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Abstract

Recently, cobalt (Co) is reported to be taken as a supplement by athletes for improving anaerobic performance. For the diagnosis of abuse, the limit of detection (LOD) of Co in the analysis should be lower than the concentrations of Co in plasma and urine of normal persons. A simple, rapid and sensitive method has been developed for the determination of Co in urine. Co was complexed with diethyldithiocarbamate (DDC) and extracted with isoamyl alcohol in the presence of citric acid. The detection of Co was achieved by injecting a 1 μl aliquot of isoamyl alcohol containing Co-DDC complex directly into an electrospray ionization tandem mass spectrometric (ESI-MS-MS) instrument without chromatographic separation. The quantification was performed using selected reaction monitoring at *m/z* 291 of the product ion $Co(C_4H_{10}NCS)_2^+$ which was produced by collision-induced dissociation from the precursor ion $Co(DDC)_2^+$ at *m/z* 355. ESI-MS-MS data were obtained in less than 10 min with an LOD of 0.05 $\mu g l^{-1}$ and a linear calibration range of 0.1 – 100 $\mu g l^{-1}$ using 10 μl of urine. The procedure was validated with certified reference materials (SRM 2670a and SRM 1643e). This method is suitable for the analysis of Co in the laboratories already equipped with an ESI-MS-MS instrument.

Key words: Cobalt; Tandem mass spectrometry; Electrospray ionization:

Diethyldithiocarbamate; Urine

1. Introduction

Cobalt (Co) is an essential element in humans necessary as a component of vitamin B_{12} that catalyzes several reactions, and $CoCl_2$ is known to enhance erythropoiesis and angiogenesis. There is an emerging evidence that Co is used as a supplement, resulting in increased erythrocyte concentrations occasionally observed in athletes [1,2]. Blood tests were introduced at the Sydney Olympic Game [3] to examine the injection of erythropoietin for athletes who were involved in endurance sports such as swimming, rowing and running. Co is, however, not currently comprehended within the World Anti-Doping Agency prohibited list [1,2], and the threshold of Co levels in plasma and urine had not been reported yet. Excessive administration of Co, however, impairs thyroid activity and myocardial function, and promotes carcinogenesis [2,4]. The limit of detection (LOD) of Co in the analysis should be lower than the concentration, ca. 0.3 $\mu g l^{-1}$, in plasma and urine of normal persons [5-10] to discriminate the persons abusing Co. However, the LOD of Co in the analysis based on conventional inductively coupled plasma (ICP) mass spectrometry (MS) was unsuitable; the LOD was 0.7 $\mu g l^{-1}$ using 10 ml of urine in the determination of 13 elements, i.e., Be, Cr, Mn, Co, Mo, Sn, Sb, Cs, Ba, W, Pt, Tl and Pb together [7], and the interferences due to polyatomic isobars such as $^{36}Ar^{23}Na$, $^{43}Ca^{16}O$ and $^{40}Ar^{18}OH$ corresponded to such a high level as 0.4 $\mu g l^{-1}$ in serum [6].

To obtain much better sensitivity using MS that can afford the decisive determination of Co, electrospray ionization (ESI) tandem mass spectrometry (MS-MS)

has been used in the present method. At first, Co was complexed with diethyldithiocarbamate (DDC, $(C_2H_5)_2NCSS^-$) and extracted with isoamyl alcohol (IAA) in the presence of citric acid. A 1 μl aliquot of IAA layer containing Co-DDC complex was directly injected into a triple quadrupole mass analyzer without chromatographic separation. The first quadrupole serves then to isolate the precursor ion $Co(DDC)_2^+$ for further analysis, the second one functions as a collision cell for collision-induced dissociation, and the third one separates the product ion $Co(C_4H_{10}NCS)_2^+$. This method was applied to the determination of Co in 10 μl of urine. The merit and demerit of the present method were compared with those of newly developed sector-field ICP-MS instruments [9] and ICP-MS instruments with a collision cell [10].

2. Experimental

IAA suitable for nucleic acid purification was obtained from Sigma-Aldrich, USA. Atomic absorption standard solutions (AASS) of Co and 20 other metals, atomic absorption grade 35 % HCl, and other reagents of analytical grade were obtained from Wako Pure Chemicals, Japan. Pure water, having a specific resistance of 18 MΩcm was used. Urine was obtained from healthy volunteers and the mixture was called as urine, hereafter. Calibration standard solutions and quality control solutions of Co were prepared daily by spiking AASS of Co at concentrations of 0, 0.1, 1, 10 and 100 $\mu g l^{-1}$ to urine or 0.15 M NaCl solution. Standard reference materials of urine (SRM 2670a) and water (SRM 1643e) were purchased from the National Institute of Standards and Technology, Gaithersburg, MD, USA.

Co was assayed as follows. To 10 μl of urine or standard solution in a polypropylene tube (Eppendorf AG, Hamburg, Germany), a 1 μl aliquot of 1 M DDC was added and mixed for 30 s with a vortex mixer. Then, a 1 μl aliquot of 2 M citric acid and 10 μl of IAA were added and mixed for 30 s, and then centrifuged at 5000 g for 30 s.

ESI-MS-MS analysis was performed on a TSQ 7000 LC-quadrupole mass spectrometer (Thermo Quest, Japan) in the positive ion mode. A syringe (1701 PTFE- coated, Hamilton Co. Reno, NV, USA) was washed with methanol more than 5 times and with IAA sample itself twice, respectively, before the injection of each sample. Using a direct-injection apparatus attached just before a detection assembly, a 1 μl aliquot of IAA sample in the syringe was injected manually within 1 s into methanol that was flowed continuously as the carrier at 200 $\mu l min^{-1}$ through the detection assembly. It means that the detection assembly is washed automatically with methanol for 59 s when a sample is injected every 60 s. The capillary temperature was set at 280 ℃. The electrospray voltage was set at 4.5 kV, the multiplier voltage at 1.3 kV and the collision voltage at 20 - 50 V, respectively. Nitrogen was used as sheath gas (469 kPa) and also as an auxiliary gas (8 units), and argon was used as collision gas (134 kPa). MS-MS spectrum was collected from *m/z* 10 to *m/z* 410 with a scan time of 0.8 s, and stored in a computer every 0.8 s continuously. Figure 1 is an example of a scan. For the quantification, the product ions in the range of *m/z* 290.7±0.2 that were derived from the precursor ion at *m/z* 354.8±0.3 were accumulated every 0.5 s. The total amounts of product ions were recorded in a computer every 0.5 s continuously, and were plotted as a function of time, forming a

peak. The peak area was integrated, and the amount of Co was quantified using a calibration curve made up with spiked matrix samples at different concentrations.

3. Results and Discussion

Stability of Co-DDC complex and interferences

At first, a suitable acid for the production of a ternary Co-DDC complex was examined, as explained in our ESI-MS work on chromate [11]. The rate of production in 0.2 M ascorbic acid, 0.2 M oxalic acid, 0.2 M HCl, 0.1 M H_2SO_4, 0.2 M HNO_3 and 0.07 M H_3PO_4 were found to be 100, 50, 40, 30, 20 and 10 %, respectively, by taking the rate in 0.2 M citric acid as 100 %. Since the rate in citric acid was almost the same in the concentration range from 0.06 M to 0.5 M, 0.2 M citric acid was selected. IAA was chosen for the extraction of Co-DDC complex since the percentages of back ground signal to the signal of 0.1 pg Co at *m/z* 291 were roughly 20, 50 and 80 % in IAA, octanol and hexanol, respectively. Co-DDC complex extracted with IAA was quite stable; 90 % of the complex remained after 24 h in the extract from a 0.15 M NaCl solution and after 5 h in the extract from urine, respectively, under room light at 25 ℃.

DDC is well known to form chelate complex with various transition metals. We confirmed, however, that neither the signal of Co at *m/z* 355 nor that at *m/z* 291 was interfered with by the following metals, i.e., Ti, V, Cr, Mn, Fe, Ni, Cu, Zn, As, Zr, Mo, Ru, Pd, Ag, Cd, W, Pt, Au, Tl and Pb under the present assay.

MS-MS spectrum

Figure 1 shows the mass spectrum of product ions with a collision voltage of 25 V. Product ions containing Co such as $Co(C_4H_{10}NCS)_2^+$ at *m/z* 291, $CoDDCH^+$ at *m/z* 208

and $CoC_4H_9NCS^+$ at *m/z* 174 were the main ions with collision voltages at 25, 27 and 30 V, respectively. Fig. 1 also shows product ions such as $C_4H_{10}NCS^+$ at *m/z* 116, $H(C_2H_5)NCS^+$ at *m/z* 88 and $C_4H_{10}N^+$ at *m/z* 72, respectively [12,13]. For the quantification of Co, a mass chromatogram of the product ion $Co(C_4H_{10}NCS)_2^+$ after dissociation from the precursor ion $Co(DDC)_2^+$ at a collision voltage of 25 V was selected because it was the most abundant ion among the three product ions containing Co.

Precision and accuracy

Calibration standard solutions of Co were prepared by spiking AASS at concentrations of 0, 0.1, 1, 10 and 100 μgl^{-1} to 0.15 M NaCl solution and urine. The peak-width increased as the concentration increased, and the increment of the peak-width in urine was larger than that in 0.15 M NaCl solution. The concentrations of Co determined from not the peak height but the peak area (y) were linear to the concentrations spiked (x) up to 100 μgl^{-1}, $y = 1.0024\ x + 0.0551$ with a correlation coefficient of 0.9999 in 0.15 M NaCl solution and $y = 0.9978\ x + 0.0867$, with a correlation coefficient of 0.9991 in urine, respectively.

A 0.15 M NaCl solution and urine that were not spiked with Co were measured 6 times and their standard deviations (σ) were calculated in pg based on the calibration, respectively. Since an LOD was defined as 3σ for the reagent blank [8], LODs in the present assay were calculated to be 0.03 μgl^{-1} and 0.05 μgl^{-1} in 0.15 M NaCl solution and urine, respectively.

Precision and accuracy were assessed by the analysis of 0.15 M NaCl solution and urine spiked at 0.1, 1, 10 and 100 μgl^{-1}, respectively, as listed in Table 1. These

samples were analyzed three times a day as well as on three different days. The coefficient of variation was less than 17.8 %, and accuracy was between 81.1 to 121.8 % for both intra-day and inter-day variations in any samples, even urine spiked at 0.1 μgl^{-1}.

To check the recovery and validate the proposed procedure, standard reference materials were examined. The certified Co levels of low level urine 2670a, high level urine 2670a and water 1643e were 0.166±0.04, 51.2±3.2 and 27.06±0.32 μgl^{-1}, and those we obtained were 0.18±0.03, 50.3±2.4 and 26.8±1.1 μgl^{-1}, respectively. The recoveries of Co were within 100±8 % in these three reference materials. These high recoveries may be due to the strong affinity of Co to DDC as well as to the high solubility of the Co-DDC complex in IAA. Likewise, complete recoveries of Co complexes with dithiocarbamate derivatives were reported in the extraction with one-volume of trichloro-trifluoroethane from 17-volumes of seawater [15] and with one volume of diisopropylketone from 4-volumes of urine [6].

Comparison with other MS methods

The LOD of the presented ESI-MS-MS method is better than that of conventional ICP-MS method [6,7] and can quantify the concentration of Co in urine of normal persons. Recently, however, new ICP-MS such as sector-field ICP-MS [9] and ICP-MS with a collision cell [10] were developed. The advantages of the present method over ICP-MS are as follows. Firstly, the required sample volume is about one hundredth of that of ICP-MS. Secondly, in the analysis of single-mass metals such as Co and As, the present method shows several lines for each metal due to product ions that provide more confidential identification, whereas ICP-MS can show

only a single line for each metal. Thirdly, ESI-MS (-MS) can determine the valence state of the metal [14,15]. However, the disadvantages of the presented ESI-MS-MS method over the new ICP-MS method are as follows. Firstly, the LOD of the presented method, 0.05 μgl^{-1} in urine, is higher than that of new ICP-MS method such as 0.02 μgl^{-1} [9] or 0.005 μgl^{-1} [10]. Secondly the presented method can analyze only several elements having similar chemical properties such as Co, chromate [14], molybdenum and ruthenium [15] together, whereas ICP-MS can detect quite a larger number of elements together [6,7,9,10].

In conclusion, this method enables the determination of metals in the laboratories equipped with only ESI-MS (-MS) instrument that is currently used for the analysis of organic molecules. The determination of metals is usually performed on ICP-MS and atomic absorption (or emission) spectroscopy, although these methods cannot be used for the analysis of organic molecules.

Table 1

Intra-day (3 times) and inter-day (3 days) variations of Co values were determined by the presented method. The observed values and coefficients of variations (C.V.) were listed for Co spiked at 0.1 -100 $\mu g l^{-1}$ in 0.15 M NaCl solution (A) and urine (B), respectively.

	Intra-day				Inter-day			
	A		B		A		B	
Spiked Co $\mu g l^{-1}$	Observed Co $\mu g l^{-1}$	C.V.	Observed Co $\mu g l^{-1}$	C.V.	Observed Co $\mu g l^{-1}$	C.V.	Observed Co $\mu g l^{-1}$	C.V.
0.1	0.096	2.9	0.122	5.5	0.120	13.5	0.081	17.8
1	1.00	7.6	1.02	2.8	0.984	2.9	0.998	9.2
10	10.1	1.0	10.1	3.4	9.98	3.6	10.0	4.1
100	101	4.0	99.2	3.7	98.8	1.4	101	2.1

Figure caption

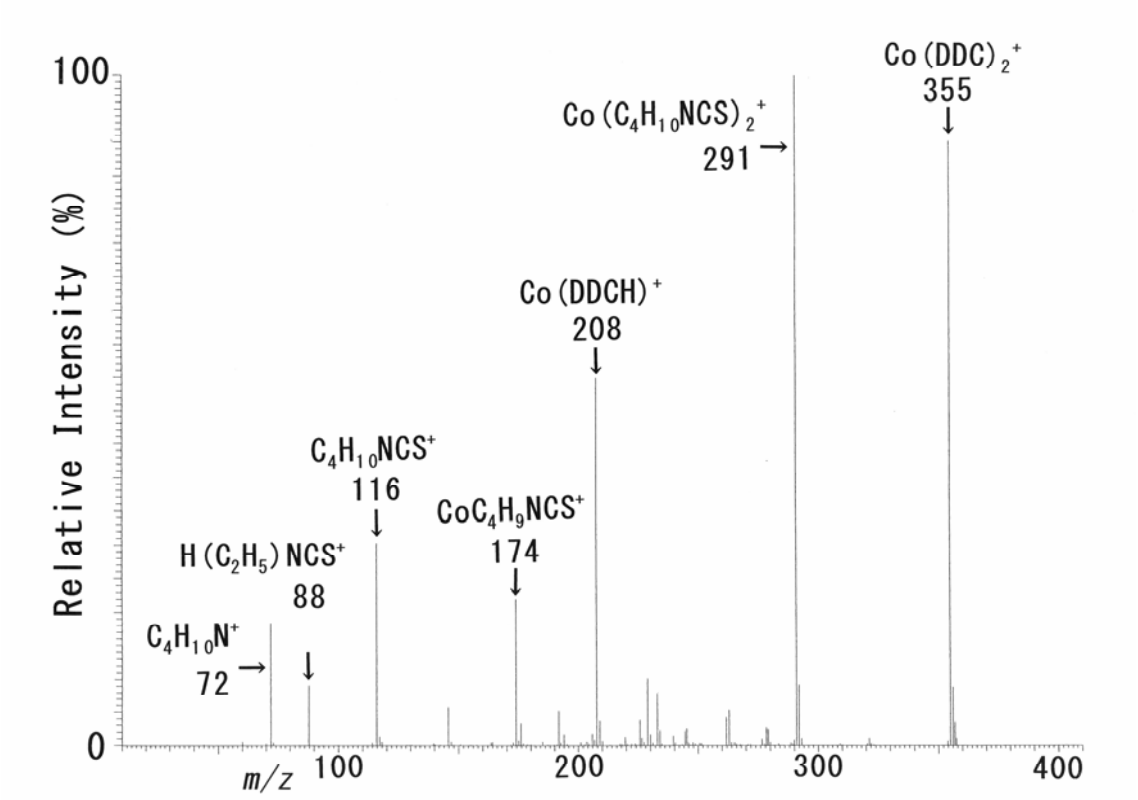

Figure 1: Mass spectrum after collision-induced dissociation of $Co(DDC)_2^+$ at the collision voltage of 25 V. Not only the ions containing Co such as $Co(DDC)_2^+$ at *m/z* 355, $Co(C_4H_{10}NCS)_2^+$ at *m/z* 291, $CoDDCH^+$ at *m/z* 208 and $CoC_4H_9NCS^+$ at *m/z* 174 but also ions such as $C_4H_{10}NCS^+$ at *m/z* 116, $H(C_2H_5)NCS^+$ at *m/z* 88 and $C_4H_{10}N^+$ at *m/z* 72 were observed.

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