ml / min. Sample metal standard solutions were mixed with 50 μl of $1 \times 10^{-3}$ mol $l^{-1}$ ABEDTA solution and 500 μl of $1 \times 10^{-3}$ mol $l^{-1}$ HEPES-NaOH pH buffer solution, and then made up to 5 ml with pure water. 100 μl of the prepared sample of was injected to the HPLC after allowing the mixture to stand for 1 minute in the dark.

## 3. Results and discussion

### *3.1. Fluorometric properties of ABEDTA complexes.*

The fluorescence spectra of the ligand, ABEDTA, and the doubly and triply charged metal complexes ($Pb^{2+}$, $Cd^{2+}$, $Hg^{2+}$, $Cu^{2+}$, $Fe^{3+}$, $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Mg^{2+}$ and $Al^{3+}$-abedta) were measured as shown in Figure 1. The metal complexes with $Pb^{2+}$ and $Cd^{2+}$ immediately formed after sample preparation (within 1 minute). The ligand and the metal complexes provide a ligand-centered emission originating from the aminobenzyl group bound with the EDTA structure. While no spectral shift of maximum emission wavelength at 355 nm for any of the metal complexes was observed with excitation at 235 nm, the intensity substantially depended on center metal ions. The analogue shapes of the excitation spectra were also observed for the metal complexes and the ligand with an excitation maximum at 235 nm. While the complexes of $Al^{3+}$, $Mg^{2+}$ and $Cd^{2+}$ have strong emissive characteristics, the emission of $Cu^{2+}$ and $Fe^{2+}$ complexes was weakened due to the paramagnetic quenching effect. With respect to other metal complexes, $Pb^{2+}$, $Hg^{2+}$, $Zn^{2+}$, $Ni^{2+}$ and $Co^{2+}$ showed a moderate intensity. For heavy metal ions in the fifth and sixth row ($Cd^{2+}$, $Pb^{2+}$ and $Hg^{2+}$), it seemed that the heavy atom effect had a strong impact, except for the Cd complex. The fluorescence intensity of $Pb^{2+}$ and $Hg^{2+}$-abedta complex is smaller than those of the Cd complex by a factor of 10. However, the fluorescence of Pb and Hg complexes did not completely quench. This is probably related to the long distance between the center-metal ion and the aminobenzyl moiety in the complexes. There is a possibility those emissive complexes can be detected in the precolumn derivatizing HPLC if separation can be made among the ligand and the complexes, and if the complexes are kinetically inactive.

### *3.2. Reversed-phase partition and ion-pair mode chromatography*

First, reversed-phase partition (RP) HPLC was examined for mutual separation with an ODS column. Not even a slight separation, however, was achieved between the complexes and the ligand or among the metal complexes at any mobile phase composition. The separation mechanism of

RP-HPLC is generally well known to be mainly based on the hydrophobic interaction between analytes and the stationary phase. For ABEDTA and its complexes, their hydrophobicity was likely to be approximately the same. Similarities in terms of the size, the charge and the chemical structure of the complexes probably lead to comparable hydrophobicity. In order to evaluate the effect of charge-charge interactions in the separation system, ion-pair mode HPLC was tested by the addition of tetra-*n*-butylammonium bromide (TBABr) as an auxiliary agent. The chromatogram with spectrophotometric UV detection ($\lambda_{abs}$ = 235 nm) was shown in Figure 2. The resolution was drastically improved compared with that in the RP system. However, no separation between the objective heavy metal ions, $Pb^{2+}$ and $Cd^{2+}$, was achieved at all. Meanwhile, no peaks for the $Zn^{2+}$, $Hg^{2+}$, $Ca^{2+}$ or $Mg^{2+}$-abedta complex were observed, and the peaks corresponding to $Cu^{2+}$, $Co^{2+}$ and $Ni^{2+}$ collapsed to one fifth or less of the peak height of $Pb^{2+}$. It is likely that these metal complexes were decomposed in the column to some extent through a spontaneous dissociation process (ML → $M^{2+}$ + L; $k_d$ $s^{-1}$) dependent on their kinetic activity. When fluorometric detection was employed, only the $Pb^{2+}$, $Cd^{2+}$ and $Al^{3+}$ complexes were selectively detectable, and the other metal complexes with $Ca^{2+}$, $Mg^{2+}$ $Zn^{2+}$ and $Hg^{2+}$ were not detected despite their fluorometric detectability. Both the emissive and kinetic properties of the complexes seem to cooperatively provide the selectivity (a typical chromatogram of $Pb^{2+}$ complex is shown in Fig. 3). Although both the $Pb^{2+}$ and $Cd^{2+}$ complexes were detectable, the $Hg^{2+}$ complex was not. This implies that the $Hg^{2+}$ complex is kinetically active in contrast to $Pb^{2+}$ and $Cd^{2+}$ complexes. It is noted that the chemical structure of mere methyl-EDTA provides interesting specificity for those three heavy metal ions. The detection rule in this HPLC seemed to be controlled by kinetics but not thermodynamics since the stability constant of $Hg^{2+}$ complex with methyl EDTA was substantially larger than those of $Pb^{2+}$ and $Cd^{2+}$ complexes (the values of log $K_{metal\text{-}medta}$ were shown in the Introduction).

### *3.3. The employment of a column coated with CTAB*

Since it was expected that the resolution between metal-abedta complexes was enhanced using the electrostatic separation mode more than the ion-pair mode, a column coated with the cationic detergent, CTAB, was employed in order to achieve complete separation between $Pb^{2+}$ and $Cd^{2+}$, where the ion exchange mechanism worked, i. e. the charge-charge interaction between the stationary phase and the complexes predominantly governed the separation. Bedsworth et al. reported that some metal-edta complexes were efficiently separated in the coated column [22]. The obtained chromatogram was shown in Figure 4. Only complexes of $Al^{3+}$, $Pb^{2+}$ and $Cd^{2+}$ and the large excess ligand were detected and mutually and completely separated. The number of theoretical plates in this system for the $Pb^{2+}$ complex improved; at 8100 it is high compared with that typically obtained by the ion-pair mode, at 5600. Interestingly, the order of retention factor, $k'$, of $Pb^{2+}$ and $Cd^{2+}$ ($k'_{Pb} < k'_{Cd}$) was reversed in comparison with that of the EDTA complex ($k'_{Cd} < k'_{Pb}$) [22]. In our system, the composition of methanol in the mobile phase effected the overall retention time, while the concentration of $Na_2SO_4$ sensitively controlled the resolution between the $Pb^{2+}$ and the $Cd^{2+}$ complex and the ligand. Both the hydrophobic partition and ion exchange

mechanism was likely to have a significant effect on retention, though the separation mechanism is yet obscure.

### *3.4. Detection limit, reproducibility and intereference*

The limit of detection was determined using the coated column based on signal to noise ratio (S / N = 3); $1.5 \times 10^{-8}$ mol $l^{-1}$ (3.1 μg $l^{-1}$, 1.5 pmol on amount basis) and $3.3 \times 10^{-9}$ mol $l^{-1}$ (0.3 μg $l^{-1}$, 0.33 pmol on amount basis) with a linear range of $5 \times 10^{-8}$ - $2 \times 10^{-6}$ mol $l^{-1}$ and $5 \times 10^{-9}$ - $2 \times 10^{-7}$ mol $l^{-1}$ ($R^2$ = 0.9991 and 0.9991, n = 5) for $Pb^{2+}$ and $Cd^{2+}$, respectively. Furthermore, good relative standard deviations of peak heights were obtained, with 0.23 % and 0.42 % for $Pb^{2+}$ and $Cd^{2+}$. The difference between the sensitivity of the two metal ions seemed to be caused by emissive characteristics (see Figure 1). The interference from other foreign metal ions was investigated. The results are summarized in Table 1 and 2. There is no marked interference even from 100-1000-fold coexisting ions, except in the case of $Zn^{2+}$. A large excess of $Zn^{2+}$ provided a greatly broadened peak around the retention time of $Pb^{2+}$. This suggests that the dissociation reaction of the $Zn^{2+}$-abedta complex does not proceed to completion and some Zn complexes are detected. Tap water passed through an old domestic tap water pipe partly made of lead was analyzed as a real sample solution as shown in Figure 5. The acidified sample by nitric acid (pH 1.0) was diluted by a factor of 1.1. The found concentration of 19.2 ± 1.0 ppb (n = 5) was comparable to that of 18.0 ± 1.9 ppb determined by ICP-MS. This coated column was amenable to use over a long period of time. We could use the column for 50 days with 700 runs with a total elution time of 350 hours without re-preparation.

## 4. Conclusion

This HPLC method has high sensitivity and selectivity, making it suitable for measuring the allowable concentrations of $Pb^{2+}$ and $Cd^{2+}$ in drinking water as defined by WHO. Our method is superior to other HPLC systems in the following ways; 1) it has a simple HPLC setup, 2) it uses direct fluorescence detection for $Pb^{2+}$ and $Cd^{2+}$ (usually spectrophotometric detection), 3) the ease of handling samples without any complex procedures such as pre-concentration (only the addition of the agent to the sample solution), and 4) it has very low running costs. It is noted that selectivity for $Pb^{2+}$ and $Cd^{2+}$ over other heavy metal ions was observed based on the dissociation kinetics of complexes with a methyl-EDTA frame. The improvement of antenna moiety in the ligand to modulate and, thus, significantly lengthen the fluorescent wavelength, is underway.

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**Legends for Figures**

Fig. 1. Fluorescence spectra of the different metal complexes and the ligand. $C_{\mathrm{ABEDTA}} = 1\times10^{-6}$ mol $l^{-1}$; $C_{\mathrm{HEPES\text{-}NaOH}} = 1\times10^{-3}$ mol $l^{-1}$ (pH 7.5); $C_{\mathrm{metal\text{-}abedta}} = 1\times10^{-5}$ mol $l^{-1}$. $\lambda_{ex} = 235$ nm. The insertion is the excitation spectrum of the ligand.

Fig. 2. Typical chromatogram of metal-abedta complexes with spectrophotometric detection. Sample, $C_{\mathrm{Pb,\ Cd,\ Hg}} = 1 \times 10^{-6}$ mol $l^{-1}$, $C_{\mathrm{Fe,\ Cu,\ Zn,\ Ni,\ Co,\ Al}} = 2 \times 10^{-6}$ mol $l^{-1}$, $C_{\mathrm{ABEDTA}} = 1 \times 10^{-4}$ mol $l^{-1}$; $C_{\mathrm{HEPES\text{-}NaOH}} = 1 \times 10^{-3}$ mol $l^{-1}$ (pH 7.5 ). Eluent, 19 % (w/w) methanol-water containing HEPES-NaOH of $1 \times 10^{-3}$ mol $l^{-1}$ (pH 7.5) and TBABr of $1 \times 10^{-4}$ mol $l^{-1}$. Flow rate = 0.8 ml / min , $\lambda_{abs} = 235$ nm.

Fig. 3. Typical chromatogram of $Pb^{2+}$-abedta complex with fluoromeric detection. Sample, $C_{\mathrm{Pb}} = 6 \times 10^{-7}$ mol $l^{-1}$; $C_{\mathrm{ABEDTA}} = 5 \times 10^{-5}$ mol $l^{-1}$; $C_{\mathrm{HEPES\text{-}NaOH}} = 1 \times 10^{-3}$ mol $l^{-1}$ (pH 7.5 ). Eluent, 16.5 % (w/w) methanol-water, $C_{\mathrm{HEPES\text{-}NaOH}} = 1 \times 10^{-3}$ mol $l^{-1}$ (pH 7.5), $C_{\mathrm{TBABr}} = 1 \times 10^{-4}$ mol $l^{-1}$. Flow rate = 1.0 ml / min, $\lambda_{ex} = 235$ nm, $\lambda_{em} = 355$ nm.

Fig. 4. Typical chromatogram of $Cd^{2+}$ and $Pb^{2+}$-abedta complexes. Sample, $C_{ABEDTA} = 1 \times 10^{-5}$ mol $l^{-1}$, $C_{Cd} = 5 \times 10^{-8}$ mol $l^{-1}$, $C_{Pb} = 5 \times 10^{-7}$ mol $l^{-1}$, $C_{HEPES\text{-}NaOH} = 1 \times 10^{-3}$ mol $l^{-1}$ (pH 7.5 ). Eluent, 15.0 % (w/w) methanol, $C_{HEPES\text{-}NaOH} = 1 \times 10^{-3}$ mol $l^{-1}$ (pH 7.5 ), $C_{Na2SO4} = 7 \times 10^{-3}$ mol $l^{-1}$. Flow rate = 1.5 ml / min, $\lambda_{ex} = 235$ nm, $\lambda_{em} = 355$ nm.

Fig. 5. Typical chromatogram of a tap water sample. Sample, a tap water of 4.5 ml was made up to 5 ml. $C_{ABEDTA} = 1 \times 10^{-5}$ mol $l^{-1}$, $C_{HEPES\text{-}NaOH} = 1 \times 10^{-3}$ mol $l^{-1}$ (pH 7.5 ). Other conditions are the same as in Fig. 4.