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Solutions Advertised as Erythropoiesis-stimulating Products were Found to Contain Undeclared Cobalt and Nickel Species

Products overtly advertised with arguably licit performance-enhancing properties have been the subject of inquests particularly in the context of human sports drug testing for several years [5,6,9,13]. In investigated products, analyses frequently revealed the presence of (undeclared) anabolic agents or stimulants, being intentionally or inadvertently consumed by athletes. Conversely, substances stimulating erythropoiesis (*e.g.* erythropoietin, hypoxia-inducible factor (HIF) stabilizers) have rarely been reported as ingredients of such products; however, mixtures of proprietary and thus undisclosed content (arguably intended for veterinary use only) have been observed in the personal environment of elite athletes recently. These mixtures, confiscated or test-purchased from Internet-based suppliers by the Center for Preventive Doping Research and different anti-doping organizations, claimed blood-building properties and were therefore forwarded to (bio)chemical analyses concerning erythropoietin (EPO) and its derivatives as well as low molecular mass organic and inorganic HIF stabilizers according to established protocols [13].

A total of 19 products was obtained *via* online order or were confiscated and subjected to various different analytical techniques including gas and liquid chromatography-high resolution/high accuracy mass spectrometry-based approaches (GC-MS, LC-HRMS/MS), gel electrophoresis, and inductively-coupled plasma/mass spectrometry (ICP/MS) as described elsewhere [13]. Tests conducted covered peptidic/proteinaceous analytes (*e.g.* EPO, growth hormone (GH), growth hormone releasing hormones (GHRHs), growth hormone releasing peptides (GHRPs), etc.), low molecular mass organic analytes such as anabolic agents, stimulants, β_2-agonists, *etc.*, and elements including cobalt (Co) and nickel (Ni). Further, data collected by ICP/MS analysis in a former study with 98 doping control urine samples and 93 urine specimens collected from students enrolled at the German Sport University Cologne were re-evaluated concerning urinary Ni concentrations and compared to literature data.

The results of the 19 analyzed products are summarized in **Table 1**. One product (**6**) and one confiscated injection syringe content (**19**) were received unlabeled; 8 products (**3**–**5**, **7**, and **11**–**14**) were advertised with beneficial effects including increased oxygen supply/transport/utilization, anti-inflammatory properties, increased competitiveness, and reduced fatigue, and nine out of 19 products (**1**, **2**, **8**–**10**, **15**–**18**) were advertised with properties such as stimulating or supporting erythropoiesis. The latter group of nine products particularly raised suspicion about the presence of substances such as erythropoietin (EPO) or other agents increasing the organism's endogenous production of EPO (*e.g.* low molecular mass HIF stabilizers such as FG-4592, FG-2216, GSK1278863 *etc.*, or transition metals such as cobalt).

None of the investigated products returned findings for peptidic/proteinaceous substances or low molecular mass organic compounds relevant for doping controls. By means of ICP-MS analysis, however, considerable amounts of cobalt (**2**, **6**, **8**–**10**, **19**) and nickel (**1**) were determined in a total of 7 products, none of which did indicate cobalt or nickel salts as ingredient. Noteworthy, among these 7 products, three bore very similar labels with identical names of the same brand; their contents, however, were substantially different with one vial (**1**) containing Co at ca. 0.1 mg/mL and Ni at ca. 7.5 mg/mL, and 2 vials (**2** and **10**) containing Co at ca. 4.8 and 3.3 mg/mL but no relevant amounts of Ni. Moreover, 2 additional vials (**17** and **18**) equipped with the same label did contain Co only at concentrations of 0.1 mg/mL, attributed to the concomitant presence of ca. 2.6 mg/mL cyanocobalamin (**Table 1**), suggesting that counterfeiting, adulteration and/or the intended distribution of differently composed products of very similar outer appearance exist. The highest Co concentration was determined with 5.5 mg/mL in remnants collected from a seized syringe (sample number **19**), indicating that the administration of products enriched with bioavailable cobalt species to athletes has evidently found its way into elite sport. 2 samples (**15** and **16**) declared Co as ingredient in the forms of cobalt gluconate and cyanocobalamin, with cobalt gluconate representing an accepted dietary additive providing bioavailable ionic cobalt (Co^{2+}) [1,18]. The indicated content of cobalt gluconate was 2.0 and 0.7 mg/mL (**Table 1**), accounting theoretically for ca. 260 and 90 µg/mL of cobalt, respectively. Interestingly, sample **15** exhibited a substantially higher Co concentration with 2.6 mg/mL, while sample **16** was found to contain Co only at 0.2 mg/mL (**Table 1**). The discrepancy was not accounted for by the declared and analytically confirmed amount of cyanocobalamin, contributing to the measured Co concentrations with ca. 5 and 15 µg/mL, respectively.

The finding of soluble and bioavailable Co and Ni species in products advertised as erythropoiesis-stimulating preparations is particularly relevant to doping controls as compounds acting as HIF stabilizers are prohibited in sports according to the Prohibited List [17] established by the World Anti-Doping Agency (WADA). Among the listed HIF stabilizers, organic (*e.g.* FG-4592) as well as inorganic compounds such as cobalt are exemplified. To the best of the authors' knowledge this is the first report on products claiming to support blood-building processes that contain significant amounts of undeclared cobalt and nickel species. Apparently, the capability of the transition metal ions Co^{2+} and Ni^{2+} to mimic hypoxic situations [2,15], as exploited also in obsolete therapeutics such as Roncovite (containing $CoCl_2$), was intended to be utilized [14]. Especially with regards to the finding of Ni, it is not excluded that this approach was chosen to undermine anti-doping measures that currently focus predominantly on Co, where pilot studies concerning urinary cobalt levels of human athletes [12] as well as racing animals [7,11] have been conducted to support future strategies towards the identification of cobalt misuse in sport. Noteworthy, despite the fact that cobalt is an essential metal to humans (*e.g.* as part of vitamin B_{12}), exposure to larger doses was shown to be associated with serious side effects [3,8], and cobalt further demonstrated to exert a considerably higher oxidative stress in cells than nickel. Nevertheless, also nickel has been associated with a variety of health issues, particularly in cases of parenteral exposure scenarios [15,16].

Table 1 Product description and analytical results.

product #	advertised effect	product formulation (suggested route of administration)	determined content relevant for doping controls (≥ 0.1 mg/mL)	declared on label	remark
1	erythropoiesis	aqueous solution (*i. v.*)	cobalt (0.1 mg/mL)	no	cyanocobalamin (ca. 2.0 mg/mL) detected, accounting for ca. 90 µg/mL cobalt
			nickel (7.5 mg/mL)	no	
2	erythropoiesis	aqueous solution (*i. v.*)	cobalt (4.8 mg/mL)	no	cyanocobalamin (ca. 1.7 mg/mL) detected, accounting for ca. 75 µg /mL cobalt
3	increased oxygen supply	aqueous solution (*i. v.*)	–		
4	counteracts fatigue	aqueous solution (injection)	–		
5	anti-inflammatory properties	gel (*i.m.* or *i. v.*)	–		
6	–	aqueous solution	cobalt (3.4 mg/mL)	no	
7	–	aqueous solution	–		
8	erythropoiesis	aqueous suspension (*i. v.*)	cobalt (1.9 mg/mL)	no	cyanocobalamin (ca. 2.6 mg/mL) detected, accounting for ca. 110 µg/mL cobalt
9	erythropoiesis	aqueous suspension (*i. v.*)	cobalt (2.2 mg/mL)	no	
10	erythropoiesis	aqueous solution (*i. v.*)	cobalt (3.3 mg/mL)	no	cyanocobalamin (ca. 3.0 mg/mL) detected, accounting for ca. 270 µg/mL cobalt
11	increased competitiveness	aqueous solution (*i. v.*)	–		
12	counteracts fatigue	aqueous suspension (*i. v.*)	–		
13	increased oxygen transport	aqueous solution (*i. v.*)	–		
14	increased oxygen utilization	aqueous solution (*i.m.*)	–		
15	erythropoiesis	aqueous suspension (*i.m.* or *i. v.*)	cobalt (2.7 mg/mL)	yes	label declares cobalt gluconate (2.0 mg/mL) accounting for ca.260 µg/mL cobalt
					label declares cyanocobalamin (0.25 mg/mL) accounting for ca. 10 µg/mL cobalt
					cyanocobalamin (ca. 0.1 mg/mL) detected, accounting for ca. 5 µg/mL cobalt
16	supports erythropoiesis	aqueous suspension (*i.m.* or *i. v.*)	cobalt (0.2 mg/mL)	yes	label declares cobalt gluconate (0.7 mg/mL) accounting for ca. 90 µg/mL cobalt
					label declares cyanocobalamin (0.15 mg/mL) accounting for ca. 7 µg/mL cobalt
					cyanocobalamin (ca. 0.3 mg/mL) detected, accounting for ca. 15 µg /mL cobalt
17	erythropoiesis	aqueous solution (*i. v.*)	cobalt (0.1 mg/mL)	no	cyanocobalamin (ca. 4.0 mg/mL) detected, accounting for ca. 175 µg/mL cobalt
18	erythropoiesis	aqueous solution (*i. v.*)	cobalt (0.1 mg/mL)	no	cyanocobalamin (ca. 3.3 mg/mL) detected, accounting for ca. 140 µg/mL cobalt
19	–	aqueous solution, residue in confiscated syringe	cobalt (5.5 mg/mL)	n/a	cyanocobalamin (ca. 5.3 mg/mL) detected, accounting for ca. 230 µg/mL cobalt

The bioavailability of Ni strongly depends on its route of administration, while the elimination of absorbed nickel is primarily renal and independent of the route of application [16]. The European Food Safety Authority (EFSA) reported normal urinary Ni concentrations below 10 ng/mL for individuals not being exposed to Ni on an occupational basis [4]. Complementary studies on children in Germany found Ni values up to 38 ng/mL in individuals growing up in close proximity to steel mills with arithmetic and geometric mean values of 1.7 ng/mL [19] and 2.8 ng/mL [10], respectively. In the context of doping controls and the potential misuse of Ni-containing products as HIF stabilizers, urinary Ni concentrations of 98 doping control samples and 93 urine specimens collected from students registered at the German Sport University Cologne were compared. The obtained results demonstrated mean values of 2.9 and 1.7 ng/mL with concentrations ranging from 0.2–26.5 ng/mL respectively 0.2–39.9 ng/mL. The urinary concentrations for both subsets of samples were found log-normal distributed (*Shapiro Wilk* test, a = 0.95) and the comparison of both groups revealed a small yet highly significant difference (t-test, $p < 0.001$, ▶ **Fig. 1**) with the mean urinary Ni concentrations of the sport students closely

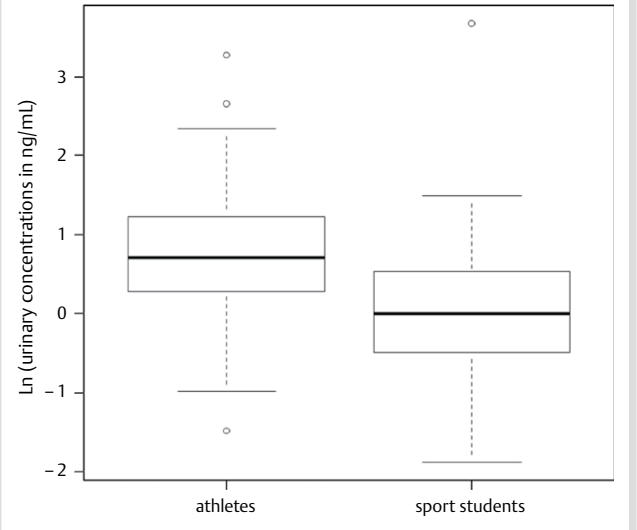

Fig. 1 Boxplot of logarithmic urinary Ni concentrations found in athletes and sport students. The mean values were found to be significantly different.

matching earlier reported data [10, 19]. In the light of the availability of Ni-containing products advertised with performance-enhancing properties and the corresponding potential misuse of Ni in sport, further studies particularly concerning reference ranges of urinary Ni concentrations in elite athletes' specimens are deemed warranted.

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References

1 *Abdel-Monem MM*. Cobalt complexes and their use as nutritional supplements. Patent Number: US4,678, 854 1987
2 *Davidson TL*, *Chen H*, *Di Toro DM*, *D'Angelo G*, *Costa M*. Soluble nickel inhibits HIF-prolyl-hydroxylases creating persistent hypoxic ignaling in A549 cells. Mol Carcinog 2006; 45: 479–489
3 *Ebert B*, *Jelkmann W*. Intolerability of cobalt salt as erythropoietic agent. Drug Test Anal 2014; 6: 185–189
4 *European Food Safety Authority*. Tolerable upper intake levels for vitamins and minerals. Available from http://www.efsa.europa.eu/sites/default/files/efsa_rep/blobserver_assets/ndatolerableuil.pdf Access date: 12-11-2015
5 *Geyer H*, *Braun H*, *Burke LM*, *Stear SJ*, *Castell LM*. Inadvertent doping. In: A-Z of nutritional supplements: dietary supplements, sports nutrition foods and ergogenic aids for health and performance – Part 22. Br J Sports Med 2011; 45: 752–754
6 *Geyer H*, *Parr MK*, *Koehler K*, *Mareck U*, *Schänzer W*, *Thevis M*. Nutritional supplements cross-contaminated and faked with doping substances. J Mass Spectrom 2008; 43: 892–902
7 *Ho EN*, *Chan GH*, *Wan TS*, *Curl P*, *Riggs CM*, *Hurley MJ*, *Sykes D*. Controlling the misuse of cobalt in horses. Drug Test Anal 2015; 7: 21–30
8 *Jelkmann W*. The disparate roles of cobalt in erythropoiesis, and doping relevance. Open J Hematol 2012; 3: 1–9
9 *Justa Neves DB*, *Caldas ED*. Dietary supplements: International legal framework and adulteration profiles, and characteristics of products on the Brazilian clandestine market. Regul Toxicol Pharmacol 2015; 73: 93–104
10 *Kasper-Sonnenberg M*, *Sugiri D*, *Wurzler S*, *Ranft U*, *Dickel H*, *Wittsiepe J*, *Hölzer J*, *Lemm F*. Prevalence of nickel sensitization and urinary nickel content of children are increased by nickel in ambient air. Environ Res 2011; 111: 266–273
11 *Knych HK*, *Arthur RM*, *Mitchell MM*, *Holser I*, *Poppenga R*, *Smith LL*, *Helm MN*, *Sams RA*, *Gaskill CL*. Pharmacokinetics and selected pharmacodynamics of cobalt following a single intravenous administration to horses. Drug Test Anal 2015; 7: 619–625
12 *Krug O*, *Kutscher D*, *Piper T*, *Geyer H*, *Schanzer W*, *Thevis M*. Quantifying cobalt in doping control urine samples – a pilot study. Drug Test Anal 2014; 6: 1186–1190
13 *Krug O*, *Thomas A*, *Walpurgis K*, *Piper T*, *Sigmund G*, *Schänzer W*, *Laussmann T*, *Thevis M*. Identification of black market products and potential doping agents in Germany 2010-2013. Eur J Clin Pharmacol 2014; 70: 1303–1311
14 *Lippi G*, *Franchini M*, *Guidi GC*. Blood doping by cobalt. Should we measure cobalt in athletes? J Occup Med Toxicol 2006; 1: 18
15 *Maxwell P*, *Salnikow K*. HIF-1 – an oxygen and metal responsive transcription factor. Cancer Biol Ther 2004; 3: 29–35
16 *Schaumlöffel D*. Nickel species: Analysis and toxic effects. J Trace Elem Med Biol 2012; 26: 1–6
17 *World Anti-Doping Agency*. The 2015 Prohibited List. Available from: https://wada-main-prod.s3.amazonaws.com/resources/files/wada-2015-prohibited-list-en.pdf Access date: 24-07-2015
18 *Wadhwa MS*. Liquid dietary supplement formulation compositions. Patent Number: WO2013/090402A1 2013
19 *Wilhelm M*, *Wittsiepe J*, *Seiwert M*, *Hünken A*, *Becker K*, *Conrad A*, *Schulz C*, *Kolossa-Gering M*. Levels and predictors of urinary nickel concentrations of children in Germany: results from the German Environmental Survey on children (GerES IV). Int J Hyg Environ Health. 2013: 2016: 163–169

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