Simultaneous Detection of [metal(II)-tpen]$^{2+}$ as Kinetically Inert Cationic Complexes Using Pre-capillary Derivatization Electrophoresis: An Application to Biological Samples

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A high resolution of doubly charged first row transition (Fe, Cu, Zn, Ni, Co, Mn) and heavy metal ions (Pb, Cd, Hg) was achieved in capillary electrophoresis (CE) with high sensitivity (sub-micro mol dm^{-3} level), using *N, N, N', N'*-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) as a pre-capillary derivatizing agent. The non-charged reagent, TPEN, was applied to capillary zone electrophoresis (CZE) for the first time. Since complete spatial separation between the complexes and the ligand was carried out in a carrier buffer, which was free of TPEN, kinetic inertness of metal complexes was necessary for the detection in this pre-capillary method. All the nine metal complexes were detected; Ca^{2+}, Mg^{2+}, Al^{3+}, Fe^{3+}, and Co^{3+} complexes were undetectable. This, interestingly, suggests that those nine cations form kinetically inert tpen complexes without strong charge-charge interactions between the metal ion and the ligand. It is expected that the hard-soft-acid-base (HSAB) principle governed the kinetics selectivity. With respect to the electrophoretic behavior, the addition of chloride ion and methanol to the carrier significantly improved the resolution. This is due to the formation of ternary complexes or ion aggregates and the solvation effect, respectively. These effects provided a satisfactory baseline resolution among the nine metal ions. An application to biological samples was demonstrated. Some metal ions in human serum and urine were successfully detected in a simple process without the need for deproteinization using a non-coated fused-silica capillary because of the differenciation in the direction of migration between organic matter and complexes.

Introduction

It is difficult to simultaneously determine trace metal ions in substances with a high matrix, like biological samples, without pretreatment. To our knowledge, there are few reports about multi-element analysis in biological samples with direct sample injection, with respect to capillary electrophoresis (CE). This is due to both the low sensitivity of absorbance detection and interference from organic substances when carrying out UV detection. Our aim in this study is to develop a CE method for the simultaneous detection of trace metal ions with simple treatment of biological samples.

Among the many CE techniques for metal ions, pre-capillary derivatizing methods without a derivatizing reagent in the carrier buffer take a leading role with respect to their selectivity and sensitivity.[1] The advantages of the method originate from the fact that only kinetically inert complexes are detectable and the baseline noise is remarkably stable in the absence of a complexing reagent, which is highly light-absorptive in the carrier buffer. Metal complexes have to be sufficiently kinetically inert on dissociation to be detected in the pre-capillary complexing CE system because a complex spatially separated from the ligand is exposed to a strong driving force to dissociate. Although this method is very useful, the selection of a metal complex candidate is difficult since the selectivity is based on dissociation activity. Several excellent pre-capillary derivatizing systems have been reported for hard metal ions,[1] such as *o*, *o*'-dihydroxyazobenzene (DHAB) derivative,[2] pyridylazoresolcinol (PAR),[3, 4, 5] α,β,χ,δ-tetrakis(4-carboxyphenyl)porphine (TCPP),[6] *N,N*'-bis(2-hydroxybezyl)ethylenediamine-*N,N'*-diacetate (HBED)[7], 8-Amino-2-[(2-amino-5-methylphenoxy)methyl]-6-methoxyquinoline-*N,N,N',N'*-tetraacetate (Quin2)[8] and 4-aminobenzyl-EDTA (ABEDTA) systems.[9] There were few simultaneous detection methods for relatively soft metal ions using kinetic inert complexes, while some methods were reported applying soft ligands to soft metal ions,[10, 11, 12] which needed detecting agents in the carrier buffer due to their kinetic activity during the dissociation process. This can be explained by the presence of a small number of an inert complex systems with soft-middle metals with all the following characteristics: high detectability, high resolution and water solubility. Furthermore, they are inapplicable to biological samples without deproteinization because of adsorption on the capillary wall of proteins and a lack of selectivity of metal ions over other organic matters.

In this work, we applied *N,N,N',N'*-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) complexes to the pre-capillary method in order to simultaneously detect relatively soft metal ions for the first time. Furthermore, we were able to analyze metal ions in biological samples without complicated pretreatment. The promising reagent, TPEN, was selected because a) TPEN exhibits large thermodynamic stability with many metal ions and forms doubly positively charged complexes with one to one compositions,[13] which might help to provide kinetic inertness, b) its pyridine ring is classified as a middle base on the hard and soft acids and bases (HSAB) principle; we expected that a polydentate ligand possessing binding sites with soft bases characteristics could be suitable for relatively soft metal ions, c) the TPEN complexes have large absorption coefficients (~17000 cm^{-1} M^{-1}) originating from their four pyridine rings, and d) TPEN has excellent solubility in water for easy handling. The ligand in the cation complex ([M-tpen]$^{2+}$) is neutral in form. This is notable in contrast with the fact that anion complexes with anionic ligands have generally used for the pre-capillary CE mode to date.

Moreover, when the CE system of TPEN complexes was applied to biological samples, no interference from organic substances was expected if the electroosmotic flow was suppressed. Most biological substances, such as sugars, lipids, vitamins, amino acids, inorganic acids and proteins, are anionic or neutral in form at relatively high pH 7-10, while the metal-tpen complexes possibly be doubly or triply positive. Most

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See http://www.rsc.org/suppdata/cc/b0/b000000a/

biological substances migrated to the opposite direction from tpen complexes and exit from a capillary tube. Therefore, it is expected that only metal complexes are able to be detected. There are no reports about the strategy like this in order to obtain tolerance to biological substances. This is because there are few kinetically stable cation complexes at neutral to high pH with high sensitivity known so far.

Experimental

Chemicals

The pre-capillary complexing agent, TPEN obtained form Dojindo Lab (Kumamoto, Japan) was dissolved in deionised water by Milli-Q SP. TOC. System (Millipore Co., Billerica, MA, USA) to make the concentration to 10^{-3} M. Standard solutions of metal ions were prepared by dissolving the chloride salts (99.9 % purity, Wako Pure Chemical Industries, Japan) in deionised water with a few drops of concentrated hydrochloric acid. Those metal ion solutions were standardized by EDTA titration. The pH buffer solution of bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris) (analytical grade, Dojindo. Lab.) was dissolved in deionised water and the pH value was adjusted to 7.0 with concentrated hydrochloride solution. All other chemicals employed were of an analytical-reagent grade.

Apparatus

The electrophoresis was performed with BioFocus 3000 capillary electrophoresis equipment (Bio Rad Laboratories Japan, Tokyo, Japan) under constant voltage mode. The temperature of the capillary column was fixed at 293K with a thermostat device in the CE equipment. A fused-silica capillary tube (0.05 mm I.D.; total length 50.0 cm; effective length to the detection window 43.4 cm) purchased from Scientific Glass Engineering Inc. (Austin, TX) was employed. Solution pH was measured with a HM26s pH meter (TOA Electrics, Tokyo, Japan). Absorption spectra were recorded on a SHIMADZU UV2400PC spectrophotometer (Kyoto, Japan).

Sample preparation and electrophoresis

The TPEN solution of 1×10^{-3} M was added to a mixed solution of metal ions and a pH value of the solution was adjusted to 7.0 by adding Bis-Tris-HCl stock solution (0.1 M). The prepared solutions were injected with nitrogen gas pressure (690 kPa) at the cathodic end. The injected volume of 9.6 nL (5 psi × sec) was calculated using the Hagen-Poiseuille equation. The complexes were detected at 260 nm with on-capillary absorbance detection. The capillary column was initiated by washing with 1.0 M NaOH (15 min) – deionized water (15 min) – a carrier buffer solution (10 min) before custom experiment. Between each of the CE runs, a washing sequence of 1.0 M NaOH (2 min) – water (2 min) – a carrier buffer (5 min) was carried out. Electrophoretic separation was performed at an applied voltage of +20 kV (a typical value of an electronic current was 72-80 μA). Electrophoretic mobility of the TPEN complexes was calculated based on the following equation:

$$t_m = \frac{l}{(\mu_{ep} + \mu_{eo})E} \quad (1)$$

where t_m, l, μ_{ep} and E represent the migration time of the objective complex, capillary length, mobility of the complex and electric field strength, respectively. The mobility of electroosmotic flow, μ_{eo}, was estimated from the migration time of the solvent.

Human serum and urine samples

Before their introduction to CE, biological samples (human serum and urine) were acidified to pH 2 with a few drops of concentrated hydrochloride solution in order to free the metal ions bounded with organic substances and a minimum amount of ascorbic acid was added to sample solution for reduction. Then 100 μL of the sample was mixed with solutions of 225 μL of 1 mM TPEN and 140 μL of 1 M Bis-Tris-HCl. The volume of the solution was made up to 500 μL with deionized water. After leaving the mixture stand for 5 minutes, it was directly injected into the CE. A dilution factor of five was set for the biological samples.

Results and Discussion

Complexation and spectroscopic property of $[M\text{-tpen}]^{2+}$.

The standard metal ion solution containing Al^{3+}, Fe^{3+}, Co^{3+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Pb^{2+}, Cd^{2+} and Hg^{2+} was mixed with the TPEN stock solution at pre-capillary reaction and the metal ions were converted into $[M\text{-tpen}]^{2+}$ complexes at pH 7. These complex formation reactions take place at a high rate (the reactions were completed within 5 minutes). The shape and magnitude of the absorption spectrum of each metal complex (λ_{max} = 252-261 nm, $\varepsilon = 1.0\text{-}1.7 \times 10^4$ cm^{-1} M^{-1}) shows hardly any differences with that of just TPEN by itself, except for the Fe^{2+} complex (see Fig. 1). Besides the peak at 252.5 nm, another absorption band was observed at 417.5 nm for the $[Fe\text{-tpen}]^{2+}$ complex. It is reasonable for multi-element detection that each metal complex has the same maximum wave length. A wavelength at 260 nm was employed for CE experiments.

Effect of pH on detection selectivity in CE

On the CE runs, the $[M\text{-tpen}]^{2+}$ complexes are detectable in rather high concentrations of electrolyte in the carrier buffer (~100 mM), while no other metal complexes could not be detected in low electrolyte concentration (~10 mM) of borate, phosphate and Bis-Tris at pH 7-10. It is presumed that the dissociation rates of the positively charged complexes are accelerated by the attack of deprotonated silanol function at the capillary wall surface. The effective activity of the deprotonated silanol group may decrease with increasing electrolyte concentration due to the formation of the electric double layer. Uehara et. al. reported some examples of interaction between the silica surface and metal complexes. [14, 15, 16]

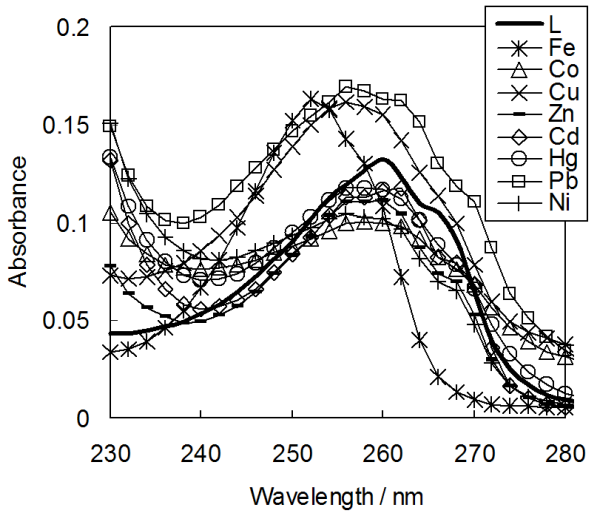

Fig. 1 Absorption spectra of TPEN and several tpen complexes in aqueous solution at pH 7. [TPEN] = 1×10^{-5} M, $[M^{2+}] = 1 \times 10^{-5}$ M, [Bis-Tris] = 10 mM (pH 7.0).

TPEN complexes with first-row transition metal ions (Fe^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+} and Mn^{2+}) and heavy metal ions (Pb^{2+}, Cd^{2+} and Hg^{2+}) were detected, providing sharp peaks at pH 7-10 with high concentrations of the carrier buffer (100 mM~). However, the peaks of the complexes of alkaline earth metal ions (Ca^{2+} and Mg^{2+}), and triply charged cations (Al^{3+}, Fe^{3+} and Co^{3+}) were not clearly observed on the CE run. This strongly suggests that the doubly charged transition and heavy metal complexes survive during the running time in the capillary column due to kinetic inertness, while triply charged cation complexes dissociate. It should be emphasized that the non-charged ligand (TPEN) was found to form doubly-charged complexes ([M-tpen]$^{2+}$) which were kinetically inert enough to be detected in CE with middle and soft metal ions. In contrast to the fact that the inert complexes employed in conventional chelating CE methods usually involve charge-charge interaction, this [M-tpen]$^{2+}$ system formed an inert complex without a strong charge-charge interaction. Rather, a charge-dipole and/or electron exchange interaction probably controlled the kinetic selectivity. With respect to Fe, this system was able to selectively detect doubly charged iron ions, while most of chelating reagents, such as polyaminocarboxylates, forcibly oxidize to triply charged ion, Fe^{3+}, during complex formation. At higher pH values than 11, the peaks of Pb^{2+}, Cd^{2+} and Hg^{2+} complexes were either broadened or completely disappeared. This is probably due to the solvolytic dissociation of the complexes in the presence of hydroxide ions. Although the stability constants of TPEN complexes with heavy metal ions are remarkably large (log $K_{Hg\text{-}tpen}$ = 25.05, log $K_{Cd\text{-}tpen}$ = 16.33, log $K_{Pb\text{-}tpen}$ = 13.98),[13] they are undetectable in high pH solutions, while other transition metal complexes possessing considerably less stable thermodynamically are detected in the CE. This indicates that it is just the dissociation kinetics which controls the detection selectivity of this system, and the equilibrium stability of the complexes does not necessarily become a criterion for detection selectivity. Sufficient mutual resolution among metal ions was not achieved with pH control only.

Effect of additive in carrier buffer on separation

In order to improve resolution among metal complexes, the introduction of an auxiliary anion in the carrier buffer solution was investigated. The anions employed were halides (F^-, Cl^-, Br^- and I^-), inorganic anions (phosphate, carbonate, borate, perchlorate and sulfate) and organic anions (citrate and succinate). Among all anions tested, only halides and citrates affected the electropherogram of [M-tpen]$^{2+}$ complexes. We found that the addition of Cl^- to the carrier especially affects the mobility of Pb^{2+}, Cd^{2+} and Hg^{2+} complexes. Their mobility substantially decreases with the increase in the concentration of KCl, compared with other metal complexes (Fig. 2). The baseline resolution of these three metals was achieved with the addition of KCl (100 mM~), implying that a dynamic ternary complex formation, [M^{2+}-tpen]$^{2+}$ + nCl^- ↔ [M^{2+}-tpen-Cl^-_n]$^{+2-n}$, or an ion association reaction, [M^{2+}-tpen]$^{2+}$ + nCl^- ↔ [M^{2+}-tpen]$^{2+}$·Cl^-_n, takes place. We suspected the ternary complex mechanism is probable for the following reasons. 1) The effect of chloride remarkably influenced on only complexes of heavy metal ions which have stronger affinity to halide than other transition metal ions. If a mere ion-association occurs, the same effect on all metal complexes should be observed. 2) In the addition of the other halides, Br^- and I^-, to the carrier, the peaks of Pb^{2+}, Cd^{2+} and Hg^{2+} complexes broadened or disappeared through on-capillary decomposition. This strongly suggests that halides nucleophilically attack the center metal ions. 3) The order of thermodynamic stability of the metal-Cl^- complexes relates to that of migration times (Hg > Cd > Pb). A similar case, in which the ternary complex dynamic equilibrium controls the resolution, was previously reported for lanthanide-aromatic polyaminocarboxylate complexes utilizing carbonate as the auxiliary reagent.[9]

Since fast electroosmotic flow (EOF) completely carried all the TPEN complexes to an anodic end before the complexes were separated, it was desirable to delay EOF. In the TPEN system, the positively charged complexes move electrophoretically to the detection window without EOF when the voltage is positive. The simultaneous addition of methanol and KCl to the carrier buffer resulted in a marked decrease in the rate of EOF, so that the detection window was extended (see Fig. 3). The migration time increased and the resolution among Fe^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+} improved with an increase in the methanol composition. A complete baseline separation of the nine metal ions was demonstrated using 10 mM Bis-Tris-HCl as a carrier buffer with 78 vol% methanol and 175 mM KCl as shown in Figure 4. Since the electroosmotic flow rate was too small to measure the mobility of the complexes under this carrier buffer conditions, the migration behavior of [M-tpen]$^{2+}$ could not be analyzed in the cases that methanol was added to the buffer solution. However, it seems that the addition of methanol also affects the resolution through the solvation of the electrolyte.[17]

Sensitivity and reproducibility

The linear detection curves for each of the metal ions were obtained up to 1.0 × 10^{-4} M. The detection limits of sub-micro

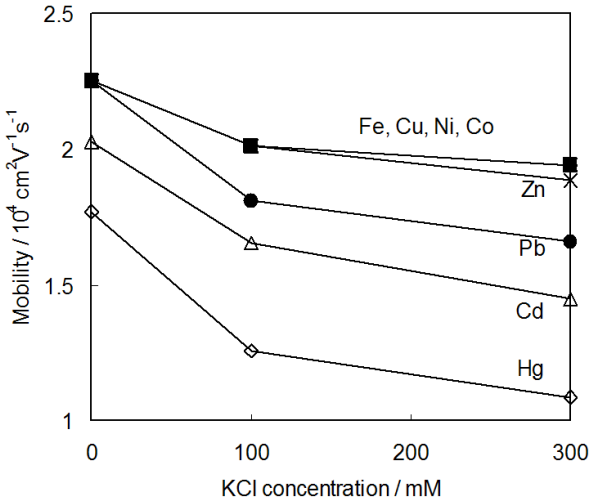

Fig. 2 Effect of KCl concentration on mobility. Carrier buffer, [Bis-Tris] = 100 mM (pH 7.0), [KCl] = 0 – 300 mM; sample, [TPEN] = 9 × 10^{-4} M, [M^{2+}] = 5 × 10^{-5} M, [Bis-Tris] = 10 mM (pH 7.0); applied voltage, 10 kV; capillary, 45.4/50 cm × 0.05 mm i.d.; detection wavelength, 260 nm; temperature, 293 K.

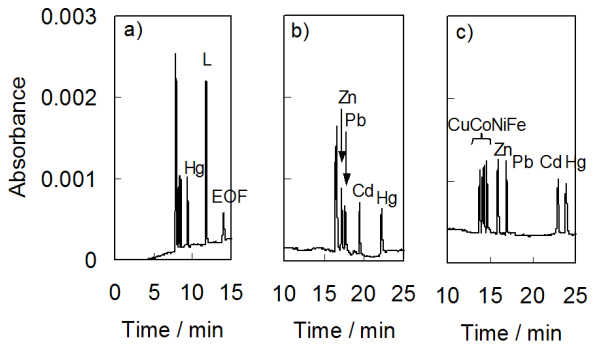

Fig. 3 Effect of MeOH in carrier buffer on mutual separation among [M-tpen]$^{2+}$ complexes. Carrier buffer, a) MeOH 0%, b) MeOH 30%, c) MeOH 80%, [Bis-Tris] = 100 mM (pH 7.0), [KCl] = 100 mM; sample, [TPEN] = 9 × 10^{-4} M, [M^{2+}] = 2 × 10^{-5} M each of Fe^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Pb^{2+}, Cd^{2+}, Hg^{2+}, [Bis-Tris] = 10 mM (pH 7.0).

molar level (2.8 – 11 fmol as amount basis) were determined for each of the nine metal ions, defined as S/N = 2 (Table 1). Satisfactory reproducibility was observed for both the peak height and the migration time; RSD (n = 5) were 1.8-6.3 % at 1.0 × 10^{-5} M of each metal ions and 1.5-2.1 %, respectively. The sensitivity of this system is superior to other conventional indirect detection CZE methods for transition and heavy metal ions (several μM level detection). [18, 19, 20] The high sensitivity of this system seems to originate from the remarkable baseline stability despite UV detection.

An application to biological samples

This CE technique was applied to both human serum and urine samples. Typical electropherograms were obtained, as shown in Figure 5. Using this method the trace metal ions of Cu^{2+}, Zn^{2+} and Fe^{2+} were able to be practically determined with very simple handling of the sample. It is emphasized that the baseline was silent and that no extra peaks originating from organic substances were observed in any of our measurements in spite of UV detection at 260 nm. However, it is noted that several unidentified peaks were observed, when the pH values of the carrier buffer solution were set at lower than 7.0. This suggested that some organic substances possessing p*I* values of about 7 were charged as cations. As schemed, the TPEN complexes behave as doubly charged cations, $[M\text{-tpen}]^{2+}$, at even relatively high pH 7-10 in contrast to anionic organic and neutral substances. The detected concentrations of Fe^{2+}, Cu^{2+} and Zn^{2+} in serum and Fe^{2+} and Zn^{2+} in urine lay in the range of higher 10^{-6} to lower 10^{-5} M (an example of the determined concentration is in the caption of Fig. 4), of which the concentration level was generally reported and reasonable. The absence of Cu ions in the urine sample was possibly due to their concentration being lower than the detection limit (usually lower than 3 × 10^{-7} M in human urine). The recovery test was also carried out for each samples. By adding 5-20 × 10^{-6} M of each metal ions, a recovery of 95 %-110 % was obtained. With respect to human serum sample, the determined values were compared with those with ICP-AES. The values were consistent within ±9 % error (86 ppb, 83 ppb for Fe, 98 ppb, 90 ppb for Cu, 72 ppb, 66 ppb for Zn with ICP-AES and this CE technique, respectively).

Conclusion

Manipulating the kinetic characteristics of the complexes and the interaction between complexes and carrier additives resulted in a highly sensitive and selective CE system. The sensitivity of this system is comparable to other eminent CE system for metal ions. Its simplicity and robustness over real samples suggest that it could be applied in practical way. Another advantage of this method is that raw fused-silica capillary tubes are useable for biological samples, instead of a coated capillary tubes. Compared with currently used techniques such as ICP-AES, this method has the advantage of being able to be run at a very low cost with low sample volumes without the need for deproteinization and extreme sample dilution. The authors believe this technique could well be applied to metal speciation since while Fe^{2+} is detected in this method, Fe^{3+} is not. Also, without the acidification of serum, the recovery of metal ions decreased, i.e. only free metal ion seemed to be detected. In addition, the dissociation kinetics of multidentate ligands with relatively soft donors is an interesting subject in inorganic chemistry. We are continuing our research in the direction to a more powerful ligand for better ultra trace detection and understanding energetics for dissociation reaction rates.

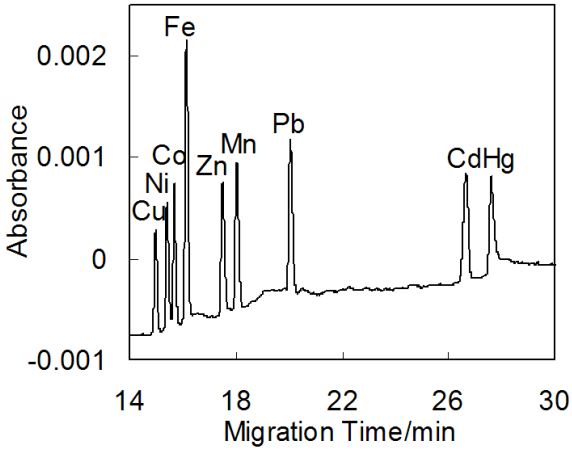

Fig. 4 Typical electropherogram of $[M\text{-tpen}]^{2+}$ complex with addition KCl and methanol in carrier buffer. Carrier buffer, [Bis-Tris] = 10 mM (pH 7.0), [KCl] = 175 mM, methanol, 78 vol%; sample, [TPEN] = 9 × 10^{-4} M, $[M^{2+}]$ = 2 × 10^{-5} M, [Bis-Tris] = 10 mM (pH 7.0). Other conditions are the same as in Fig. 2.

Table 1 Detection limit of $[M\text{-tpen}]^{2+}$ system (S/N = 2)[a]

Metal ion	Detection limit / mol dm^{-3}
Cu^{2+}	2.9 × 10^{-7}
Ni^{2+}	6.4 × 10^{-7}
Co^{2+}	2.9 × 10^{-7}
Fe^{2+}	3.8 × 10^{-7}
Zn^{2+}	5.2 × 10^{-7}
Mn^{2+}	3.9 × 10^{-7}
Pb^{2+}	4.1 × 10^{-7}
Cd^{2+}	3.9 × 10^{-7}
Hg^{2+}	1.1 × 10^{-6}

[a] Experimental conditions are same as those of Fig. 4.

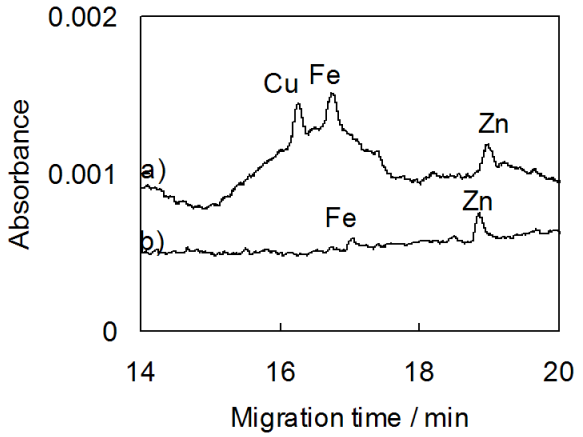

Fig. 5 Typical electropherogram of biological samples. Sample, [TPEN] = 2 × 10^{-4} M, [Bis-Tris] = 140 mM (pH 7.0), a) human urine 20 vol%, b) human serum 20 vol%. Samples were acidified by nitric acid to pH 2 before pre-capillary derivatization. Calculated metal concentrations in the original samples: $[Cu^{2+}]$ = 1.7 × 10^{-5} M, $[Fe^{2+}]$ = 2.2 × 10^{-5} M and $[Zn^{2+}]$ = 2.9 × 10^{-5} M in serum, $[Fe^{2+}]$ = 7.4 × 10^{-6} M and $[Zn^{2+}]$ = 2.6 × 10^{-5} M for urine.

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