

Controlling the misuse of cobalt in horses

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Cobalt is a well-established inducer of hypoxia-like responses, which can cause gene modulation at the hypoxia inducible factor pathway to induce erythropoietin transcription. Cobalt salts are orally active, inexpensive, and easily accessible. It is an attractive blood doping agent for enhancing aerobic performance. Indeed, recent intelligence and investigations have confirmed cobalt was being abused in equine sports. In this paper, population surveys of total cobalt in raceday samples were conducted using inductively coupled plasma mass spectrometry (ICP-MS). Urinary threshold of 75 ng/mL and plasma threshold of 2 ng/mL could be proposed for the control of cobalt misuse in raceday or in-competition samples. Results from administration trials with cobalt-containing supplements showed that common supplements could elevate urinary and plasma cobalt levels above the proposed thresholds within 24 h of administration. It would therefore be necessary to ban the use of cobalt-containing supplements on raceday as well as on the day before racing in order to implement and enforce the proposed thresholds. Since the abuse with huge quantities of cobalt salts can be done during training while the use of legitimate cobalt-containing supplements are also allowed, different urinary and plasma cobalt thresholds would be required to control cobalt abuse in non-raceday or out-of-competition samples. This could be achieved by setting the thresholds above the maximum urinary and plasma cobalt concentrations observed or anticipated from the normal use of legitimate cobalt-containing supplements. Urinary threshold of 2000 ng/mL and plasma threshold of 10 ng/mL were thus proposed for the control of cobalt abuse in non-raceday or out-of-competition samples. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: cobalt; inductively coupled plasma-mass spectrometry; urine; plasma; horse; threshold

Introduction

Cobalt is a well-established chemical inducer of hypoxia-like responses and had been used to treat anaemia in pregnant women, infants, and patients with chronic anaemia.^[1] Hypoxia causes gene modulation at the hypoxia inducible factor (HIF) pathway, leading to cell and tissue adaptation to the low oxygen conditions. The main mediator hypoxia inducible factor 1 α (HIF1 α) activates genetic sequences, including those of the erythropoietin (EPO) gene, which promotes efficient adaptation to hypoxia.^[2] Apart from the haematopoietic effects, cobalt also induces the pleiotropic and non-haematopoietic effects of erythropoietin, including modification of several parameters of lipid and glucose metabolism.^[3] The seminal studies of the effects of inorganic cobalt administration in healthy men revealed that a daily intake of 150 mg of cobalt chloride would produce an increase in red blood cell (RBC) counts by about 1 million cells per microlitre of blood within 7 to 22 days. The high RBC counts would return to normal 9 to 15 days after cobalt administration.^[4] Nevertheless, cobalt salt is no longer used for anti-anaemia treatment due to its adverse effects.^[3,5] The role of cobalt in erythropoiesis is disparate. Cobalamin deficiency can result in anemia. However, supplementing with cobalamin does not benefit performance unless there is a nutritional deficit.^[6] Inorganic cobalt ion (Co²⁺) stimulates erythropoiesis through the stabilization of HIF as discussed, with increased expression of the EPO gene even in non-anemic subjects. Indeed, the activity of an International EPO Unit (IU) was originally referenced against the biological effect of 5 μ M of cobalt chloride.^[7]

Cobalt is an essential micronutrient in the form of vitamin B12 (cobalamin), but inorganic cobalt as such is not required in the human diet. Cyanocobalamin is the synthetic form of vitamin B12 and

the form commonly available in vitamin B12 supplements. The daily nutritional requirement of an adult amounts to 2 to 3 mg of cobalamin. Inorganic cobalt is also obtained from the diet. The normal daily intake is on average about 7.5 μ g.^[8] Cobalt is acutely toxic in larger doses; cobalt ions and cobalt metal (nanoparticles) are cytotoxic and induce apoptosis and at higher concentrations necrosis with inflammatory response. There is evidence suggesting that cobalt salt may cause severe gastrointestinal, endocrine, cardiovascular, haematological, reproductive, neurological, and immunological responses.^[9] Cobalt metal and salts are also genotoxic, mainly resulting from oxidative DNA damage by reactive oxygen species. Cobalt salt was further shown to inhibit thyroidal iodide uptake,^[10] and chronic cobalt chloride ingestion can cause hypothyroidism and goiter.^[11] This may be the reason why the administration of cobalt chloride for performance enhancement is suspected to be supplemented with thyroid hormone. More impor-

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tantly, the cobalt-induced activation of HIF, present in almost all animal cells, with transcription of a range of hypoxia responsive HIF-target genes, probably promotes tumor development and growth.^[12,13]

Considering that cobalt salts are low cost, readily available, orally active, and effective in boosting endogenous erythropoietin production, they are attractive blood doping agents to enhance aerobic performances. Indeed, gene therapy targeting the HIF pathway has been reported as an attractive alternative to traditional techniques of blood doping since the last decade.^[14–16] The stimulated erythropoietin production and increased erythropoiesis increase the oxygen-carrying capacity of blood. Moreover, preconditioning with cobalt salts promotes tissue adaptation to hypoxia, improves hypoxic/ischemic tolerance, protects skeletal muscles from exercise-induced oxidative damage and enhances physical endurance performance.^[3,17] It has also been proposed that cobalt preconditioning could possibly avoid high altitude-induced oxidative stress and ameliorate mountain sickness. While there has been no reported study confirming the effect of cobalt on the performance of racehorses, recent intelligence from the USA and investigations of overseas out-of-competition and post-competition samples in the authors' laboratory, as well as a number of reported cases in Australia, have confirmed that cobalt is being abused in equine sports.

Due to the ability of cobalt to act as an erythropoietic agent in equine sports, a method to control cobalt misuse is needed. Inductively coupled plasma mass spectrometry (ICP-MS) is by far the best technique to quantify elements other than C, H, O, F, and the inert gases in biological samples. Besides its high sensitivity and fast turnaround time, another major advantage of using ICP-MS to quantify total cobalt in biological samples is the simple sample preparation required. Blood and urine can often be analyzed directly after dilution with acid.^[18,19] This paper describes ICP-MS methods for the quantification of total cobalt in equine plasma and urine. Equine plasma was first protein-precipitated. An aliquot of the deproteinated plasma or a portion of urine was then diluted with nitric acid and submitted directly to ICP-MS analysis. The total cobalt concentration was determined from a multi-point linear regression calibration curve using Germanium (Ge) as the internal standard.

As cobalt is naturally occurring in equine biological samples, a threshold is necessary to control its misuse in horses. With a threshold established, any equine sample is deemed to be positive for a prohibited substance if its cobalt concentration exceeds the respective threshold and if its presence in the sample can be independently confirmed using an unequivocal identification method. A few papers have reported the qualitative identification of the presence of cobalt by the formation of metal-complexes that can be analyzed by gas chromatography–mass spectrometry (GC-MS)^[20] or electrospray ionization mass spectrometry (ESI-MS).^[21,22] Based on the study reported by Minakata *et al.*,^[21] definitive liquid chromatography–mass spectrometry (LC-MS) methods for confirming the presence of cobalt in equine plasma and urine were developed by monitoring its diethyldithiocarbamate complex.

Materials and methods

Materials

Reference standard solutions of cobalt (Co) and germanium (Ge), with certified values traceable to the respective Standard Reference Material (SRM) 3113 and 3120a of the National Institute of Standards

and Technology (NIST), were obtained from High Purity Standards (Charleston, SC, USA). Triton-X (Ultra), diethyldithiocarbamate (DDC) and isoamyl alcohol (IAA) were purchased from Sigma-Aldrich (St Louis, MO, USA). Nitric acid (Suprapur grade; 65%), trichloroacetic acid (pro analysi grade), citric acid (pro-analysi grade) and sodium chloride (pro analysi grade) were obtained from Merck (Darmstadt, Germany). High purity deionised water was obtained from a Milli-Q Element A10 water purification system (Milli-Q, Molsheim, France). Blank plasma and urine samples were taken from post-race samples collected from horses after their races in Hong Kong.

Working standard solutions

The working standard solutions of Co and Ge were prepared from the respective reference standard solutions by dilution with 3.25 % (v/v) nitric acid. Only plastic containers and labware were used for all ICP-MS analyses.

Sample preparation for ICP-MS analyses

Blood

Blood samples (collected in lithium heparin tubes) were centrifuged at 3000 rpm (~1650 g) for 10 min and the plasma fraction was isolated. Germanium standard solution (40 ng) was added as an internal standard to plasma (80 µL). The concentration of the internal standard in the plasma sample was equivalent to 500 ng/mL. The mixture was deproteinated by the addition of trichloroacetic acid (300 µL; 10 g trichloroacetic acid and 120 mg NaCl in 100 mL deionized water) and nitric acid (3.25 %) to give a total volume of 4 mL. The mixture was vortexed briefly and left standing at room temperature for 10 min and then centrifuged at 2000 rpm (~750 g) for 10 min. The supernatant (3.6 mL) was transferred to an ICP-MS autosampler tube (a 4-mL polypropylene tube) and then infused *via* an autosampler to the ICP-MS.

Urine

Urine samples were centrifuged at 3000 rpm (~1650 g) for 10 min. Germanium standard solution (40 ng) was added as an internal standard to urine (80 µL). The sample was then diluted with nitric acid (3.25 %) to give a total volume of 4.0 mL. The diluted sample was then infused *via* an autosampler to the ICP-MS.

Protein precipitation for plasma sample for confirmation by LC-MS

Blood samples were centrifuged at 3000 rpm (~1650 g) for 10 min and the plasma fraction was isolated. Trichloroacetic acid (50 µL, 10 %, w/v) was added to an aliquot of plasma (300 µL). The mixture was then vortexed for 1 min. After standing for 10 min, the mixture was centrifuged at 14 000 rpm (~13 000 g) for 1 min. Two hundred microlitres of the supernatant was then subjected to complex formation.

Sample preparation for confirmation by LC-MS

Diethyldithiocarbamate (DDC; (C₂H₅)₂NCSS⁻, 20 µL, 1 M) was added into either deproteinated plasma (200 µL) or urine (200 µL). Internal standard was not used. The mixture was then vortexed for 1 min and shaken at 1400 rpm at 25 °C (in a thermo-mixer) for 10 min. Citric acid (20 µL, 0.2 M) and isoamyl alcohol (IAA, 500 µL) were added to the mixture. After further mixing in the thermo-mixer at 1400 rpm at 25 °C for 10 min, the mixture was centrifuged at 14

000 rpm (~13 000 g) for 1 min. The supernatant was taken out and mixed with 100 μ L of methanol to facilitate evaporation. The mixture was blown down to dryness at 60 °C with nitrogen. The dry residue was then reconstituted with methanol (40 μ L) and subjected to LC-MS analysis.

Instrumentation

ICP-MS analyses were performed on an Agilent 7500ce inductively coupled plasma mass spectrometer equipped with a G3160A integrated autosampler and a MicroMist nebulizer (Agilent Technologies, Santa Clara, CA, USA). LC-MS analyses were performed on a Thermo Finnigan TSQ Quantum Classic mass spectrometer (Thermo Finnigan, San José, CA, USA) equipped with a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA).

ICP-MS conditions

An RF power of 1400 W was employed. The argon carrier gas flow rate was set at 1.05 L/min. The spray chamber temperature was set at 2 °C. Helium (4.0 mL/min) was used as the collision gas. The peristaltic pump speed was set at 0.2 revolutions per sec (rps) during analysis. The sample uptake rate was about 0.8 mL/min, and the sample uptake time was set at 30 s. The isotopes to be monitored for Co and Ge were m/z 59 and m/z 72, respectively. All data acquisitions were performed in Spectrum Analysis mode with triplicate measurements. Peak Area Integration mode was used, and the integration time per mass was 2 s for Co, and 1 s for Ge. The total acquisition time per sample was about 3 min. After each injection, the autosampler probe was rinsed with deionized water for 5 s in the rinse port and 5 s in the rinse vial, followed by intelligent rinse with 0.07 % Triton-X for a maximum of 100 s to minimize carry over. The autosampler probe was finally rinsed with deionized water for 20 s before the next infusion.

LC-MS conditions for confirmation of cobalt

A reversed-phase UPLC column (Waters; Acquity BEH C18; 10 cm L \times 2.1 mm ID; 1.7 μ m particle size) was used for the analysis of cobalt diethyldithiocarbamate (Co-DDC) complex. The mobile phase was composed of 5 mM ammonium formate (pH 3) in deionized water as solvent A and 0.1% formic acid in acetonitrile as solvent B. A linear gradient was run at a constant flow rate of 350 μ L/min, with 100 % solvent A at the initial condition ($t = 0$ min), decreasing to 0 % solvent A from $t = 1$ min to $t = 6$ min, and held for 0.1 min (until $t = 6.1$ min). The gradient was then returned to 100 % solvent A from $t = 6.1$ min and equilibrated until $t = 10$ min before the next injection. The injection volume was 5 μ L.

Detection of the Co-DDC complex was performed in positive electrospray ionisation mode in a single time segment using selected reaction monitoring (SRM). Spray voltage of 3800 V and capillary temperature of 320 °C were employed. The nitrogen sheath and auxiliary gas flow rates were set at 60 and 10 arbitrary TSQ Quantum units respectively. The selected precursor ion of the Co-DDC complex was m/z 355, while the product ions monitored were m/z 116, 174, 208, and 291. The collision offset voltages were set from 25 V (for m/z 116, 174) to 35 V (for m/z 208, 291). Argon was used as the collision gas and set at 1.2 mTorr. The peak widths for the precursor and product ions in respectively Q1 and Q3 were set at 0.7 amu (FWHM). The scan width for the product ions was set at 0.01 amu and

the scan time for each transition was 50 msec. Data processing was performed using the Thermo Finnigan Xcalibur software (Version 2.0.6).

Calibrators and quality control samples for ICP-MS analyses

Calibration curves were established by analyzing a set of cobalt calibrators at concentrations of 0, 2, 4, 6, 8, and 10 ng/mL and 0, 30, 60, 90, 120, and 150 ng/mL in deionized water for equine plasma and urine, respectively. Quality control (QC) samples at 1 and 4 ng/mL (for plasma) and 60 ng/mL (for urine) were prepared in duplicate by spiking cobalt standard to blank plasma and blank urine, respectively. The calibrators, QC samples, and their corresponding blank matrices were analyzed alongside each batch of test samples using identical procedures. As a QC measure, the calibrators and QC samples were made up from Co working standard solutions that had been prepared separately. The peak area count ratios of cobalt to the internal standard (Ge) versus the spiked Co concentrations were fitted using linear regression to obtain the calibration curve. Concentrations of total cobalt in the test samples were interpolated from the calibration curve using standard ChemStation quantification software. For the QC samples, the actual recovered concentration of cobalt was derived by subtracting the concentration of the corresponding blank matrix from the total concentration determined.

Statistical analysis

Statistical analysis was performed with Minitab computer software version 13.32 (2000) (Minitab Inc., State College, PA, USA). The Kolmogorov-Smirnov normality test was used to compare the observed frequencies with the calculated distribution. Outliers in a data set were identified using the standard function from Microsoft Excel. Any number in a data set with the absolute value of Z-score exceeding 3.5 is considered an outlier.

Drug administration experiments (at manufacturers' recommended daily dosages)

Hemo-15

Hemo-15[®] (10 mL each, Virbac, Milperra, NSW, Australia) was administered daily by intravenous injection to 3 thoroughbred geldings for 3 consecutive days.

VAM[®] Injection

VAM[®] Injection (11 mL each, Nature Vet, NSW, Australia) was administered twice on alternate days by intramuscular injection to 2 thoroughbred geldings.

Farrier's Formula[®]

Farrier's Formula[®] (1.5 cups, ~255 g, Life Data Labs, Inc., Cherokee, Alabama, USA) was administered daily by stomach tubing to 1 thoroughbred gelding for 3 consecutive days.

Twydil[®] Hemopar

Twydil[®] Hemopar (60 mL each, PAVESCO AG, Basel, Switzerland) was administered daily by mixing with the daily feed to 2 thoroughbred geldings for 3 consecutive days.

Twydil® Hematinic

Twydil® Hematinic (40 mL each, PAVESCO AG, Basel, Switzerland) was orally administered twice daily to 2 thoroughbred geldings for 3.5 consecutive days (totally 7 times per horse).

Blood and urine samples were collected before and after administration. Blood samples were collected in lithium heparin tubes and centrifuged upon receipt and the corresponding plasma samples were kept at below -60°C until analysis. Approval of the drug administration experiments in this study has been obtained from the Animal Ethics Committee of the Hong Kong Jockey Club (reference HKJC-ERC004).

Method validation

Quantification method by ICP-MS

The inter-day accuracy and precision were assessed by analyzing the QC samples with cobalt spiked at various concentrations (1 and 4 ng/mL in plasma and 60 ng/mL in urine and the corresponding blank samples). The limit of detection (LoD) and limit of quantification (LoQ) of cobalt in equine urine and plasma were estimated by replicate analyses of blank sample matrices ($n = 6$ each). The impact on method recovery of the different common forms of cobalt (inorganic cobalt, cyanocobalamin and cobalt gluconate) found in some cobalt-containing supplements was evaluated by analyzing different untreated plasma samples spiked with different forms of cobalt (equivalent to 1 ng/mL cobalt) and their corresponding sample blanks. Recoveries were corrected by subtracting the cobalt concentration in the corresponding blank matrix from the total cobalt concentration determined for the spiked sample. Pairs of plasma and serum samples isolated from blood collected (with and without anticoagulant respectively) from the same horses ($n = 6$) were analyzed in quadruplicate for total cobalt to assess the method applicability to serum samples.

Confirmation method by LC-MS

The inter-day precisions of area count and retention time were assessed by analyzing cobalt spiked urine sample at 100 ng/mL and spiked plasma sample at 2 ng/mL. The detection sensitivity was evaluated by analyzing post-administration samples with their concentrations pre-determined by the ICP-MS method. Matrix suppression was studied by comparing the area counts obtained from matrix-spiked samples (corrected for contribution from their corresponding blank matrices) with those from the water spiked samples at the same cobalt concentrations. Method applicability to other forms of cobalt was studied by analyzing spiked plasma with cyanocobalamin and cobalt gluconate at cobalt equivalent concentrations of 1 and 4 ng/mL.

Results and discussion

Validation of ICP-MS quantification method for total cobalt in equine urine and plasma

It is well known that polyatomic isobars ($^{36}\text{Ar}^{23}\text{Na}$, $^{43}\text{Ca}^{16}\text{O}$ and $^{40}\text{Ar}^{18}\text{OH}$) could interfere with ICP-MS analysis of cobalt. The contribution of various polyatomic interferences was reported to be equivalent to about 0.4 ng/mL of cobalt in human serum.^[23] Collision/reaction cell (CRC) technology has been shown to be a very effective tool to remove isobaric interferences from polyatomic species.^[24] This technique was employed in the present study using helium as the collision gas. The inter-day accuracy

and the precision of the method were determined to be within $\pm 5\%$ and $\leq 9\%$ RSD, respectively, at all QC levels in both matrices (Table 1). The LoD and LoQ were found to be 0.16 ng/mL (equivalent to $3 \times \text{SD}$) and 0.52 ng/mL (equivalent to $10 \times \text{SD}$) in equine urine, and 0.06 ng/mL and 0.21 ng/mL in equine plasma. Calibration curves were linear within the concentration range, with correlation coefficients (r) greater than 0.99 in all cases.

Method recovery based on untreated plasma samples spiked with inorganic cobalt (equivalent to 1 ng/mL cobalt) was found to be 101%, while slightly lower method recoveries of about 90% were observed for untreated plasma spiked with other forms of cobalt (cyanocobalamin and cobalt gluconate) at equivalent cobalt concentration. As the magnitude of the negative recovery (about -10%) for the non-salt forms is not significantly different from the method precision (about 9% RSD at 1 ng/mL), the impact was not considered significant for the purpose of population surveys, particularly when a high confidence limit would be used to establish the thresholds.

There was no significant difference between the total cobalt concentrations determined in plasma and serum collected from the same horse ($n = 6$), as shown in Table 2. Therefore, population data of total cobalt in either equine plasma or equine serum can be considered to be equivalent.

Total cobalt population survey in post-race equine urine

In Hong Kong, the screening of total cobalt in equine urine samples has started since 2006. Equine urine was analyzed by ICP-MS after a simple dilution with nitric acid. Germanium (Ge) was used as the internal standard because there was essentially no interference with cobalt determination. In addition, Ge is not normally present in horse urine and blood, and has a mass (72 Da) close to that of Co (59 Da), minimizing possible mass-dependent difference in detector sensitivity. The total cobalt concentration in a urine sample was interpolated directly from a multi-level linear regression calibration curve constructed using calibrators prepared from water spiked with various

Table 1. Precision and accuracy of spiked quality control (QC) samples

QC sample (ng/mL)	Number of QC samples analyzed	Precision (%RSD)	Accuracy (%)
Equine urine			
60	550	4.7	95
Equine plasma			
1	21	9.0	101
4	19	3.2	98

Table 2. Total cobalt concentrations in plasma and serum samples collected from the same horse ($n = 6$)

Horse	Total cobalt in plasma (ng/mL)	Total cobalt in serum (ng/mL)
G118	0.24	0.26
G312	0.30	0.32
L098	0.34	0.35
M113	0.35	0.34
M266	0.28	0.26
N093	0.30	0.28

concentrations of cobalt. A cobalt threshold in equine urine could be established based on a population survey of 7462 post-race urine samples collected from horses after their races in Hong Kong. The population mean \pm standard deviation (SD) was 5.5 ± 5.0 ng/mL. This set of data showed a skewed distribution and could not be used directly to establish a threshold. A normal distribution could be obtained for the whole set of data after a logarithm transformation (Figure 1a). The transformed data were then subjected to the Kolmogorov-Smirnov normality test, resulting in an acceptable significance level of 0.05. A possible threshold could then be set at a level equal to the untransformed 'mean + 3.72 SD' value of 74.5 ng/mL, representing a risk of 1 in 10 000 (assuming the degree of freedom to be infinity) for a normal sample to exceed this threshold. Based on this approach which is often used to establish internationally-accepted thresholds in the horseracing industry,^[18,19,25–27] a 'rounded-up' threshold of 75 ng/mL of total cobalt in raceday equine urine samples was proposed. The risk associated with this threshold was about 1 in 10 315 (with a degree of freedom of 7461). This proposed threshold could be used to control total cobalt concentration in a raceday urine sample.

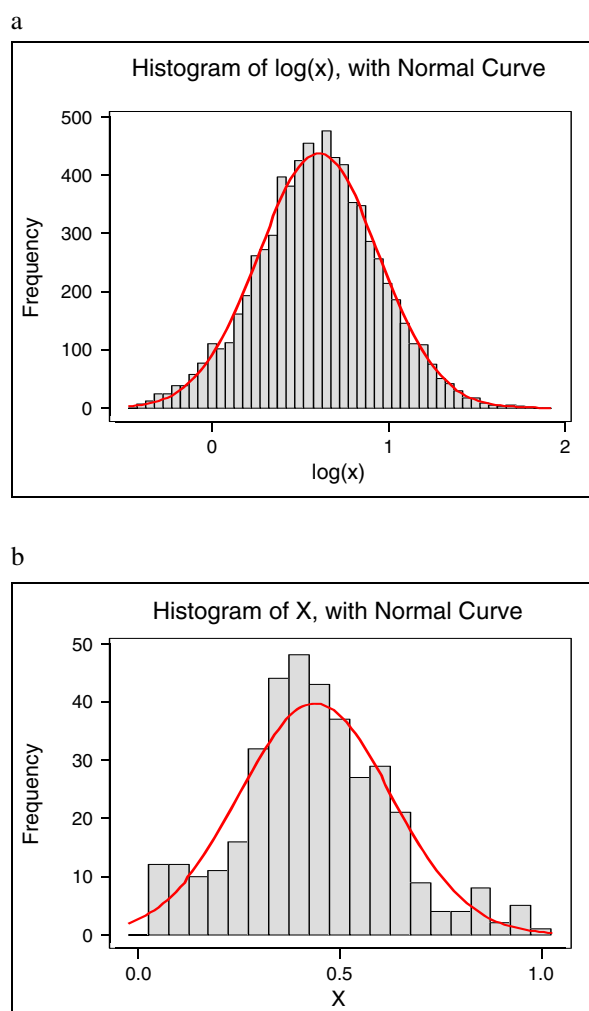


Figure 1. (a) Total cobalt concentration in equine urine samples after logarithm transformation (b) Total cobalt concentration in equine plasma samples (without transformation).

Total cobalt population survey in post-race equine plasma

Owing to the increasing popularity of using blood samples for doping control testing, the authors have also started monitoring total cobalt in blood samples since April 2013. The sample preparation procedures for blood samples were similar to those for urine samples, except that an additional protein precipitation step was included. Total non-protein-bound cobalt in plasma was measured. A proposed cobalt threshold in equine plasma could be established based on a population of 375 post-race blood samples using the same approach described above for equine urine. The population mean \pm SD was 0.44 ± 0.19 ng/mL. This whole set of data fits a normal distribution as shown Figure 1b, resulting in an acceptable significance level of 0.068 in the Kolmogorov-Smirnov normality test. A possible threshold could then be set at the 'mean + 3.72 SD' value of 1.14 ng/mL, representing a risk of 1 in 10 000 (assuming the degree of freedom to be infinity) for a normal sample to exceed this threshold. As a relative small population was used to derive the threshold, a 'rounded-up' threshold of 2 ng/mL of total cobalt in raceday equine plasma samples was proposed. The risk associated with this threshold was about one in 1000 trillion (with a degree of freedom of 374).

The proposed total cobalt threshold in equine plasma was verified using an independent population of 109 raceday blood samples from the Emirates Racing Authority (ERA) analyzed in Hong Kong using the same quantification method. Blood samples from ERA are a good choice for cobalt population survey because, like in Hong Kong, injections are not allowed to be given on raceday, thus minimizing the risk of samples being affected by injection with cobalt-containing supplements. The mean \pm SD for the plasma total cobalt in the ERA population was determined to be 0.70 ± 0.44 ng/mL. Among these 109 samples, 6 (with plasma total Co levels at 1.5–2.8 ng/mL) were considered outliers. These 6 samples were reportedly collected from horses belonging to two trainers and the elevated cobalt levels in these 6 samples were probably remnants of earlier treatments with cobalt-containing supplements. This set of data gave, after removal of the outliers, a mean \pm SD value of 0.61 ± 0.19 ng/mL and a normal distribution after a square-root transformation with an acceptable significance level of 0.05. The untransformed 'mean + 3.72 SD' value was 1.47 ng/mL, which was below the proposed threshold of 2 ng/mL, suggesting that the proposed plasma total cobalt threshold may also be applied to raceday blood samples from other countries.

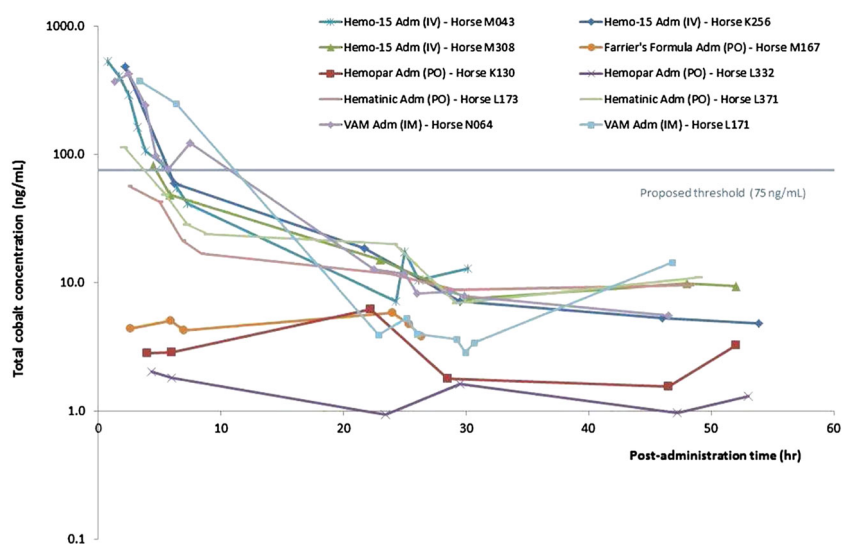
Administration trials with cobalt-containing supplements

In order to evaluate the impact of legitimate cobalt-containing supplements on the proposed thresholds, administration trials were conducted with 2 cobalt-containing injectables (Hemo-15 and VAM® Injection) and 3 cobalt-containing oral supplements (Farrier's Formula®, Hemopar, and Hematinic). The dose regimens used in the trials were based on those recommended by the manufacturers. Details of the listed cobalt ingredients and the cobalt equivalent in a daily dose for each supplement are summarized in Table 3, and the elimination profiles of total cobalt in urine and plasma are shown in Figures 2 and 3, respectively.

Elevated total cobalt levels in urine and plasma above the respective proposed thresholds were observed for the two injectables (Hemo-15 and VAM® Injection) and observed only in urine for the oral supplement with the highest daily dose of cobalt (Hematinic). Peak urinary and plasma total cobalt levels for these three products were all observed within 2 h of the last administration. Despite a

Table 3. Listed cobalt ingredients and comparison of Co equivalent per daily dose

Cobalt-containing supplement	Cobalt ingredient as listed	Recommended daily dose	Cobalt equivalent per daily dose (mg)
<i>Injections</i>			
Hemo-15	Cyanocobalamin 150 µg/mL Cobalt gluconate 0.7 mg/mL	10 mL	0.99
VAM® Injection	Cyanocobalamin 150 µg/mL Cobalt sulphate 240 µg/mL	11 mL	1.08
<i>Oral supplements</i>			
Farrier's Formula	Cobalt carbonate 1.9 mg/cup (cup = 170 gram of product)	1.5 cup (=255 gram)	1.41
Hemopar	Cyanocobalamin 800 µg/L Cobalt sulphate monohydrate 9 mg/L	60 mL	0.19
Hematinic	Cyanocobalamin 180 mg/L Cobalt carbonate 110 mg/L	80 mL (40 mL twice)	4.99 (2.5 twice)

**Figure 2.** Urinary total cobalt following administration of various forms of cobalt-containing supplements to horses.

much higher last dose of the oral supplement Hematinic than that of the two injectables, its peak total cobalt concentrations in urine and plasma was lower than those for the injectables, indicating that absorption of cobalt by the oral route is far less efficient than by way of injection. For the other two lower-dose oral supplements (Farrier's Formula® and Hemopar), no significant change in urinary and plasma total cobalt levels was observed in post-administration samples, with levels below the respective proposed thresholds at all times. Based on the proposed thresholds in urine (75 ng/mL) and plasma (2 ng/mL), VAM® Injection showed the longest detection time in urine of about 12 h, while both VAM® Injection and Hemo-15 had the longest detection time in plasma of about 6 h (Table 4). The results from these administration trials would suggest that legitimate cobalt-containing injectables should be banned not just on raceday but preferably on the day before racing in order to ensure that the proposed thresholds are not inadvertently breached in raceday samples. The use of oral supplements containing relatively high cobalt content should also be restricted to non-racedays.

The initial elimination half-life for plasma total cobalt was observed to be about 2–6.4 h, and the terminal elimination half-life

was found to be about 42–68 h. Similar to plasma, urinary total cobalt levels decreased rapidly and dropped below the proposed threshold of 75 ng/mL within 12 h of the last administration. Our findings were broadly in line with those observed in rats and human. The elimination profile of plasma cobalt appeared to be triphasic in rats with an absorption half-life of 0.9 h, an elimination phase half-life of 3.9 h, and a terminal elimination half-life of 22.9 h.^[28] In human studies, it has been reported that cobalt concentration in blood and serum was initially high but decreased rapidly due to tissue uptake combined with urinary excretion.^[8] The renal excretion reported for human was initially rapid but decreasing over the first days, followed by a second slow phase lasting several weeks, and with retention in tissues for several years.^[29,30]

Control of cobalt misuse in non-raceday samples

The control of cobalt misuse in non-raceday samples would require a different approach since numerous legitimate cobalt-containing supplements are allowed to be used during training. Indeed, cobalt levels in excess of 30 ng/mL (with one exceeding 1000 ng/mL!) have been observed by the authors' laboratory in

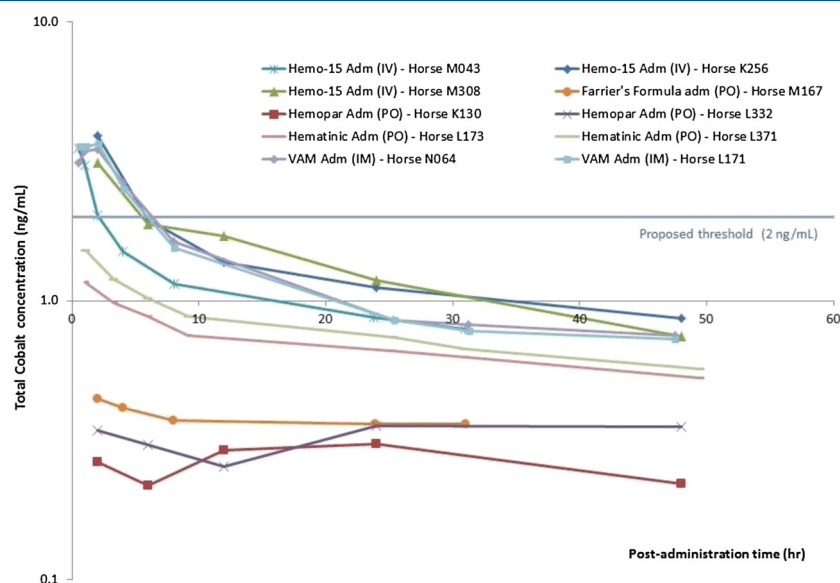


Figure 3. Plasma total cobalt following administration of various forms of cobalt-containing supplements to horses.

several non-raceday blood samples from overseas. A pragmatic approach would be to set a threshold for non-raceday samples in excess of the maximum urinary or plasma total cobalt concentrations expected to be attainable by the use of common *bona fide* cobalt-containing supplements. Based on our administration trials with various products, it would appear that administering a supplement with the largest recommended dose intravenously would provide the highest possible maximum concentration (C_{max}) in both urine and plasma to be considered as thresholds for non-raceday samples. A search on the Internet revealed that Hemo-15 was the *bona fide* cobalt-containing equine supplement with the highest recommended IV dose. Based on our trials with Hemo-15, the highest C_{max} (by extrapolation to time=0) would be from Horse K256 at respectively 6 ng/mL in plasma and 1600 ng/mL in urine. Total cobalt thresholds for non-raceday samples could thus be proposed at 10 ng/mL in plasma and 2000 ng/mL in urine. The suitability of these thresholds might warrant further verification by conducting administration trials with other legitimate cobalt-containing supplements not included in the present study.

Confirmation of cobalt in equine urine and plasma by LC-MS

When a regulatory sample is shown to have a total cobalt concentration exceeding the relevant threshold, the presence of cobalt in

the sample should ideally be established unequivocally and independently using a confirmation method. The confirmation method adopted was based on the mass-spectrometric method reported by Minakata *et al.*,^[21] with additional liquid-chromatographic separation to enhance the degree of proof. Cobalt in either urine or deproteinated plasma was complexed with diethyldithiocarbamate (DDC) and extracted with isoamyl alcohol (IAA) in the presence of citric acid. The resulting Co-DDC complex was analyzed by LC-MS in ESI mode. The precursor ion at m/z 355 corresponds to the Co-DCC complex $[\text{Co}(\text{DDC})_2]^+$, while the characteristic product-ions monitored were m/z 291 $[\text{Co}(\text{C}_4\text{H}_{10}\text{NCS})_2]^+$, m/z 280 $[\text{Co}(\text{DDCH})^+]$, m/z 174 $[\text{CoC}_4\text{H}_9\text{NCS}^+]$, and m/z 116 $[\text{C}_4\text{H}_{10}\text{NCS}^+]$. Cobalt could be easily confirmed in a blood sample collected 8.1 h after the last Hemo-15 administration (Figure 4). Both the retention time and mass spectrum of Co-DDC complex obtained from the post-administration sample matched well with those from the cobalt standard. These LC-MS data met the criteria stipulated in the *AORC Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry*.^[31] The total cobalt concentration in this sample had been determined to be about 1.2 ng/mL by the ICP-MS quantification method, suggesting that this LC-MS method has adequate sensitivity to confirm the presence of cobalt in horse plasma exceeding the threshold of 2 ng/mL. Similarly, cobalt was confirmed in a post-administration urine sample collected 6.4 h

Table 4. Comparison of peak total cobalt concentrations observed and maximum detection times based on the respective proposed thresholds in urine and plasma

Administration with cobalt-containing supplement	Urine		Plasma	
	Peak total cobalt level observed (ng/mL)	Maximum detection times (hrs)	Peak total cobalt level observed (ng/mL)	Maximum detection times (hrs)
<i>Injectables</i>				
Hemo-15	81–530	6.1	3.1–3.9	5.9
VAM® Injection	374–424	11.6	3.5–3.6	6.3
<i>Oral supplements</i>				
Farrier's Formula		Elevated total cobalt level not observed		
Hemopar		Elevated total cobalt level not observed		
Hematinic	56–113	3.8	1.2–1.5	—

after the last Hemo-15 administration, and the total cobalt concentration in the sample was about 55 ng/mL and less than the proposed threshold in urine (Figure 5). Since cobalt is endogenous in the horse, small amount of cobalt was also detected in pre-administration samples (Figures 4a and 5a).

The inter-day precisions on area count and retention time were determined to be 21 % and 0.13 %, respectively, in urine, and 25 % and 0.1 %, respectively, in plasma, indicating that the confirmation method has adequate precision for qualitative identification. Significant ion-suppressions (31 % in urine and 75 % in plasma) were observed in the LC-MS analyses of both urine and plasma spiked samples compared with water spikes. Nevertheless, the method could still serve its purpose as it could reliably confirm the presence of cobalt at or below the respective proposed thresholds for raceday samples. This LC-MS confirmation method has also been verified to be applicable to cyanocobalamin and cobalt gluconate, suggesting that the presence of cobalt could be confirmed regardless of the form of cobalt present in the samples.

Conclusion

ICP-MS quantification methods for total cobalt in equine urine and plasma samples were developed and validated. Urine and deproteinated plasma samples were analyzed directly by ICP-MS after simple dilution with nitric acid. Endogenous total cobalt levels in post-race urine ($n=7462$) and plasma ($n=375$) samples were determined with the aim to establish thresholds to control the misuse of cobalt in

horses. Plasma total cobalt was found to have a normal distribution, while a logarithm transformation is required for urinary total cobalt to achieve a normal distribution. Urinary and plasma thresholds of 75 ng/mL and 2 ng/mL, respectively, were proposed for raceday samples with a risk factor of less than 1 in 10 000. Results from administration trials showed that cobalt-containing supplements, especially injectables, could cause urinary and plasma total cobalt levels to exceed the respective thresholds within the first 24 h. Therefore, the use of cobalt-containing injectables should be prohibited starting on the day before racing and the use of oral supplements containing relatively high cobalt content should be restricted to non-racedays in order to successfully institute the proposed thresholds for raceday samples. Since cobalt-containing supplements could be used during training, different urinary and plasma cobalt thresholds would be required to control the misuse of cobalt for non-raceday samples. This could be achieved by setting the thresholds in excess of the maximum urinary and plasma total cobalt concentrations anticipated from the use of common bona fide cobalt-containing supplements. Results from administration trials and Internet search suggested that Hemo-15, a high-dose supplement administered intravenously, was a good model for establishing cobalt thresholds in non-raceday samples. Based on results from the Hemo-15 administration trials, thresholds of 2000 ng/mL in urine and 10 ng/mL in plasma for total cobalt in non-raceday samples were proposed. The presence of cobalt in the test samples could be confirmed unequivocally and independently by forming a cobalt-diethyldithiocarbamate complex followed by LC-MS analysis. While the diet seems to be a major factor that can influence the observed levels of cobalt in horses, there is still not much known regarding other factors, such as

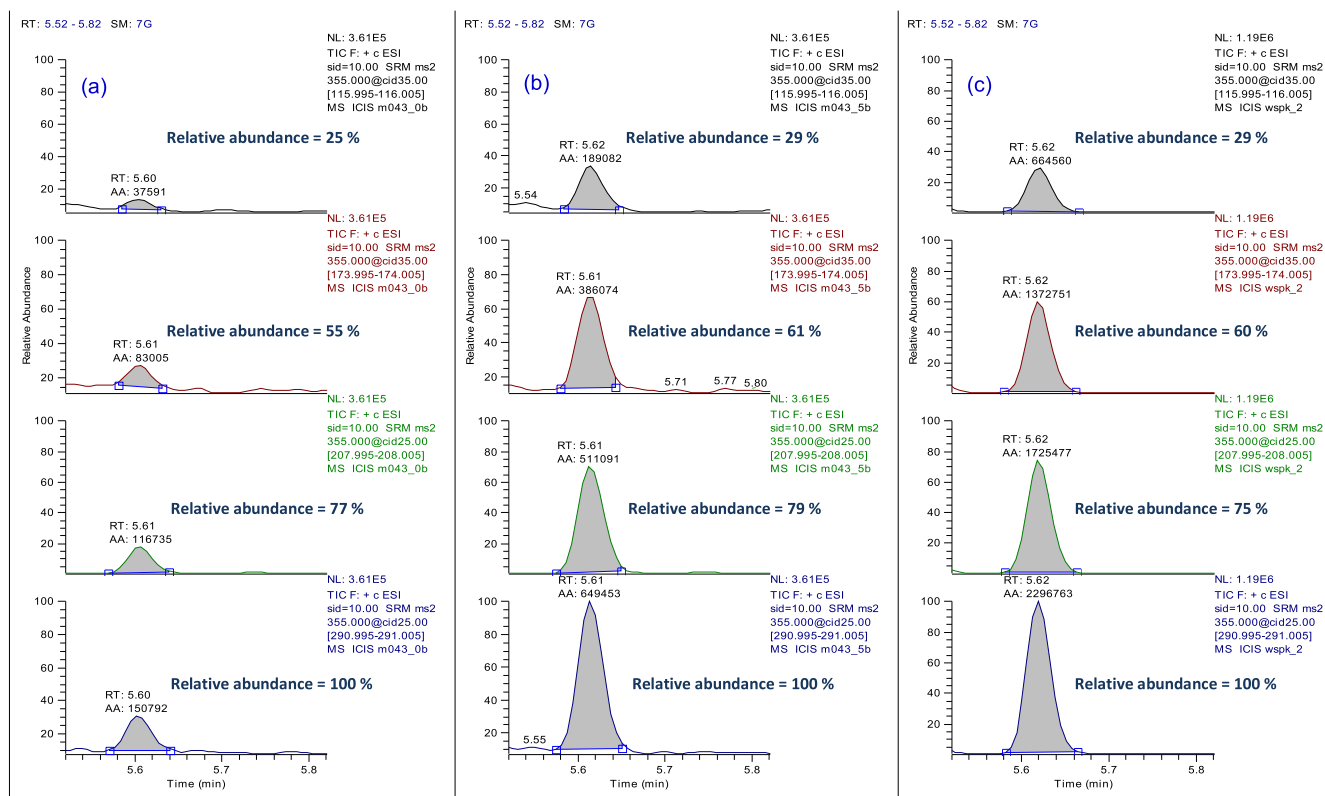


Figure 4. LC-MS product-ion chromatograms of Co-DDC obtained from (a) a pre-administration plasma sample, (b) a post-administration blood sample collected 8.1 h after IV administration of 10 mL of Hemo-15 daily for three days to a horse, and (c) a standard solution of cobalt in water.

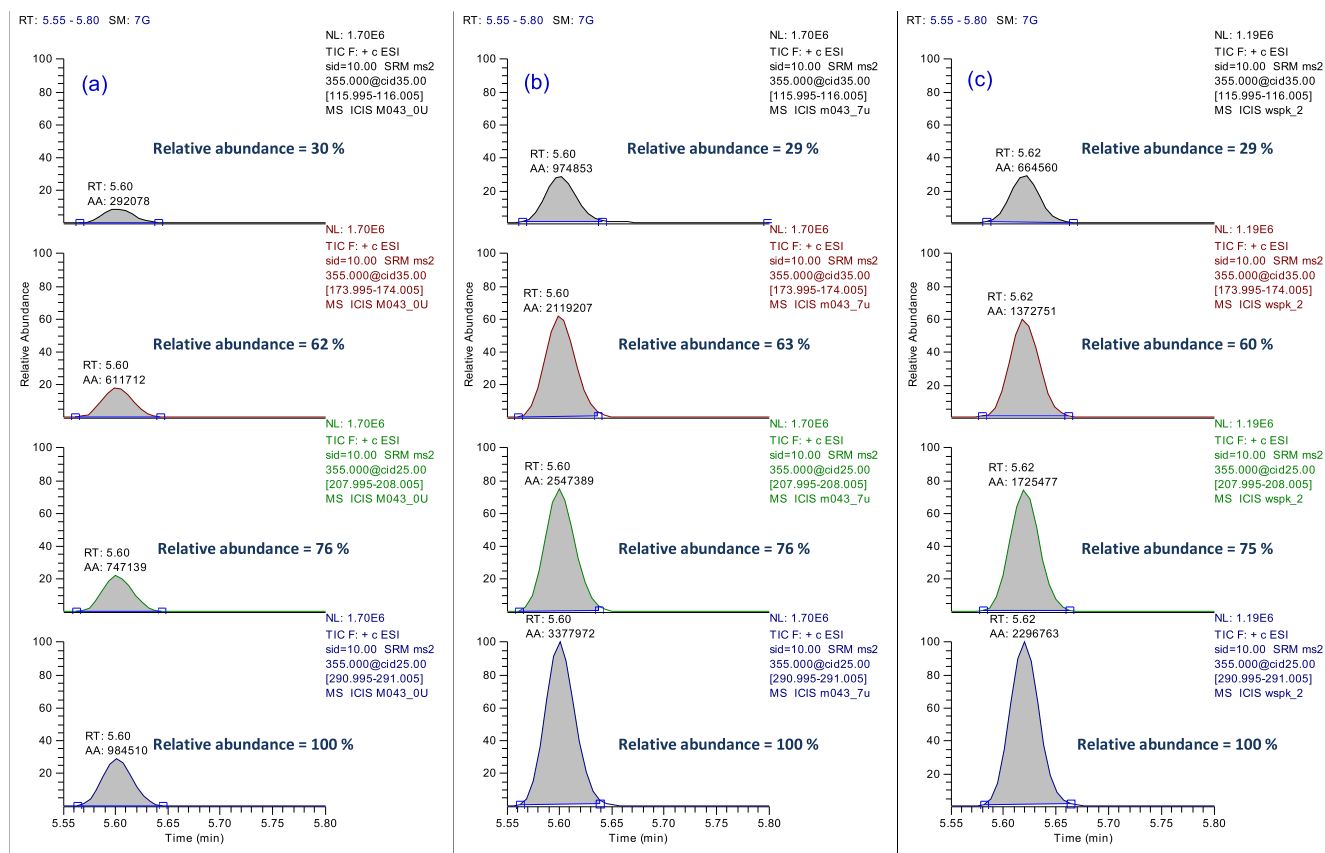


Figure 5. LC-MS product-ion chromatograms of Co-DDC obtained from (a) a pre-administration urine sample, (b) a post-administration urine sample collected 6.4 h after IV administration of 10 mL of Hemo-15 daily for three days to a horse, and (c) a standard solution of cobalt in water.

clinical or pathological conditions, that can influence the pharmacokinetics, and hence the observed levels, of cobalt in horses. In order to further improve the control of the misuse of cobalt in equine sports, a database of basal values of total cobalt in samples from a significant number of untreated horses in different regions should be established. In addition, more administration trials should be conducted with legitimate cobalt-containing equine supplements commonly used in different countries. This objective would require further international collaboration.

Acknowledgements

The authors wish to thank Chris Szeto, Shirley Tang, and Stephen Cheung of the Racing Laboratory of The Hong Kong Jockey Club for their technical assistance.

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