Pharmacokinetics of inorganic cobalt and a vitamin B_{12} supplement in the Thoroughbred horse: differentiating cobalt abuse from supplementation

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Summary

Background: Whilst cobalt is an essential micronutrient for vitamin B_{12} synthesis in the horse, at supra-physiological concentrations it has been shown to enhance performance in humans and rats and there is evidence that its administration in high doses to horses poses a welfare threat. Animal sport regulators currently control cobalt abuse via international race day thresholds, but since cobalt may be present in physiological concentrations this work was initiated to explore means of potentially adding to application of those thresholds.

Objectives: To devise a scientific basis for differentiation between presence of cobalt from *bona fide* supplementation and cobalt doping through the use of ratios.

Study design: Six Thoroughbred horses were given 10 ml vitamin B_{12}/cobalt supplement (Hemo-15®[a], 1.5 mg B_{12}, 7 mg cobalt gluconate = 983 µg total Co) as an intravenous bolus then an intravenous infusion (15 minute) of 100 mg cobalt chloride (45.39 mg Co) six weeks later. Pre-and post-administration plasma and urine samples were analysed for cobalt and vitamin B_{12}.

Methods: Urine and plasma samples were analysed for vitamin B_{12} using an immunoassay and cobalt concentrations were measured via ICP-MS. Baseline concentrations of cobalt in urine and

plasma for each horse were subtracted from their cobalt concentrations post administration for the PK analysis. Compartmental analysis was used for the determination of plasma PK parameters for cobalt using commercially available software.

Results: On administration of a vitamin B_{12}/cobalt supplement, the ratio of cobalt to vitamin B_{12} in plasma rapidly increased to approximately three and then rapidly declined below a ratio of one and then back to near baseline over the next week. On administration of 100 mg cobalt chloride the ratio initially exceeded ten in plasma and then declined with the lower 95% confidence interval remaining above a ratio of one for seven days. For two horses with extended sampling the plasma ratio remained above one for approximately 28 days after cobalt chloride administration.

The effect of the administration of the vitamin B_{12}/cobalt supplement on the urine ratio was transient and reached a peak value of ten which then rapidly declined. However, a urine ratio of ten was exceeded, with the lower 95% confidence interval remaining above a ratio of ten for seven days after cobalt chloride administration. For the two horses with extended sampling, the urine ratio remained above ten for about 18 days (442 hours) after cobalt chloride administration even though the absolute cobalt urine concentration had dropped below the international threshold of 100 ng/ml after 96 hours.

Main limitations: Only one vitamin B_{12}/cobalt product was evaluated, a limited number of horses were included, the horses were not in full race training and the results may be specific to this population of horses.

Conclusions: The results provide the basis for a potential strategy for allowing supplementation with vitamin B_{12} products whilst controlling the misuse of high doses of cobalt, through a combination of international thresholds and ratios of cobalt to vitamin B_{12}, in plasma and urine.

Introduction

Cobalt is an essential micronutrient needed for vitamin B_{12} synthesis in the equine caecum and colon. Under normal circumstances, adult horses obtain sufficient cobalt from their diet to meet their daily demand and supplemental cyanocobalamin, a synthetic form of vitamin B_{12}, is only beneficial if there

is vitamin B_{12} deficiency [1] manifesting as weakness and megaloblastic anaemia [2]. Such supplements are currently permitted in horses in training. There is evidence that supra-physiological concentrations of cobalt enhance performance in humans and rats by protecting muscle groups against oxidative stress and stabilising HIF2α, subsequently increasing erythropoiesis and therefore aerobic capacity and athletic performance [3,4]. However, cobalt compromises equine welfare at high doses [5], and there is a need to regulate it in racehorses.

Global surveys of urine and plasma cobalt concentrations in racehorse populations have led to the agreement of race day cobalt thresholds of 100 ng/mL (urine) and 25 ng/mL (plasma) by the International Federation of Horseracing Authorities (IFHA). This has been complemented by collaborative pharmacokinetic (PK) studies involving cobalt containing products [6,7]. Many of the studies have involved single administrations and there are limited data with regard to multiple dosing regimens of cobalt supplements which may result in cobalt accumulation. Cobalt supplements for intravenous administration usually comprise cyanocobalamin, containing approximately 4% cobalt in the trivalent state Co (III), and inorganic cobalt in the divalent state (II); both must be considered. If cobalt, included with preparations also containing vitamin B_{12}, is regularly supplemented in the horse's diet, it is hypothesised that as well as cobalt increasing in the circulation, increased vitamin B_{12} concentrations will be observed as a marker of administration. A ratio approach to cobalt versus vitamin B_{12} in conjunction with total cobalt 'dose' may help distinguish a horse receiving vitamin B_{12} supplementation from one administered inorganic cobalt salts, enabling horseracing authorities to regulate the abuse of inorganic cobalt whilst allowing *bona fide* supplementation with cobalt containing products. The aims of the study were therefore, (i) To analyse plasma and urine samples for vitamin B_{12} and cobalt before and after the administration of a vitamin B_{12}/cobalt supplement then cobalt chloride to six horses, determining excretion profiles, and (ii) to establish whether the administration of a vitamin B_{12}/cobalt supplement can be distinguished from administration of cobalt chloride by comparing the post-administration ratios of vitamin B_{12} and cobalt.

Materials and Methods

Horse Phase

Six Thoroughbred horses (five geldings and one filly aged 4-10 years and weighing 490-542 kg) fed a normal racehorse diet and housed at the British Horseracing Authority's Centre for Racehorse Studies (Newmarket, UK) were used for this study. They were exercised daily according to a programme typical of a training yard to include sessions on the horse walker, trot and canter work over varying distances depending on the individual horse and their level of fitness. Control blood and urine samples were taken on each of five consecutive days in the week preceding administration on Day 1. Each 'blood sample' comprised 4 x 10 ml lithium heparin vacutainers being filled with venous blood. The vacutainers were centrifuged and plasma harvested and frozen at nominally -20°C immediately after collection. Each urine sample comprised 165 ml urine taken from the total voided by free flow at each time point.

On Day 1, an intravenous catheter (Milacath®[b]) was placed into the left jugular vein of each horse. A sixth control blood sample was taken via the catheter immediately prior to 10 ml of a vitamin B_{12}/cobalt supplement (Hemo-15®[a]) (stated by manufacturer to contain 1.5 mg B_{12}, 7 mg cobalt gluconate = 983 µg cobalt) being administered intravenously into the contralateral jugular vein at 9 am. Blood samples were collected via the catheter at 15, 30, 60, 90, 120, 180, 240, 300, 360, 420, 540, 660, 780, 900, 1020, 1140, 1260, 1380, 1500, 1620, 1740 and 1860 minutes post dose (Supplementary Item 1). Urine samples voided through the day were collected from the time of dosing until 900 hours post dose. On Day 2, one urine sample was collected as close to 09.00 h and then 17.00 h as possible. On Day 3, one blood sample was taken via the catheter at 09.00 h and one at 17.00 h then catheters removed. One urine sample was collected as close to 09.00 h and 17.00 h as possible. On Days 4, 5, 6, 8, 15, 22, 29 and 36, one blood sample was taken by venepuncture each morning at 09.00 h with one urine sample taken each day as close to this time as possible.

On Day 43, 6 weeks after the administration of the vitamin B_{12}/cobalt supplement, an intravenous catheter was again placed into the left jugular vein of each horse. A control blood sample was taken via the catheter immediately prior to intravenous infusion of 100 mg cobalt chloride (45.39 mg cobalt[c] in 250 ml normal saline (400 µg/ml cobalt) over 15 minutes. Blood samples were collected via the catheter at 15, 30, 60, 90, 120, 180, 240, 300, 360, 420, 540, 660, 780, 900, 1020, 1140, 1260, 1380, 1500, 1620, 1740 and 1860 minutes post dose. Urine samples voided through the day were collected up until 900 minutes post dose. On Day 44**,** one urine sample was collected as close as possible to 09.00 h and then 17.00 h. On Day 45, one blood sample was taken via the catheter at 09.00 h and

one at 17.00 h before the catheters were removed and one urine sample was collected in the morning and one in the afternoon, again as close as possible to 09.00 h and 17.00 h respectively. One blood and one urine sample were collected on Days 46, 48 and 50, with just urine on the intervening days until Day 51. Collection of blood and urine was extended by a further three weeks, with twice weekly sampling for the fifth and sixth horses in the study owing to refinement of the study protocol from data produced in the first horses.

Laboratory Analysis

All samples were analysed at LGC (Newmarket, UK). Ultra-pure water was provided by a water system (Ultra Clear Duo[d]). The Immulite® vitamin B_{12} assay kit included components of the working solution, sample diluent, substrate solution, probe wash concentrate and Immulite® sample cups[e]. Elemental stocks were provided at 1,000 ppm (1 mg/ml) from Romil[f] and VHG Laboratories[g]. Solutions of nitric acid (HNO_3) at 3.4% and 0.68% were prepared from a Super Purity Acid grade (67-69%) concentrated solution (Romil[f]). All consumables were plastic.

Vitamin B_{12} analysis (urine and plasma)

The analysis of all urine and plasma samples for vitamin B_{12} was carried out using a clinical analyser (Immulite® 1000[e]), according to the protocol provided with the assay kit. Before starting sample analysis, the protocol required the analysis of adjuster solutions to be within criteria set by the manufacturer. The purpose of the adjustment was to align the internal instrument calibration with the master curve for each kit lot number which was generated by the manufacturer; eliminating the requirement to process standard curves during each batch of samples. The adjusters were also run as quality control (QC) samples at the beginning and end of each analytical batch to monitor the performance of the assay.

Pre- and post-administration urine and plasma samples were defrosted overnight at nominally 4°C prior to analysis. For each experimental batch, a working solution was prepared by combining 1,000 µL borate-potassium cyanide buffer with 20 µL dithiothreitol per sample, and was scaled up as appropriate. A volume (1,000 µL) of working solution was added to 200 µL dilute sample (diluted with Immulite® sample diluent). The typical starting dilution for both the urine and plasma samples was 1:5; however, some samples were found to be outside of the assay concentration range (150 – 1200

pg/mL) so required re-testing at an alternative dilution factor. The range of dilutions required for analysis of urine samples was between 1:2 and 1:800, whilst the plasma samples were diluted between 1:5 and 1:40.

The sample was boiled at 100°C for 15 minutes to inactivate vitamin B_{12}-binding proteins, before being incubated in a room-temperature water bath for a further five minutes. A portion of the prepared sample was aliquoted into a sample cup, which was placed on the instrument for analysis followed by a vitamin B_{12} test unit. The remainder of the assay was fully automated with the raw data generated by the Immulite® 1000 being in the form of a print-out indicating the concentration of vitamin B_{12} in each sample, measured in pg/mL.

Elemental analysis (including cobalt): urine

Pre- and post-administration urine samples were defrosted overnight at nominally 4°C prior to analysis. An aliquot from each was centrifuged for 15 min at 1,500 *g*. For each experimental batch, a series of calibration samples was prepared in HNO_3 (3.4%) containing 12 elemental analytes at concentrations of 0.0, 0.1, 0.5, 1.0, 5.0, 10 and 50 ng/mL; bromine and magnesium were spiked at 0.0, 1.0, 5.0, 10, 50, 100 and 500 ng/mL. A QC sample was independently prepared containing 5 ng/mL of each element (50 ng/mL bromine and magnesium) in the dilute acid.

Additionally, positive control samples were prepared in duplicate: 10 mL of pooled control equine urine was spiked at the respective cobalt and arsenic concentrations of 100 and 300 ng/mL; a volume (1 mL) of this was then diluted 50-fold in 3.4% HNO_3. These positive control samples were analysed throughout each experimental batch to demonstrate that matrix components were not adversely affecting the assay. Two further aliquots (200 µL) of the same urine pool were prepared for analysis (un-spiked) by 50-fold dilution into 3.4% HNO_3. These samples permitted adjustment of the positive control results for the endogenous urinary concentrations of the two spiked analytes. A volume (200 µL) of the relevant administration urine samples were diluted 50-fold in 3.4% HNO_3. An internal standard solution (bismuth, germanium and indium) in 3.4% HNO_3 was added to each calibration, QC, positive control, un-spiked matrix and administration sample at a final concentration of 10 ng/mL. Finally, samples enabling calculation of the instrumental limit of detection and lower limit of quantification were analysed (in sextuplicate) alongside each batch, consisting of 10 ng/mL internal standard mix in 3.4% HNO_3. The assay of all urine samples was carried out using a 7500ce ICP-MS

operating with ChemStation G1834B software version B.04.00[h], coupled with a Cetac ASX-520 autosampler[i].

A radio frequency power of 1550 W was employed. The argon carrier gas flow rate was set at 1.06 L/min and the spray chamber temperature was set at 2°C. Helium (3.2 mL/min) was used as the collision gas. The peristaltic pump speed was set at 0.1 revolutions per second during analysis. After each injection, the autosampler probe was rinsed with 0.68% HNO_3 (v/v) for 30 s in the rinse port and washed before each new set of samples in 3.4% HNO_3 (v/v) for 40 s to minimise contamination from carry over. Data acquisitions were performed in spectrum analysis (multi tune) mode.

Elemental analysis (including cobalt): plasma

Administration plasma samples were defrosted overnight at nominally 4°C prior to analysis. For every batch, calibration samples were prepared in HNO_3 (0.68%) containing 12 elemental analytes at concentrations of 0.0, 0.1, 0.5, 1.0, 5.0, 10 and 20 ng/mL; bromine and magnesium were spiked at 0.0, 1.0, 5.0, 10, 50, 100 and 200 ng/mL. A QC sample was independently prepared containing 5 ng/mL of each element (50 ng/mL bromine and magnesium) in the acid solution.

Analogous to urine, duplicated plasma positive control samples were prepared: 2 mL of pooled control equine plasma was spiked at the respective cobalt and arsenic concentrations of 25 and 75 ng/mL; a volume (1 mL) of this was then diluted 50-fold in 0.68% HNO_3. Two further aliquots (200 µL) of the plasma pool were diluted (un-spiked) by 50-fold into 0.68% HNO_3 allowing background concentrations of cobalt and arsenic to be accounted for. Preparation of QC and limit of detection/lower limit of quantification assessment samples, in addition to that of the administration plasma samples themselves, was performed as described for urine but using 0.68% HNO_3. Internal standard solution in 0.68% HNO_3 was added to each sample at a final concentration of 10 ng/mL. The assay of plasma samples was performed as for urine.

Pharmacokinetic Analysis

Baseline concentrations of cobalt (average of pre-administration samples) in urine and plasma for each horse were subtracted from their cobalt concentrations post administration for the pharmacokinetic (PK) analysis. Compartmental analysis was used for the determination of plasma

pharmacokinetic parameters for cobalt using commercially available software (Phoenix WinNonlin Version 6.0[j]). A compartmental model was chosen based on the coefficient of variation, Akaike Information Criterion and visual inspection of the residual plots. The area under the curve for cobalt concentration in urine was calculated using the trapezoidal method via a non-compartmental analysis (NCA) using the same software. The ratio of cobalt to vitamin B_{12} was determined by dividing the measured concentration of cobalt in ng/mL by the measured concentration of vitamin B_{12} in ng/mL i.e. baseline concentrations were not subtracted as the ratio is meant to represent what would be expected from a sample taken on race day or while the horse is in training. The 95% confidence interval was generated for the ratios using GraphPad Prism version 7.00 for Windows[k].

Results

Following urine elemental analysis, all acid QCs were found to be within ± 10% for cobalt in terms of both intra-batch precision (coefficient of variation, CV) and relative error (RE). All cobalt positive control samples demonstrated intra-batch CV measurements within 10% and fell within the calculated 99% confidence interval (± 11%). Across eight batches, the mean cobalt limit of detection was calculated to be 0.44 ng/mL (in undiluted urine) whilst the calculated lower limit of quantification was not greater than the bottom calibration sample (equivalent to 5 ng/mL in undiluted urine).

During plasma elemental analysis, all acid QCs were within ± 10% for cobalt in terms of both intra-batch precision (CV) and RE. Cobalt positive control samples revealed intra-batch CV and RE measurements within 10% and ± 15%, respectively. The mean cobalt limit of detection over 13 analytical batches was 0.57 ng/mL (in undiluted plasma) and the calculated lower limit of quantification did not exceed the bottom calibration sample (equivalent to 5 ng/mL in undiluted plasma).

Figure 1 displays the mean plasma and urine total cobalt concentration for the single administration of a vitamin B_{12}/cobalt supplement (983 µg Co) followed by cobalt chloride administration (45.39 mg) at time equals zero and 1008 hours, respectively. Plasma and urine cobalt physiological baseline levels (pre-administration) were approximately 1 and 2 ng/mL, respectively. There was an increase in cobalt

concentration in both plasma and urine after the vitamin B_{12}/cobalt supplement, more detail of which is seen in the expanded view in Supplementary Item 2. Supplementary Item 2 displays the plasma and urine total cobalt concentrations for the first 24 hours after the single administration of a vitamin B_{12}/cobalt supplement. Plasma and urine cobalt concentrations returned to near baseline levels approximately 672 hours after the vitamin B_{12}/cobalt supplement administration compared with approximately 1176 hours for the cobalt chloride administration.

Plasma cobalt levels dropped below the 25 ng/mL plasma threshold within 2 hours post administration and by 4 hours it was below 10 ng/mL in all horses whereas urine cobalt concentrations peaked above the threshold of 100 ng/mL at about 2 hours with all horses' concentrations below it by 8 hours post administration. After returning to near baseline levels cobalt concentrations in urine and plasma remained unchanged until the administration of cobalt chloride (Fig 1).

Baseline concentrations of cobalt (pre-administration samples) for each horse were subtracted from their cobalt concentrations post administration of the vitamin B_{12}/cobalt supplement and the resulting data set, up to the administration of cobalt chloride, was analysed using compartmental analysis. Based on coefficient of variation, Akaike Information Criterion and visual inspection of the residual plots, a three-compartment bolus model (Supplementary Item 3) with a weighting factor of $1/\hat{y}$ gave the best fit to the cobalt concentration data points from individual animals. Table 1 displays the PK parameters using a 3-compartmental model which gave a low mean clearance of 0.028 mL/min/Kg and a moderate steady-state volume of distribution (Vss) of 0.635 L/Kg. The terminal (Gamma) plasma half-life was very long with a geometrical mean value of 319 hours.

Supplementary Item 4 displays the plasma and urine total cobalt concentration for the single administration of cobalt chloride (45.39 mg Co) for the first 21 days after administration. Cobalt chloride administration caused a sharp increase in cobalt concentration in both plasma and urine, and peaked at maximums of 700 ng/ml and 20 µg/ml, respectively. Urine cobalt concentrations were higher than plasma for the first 24 hours after administration but then fell below the plasma concentrations at 72-96 hours with the rate of decline being much faster in urine, and urine concentrations thereafter were lower than plasma. The two horses sampled for longer showed cobalt plasma concentrations flattening at 1344 hours post administration of cobalt chloride but did not quite return to baseline at the point where sampling stopped (Fig 1).

Baseline concentrations of cobalt (pre-administration samples) were subtracted from cobalt concentrations post administration of cobalt chloride and the resulting data set was analysed using compartmental analysis. Based on coefficient of variation, Akaike Information Criterion and visual inspection of the residual plots, a three-compartment infusion model with a weighting factor of $1/\hat{y}$ gave the best fit to the cobalt concentration data points from individual animals. Table 2 displays the PK parameters using a 3-compartmental model which gave a low mean clearance of 0.07 mL/min/Kg and a moderate steady-state volume of distribution (Vss) of 0.711 L/Kg. The terminal (Gamma) plasma half-life was long with a geometrical mean value of 132 hours.

The average concentration, based upon a once a day vitamin B_{12}/cobalt supplement regimen, from first dose to steady-state is expected to increase by 8.3 and 1.8 fold for plasma and urine, respectively (Table 3). The extent of accumulation is expected to be slightly less for the once a day administration of cobalt chloride where the average cobalt concentration is expected to increase by 5.5 and 1.3 fold once steady-state has been reached for plasma and urine, respectively (Table 3).

Figure 2 shows that both plasma and urine concentrations of vitamin B_{12} peaked then decreased after administration of the vitamin B_{12}/cobalt supplement but at a slower rate of decrease than the cobalt (Supplementary Item 2). As shown in Supplementary Item 5, an expanded view of the first 168 hours after administration, vitamin B_{12} concentrations reverted to baseline in plasma within eight hours and urine concentrations fell to background concentrations of approximately 1 ng/ml within 72 hours. Supplementary Item 6 shows, as would be expected, that administration of cobalt chloride had no effect on plasma or urine B_{12} concentrations. Supplementary Items 7 and 8 show the individual horse data used to calculate the average plasma and urine concentrations of, respectively, cobalt and vitamin B_{12} in Supplementary Items 2, 4, 5 and 6.

Based upon background levels within these horses there was approximately ten times more vitamin B_{12} than cobalt in plasma when using units of ng/mL (Fig 3a). On administration of a vitamin B_{12}/cobalt supplement, the ratio of cobalt to vitamin B_{12} rapidly increased to approximately 3 owing to the relative quantities of cobalt gluconate to vitamin B_{12} in the product and then rapidly declined below a ratio of 1 and then back to near baseline over the next week. On administration of 100 mg cobalt chloride, because only cobalt and not vitamin B_{12} was given, the ratio initially exceeded ten in plasma and then declined with the lower 95% confidence interval remaining above a ratio of 1 for 7 days. For the two

horses with extended sampling the ratio remained above 1 for approximately 28 days after cobalt chloride administration (Fig 3a).

Based upon background levels within these horses there was approximately 1-2 times more cobalt than vitamin B_{12} in urine (Fig 3b). As in plasma, the effect of the administration of the vitamin B_{12}/cobalt supplement on the urine ratio was transient and reached a peak value of 10 which then rapidly declined. However, a urine ratio of 10 was exceeded, with the lower 95% confidence interval remaining above a ratio of 10 for 7 days after cobalt chloride administration. For the two horses with extended sampling the urine ratio remained above 10 for about 18 days (442 hours) after cobalt chloride administration even though the absolute cobalt urine concentration had dropped below the threshold of 100 ng/ml after 96 hours.

Discussion

The PK parameters obtained following cobalt chloride administration in the current study are consistent with those obtained in a previous study by Knych *et al.* [6] that investigated the PK of a single administration of cobalt chloride (49 mg Co; Table 2). In the previous study, cobalt chloride was infused i.v. over a 10-minute period and in this situation it is imperative that PK samples are taken during the infusion period. Based upon the alpha half-life found previously [6] it was estimated that a reasonable back extrapolation for the PK parameter, V1, would be obtained from a first sample point taken 15 minutes post bolus administration so as to reduce the number of blood samples taken. The mean clearance value obtained by Knych *et al*. [6] was slightly higher than found in the current $CoCl_2$ study which would not be the case if the early portion of the AUC was missed. In both the current and previous studies, cobalt chloride clearance and terminal half-life parameters (Table 2) are larger (3 x) and shorter (2 x), respectively, compared with the parameters obtained for the lower cobalt 'dose' present in the vitamin B_{12}/cobalt preparation administration (Table 1). The higher clearance value for the single administration of cobalt chloride (49 mg Co) relative to the administration of the vitamin B_{12}/cobalt preparation used in the current study may be due to a reduction in cobalt re-absorption from the kidney back to blood. The dose used for the vitamin B_{12}/cobalt preparation was the recommended dose of this product, licensed for use in many countries. The single dose of cobalt chloride was representative of a 'doping' dose that could affect performance and have adverse side

316 effects. This ‘doping’ dose was based upon doses in human which have shown increases in EPO and
317 was the same as the dose used in the Knych (2012) study. The pharmacokinetic argument put
318 forward is that there is only one possible explanation for the clearance to be higher for the higher
319 dose cobalt chloride administration relative to the vitamin B_{12}/cobalt preparation i.e. the reabsorption
320 mechanism from urine to blood is saturated for high concentrations of cobalt in the urine but for lower
321 doses (and lower urine levels) reabsorption returns to first order kinetics.

322

323 In plasma, the threshold of 25 ng/ml is only exceeded for a couple of hours after the administration of
324 the current vitamin B_{12}/cobalt preparation whereas urine concentrations drop below the 100 ng/mL
325 threshold by approximately 8 hours. Based upon the predicted accumulation (Table 3) it would be
326 highly unlikely that any increase in urine concentration would exceed a 100 ng/mL urine screening
327 limit with a once a day regimen of the current vitamin B_{12}/cobalt preparation or equivalent dose cobalt
328 supplement. However, based on a Monte Carlo simulation (data not shown), the 25 ng/mL plasma
329 threshold could potentially be breached in a population of horses with a once a day regimen of a
330 vitamin B_{12}/cobalt supplement or equivalent dose cobalt supplement. Therefore, practitioners should
331 continue to advise caution when using these products in racehorses.

332 Following administration of cobalt chloride alone the plasma threshold of 25 ng/ml was exceeded for
333 at least 7 days and in the 2 horses with extended sampling, up to 15 days (360 hours) post
334 administration, whereas urine concentrations drop below the 100 ng/mL threshold by approximately
335 120 hours (5 days).

336 Although the total cobalt concentration was above 25 ng/mL in plasma for at least 7 days after a 100
337 mg cobalt chloride administration, the use of a cobalt to vitamin B_{12} ratio may potentially extend the
338 period of detection for this doping practice and allow regulators to differentiate it from administration of
339 a typical cobalt/vitamin B_{12} supplement with total cobalt dose of approximately 1mg, such as the
340 vitamin B_{12}/cobalt supplement in this study. The current study demonstrated that cobalt originating from
341 the administration of a product containing cobalt and vitamin B_{12} could be distinguished from cobalt
342 originating from the administration of exclusively inorganic cobalt salts by the differences in the ratio of
343 cobalt to vitamin B_{12} associated with each, in both plasma and urine. There were a number of
344 limitations of the study which included; only one vitamin B_{12}/cobalt product was evaluated, however a

number of commercial B_{12} products are available with variable cobalt content; only a single dose was administered and multiple doses are required to confirm the estimated extent of accumulation; only six horses were investigated, which were not in full race training and therefore the results may be specific to this population of horses.

A strategy for allowing supplementation with cobalt/vitamin B_{12} containing products whilst controlling the misuse of high doses of inorganic cobalt could be based on using a combination of such a ratio of cobalt to vitamin B_{12}, in plasma and urine with the existing IFHA thresholds for cobalt of 100 ng/ml in urine and 25 ng/ml in plasma. Such a strategy, summarised in Table 4, would require two phases; i.e. control within 24 hours of an administration, which would cover a race day, and control for longer than 24 hours from administration in the case of training surveillance.

It should be noted that the pharmacokinetics of cobalt resulting from the administration of legitimate cobalt/B_{12} supplements, such as the vitamin B_{12}/cobalt supplement used here, suggest that accumulation of cobalt in plasma as a result of multiple daily administrations may exceed the international threshold of 25 ng/mL within a population of horses. Therefore, more studies are required that investigate the extent of accumulation in plasma resulting from a multi-dose regimen of cobalt supplements. No significant accumulation of cobalt concentration is expected within urine.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

The study was approved by the British Horseracing Authority's Centre for Racehorse Studies Animal Welfare and Ethics Review Board, with the horses and personnel involved licensed under the UK's Animals (Scientific Procedures) Act.

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Authorship

All authors contributed equally to the study design, preparation and final approval of the manuscript with the process being led by L. Hillyer. L. Hillyer, Z. Ridd, P. Hincks and S. Fenwick were responsible for the study execution. S. Paine and L. Hillyer were responsible for the data analysis and interpretation. All authors have approved the final version of the manuscript.

Manufacturers' addresses

[a]Vetoquinol, Buckingham, Buckinghamshire, UK.

[b]MILA International, Inc., Florence, Kentucky, USA.

[c]Sigma-Aldrich Company Limited, Dorset, UK.

[d]Triple Red Ltd., Buckinghamshire, UK.

[e]Siemens Healthcare Diagnostics, Camberley, Surrey, UK.

[f]Romil, Cambridge, UK.

[g]VHG Laboratories, LGC Standards, Manchester, UK.

[h]Agilent, Manchester, UK.

[i]PerkinElmer, Seer Green, UK.

[j]Pharsight, Cary, North Carolina, USA.

[k]GraphPad Software, La Jolla, California, USA, www.graphpad.com.

Figure legends:

Fig 1: Average (6 horses) plasma (red) and urine (green) cobalt concentrations (± s.d. for n>2 common time points) during the sequential administrations of a vitamin B_{12}/cobalt preparation (t = 0 hours) and cobalt chloride (t = 1008 hours), with IFHA adopted thresholds of 25 and 100 ng/mL respectively

Fig 2: Average (6 horses) plasma (red) and urine (green) vitamin B_{12} concentrations (± s.d. for n>2 common time points) during the sequential administrations of a vitamin B_{12}/cobalt preparation (t = 0 hours) and cobalt chloride (t = 1008 hours)

Fig 3a and **3b:** Average (6 horses) plasma (red) and urine (green) ratio of cobalt to vitamin B12 concentrations (95% CI for n>2 common time points) during the sequential administrations of a vitamin B_{12}/cobalt preparation (t = 0 hours) and cobalt chloride (t = 1008 hours).

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Tables

Table 1: Pharmacokinetic parameters of cobalt following a single intravenous administration of a vitamin B_{12}/cobalt preparation (983 µg Co) to 6 exercised Thoroughbred horses using a 3-compartment bolus PK model. See Supplementary Item 3 for parameter definitions.

Horse	Weight (Kg)	V1 (L/Kg)	V2 (L/Kg)	V3 (L/Kg)	Alpha HL (h)	Beta HL (h)	Gamma HL (h)	MRT (h)	Vss (L/Kg)	CL (mL/min/kg)
1	504	0.0692	0.105	0.363	1.14	14.5	245	307	0.537	0.029
2	542	0.126	0.175	0.562	0.810	13.7	432	585	0.863	0.025
3	508	0.0094	0.0734	0.453	0.731	25.8	231	223	0.536	0.040
4	532	0.088	0.140	0.213	1.28	67.6	463	530	0.441	0.014
5	498	0.128	0.130	0.511	0.981	12.9	348	463	0.768	0.028
6	490	0.127	0.123	0.415	1.00	13.2	266	349	0.666	0.032
Mean	**512**	**0.0912**	**0.124**	**0.419**	**0.973***	**19.7***	**319***	**410**	**0.635**	**0.028**
Median	**506**	**0.107**	**0.126**	**0.434**	**0.992**	**14.1**	**307**	**406**	**0.601**	**0.028**

*Geometric mean

Table 2: Pharmacokinetic parameters of cobalt following a single intravenous administration of 45.39 mg cobalt as cobalt chloride to 6 exercised Thoroughbred horses using a 3-compartment infusion PK model. See Supplementary Item 3 for parameter definitions.

Horse	Weight (Kg)	V1 (L/Kg)	V2 (L/Kg)	V3 (L/Kg)	Alpha HL (hrs)	Beta HL (hrs)	Gamma HL (hrs)	MRT (hrs)	Vss (L/Kg)	CL (mL/min/kg)
1	504	0.214	0.248	0.302	1.41	14.3	100	126	0.764	0.100
2	542	0.188	0.165	0.367	1.41	12.0	150	195	0.720	0.061
3	508	0.168	0.0119	0.358	0.0671	0.629	97.2	138	0.538	0.065
4	532	0.156	0.141	0.544	0.567	5.79	122	159	0.840	0.088
5	498	0.149	0.311	0.335	1.61	24.3	182	230	0.795	0.058
6	490	0.0152	0.199	0.393	0.0364	8.99	166	212	0.608	0.048
Mean	**512**	**0.148**	**0.180**	**0.383**	**0.405***	**7.18***	**132***	**177**	**0.711**	**0.070**
a	494-626	0.130	0.162	0.639	0.434*	6.80*	149*	200	0.940	0.083
Median	**506**	**0.162**	**0.182**	**0.362**	**0.988**	**10.5**	**136**	**177**	**0.741**	**0.063**
b	494-626	0.132	0.156	0.630	0.660	6.31	143	184	0.924	0.084

*Geometric mean; [a,b]Knych *et al*. (2015) mean and median data

448 **Table 3:** Average ± s.d. estimated ratio of the area under the curve (AUC) in the first 24 hours relative
449 to the total AUC extrapolated to infinity and the predicted accumulation of the average cobalt
450 concentration upon administration every 24 hours until steady-state has been reached.
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Administration	Matrix	AUC_{0-24}/AUC_{0-inf} ± s.d.	Accumulation (SS) ± s.d.
vitamin B_{12}/cobalt preparation	Plasma	0.153 ± 0.100	8.28 ± 3.43
	Urine	0.615 ± 0.209	1.84 ± 0.78
Cobalt Chloride	Plasma	0.186 ± 0.031	5.48 ± 0.82
	Urine	0.778 ± 0.075	1.30 ± 0.12

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Table 4: Anticipated testing outcomes relating to scenarios involving the administration of a vitamin B_{12}/cobalt product or cobalt chloride relative to the international cobalt thresholds and cobalt to vitamin B_{12} ratios (h or d is the hours or days a positive result persists based upon the average horse concentration or ratio).

Cobalt Administration	Time Period (hours)	Plasma Threshold (>25 ng/mL)	Urine Threshold (>100 ng/mL)	Plasma Co/B_{12} Ratio > 1	Urine Co/B_{12} Ratio > 10
Routine use of a vitamin B_{12}/cobalt preparation	<24	positive (2 h)	positive (8 h)	positive	positive
	>24	negative	negative	negative	negative
Doping with Cobalt Chloride (100 mg dose)	<24	positive	positive	positive	positive
	>24	positive (15 d)	positive (4 d)	positive (15 d)	positive (18 d)