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Analysis of cobalt for human sports drug testing purposes using ICP- and LC-ICP-MS

Andre Knoop[1] | Peter Planitz[2] | Bernhard Wüst[2] | Mario Thevis[1,3]

[1]Institute of Biochemistry, Center for Preventive Doping Research, German Sport University Cologne, Cologne, Germany

[2]Agilent Technologies GmbH und Co. KG, Waldbronn, Germany

[3]European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany

Correspondence
Andre Knoop, Institute of Biochemistry, Center for Preventive Doping Research, German Sport University Cologne, Am Sportpark Müngersdorf 6, Cologne 50933, Germany.
Email: a.knoop@biochem.dshs-koeln.de

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Abstract

Due to the current demands in the fight against manipulation of blood and blood components, commonly referred to as "blood doping" in sports drug testing, specific and sensitive detection methods enabling the detection of prohibited substances and methods of doping are required. Similar to illicit blood transfusions, erythropoiesis stimulating agents have been shown to be misused in sport, aiming at improving an athlete's aerobic capacity and endurance performance. Amongst other strategies, the administration of ionic cobalt (Co^{2+}) can increase the number of erythrocytes by stimulating the endogenous erythropoietin (EPO) biosynthesis. Conversely, several organic Co-containing compounds such as cyanocobalamin (vitamin B12) are not prohibited in sports, and thus, an analytical differentiation of permitted and banned contributions to urinary Co-concentrations is desirable. An excretion study with daily applications of either 1 mg of $CoCl_2$ or 1 mg of cyanocobalamin was conducted with 20 volunteers over a period of 14 consecutive days. Urine, plasma, and concentrated red blood cells were analyzed for their cobalt content. The samples were collected starting 7 days before the administration until 7 days after. Total Co concentrations were analyzed by using inductively coupled plasma mass spectrometry (ICP-MS), which yielded significantly elevated levels exclusively after inorganic cobalt intake. Furthermore, a liquid chromatography (LC)-ICP-MS approach was established and employed for the simultaneous determination of organically bound and inorganic cobalt by chromatographic separation within one single run. The analytical approach offers the option to further develop detection methods of illegal Co^{2+} supplementation in sport.

KEYWORDS
cobalt, doping, ICP-MS, LC-ICP-MS, sports

1 | INTRODUCTION

As blood doping in elite sports is a much discussed topic, cobalt has returned to the spotlight of ongoing debates. By stabilization of the erythropoietic transcription factor HIF-α (hypoxia-inducible factor) even under normoxic conditions, cobalt is well known to act as an erythropoiesis stimulating agent (ESA).[1–5] With regard to its polycythemic properties, ionic cobalt, in particular cobalt chloride ($CoCl_2$), was used

to treat anemia for many years since the late 1950s.[6,7] Although its application was associated with various adverse effects,[1,5,8] only since the development of recombinant EPO and its clinical approval in 1989,[4,9] an effective replacement therapy was at hand.

Due to its potential to enlarge the hemoglobin-related aerobic capacity along with an increase of the red blood cell count, cobalt was included into the World Anti-Doping Agency's (WADA) Prohibited List[10] in Section 2.1 "Erythropoietins and agents affecting erythropoiesis" in 2015. With regard to doping control testing procedures, it is important to distinguish the legitimate presence of organically bound cobalt in the form of cyanocobalamins (e.g., vitamin-B_{12}) from atypical levels of inorganic Co^{2+} in human urine. To date, in contrast to horse racing federations,[11] no threshold value or reporting level has been established for the interpretation of measured (urinary) cobalt concentrations in human doping control samples whilst numerous nutritional supplements containing inorganic cobalt are readily available.[12] Within a pilot-study in 2014,[3] reference populations for doping control scenarios were tested, and the excretion of total Co after the intake of $CoCl_2$ and cyanocobalamin was evaluated for the first time for human sports drug testing purposes employing inductively coupled plasma-mass spectrometry (ICP-MS). Further studies investigated the effects of low-dose $CoCl_2$ administrations on hematological parameters and its impact on aerobic performances.[2,13] Urinary cobalt reference concentrations of different populations were described in ranges below 2.2 ng/ml[1,3,14] but can be significantly elevated in cases of extraordinary cobalt exposure as, for example, in case of miners and residents in close proximity to cobalt mines, employees of cobalt processing industries,[15–17] or patients with cobalt-containing endoprotheses.[18] In addition, elevated urinary Co concentrations were recently shown to result also from vitamin supplementation (24.3 ng/ml Co corresponding to ca. 560 ng/ml cyanocobalamin),[19] which warranted follow-up studies as presented in this communication. With regard to the scope of doping controls, Popot et al.[20] presented interlaboratory data (ring tests) for urine und plasma measurements of total Co by ICP-MS for equestrian sports. Furthermore, the International Federation of Horseracing Authorities (IFHA) have set a threshold value of 100 μg Co per liter of urine.[21]

Excretion studies with either $CoCl_2$ or cyanocobalamin were conducted with two separate groups of healthy volunteers, and urine as well as EDTA whole blood samples were frequently collected over a period of 30 days. In this proof-of-concept study, a test method enabling the separation and detection of cobalt-containing organic analytes and unbound cobalt in human urine within one single analytical run was adapted as a simplified screening from former publications[21–24] utilizing high performance liquid chromatography (HPLC) combined with ICP-MS. The approach aims at facilitating the immediate attribution of measured urinary cobalt concentrations to either the legitimate use of cyanocobalamin or the prohibited use of, for example, cobalt salts. Furthermore, as Simonsen et al.[25] described the incorporation of Co^{2+} into red blood cells, total Co was measured by ICP-MS in EDTA plasma and, moreover, in erythrocytes.

2 | MATERIALS AND METHODS

2.1 | Study design of co excretion study

Following approval (#015/2019) by the local ethical committee of the German Sport University Cologne, 20 healthy female and male volunteers (n = 10 + 10) without any self-reported diseases or medications participated in this cobalt excretion study and provided written informed consent. Regarding the intake of two different cobalt formulations, tested subjects were divided into two groups, each comprising five women and five men. The study participants receiving cyanocobalamin served as control group (representing a cohort of legitimate whilst comparably high daily intake of cyanocobalamin), who administered daily two ready-for-use ampoules of "Vitasprint" (Pfizer Inc.). These ampoules are available without prescription in local pharmacies and are marketed as a nutritional supplement for oral administration, with each ampoule containing a single 500-μg cyanocobalamin dose (recommended daily dose). For the second group (administration group), cobalt (II)-chloride (99.999%) was purchased from Sigma Aldrich/Merck (Darmstadt, Germany) and dissolved and diluted with drinking water. Here, the volunteers were asked to orally administer a dose of 1 mg of $CoCl_2$ per day. The amount of cobalt ingested via the provided $CoCl_2$ solution and cyanocobalamin corresponded to 454 and 43.5 μg, respectively, and the daily intake was recommended to be completed with the first meal over a predetermined period of 14 consecutive days. The age of the participants ranged between 22 and 45 years (median: 30.5 years) for cyanocobalamin and between 23 and 53 years (median: 44.0 years) for $CoCl_2$.

2.1.1 | Sample collection and storage

The sampling of daily morning urine and selected venous EDTA-blood drawings started 7 days before initiation of the administration period and continued until 7 days postadministration (30 days in total). Polyethylene bottles were provided for the collection of daily morning urine samples, and Sarstedt blood collection sets (Saftey-Multifly®-Needle and 2.7-ml Monovette® EDTA K2E tubes) (Nümbrecht, Germany) were utilized for venous blood drawings. In total, 10 blood samples were collected at days −7, 0, 1, 3, 7, 10, 14, 17, and 21. Additionally, another blood sample was drawn in week 9 after starting the administration from 17 participants. Although urine samples were stored frozen at −20°C until analysis, the obtained EDTA blood samples were immediately processed to plasma samples (by centrifugation) before being stored at −20°C.

2.2 | (HPLC-)ICP-MS

2.2.1 | Chemicals

For all aqueous solutions, ultrapure water was obtained from a Milli-Q system (Milli-Q Integral 3 coupled to a Millipore Q-POD® Element)

TABLE 1 ICP-MS instrument settings

	ICP-MS	LC-ICP-MS
ID plasma torch	2.5 mm	1.5 mm
RF power	1600 W	1500 W
RF matching	1.62 V	1.84 V
Sample depth	8.0 mm	7.0 mm
Nebulizer gas (Ar) flow	1.06 L/min	0.5 L/min
Nebulizer pump speed	0.1 rps (approx. 200 µl/min)	0.2 rps
Ion lenses model	x-lens	
Lens extract 1 voltage	−10 V	−3.9 V
Lens extract 2 voltage	−130 V	−150 V
Omega bias voltage	−120 V	−120 V
Omega lens voltage	7.2 V	7.2 V
Cell entrance voltage	−30 V	−30 V
Cell exit voltage	−50 V	−50 V
Deflect voltage	10.2 V	11.4 V
Plate bias voltage	−35 V	−35 V
OctP bias voltage	−8.0 V	−8 V
OctP RF voltage	130 V	200 V
Energy discrimination voltage	5.0 V	5.0 V

from Merck (Darmstadt, Germany). Also, methanol of analytical grade, Suprapur® nitric acid (65%), CertiPUR® cobalt standard solution (1 mg/ml), and SUPELCO neat cyanocobalamin were also purchased from Merck. As internal standard (ISTD), an Indium plasma emission standard (1 mg/ml) from VWR International GmbH (Bruchsal, Germany) was used. An ICP-MS stock tuning solution containing 10 µg/ml Ce, Co, Li, Ti, and Y in 2% HNO_3 was from Agilent Technologies (Waldbronn, Germany). Polypropylene Falcon tubes of 15 and 50 ml as sampling vessels were obtained from Sarstedt (Nümbrecht, Germany) and VWR (Darmstadt, Germany).

2.2.2 | Sample preparation and ICP-MS measurements

Aliquots of all samples were diluted in 1% HNO_3 in 15 ml Falcon tubes. To 2 ml of urine, 8 ml of HNO_3 (1:5) and to 100 µl of plasma/cell concentrate 1900 µl HNO_3 (1:20) were added, respectively. Quantification measurements for cobalt (*m/z* 59) and indium (*m/z* 115) were performed using an Agilent 7900 ICP-MS system (Waldbronn, Germany) applying a standard resolution setup. For all direct injection measurements (urine, plasma, and blood cell concentrate), an Agilent SPS4 autosampler was interfaced to the ICP-MS. The prepared samples as well as a coinfused 20-ng/ml indium ISTD solution were

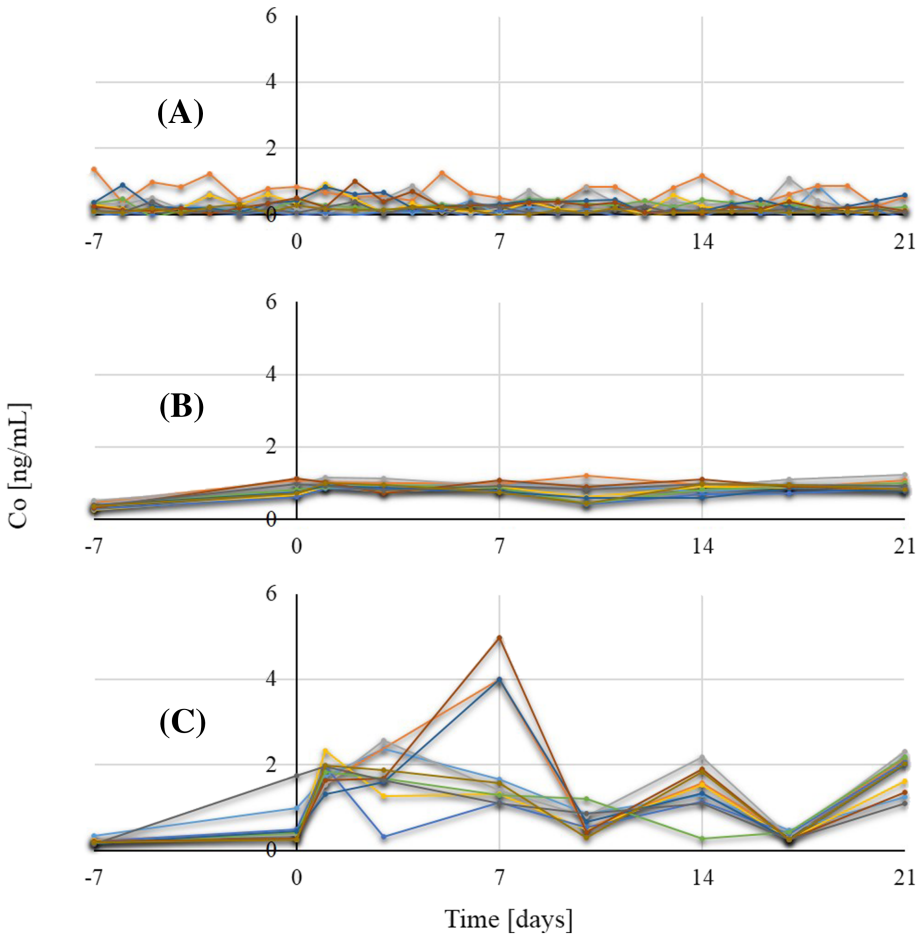

FIGURE 1 Total cobalt concentrations in subjects of the cyanocobalamin control group measured in urine (A), plasma (B), and concentrated erythrocytes (C)

promoted by a peristaltic pump (PeriPump). A concentrically borosilicate MicroMist nebulizer (Meinhard-type, 600 µl/min) from Glass Expansion (Pocasset, MA, USA) was introduced to a fused silica Scott-type Spray Chamber cooled to −5°C. Herein, the plasma ignition mode was set to aqueous solution. All parameters of the acquisition method were individually tuned and final settings are given in Table 1.

Additionally, urine samples were analyzed by an HPLC-coupled ICP-MS approach, enabling the assignments of chromatographic peaks to either unbound or cyanocobalamin-derived cobalt. Here, two Agilent 1290 Infinity HPLC systems each consisting of a high speed pump, a multisampler, and a multicolumn thermostat were used. For a fast and preparative chromatographic separation, a Poroshell Ec-C18 (2.1 × 50 mm, 2.7 µm) analytical column from Agilent was employed. The column was loaded by injection of 50 µl of neat urine. Gradient elution was carried out using 0.1% formic acid (FA) and methanol containing 0.1% FA as eluents A and B, respectively. The rapid overall gradient lasted 4 min with a constant flow rate of 400 µl/min whereas the thermostat (column oven) was set to 35°C. The gradient started at 20% B, which was increased to 50% within 2 min. A further increase to 80% within 0.5 min was followed by a constant hold for 1 min. After instant restoring of the starting conditions, the system was equilibrated for another 0.5 min.

A mixed calibration curve was prepared by spiking of a blank urine with aqueous Co and freshly prepared cyanocobalamin standards. Subsequently, nine concentration levels were generated by serial dilution (R^2 > 0.99): blank, 0.1, 0.5, 1, 5, 10, 50, 100, 500, and 1000 ng/ml of Co and 2.3, 11.5, 22.9, 114.9, 229.9, 1149, 2299, 11,490, and 22,990 ng/ml of cyanocobalamin, respectively). A quality control (QC) at 5-ng/ml Co (corresponding to approx. 11-ng/ml $CocCl_2$) and 114.0-ng/ml cyanocobalamin, respectively, was measured after each set of 58 samples.

For the stability of the plasma generated in the ICP-MS system, a constant proportion of organic solvent of less than 50% was preferred. Furthermore, the total flow rate should not exceed 400 µl/min. Accordingly, a second HPLC with an inverse but directly proportional gradient was linked via a T-connector to the chromatographic eluate. Finally, the organic load was fixed at 50%, and by means of a second T-connector, the flow was split to a total of 400 µl/min entering the ICP-MS system. Plasma ignition was set to organic solvent mode.

3 | RESULTS AND DISCUSSION

3.1 | Total cobalt by ICP-MS

The amounts of total cobalt were measured in all three available matrices including urine, plasma, and blood cell concentrate of all participants. Concentrations were calculated by additional calibration curves of aqueous Co standard solutions spiked at eight concentration levels (blank, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 50 ng/ml). Corresponding peak area ratios were found to be linear over the

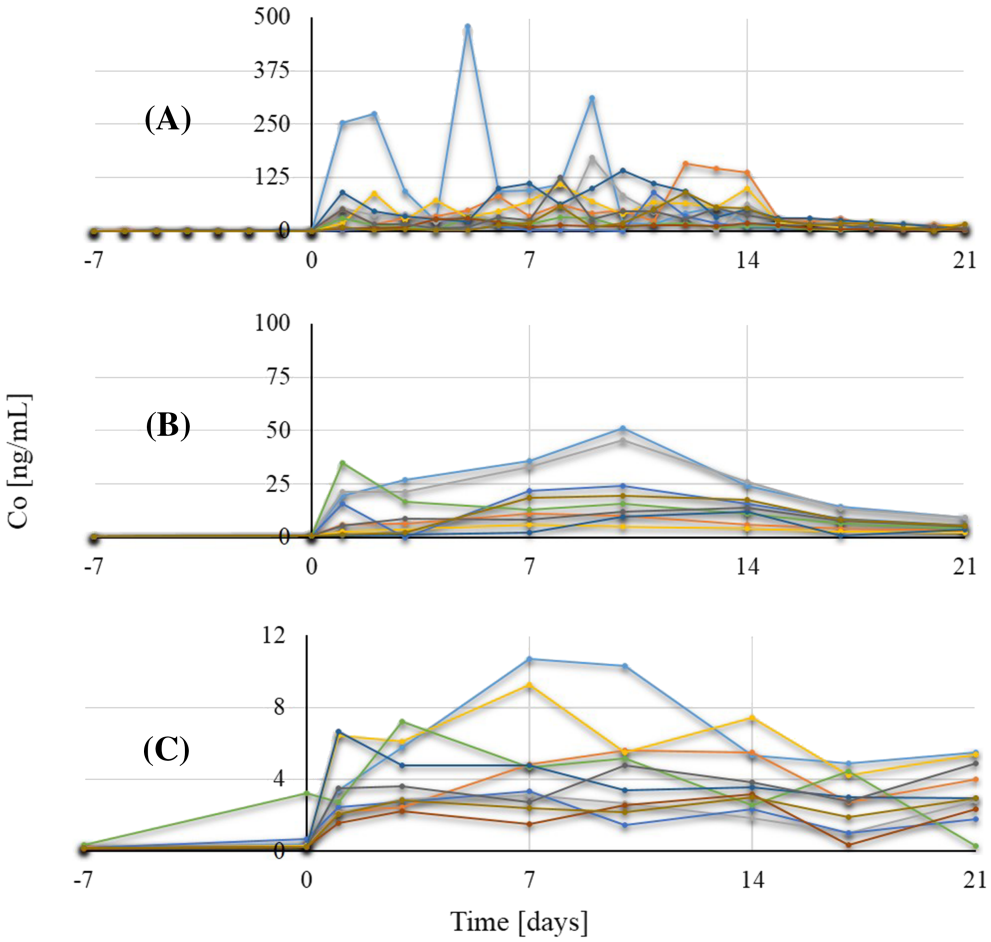

FIGURE 2 Total cobalt concentrations in subjects of the $CoCl_2$ group measured in urine (A), plasma (B), and concentrated erythrocytes (C)

entire working range with coefficients of correlation of 0.998–1.0. The method was precise with relative standard deviations below 1% tested for low (0.1 ng/ml), medium (1 ng/ml), and high (10 ng/ml) concentration levels in urine (blank urine spiked with cobalt standard).

Consistently, all measured Co levels were much higher for subjects of the $CoCl_2$ administration group compared to those of the cyanocobalamin control group as expected due to the approximately tenfold higher Co content per dose. For the control group, an increase of cobalt during or after the administration phase was exclusively observed for concentrated erythrocytes (Figure 1). Although Co levels in urine and plasma did not exceed 1.5 ng/ml, the concentration range as well as the absolute amounts in concentrated blood cells (notably erythrocytes) were slightly higher with maximum concentrations of 5 ng/ml.

By contrast, the administration of $CoCl_2$ (administration group) led to significantly increased Co concentrations in all tested matrices (Figure 2). Although the samples collected 1 week before the intake yielded cobalt levels similar to those of the control group, Co concentrations immediately increased from day one of the administration study. Maximum values of up to 478 and 51 ng/ml were detected in urine and plasma samples, respectively. The amounts of Co in blood cell concentrates were slightly higher compared to the cyanocobalamin control group with regard to the administration phase (up to 10 ng/ml). Despite of the conserved elevated levels at the end of the 4-week sampling period, Co levels had normalized in all samples additionally tested in week 9 after the first dosing.

3.2 | HPLC-ICP-MS analysis

With regard to routine doping control testing, fast and simple sample preparation protocols are desirable for high-throughput scenarios. Therefore, a preparative HPLC was coupled to the ICP-MS system and its capability to support distinguishing prohibited inorganic (here $CoCl_2$) and permitted organic (cyanocobalamin) cobalt sources was assessed.

As illustrated in Figure 3 by means of a spiked urine sample (QC), organic cyanocobalamin was observed at a retention time of ca. 1.5 min, baseline-separated from nonretained inorganic cobalt, coeluting with the injection peak (at approximately 0.5 min). In all blank samples from participants of both groups (urine samples collected within the first week of the study, prior to the first product administration), no interfering signals were observed. Immediately from day one of oral dosing, all urine samples of the $CoCl_2$ administration group did show a significant increase of the corresponding Co signal for the entire administration phase, followed by rapid clearance. Furthermore, congruent to the ICP-MS measurements, the daily ingestion of 1 mg of cyanocobalamin (corresponding to approx. 44 μg of Co) did not yield detectable signals in the corresponding chromatograms (data not shown).

The excretion profile (amounts of Co in each participants' sample) was in good correlation between both detection methods. Absolute signal intensities as well as the intrasubject variation of the daily Co excretion were comparable to the corresponding ICP-MS data as shown in Figure 2. For a statistical lineup, a Bland-Altman Plot is shown in

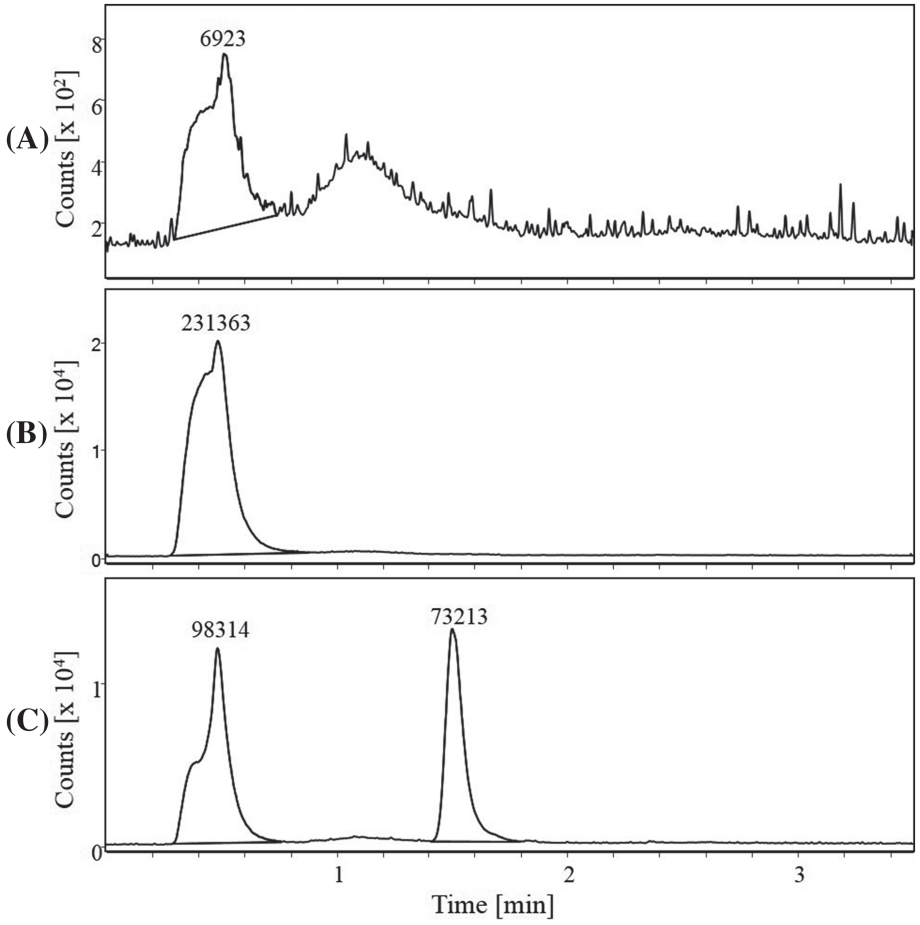

FIGURE 3 ICP total ion chromatograms (TICs) for elementary Co (*m/z* 59) obtained by HPLC separation (peak area labeled) of inorganic Co^{2+} (approx. 0.5 min) and cyanocobalamin (approx. 1.5 min) in human urine samples are shown. In the top and middle pane, urine samples of the same participant before (A) and after the first dose of $CoCl_2$ (B) are depicted. In the bottom pane (C), a spiked blank urine as a quality control (5-ng/ml Co and 114.0-ng/ml cyanocobalamin) is given

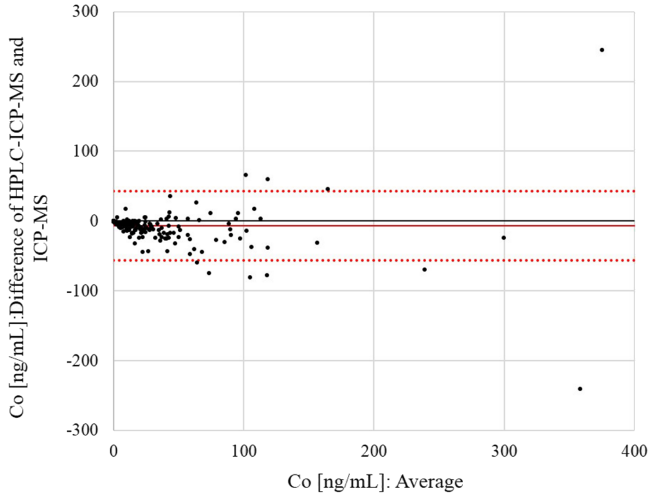

FIGURE 4 Bland-Altman plot for visualization of the agreement of both assays: HPLC-ICP-MS versus ICP-MS detection of Co in human urine. The continuous red line represents the average of all differences (mean: −6.6), whereas both dotted red lines indicate the calculated limits as the product of 1.96 and the standard deviation (±25.3), which is added to or subtracted from the bias of all data differences (corresponding to 95%), respectively

Figure 4 (data tested for normality by Shapiro-Wilk test, $p < 0.5$). Here, the average of differences is −6.6 ng/ml (mean), and the calculated limits were given as the 1.96-fold standard deviation (±25.3 ng/ml). This represents the 95% confidence interval of all data differences.

4 | DISCUSSION AND CONCLUSION

Test methods enabling the detection of the misuse of inorganic cobalt salts, for example $CoCl_2$, are critical to modern doping controls and highly sensitive ICP-MS techniques have been used for several years for that purpose. Hitherto, ICP-MS has been used for human doping controls concerning Co in urine specimens for monitoring purposes but, due to the purely element-specific operating principle that is unable to distinguish different molecular origins,[3,19] the determined urinary Co concentration represents the sum obtained from all Co-containing analytes. To account for permitted sources of Co such as cyanocobalamin, urine samples with atypically elevated concentrations of Co were forwarded to follow-up analysis to test for cyanocobalamin by LC-HRMS/MS.[19] For a better estimation of typical reference levels and those resulting from vitamin B_{12}-containing nutritional supplement[22] use, additional excretion studies including different formulations and application (i.e. injections) and complementary analytical approaches have been desirable.

The aims of this study were to provide proof-of-concept for the simultaneous detection of inorganic and organically bound cobalt in different body fluids applicable to doping controls and to contribute to the development of threshold values for urinary Co concentrations for sports drug testing purposes in general. HPLC-ICP-MS was found to allow for high throughput urine sample analyses concerning cobalt, facilitating the differentiation of permitted cobalt-containing species such as cyanocobalamin from inorganic cobalt. The feasibility was assessed by qualitative measurements of postadministration samples and the estimation of analyte concentrations using calibration curves and spiked urine quality control samples.

The levels of Co detected in urine and plasma of the study participants were significantly higher for all volunteers who administered $CoCl_2$ than for the cyanocobalamin supplementing control group. In fact, peaks corresponding to cyanocobalamin were not detected by LC-ICP-MS in the excretion study urine samples collected after oral administration of 1000 μg of cyanocobalamin per day over a period of 14 days, which can be attributed to biochemical conversion, photolytic degradation, and the limited assay sensitivity. In addition, inter-individual variation of urinary Co excretion has also been observed in earlier studies[2,13] (due to poor absorption and/or excretion mainly via feces[26]), and elimination half-lives for ionic Co in plasma were described to range between 4 and 12 h.[2] Cobalt concentrations measured from postadministration blood cell concentrates were found to be elevated; yet the observed absolute changes and the apparent absence of cobalt accumulation under the chosen experimental conditions did not allow for an unambiguous detection of Co^{2+} intake. However, in former studies and medical settings, considerably higher doses of 25–300 mg[2] were used for the treatment of anemia (before rhEPO became available), which might manifest larger intracellular cobalt levels in erythrocytes.

4.1 | Limitations/outlook

For accurate quantitative (doping control) analyses, additional aspects necessitate validation for adequate method characterization and, ideally, chromatographic performance supporting the separation of additional cobalt-containing organic species and the implementation of one or more internal standards need to be addressed. Different variants of cyanocobalamin and respective stabilities, especially with regard to photolytic degradation and possible conversions,[23] will be of prospective interest but are not a decisive factor as long as inorganic cobalt is chromatographically separated as only Co^{2+} concentrations attributed to unbound cobalt and exceeding future threshold levels will be regarded as antidoping rule violations. Owing to the fast and preparative chromatographic run, a separation of different cobalamins was not expected/intended; rather, the presented approach offers an initial testing tool in order to identify those doping control samples that justify subsequent accurate cobalt quantification.

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CONFLICT OF INTERESTS

The authors have no conflicts of interests to declare.

ORCID

Andre Knoop https://orcid.org/0000-0003-0040-8754
Mario Thevis https://orcid.org/0000-0002-1535-6451

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