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Determination of Molybdenum and/or Ruthenium in Urine Using Electrospray Ionization Mass Spectrometry

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Molybdenum (Mo) is utilized as a cofactor of the enzymes essential for incorporation of inorganic nitrogen in the synthesis of proteins, nucleic acids and other cellular nitrogenous constituents. Usually Mo takes a diamagnetic +6 valence state in liver, urine and seawater but it turns to be a paramagnetic +5 valence state during turnover of the enzymes [1,2]. The complex of Mo and diethyldithiocarbamate (DDC, $(C_2H_5)_2NCSS^-$) in a paramagnetic Mo-DDC state [3] was considered as a model of Mo-cofactor [1,2]. To determine the ratio of both constituents, we noticed that electrospray ionization mass spectrometry (ESI-MS) developed for the analysis of weakly charged species like proteins and peptides, was quite suitable for the analysis of weakly charged species like $Mo(DDC)_4^+$. $Mo(DDC)_4^+$ is soluble in polar solvents like alcohols and quite stable even for 1 week, nevertheless it is a paramagnetic substance. Furthermore, the extraction of the compound with isoamyl alcohol (IAA) results in not only its concentration but also the elimination of most substances contained in biological fluids that enables to inject sample in the direct mode without staining the capillary in ESI-MS.

Mass dependent determination of Mo was introduced previously using inductively coupled plasma mass spectrometry (ICP-MS) [4,5,6]. ICP-MS, however, required large sample volume, say 1 mL, per analysis with detection limit of 90 fg/ μ L [5,6], whereas present method required only 1 μ L per analysis with detection limit of 20 fg/ μ L. Furthermore, determination of Mo by ICP-MS was interfered with by isobars. That is, monoatomic interference came from zirconium (Zr) and ruthenium (Ru) [4,5] and polyatomic interference, from matrix materials such as Ar_2OH , $ArKO$, K_2O , Ca_2OH and BrO [6]. These interferences were not observed in the present ESI-MS method. That is, Zr^{4+} did not show peaks and matrix materials showed peaks at low m/z region whereas Mo and Ru showed peaks at high m/z region as 690, $Mo(DDC)_4^+$ and 944, $Ru_2(DDC)_5^+$, respectively. Therefore, Ru can be used as an internal standard (IS) for Mo in urine, since the contents of Ru are quite low in biological samples.

ESI-MS can be applied for a sensitive quantitation of metals when a compound is produced completely and detected efficiently as in the present case of $\text{Mo}(\text{DDC})_4^+$, although it is usually used for clarifying configurations of compounds as well as valence states of metals that cannot be done by ICP-MS.

Materials and methods

Atomic absorption (AA) grade solution of Ru was obtained from Aldrich Chemical Co. Inc., USA and used as IS of Mo. AA grade solutions of Mo and other transition metals examined, AA grade of DDC and IAA, and analytical grade of other chemicals were obtained from Wako Pure Chemical Ltd., Japan. Ultra-pure water having specific resistance of 18 M Ω cm was used. Calibration standard solutions containing Mo at 0.02 – 200 pg/ μL and/or Ru at 2 – 200 pg/ μL in 0.15 M NaCl were prepared every day from AA grade stock solutions containing Mo and Ru at 1 $\mu\text{g}/\mu\text{L}$, respectively. DDC reacts with Mo^{6+} and Ru^{3+} quickly at high pH and decomposes gradually at pH lower than 4. Oxalic acid (OA) can reduce Mo^{6+} to Mo^{5+} efficiently at pH ca.1. Therefore, pH of sample should be adjusted to pH 4 – 8 with either 1 M NaOH or 1 M HCl using pH test paper. The pH of normal urine is in the range of 5 - 8.

Mo^{6+} and/or Ru^{3+} were determined as follows. To 5 μL of urine or standard solution, 0.5 μL of 1 M DDC was added and mixed for 10 sec by a vortex mixer, and 2 μL of 2 M OA preheated at 40 $^\circ\text{C}$ was added and mixed for 5 sec. Five μL of IAA was added and mixed for 5 sec and centrifuged at 500 x g for 30 sec. An aliquot of 1 μL IAA layer was subjected to the analysis.

ESI-MS was performed by using a TSQ 7000 LC-quadrupole mass spectrometer (Thermo Quest, Japan) in the positive ion mode. One μL of IAA layer was injected manually in the direct mode. Methanol was flowed as the mobile phase at 200 $\mu\text{L}/\text{min}$. The capillary voltage was set to be 4.5 kV and the desolvation temperature was varied from 170 to 290 $^\circ\text{C}$ to find out the best condition. Data were collected

in the range of m/z 50 – 1000 and the quantitative analysis was conducted using molecular ions in the selected ion monitoring (SIM) mode.

Results and discussion

Figure 1 showed an ESI-MS of 1 μ L of IAA solution containing 200 pg Mo measured at the desolvation temperature of 170 $^{\circ}$ C. A cluster of peaks around m/z 690 corresponds to $\text{Mo}(\text{DDC})_4^+$, that around m/z 542, to $\text{Mo}(\text{DDC})_3^+$ and peaks less than m/z 300, mainly to solvents. Metal ions show clusters of peaks with their isotopes, and the shapes of the clusters are quite helpful for their decisive determination. The quantitation was performed at the desolvation temperature of 210 $^{\circ}$ C since the peaks around m/z 690 increased at first and decreased subsequently with raising the temperature, showing the maximum at 210 $^{\circ}$ C. The highest peak at m/z 690 corresponds to $^{98}\text{Mo}(^{12}\text{C}_5^1\text{H}_{10}^{14}\text{N}^{32}\text{S}_2)_4$ and other peaks, to the mixture of isotopes (Mo of 92, 94, 95, 96, 97, 98, 100 and S of 32, 33, 34) with their natural abundances as shown in Fig. 1 (b). The same compound, $\text{Mo}(\text{DDC})_4^+$, was reported to be synthesized from $\text{Mo}(\text{CO})_6$, tetrabutylammoniumhalogenide and tetraethylthiuramdisulphide [7].

Next, we tried to find out a suitable metal ion for IS that gives distinctive signal from that of Mo under the same condition, and its concentration in biological materials is lower than 1/100 that of Mo. Ru showed peaks of $\text{Ru}(\text{DDC})_3^+$ around m/z 546 (not shown) and $\text{Ru}_2(\text{DDC})_5^+$ around m/z 944 as shown in Fig. 2 (a) where 20 pg of Ru was injected. Since peaks of $\text{Ru}(\text{DDC})_3^+$ around m/z 546 overlapped with those of $\text{Mo}(\text{DDC})_3^+$ around m/z 542, only peaks of $\text{Ru}_2(\text{DDC})_5^+$ around m/z 944 could be used as IS for $\text{Mo}(\text{DDC})_4^+$ around m/z 690 and vice versa. The peak height of 1 pg of Mo in the form of $\text{Mo}(\text{DDC})_4^+$ was the same as that of 5 pg of Ru in the form of $\text{Ru}_2(\text{DDC})_5^+$. Fig. 2 (a) indicates the detection limit of Mo in the MS using 1 μ L IAA solution containing 2 pg Mo. In the negative ion mode, however, Mo and Ru did

not show any peaks corresponding to their compounds under the same treatment as that in the positive ion mode.

Effects of chelating agents as well as other transition metals were compared. Ammonium pyrolidine dithiocarbamate showed their corresponding peaks in ESI-MS but SCN^- did not under the same condition as that of DDC, although both chelating agents produced respective paramagnetic Mo^{5+} compounds [3]. Previously we observed that DDC reacted with Mo 900 times more efficiently than SCN^- [3]. This fact might be one of the reasons why Mo-SCN complex could not be observed in ESI-MS, although SCN^- was used previously as a reagent in colorimetric determination of Mo. Mo in urine tends to react with 0.1 M DDC completely although it may bound weakly with some compounds in urine. This was proved by the following observation that urine untreated had the same Mo level as that of the urine wet-ashed by HNO_3 at 85 °C for 8 h or that of the urine oxidized with 10 % H_2O_2 at 25 °C for 18 h. The ESI-MS of 1 μL urine is shown in Fig. 3. The peaks observed at smaller than m/z 502 came from matrix materials and the highest peak in the range m/z 502–1000 was that of $\text{Mo}(\text{DDC})_4^+$. Therefore, Mo at m/z 690 could be quantitated using IS at m/z 944, $\text{Ru}_2(\text{DDC})_5^+$.

Transition metals such as Zr^{4+} , Fe^{3+} , Cu^{2+} , Mn^{2+} , Ti^{4+} , V^{5+} , Cr^{6+} and Cd^{2+} at 10^{-4} M, i.e., about 10 ng each, did not show peaks from m/z 650 to 1000 corresponding to their compounds under the same condition as that of Mo and Ru. This fact was confirmed by the study on urine as shown in Fig. 3. That is, only the peaks of $\text{Mo}(\text{DDC})_4^+$ were observed in urine although it contained several metals such as Fe and Cu reacting with DDC at higher concentrations than Mo.

Figure 2 (b) indicates chromatogram of $\text{Mo}(\text{DDC})_4^+$ of Mo standard solutions in SIM mode at m/z 690. Since a weak peak was observed in the IAA solution without Mo, i.e. blank, the detection limit was found to be 20 fg, i.e., 1 μL solution at 2×10^{-10} M Mo. The calibration curve was obtained by plotting the amount calculated by

the peak area ratio of Mo to IS (y) versus the amount of Mo (x) at six concentrations with six times determinations. With the limit of quantitation of 200 fg, good linearity was observed over the range from 200 fg to 200 pg for standard solutions, $y = 0.988x + 23$, $r^2 = 0.999$. The recoveries of Mo from urine were determined six times respectively by adding Mo at 0, 2, 20 and 200 pg/ μ L and Ru at 200 pg/ μ L to either urine or water. The recoveries were 92, 94 and 98 % at three concentrations mentioned above, respectively. Since sample could be injected every one min as shown in Fig. 2 (b), Mo levels of six persons were found to lie between 8 – 45 pg/ μ L urine within ten minutes. These values were confirmed with the values determined by electron paramagnetic resonance using 10 μ L of urine [3]. In the present ESI-MS, 1 μ L of urine showed the same isotopic abundances as those shown in Fig. 1 (b). Within-day and day-to-day (for 5 days) assays were performed using six urine samples stored at 4 °C, and variations of both assays were not greater than 10 %.

ESI-MS was found to be quite suitable for the sensitive quantitation of Mo and Ru as well as the analysis of coordination [1,2] of Mo and Ru. Irving and Williams proposed the order of complexation constants of transition metals to be $Fe \ll Cu$ [8]. Although present treatment did not show appreciable signals of Fe and Cu owing to their low sensitivity, a different treatment showed that the signal of $Fe \ll$ the signal of Cu, whereas another treatment, a strong signal of Fe. Therefore, this method can be applied to other metals and chelating agents by choosing reductants, oxidants and solvents that can produce weakly charged species suitable for ESI-MS.

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FIGURE CAPTIONS

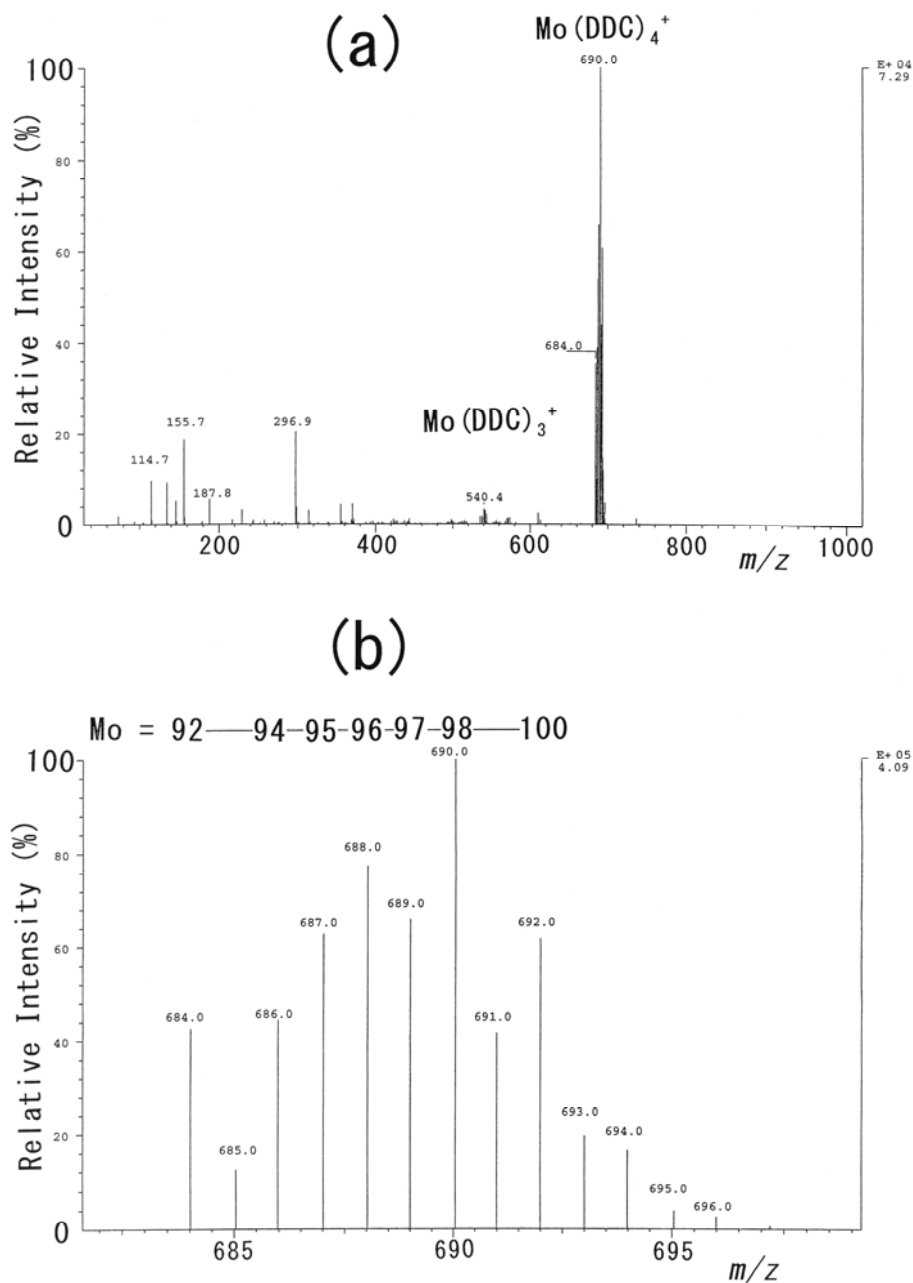


Figure 1. Mass spectrum of 1 μL of isoamyl alcohol containing 200 μg Mo measured at desolvation temperature of 170 $^\circ\text{C}$ and recorded from m/z 50 to 1000 (a), and that recorded from m/z 680 to 700 (b).

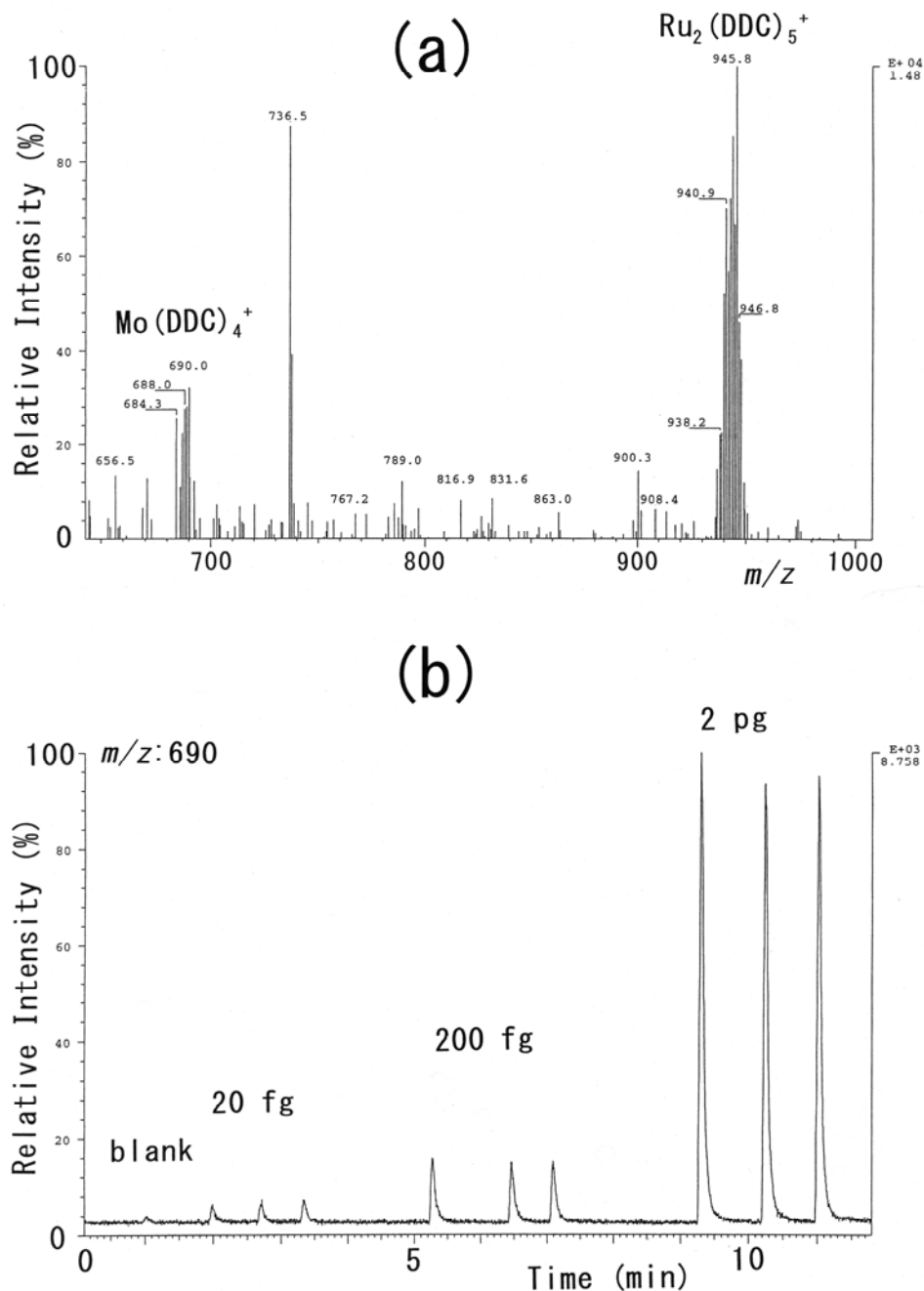


Figure 2. (a): Mass spectrum of 1 μL of isoamyl alcohol containing 2 pg Mo and 20 pg Ru measured at desolvation temperature of 210 $^{\circ}\text{C}$ and recorded from m/z 650 to 1000, indicating that the detection limit was 2 pg Mo in mass spectrum mode. (b): Chromatogram of Mo(DDC)_4^+ in selected ion monitoring mode at m/z 690 measured at desolvation temperature of 210 $^{\circ}\text{C}$. The applied amounts of Mo were 0, 20 fg, 200 fg and 2 pg, respectively, indicating that the detection limit was 20 fg Mo in selected ion monitoring mode.

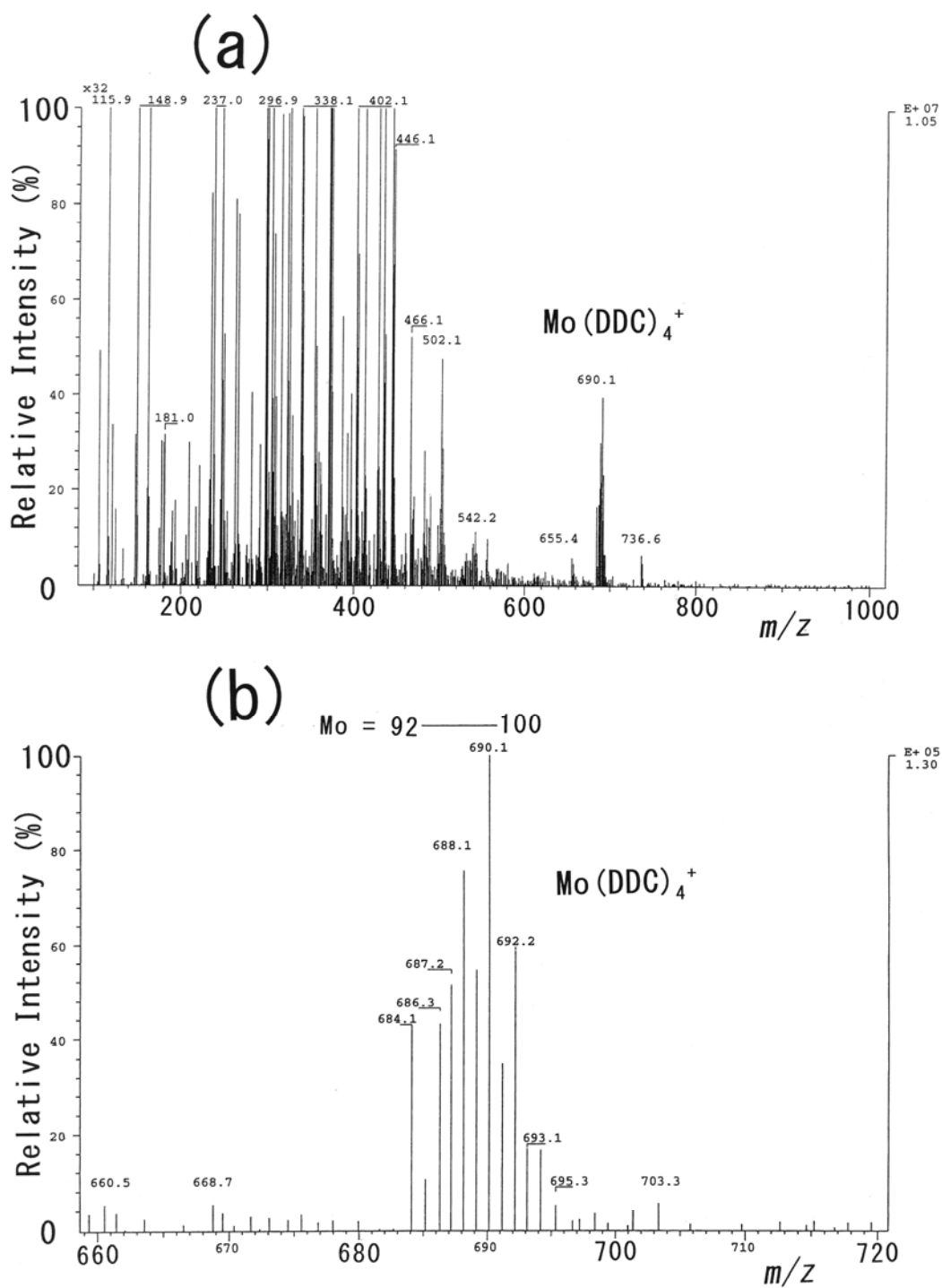


Figure 3. Mass spectrum derived from 1 μL of urine of a healthy man measured at desolvation temperature of 210 $^{\circ}\text{C}$ and recorded from m/z 100 to 1000 (a), and that recorded from m/z 660 to 720 (b). The content of Mo was 45 μg in 1 μL of urine.