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LETTER TO THE EDITOR

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Elevated urinary cobalt concentrations identified in routine doping controls can originate from vitamin B_{12}

The transition metal cobalt is categorized as an essential trace mineral. In the context of human nutrition, cobalt is critical to the formation of the organometallic complexes referred to as cyanocobalamins. The most important member of this group is vitamin B_{12}. In addition, cobalt salts have been reported as constituents of dietary supplements administered orally at approximately 1 mg/day.[1] Ionic cobalt (Co^{2+}) acts as an efficient erythropoiesis stimulating agent due to its hypoxia-inducible factor-activating effects. Before recombinant erythropoietin became available in 1987,[2] inorganic cobalt in the form of cobalt(II) chloride ($CoCl_2$) was commonly prescribed and orally administered at dosages of up to 300 mg/day for the treatment of anemia.[3] Various studies demonstrated the short-term polycythemic effects of $CoCl_2$[4,5] and, consequently, inorganic cobalt became the subject of intensive debate regarding its potential role as a doping agent.[6-8]

In 2015, cobalt was added to the World Anti-Doping Agency's (WADA's) Prohibited List,[9] and the use of products resulting in the uptake of pharmacologically relevant amounts of inorganic cobalt has been banned for athletes at all times (in- and out-of-competition). By contrast, the supplementation of organically bound cobalt is permitted. To date, doping control urine samples are commonly analyzed for total cobalt concentrations by means of inductively coupled plasma mass spectrometry (ICP-MS). Although no threshold or reporting level has yet been established above which urinary cobalt concentrations are to be considered as an adverse analytical finding, urinary reference concentrations were reported to range between 0.1 and 2 ng/mL.[10-14] Exceptionally high levels were found in urine samples of miners and inhabitants of the immediate environment of cobalt mines and workers of hard metal factories.[15-17] Furthermore, individuals having received endoprotheses were shown to exhibit elevated blood and/or urine cobalt concentrations, commonly referred to as arthroprosthetic cobaltism.[1,18-20]

In June 2019, a routine doping control urine sample was tested for cobalt and returned an atypically high level of cobalt of 24.3 ng/mL, which was neither in agreement with pilot study data on a small number of athletes[10] nor did the tested individual belong to a specific subgroup of the aforementioned categories that occupationally tend to present elevated urinary cobalt concentrations. Therefore, follow-up studies aiming at differentiating inorganic and organically bound cobalt were initiated. The urine sample was therefore subjected to solid-phase extraction (SPE, Oasis® HLB, Waters, Eschborn, Germany) to deplete potentially contributing cyanocobalamin, and the flow-through was analyzed using ICP-MS. The cobalt concentration of the SPE-depleted specimen was found below the assay's limit of quantification (< 0.5 ng/mL), and a separate aliquot of the original urine sample was reanalyzed for cyanocobalamin by high-performance liquid chromatography (HPLC) coupled to high-resolution/high accuracy tandem mass spectrometry (HRMS/MS). Here, an Accela HPLC system (Pump 1250 and Open AS) and a Q Exactive mass spectrometer from Thermo Scientific (Bremen, Germany) were used. Chromatographic separation was accomplished using a reversed-phase Nucleodur EC C18-Pyramid (50 × 2 mm; 1.8 µm particle size) analytical column from Macherey-Nagel (Düren, Germany), and gradient elution was carried out with 0.1% formic acid as solvent A and acetonitrile as B. The injected sample volume was set to 5 µL, and the flow rate was set to 200 µL/min. The gradient program started at 100% A, maintained at 100% A for 1 min, decreased to 60% A within 4 min, and decreased further to 10% A within 3 min. Finally, re-equilibration at 100% A was conducted for 3 min at an increased flow rate of 350 µL/min.

For the mass spectrometric detection of cyanocobalamin, full-MS and targeted-MS^2 experiments were performed in positive electrospray ionization (ESI+) mode with a resolving power of 35 000 full width at half maximum. Full-MS experiments were performed from *m/z* 100 to 800 and conducted with an automatic gain control (AGC) target of $5e^6$. In addition, targeted-MS^2 experiments using the doubly protonated molecule at *m/z* 678.29 were carried out using an inclusion list with an isolation width of 1.5 *m/z* and an AGC target of $2e^5$. Best results for specific product ions were obtained with a normalized collision energy of 30%. For the estimation of cyanocobalamin amounts in the suspicious sample, a matrix-matched

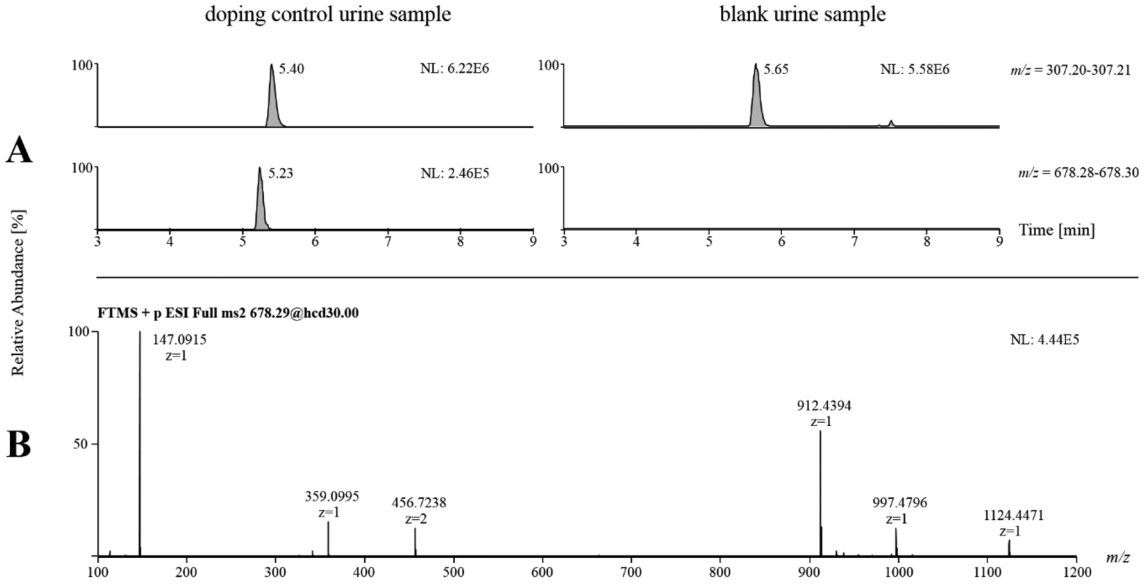

FIGURE 1 High-resolution high accuracy mass spectrometric (HRMS) detection of doubly charged cyanocobalamin at *m/z* 678.2913 is presented. A, extracted ion chromatograms (electrospray ionization +, full-mass spectrometry [full-MS]) of the suspicious urine sample (top-left) and a blank sample (top-right). Chromatograms of the ISTD (*m/z* 307) are depicted in the first row; chromatograms of cyanocobalamin (*m/z* 678) are illustrated below. B, the corresponding HRMS product ion mass spectrum with diagnostic product ions of the intact organic vitamin B_{12} complex (athlete's urine sample), which are in line with earlier published MS data[21]

calibration curve prepared with a reference standard (cyanocobalamin from Merck, Darmstadt) at seven concentration levels (0, 50, 100, 200, 500, 1000, and 2000 ng/mL) was employed. In consideration of the initially ICP-MS-measured total Co^{2+}concentration of 24.3 ng/mL, ca. 560 ng/mL of cyanocobalamin would be required to account for the observed total cobalt level.

Cyanocobalamin was unequivocally identified in the doping control urine sample by HRMS (Figure 1). The intact molecular ion was observed in full-MS measurements, and diagnostic product ions obtained by collision-induced dissociation were detected and matched previous publications.[21] It is noteworthy that a concentration of 565 ng/mL was calculated using the prepared calibration curve, computed by signal area ratios of the target analyte and the employed internal standard (fivefold deuterated isoxsuprine, ISTD).

The presented data indicate the importance of the careful assessment of total urinary cobalt concentrations in the context of routine sports drug testing programs[22,23] and, potentially, also in other clinical applications. The determination of inorganic cobalt and the differentiation from cyanocobalamin can be accommodated by simple SPE-based depletion of the cobalt-containing organic molecule and subsequent analysis of the extract's flow-through by ICP-MS. Alternatively, routine tests by means of LC-ICP-MS and/or concomitant analyses of urine samples for vitamin B_{12} are recommended for adequate result interpretation and management.

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