Trace Determination of Cyanocobalamin (Vitamin B₁₂) by Analyzing Cobalt Using a Portable Total-Reflection X-ray Fluorescence Spectrometer

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A method is presented for the detection of a trace amount of cyanocobalamin (vitamin B₁₂) using a combination of solid-phase extraction and the measurement of cobalt in vitamin B₁₂ by a portable total-reflection X-ray fluorescence spectrometer. Using this combination, a detection limit of 1 ng/mL was achieved for vitamin B₁₂.

Keywords Cobalt, solid-phase extraction, portable, total reflection X-ray fluorescence, vitamin B₁₂

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Introduction

Vitamin B₁₂ (cyanocobalamin) (molecular weight 1355.4) is a complex containing a cobalt ion, and is an essential nutrient for humans. Dietary supplements and pharmaceuticals to which vitamin B₁₂ is added are useful to compensate for a deficiency of vitamin B₁₂, and it is important to analyze this substance in these products in order to evaluate their quality. A microbiological assay is usually used to analyze vitamin B₁₂. For example, Kelleher *et al.*¹ analyzed vitamin B₁₂ in serum samples using a microbiological assay, and reported that the detection limit was below 20 ng/L. Although highly sensitive vitamin B₁₂ analysis is performed by a microbiological assay, this analytical method is time-consuming because culturing microorganisms is required for the determination of vitamin B₁₂. High-performance liquid chromatography (HPLC) methods are also employed for analyzing cobalamin species, such as cyanocobalamin (vitamin B₁₂) and hydroxocobalamin. Li *et al.*² analyzed vitamin B₁₂ using HPLC with fluorescence detection, and achieved a detection limit of 0.1 ng/mL. Makarov *et al.*³ performed HPLC with inductively coupled plasma mass spectrometry (ICP-MS) detection for the analysis of cobalamin species, and obtained detection limits of 4–8 ng/mL for them. Marszałł *et al.*⁴ performed HPLC with coulometric electrochemical detection for the determination of vitamin B₁₂, and achieved a detection limit of 0.08 ng/mL. HPLC methods make it possible to rapidly perform speciation analysis of cobalamin species, although the detection limits of vitamin B₁₂ obtained with these methods are higher (worse) than that obtained with a microbiological assay.

Total-reflection X-ray fluorescence (TXRF) analysis^{5–7} is a suitable method for the trace elemental analysis of solution samples. Portable TXRF spectrometers,^{8–10} which can be applied to on-site trace elemental analysis, have been developed since

2006. The dry residue of a solution sample on a sample holder, such as a quartz glass substrate, a diamond-like carbon (DLC) coated quartz glass substrate (DLC sample holder),¹¹ or a collodion film,^{12,13} is analyzed by these portable spectrometers. A detection limit of 8 pg,¹⁰ which was equivalent to 8 ng/mL in 1 µL of a sample solution, was obtained for chromium by using a portable TXRF spectrometer. A trace amount of cobalt in vitamin B₁₂ is able to be determined by these portable TXRF spectrometers, and therefore they make it possible to determine trace amounts of vitamin B₁₂. However, the separation of vitamin B₁₂ is needed to be performed before any TXRF measurement in order to selectively detect cobalt originating from vitamin B₁₂. Solid-phase extraction is a simple method for the separation and enrichment of target substances, and it is used as a pretreatment for vitamin B₁₂ analysis. Kuroda *et al.*¹⁴ conducted solid-phase extraction of vitamin B₁₂ using a phenyl group bonded silica gel before a microbiological assay. Moradi *et al.*¹⁵ performed solid-phase extraction using graphene oxide for the collection and enrichment of vitamin B₁₂, and achieved a detection limit of 20 µg/L for vitamin B₁₂ by measuring the Co Kα line originating from enriched vitamin B₁₂ using X-ray fluorescence (XRF) analysis. In the present study, solid-phase extraction was performed as a pretreatment for the detection of a trace amount of vitamin B₁₂ using a portable TXRF spectrometer. We show that the detection limit of vitamin B₁₂ obtained in the present study was comparable to those obtained by HPLC methods.

Experimental

The details of a portable TXRF spectrometer used in the present study were reported in a previous paper.¹⁰ However, the setup of the spectrometer was changed in the present study, as summarized here. An X-ray tube 50 kV Magnum (Moxtek Inc., Orem, UT) with a tantalum anode was operated at 25 kV and 0.2 mA. An X-ray waveguide consisting of two parallel silicon

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wafers was used as the collimator. The size of the entrance aperture of the waveguide for X-rays was 0.03 mm in height and 1 cm in width, and the size of the exit aperture for X-rays was the same as that of the entrance aperture. A silicon drift detector VITUS-SDD (Ketek GmbH, Munich, Germany) with an active area of 7 mm² was used. Sample solutions were placed and dried on DLC sample holders. The size of quartz glass substrates (Sigmakoki Co., Ltd., Hidaka, Japan) was 30 mm in length, 30 mm in width, and 5 mm in thickness, and their flatness was $\lambda/20$ ($\lambda = 632.8$ nm). The thickness of DLC films on the quartz glass substrates was 1 μ m in the present study, and DLC coating was conducted by Nanotec Co. (Kashiwa, Japan). The sample holder on which the sample residue was present was tilted at 0.04° to the horizontal during the measurement.

A stock solution of vitamin B₁₂ was prepared by dissolving 10.13 mg of vitamin B₁₂ (cyanocobalamin) (FUJIFILM Wako Pure Chemical, Co. (formerly Wako Pure Chemical Industries, Ltd.)) into 100 mL of distilled water. Solutions containing 0.4, 0.1, and 0.04 μ g/mL of vitamin B₁₂ were prepared by diluting the stock solution of vitamin B₁₂ with distilled water. A stock solution of 0.4 μ g/mL of cobalt was prepared from a 1000 mg/L cobalt standard solution (Co(NO₃)₂ in 0.1 mol/L HNO₃) (FUJIFILM Wako Pure Chemical, Co.) and distilled water. A 10- μ L drop of the stock solution of 0.4 μ g/mL of cobalt was placed and dried on a DLC sample holder. Bond Elut PH cartridges (bed mass 100 mg; volume 10 mL) (Agilent Technologies, Inc., CA, USA), which are phenyl group bonded silica based columns, were used to collect and enrich vitamin B₁₂. The following procedure, which was partly changed from the procedure reported by Kuroda *et al.*,¹⁴ was used for solid-phase extractions of the solutions containing 0.4, 0.1, and 0.04 μ g/mL of vitamin B₁₂ and the stock solution of 0.4 μ g/mL of cobalt.

1. Two milliliters of 40% ethanol were passed through the solid-phase extraction column.
2. Two milliliters of distilled water were passed through the column.
3. Ten milliliters of a sample solution were passed through the column, and then the column were washed with 10 mL of distilled water.
4. Chemical species captured on the column were eluted with 0.4 mL of 40% ethanol, and the eluate was collected.

The pressure of the bottom side of the solid-phase extraction column was reduced by using a diaphragm pump during solid-phase extraction. Ten microliter drops of the eluates obtained after solid-phase extractions were placed and dried on DLC sample holders. The dry residue of a total volume of 50 μ L of the eluate obtained after solid-phase extraction of the solution containing 0.04 μ g/mL of vitamin B₁₂ was also prepared by placing and drying a 10- μ L drop of it on a DLC sample holder five times. The spectra of all analytes were measured in air.

Results and Discussion

Figure 1 shows the TXRF spectra of the dry residues of 10 μ L drops of the stock solution of 0.4 μ g/mL of cobalt and the eluate obtained after solid-phase extraction of this solution. These spectra were measured for 600 s. A strong Co *K* α line appears in Fig. 1a and a weak Co *K* α line can be seen in Fig. 1b. The Si peaks from the sample holders were also detected. Argon was detected because the measurements were performed in air containing 0.9% of argon. The Ta L lines appear in Fig. 1

Fig. 1 Spectra of the dry residues of 10 μ L drops of (a) a stock solution of 0.4 μ g/mL of cobalt and (b) the eluate obtained after solid-phase extraction of this solution. The inset in Fig. 1b shows an enlarged spectrum in the X-ray energy range from 5 to 10 keV.

because scattering of the Ta L lines from the X-ray tube reached the X-ray detector. The S, Cl, K, Ca, Ti, Fe, and Zn peaks would originate from contamination during sample preparation. A weak Ni peak was also detected in Fig. 1b. Figure 2 shows the TXRF spectra of the dry residues of 10 μ L drops of the eluates obtained after solid-phase extractions of the solutions containing 0.4, 0.1, and 0.04 μ g/mL of vitamin B₁₂. These spectra were measured for 600 s. Figure 3 shows the relationship between the net intensity of the Co *K* α line and the concentration of vitamin B₁₂. As shown in Fig. 2, cobalt originating from vitamin B₁₂ was detected. The net intensities of the Co *K* α lines in Figs. 2a, 2b, and 2c were 10157 counts/600 s, 2798 counts/600 s, and 914 counts/600 s, respectively, and the net intensity of the Co *K* α line was approximately linear with respect to the concentration of vitamin B₁₂, as shown in Fig. 3. The detection limit (DL) (ng/mL) for cobalt can be calculated using the following equation:¹⁶

$$DL = \frac{3C}{I_{\text{Net}}} \sqrt{I_{\text{BG}}}, \quad (1)$$

where *C* is the concentration of cobalt (ng/mL), *I*_{Net} is the net intensity of the Co *K* α line (counts), and *I*_{BG} is the background intensity of the Co *K* α line (counts). In the present study, the detection limit for vitamin B₁₂ was calculated by substituting the

Fig. 2 Spectra of the dry residues of 10 μL drops of the eluates obtained after solid-phase extractions of solutions containing (a) 0.4 $\mu\text{g/mL}$, (b) 0.1 $\mu\text{g/mL}$, and (c) 0.04 $\mu\text{g/mL}$ of cyanocobalamin (vitamin B₁₂). The inset in Fig. 2c shows an enlarged spectrum in the X-ray energy range from 5 to 10 keV.

vitamin B₁₂ concentration instead of the cobalt concentration into Eq. (1). A detection limit of vitamin B₁₂ obtained from Fig. 2c was 6 ng/mL. Figure 4 shows the TXRF spectrum of the dry residue of a total volume of 50 μL of the eluate obtained after solid-phase extraction of the solution containing 0.04 $\mu\text{g/mL}$ of vitamin B₁₂, which was measured for 1800 s. A detection limit of vitamin B₁₂ obtained from Fig. 4 was 1 ng/mL.

The intensity of the Co K α line in Fig. 1b was much lower than that in Fig. 1a. This was because the ratio of the amount of

Fig. 3 Relationship between the net intensity of the Co K α line and the concentration of vitamin B₁₂.

Fig. 4 Spectrum of the dry residue of a total volume of 50 μL of the eluate obtained after solid-phase extraction of the solution containing 0.04 $\mu\text{g/mL}$ of vitamin B₁₂.

cobalt collected into the eluate obtained by solid-phase extraction to the total amount of cobalt in the solution of inorganic cobalt species was significantly low. The net intensity of the Co K α line in Fig. 2a was about 0.7-times as high as that in Fig. 1a. The difference between the net intensities of the Co K α lines in Figs. 1a and 2a was small, although the concentration of cobalt in the solution containing 0.4 $\mu\text{g/mL}$ of vitamin B₁₂ was calculated to be about 0.04-times as high as 0.4 $\mu\text{g/mL}$. This was because vitamin B₁₂ was successfully collected into the eluate obtained by solid-phase extraction, and it was enriched. The concentration of cobalt in the solution containing 0.04 $\mu\text{g/mL}$ of vitamin B₁₂ was calculated to be about 0.004-times as high as 0.4 $\mu\text{g/mL}$. However, the intensity of the Co K α line in Fig. 2c was higher than that in Fig. 1b. This result indicates that vitamin B₁₂ and dissolved inorganic cobalt species can be separated with high efficiency by using a solid-phase extraction column. The net intensity of the Co K α line almost linearly increased with the increase in the concentration of vitamin B₁₂, as shown in Fig. 3. This result shows that the recovery rate of vitamin B₁₂ was approximately constant without depending on its concentration. The measurement of cobalt in the eluate obtained by solid-phase extraction using the portable

spectrometer makes it possible to determine the concentration of vitamin B₁₂ with high accuracy in the range of the vitamin B₁₂ concentration from 0.04 to 0.4 µg/mL. The Fe Kβ line (7.06 keV) that partially overlaps with the Co Kα line (6.93 keV) can cause aggravation of the accuracy of the quantitative value of vitamin B₁₂. For example, a peak separation method using curve fitting should be employed to determine the net intensity of the Co Kα line when the Fe Kβ intensity is high enough not to be negligible, as shown in Fig. 1b. The detection limit of vitamin B₁₂, which was obtained by the combination of solid-phase extraction and the measurement of cobalt using the portable spectrometer, was improved by extending the measurement time and increasing the volume of the sample solution dried on the sample holder. The detection limit obtained from Fig. 4 was comparable to those obtained by HPLC methods with fluorescence,² ICP-MS,³ and electrochemical⁴ detections.

Conclusions

In the present study, solid-phase extraction was performed as a pretreatment in order to detect a trace amount of vitamin B₁₂ using a portable TXRF spectrometer, and a detection limit of 1 ng/mL was achieved. This detection limit was comparable to those obtained by HPLC methods. A combination of solid-phase extraction for the separation and enrichment of vitamin B₁₂ and TXRF analysis of cobalt in vitamin B₁₂ using the portable spectrometer can be applied to quality evaluations of dietary supplements and pharmaceuticals to which vitamin B₁₂ is added in the production process.

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