Laboratory Investigation of Cobalt Regulation in Horses

by Ross Gregory Wenzel

Thesis submitted in fulfilment of the requirements for the degree of

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under the supervision of Professor Philip Doble

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Ross Gregory Wenzel declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Mathematical and Physical Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by the Australian Government Research Training Program.

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The list of manuscripts is provided in Appendix 9.9.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

COMMON ABBREVIATIONS

Short form	Description
ABEC	Agnes Banks Equine Clinic
AMU	Atomic mass units
AR	Aqua regia (4-parts HNO_3 to 1-part HCl by volume)
B12	Vitamin B12, cyanocobalamin, cobalamin or other cobalt containing molecules based on a corrin structure
Co	Cobalt
Cr.	Creatinine
CRI	Collision reaction interface
c s^{-1}	Counts sec^{-1} as measured by the ICP-MS detector
EDTA	Ethylenediaminetetraacetic acid
Epo	Erythropoietin
HIF	Hypoxia-inducible factor
HPLC	High performance liquid chromatography
HRNSW	Harness Racing New South Wales
ICP-MS	Inductively coupled plasma - mass spectrometer
ID	Internal diameter
IFHA	International Federation of Horse Racing Authorities
IM	Intramuscular
IV	Intravenous
LSD	Least significant differences
MCN	Micro-concentric glass nebuliser
NATA	National Association of Testing Authorities
NIST	National Institute of Standards and Technology
RCPAQAP	Royal College of Pathologists of Australasia Quality Assurance Program
RELM	Restricted maximum likelihood
RNSH	Royal North Shore Hospital
PHD	Prolyl hydroxylase
QMEQAS	Quebec Multi-Element Quality Assurance Scheme

SG	Specific gravity
SRM	Standard reference material
VHL	von Hippel-Lindau protein
WADA	World Anti-Doping Agency

ABSTRACT

It has been proposed that cobalt (Co) can be used as a performance enhancing drug for endurance athletes. The basis for enhanced performance stems from the 1950's, 60's and 70's, when some institutions advocated the use of high doses of Co to increase red blood cell production in patients with anaemia associated with renal disease. With increased red cell production, Co was thought to provide an advantage to athletes in endurance sports where a higher red cell mass would result in improved oxygen carrying capacity. Further research using in vitro cell lines elucidated a mechanism by which divalent cations, such as Co, could reduce the rate of degradation of a protein capable of inducing erythropoietin production and thereby red blood cell production.

Subsequently, some sporting bodies advised athletes of Co misuse investigation, and the international horse racing association introduced a threshold Co level. This thesis evolved after I was approached by a legal team to determine equine urinary Co concentrations. Given my experience investigating Co in patients with failed metal-on-metal prosthetic hips, it was immediately apparent that there were deficiencies in the approach being taken by the horse racing industry to manage Co misuse.

With the backing of literature reviews and original research, this thesis concludes that there was scant evidence to support the hypothesis that Co can enhance the athletic performance of racing horses. The research identified the need to differentiate inorganic Co from vitamin B12 when testing urine to assess Co misuse. Using original research to evaluate the cumulative nature of Co, this thesis demonstrates that urinary Co is an ineffective and unreliable means of screening for Co abuse in horses.

1. GENERAL INTRODUCTION

1.1 Summary

This Chapter describes:

- The rationale behind the introduction of rules regulating cobalt (Co) intake.
- The processes used to set a threshold level for Co in racing horses.
- A limited selection of legal challenges and media reports of cases where trainers had breached the Co threshold. These cases were chosen to provide context for this thesis and highlight the significance of the research presented.
- The objectives and research plan for investigating questions that have arisen in the prosecution these cases.

1.2 Background and scope

Cobalt has been described as having the potential to act as a performance enhancing drug in athletes, with implications that high doses can increase aerobic capacity (Lippi, Franchini & Guidi 2005; Mørkeberg 2013). The first description of Co use in horses was with the Ontario Racing Commission issuing a warning over the potential unsafe use of Co (Ontario Racing Commission 2009). Four years later, a regulatory threshold for Co in urine originated in Australia. Harness Racing New South Wales (HRNSW) enacted a rule where Co was listed as a prohibited substance to be maintained below a defined threshold. Any urine samples collected since 11 September 2013, with a cobalt chloride level at or above 200 µg L^{-1}, were deemed to be in breach of the local rule (Harness Racing New South Wales 2013b). Other Australian racing authorities gradually followed suit so that by 1 January 2015 the Co threshold was applied as a national rule.

The steps taken to establish this world first Co threshold were published by Racing.com (Anderson 2015) and it was the absence of validation of the approaches described here that have plagued the enforcement and outcomes of Co control measures ever since. The threshold was adopted on the basis of just 80 post-race urine samples where cobalt chloride administration was the sole interest of all concerned. In establishing this threshold there was no mention of vitamin B12 or other possible variables that could

influence the concentration of Co in urine samples, such as urine concentration and medications. No mention was made of steps taken (if any) to validate the assumption that 50 μg L^{-1} was to be considered the upper level of a 'normal' population of urine samples. Instead, when the enforcement of the Co threshold was first challenged in court, it was stated by the statistician referring to his calculated upper limit for a normal urine Co that: 'without independent studies on a known control (not administered) population and an administration study to find out levels reached after a typical administration, the analysis given here should be regarded as provisional' (Adamson 2014). Those independent studies have never been undertaken. Furthermore, there is no evidence that the approaches taken then, or subsequently, to define a Co threshold were fit for purpose.

A threshold for blood Co originated in the United States when a New Jersey racetrack suspended 2 trainers in 2014 after they were found to have presented horses with excessive blood Co levels. A blood Co threshold was set for New Jersey and New York racetracks The Meadowlands, Tioga Downs and Vernon Downs, so that if a blood sample revealed a horse had a Co level higher than 4 times the standard deviation above the normal level, the trainer of that horse was deemed unable to participate (The Meadowlands 2014). By January 2016, the International Federation of Horseracing Authorities (IFHA) had set a Co threshold of 100 μg L^{-1} in urine for samples collected on race day (International Federation of Horseracing Authorities 2016) with a threshold to include blood Co added the following year (International Federation of Horseracing Authorities 2017). Racings' current interpretation of Co states: 'A *trace element that is naturally occurring in the environment and is present in water, animal feed and some dietary supplements. It is also part of the structure of vitamin B12 (cobalamin). Cobalt can be misused in an effort to improve the performance of a racing animal by better regulating blood cell production. It is therefore regulated in respective rules of racing by way of thresholds in urine and blood samples.*' (Queensland Racing Integrity Commission 2019).

At face value, regulation through a threshold would seem an appropriate response. However, inadequate research on numerous facets of Co disposition have left this ruling fraught with controversy. From the outset, very few studies had assessed the effects of

Co in horses. The uptake and distribution of Co were principally studied in laboratory animals and humans, where it accumulated primarily in liver, kidney, pancreas, and heart, with bone and muscle levels increasing with time (Simonsen, Harbak & Bennekou 2012). A relatively small fraction of the total blood Co was found to be present as the free ionised salt – 5-12 % in healthy human volunteers (Kerger et al. 2013), while the main fraction of plasma Co was bound tightly to albumin and other plasma proteins. Over time, with continued exposure, increasing amounts of Co enter the red blood cells via transfer proteins. Once bound within the red cell the Co is retained for the life of the cell, i.e. approximately 120 days (Simonsen et al. 2011).

Parenterally administered Co has no known natural function in the horse, but laboratory studies have demonstrated its potential as a hypoxia-inducible factor (HIF) activator (Simonsen, Harbak & Bennekou 2012), although in a literature review (Mobasheri & Proudman 2015) found no evidence of any potential performance enhancing effect in horses. No effect on red cell count or erythropoietin levels was observed after the single administration of a 49 mg intravenous (IV) dose of Co as cobalt chloride to each of 16 horses (Knych et al. 2014). Signs of acute toxicity were described when large IV Co doses (up to 4 mg kg^{-1} or 2000 mg per horse) were administered weekly for 5 weeks (Burns et al. 2018). A comprehensive review of Co supplementation in racing horses stressed the need for further work to clarify the pharmacodynamics of long-term exposure (Kinobe 2016). This review also noted that there was no evidence of either performance enhancing or toxic effects of Co in horses and emphasised that, with a compartmental excretion pattern and long elimination half-life, the cumulative effects of repeated Co dosing were unknown.

Several intravascular factors known to influence urinary levels and excretion rate of Co in humans and other animal species were not investigated when setting a Co threshold.

These include:

- Competition and displacement of Co from protein binding sites by other metals such as iron, selenium, calcium and magnesium (Flora & Pachauri 2010; Llobet, Domingo & Corbella 1986; Yang & Black 1994).

- Reduced affinity for Co binding by ischaemia-modified albumin (Apple et al. 2002; Lippi, Montagnana & Guidi 2006).
- Intravascular haemolysis caused by racing or transport stress leading to premature death of red cells (Hanzawa & Watanabe 2000; Yaqub, Mshelia & Ayo 2014). With Co tightly bound in red cells any factors leading to increased uptake and degradation of red cells by the reticuloendothelial system can alter the rate of Co excretion.

Despite the regulated maximum Co threshold having been in effect for several years, many trainers are still presenting horses at race meetings with excessive urinary Co concentrations. This is contrary to the expectation that the introduction of a regulatory threshold enforced by harsh penalties would see instances of non-compliance disappear. Cobalt infringements have, however, continued unabated. This study investigates the premise for considering Co as a performance enhancing drug and investigates factors that may mitigate the effectiveness of the current urinary Co threshold. Knowledge obtained in this thesis from the original research conducted through administration trials has advanced understanding of Co accumulation and challenged the reliability of using urine Co to detect Co misuse. By reviewing individual cases of trainers penalised for Co misuse, several sources of inadvertent Co exposure were identified. The scope of these investigations includes: a review of Co as a performance enhancing drug; identification of sources of Co; development of laboratory methods to determine Co; and application of these methods to define factors influencing Co excretion. It should also be noted that even though these experiments were conducted in horses, many of the conclusions drawn would be equally valid when considering the application of a Co threshold to greyhound racing or human athletes.

1.3 Cobalt – origin, occurrence, and properties

Cobalt is an element with the atomic weight 58.933. It has been used as a blue colouring agent for pottery, jewellery, and glass by Egyptian and Persian civilisations from around 2000 BC. Cobalt was first isolated by Swedish chemist George Brandt in the 18th Century and identified as an element by T.O. Bergman in 1780 (Hamilton 1994). The name Co

was derived from the German word *kobalt*, from kobold meaning goblin, a superstitious term used by Co ore miners as primary Co ores contain arsenic, released as the toxic arsenic oxide during smelting.

Cobalt is a relatively rare metal comprising 0.0029 % of the Earth's crust. Australia has the world's second highest reserves of Co (U.S. Geological Survey 2020). It is a magnetic element with similar chemical properties to iron and nickel, differing in atomic structure from iron by only the position of one electron in one of the orbits. Cobalt has two valence states, cobaltous (II) and cobaltic (III), with the cobaltous state most used in industry. On a global basis, the leading use of Co is in rechargeable battery electrodes. Another major use of Co is to create high-temperature, corrosion-resistant superalloys to make parts for the gas turbine jet engine. Cobalt is also used to make: car airbags; catalysts for the petroleum and chemical industries; cemented carbides (also called hard metals) and diamond tools; corrosion and wear resistant alloys; drying agents for paints, varnishes, and inks; dyes and pigments; ground coats for porcelain enamels; high-speed steels; magnetic recording media; magnets; and steel-belted radial tyres (U.S. Geological Survey 2020).

For the general population, diet is the main source of Co with drinking water typically containing very little Co. The average intake of Co was estimated at 11 μg Co day^{-1} with relatively high concentrations of Co in green leafy vegetables and unrefined cereals (Dabeka & Mckenzie 1995). Occupational exposure occurs primarily during the production of Co powders. The average Co concentration of the earth's crust is 25 mg Co kg^{-1}. Soils that contain less than 3 mg Co kg^{-1} are considered to have insufficient Co to meet the nutritional demands of grazing cattle. Cobalt is therefore a frequent addition to fertiliser used on agricultural soils (Barceloux & Barceloux 1999).

1.4 Cobalt as an essential trace element

Cobalt is essential to the metabolism of all multicellular eukaryotic organisms as a key constituent of cobalamin, also known as vitamin B12 (Yamada 2013). Vitamin B12 is the largest and structurally most complex of all the vitamins (Figure 1.1). It can only be produced by bacteria or archaea (prokaryotic single cell microorganisms, i.e. cells without

nuclei). Consequently, all animals owe their continuing existence to these primitive organisms. Though vitamin B12 contains Co, it should not be considered a source of inorganic Co as it is tightly bound within the B12 molecule. In this form its biological activity is strictly limited to B12 function. There is no evidence or suggestion that biochemical pathways exist in mammals to metabolise B12 to release inorganic Co. Indeed, given its requirement as an essential nutrient that is only available through complex microbial synthesis, the evolution of biochemical pathways for its degradation would be counterproductive.

Vitamin B12 is frequently promoted as an energy and performance enhancer, a reputation likely based on the fact that correcting the megaloblastic anaemia caused by vitamin B12 deficiency alleviates the associated fatigue and weakness. However, vitamin B12 supplementation has no proven beneficial effect on performance in the absence of a nutritional deficit. Vitamin B12 is not a prohibited substance any more than are water, salt and glucose which only act on mammalian body systems when there are deficiencies. When discussing vitamin B12 in the context of Co regulation the following points should be noted:

- It is not a performance enhancing substance because it is not a source of inorganic cobalt and it is not metabolised in the body to release 'free' cobalt.
- It is not a prohibited substance according to the World Anti-Doping Agency (WADA) (World Anti-Doping Agency 2015a).
- It is not an erythropoiesis-stimulating agent, not a non-erythropoietic EPO-receptor agonists, not a HIF stabiliser, not a HIF activator, not an allosteric effector of haemoglobin, not an oxygen carrier, and not a haematopoietic growth factor.
- It is only capable of acting on mammalian body systems when there is a vitamin B12 deficiency, something that has never been reported in horses.

Non-ruminants require a dietary source of B12 whereas rumen bacteria are capable of synthesizing B12 for their host, provided adequate Co and phosphorus are present in the diet (Stillions, Teeter & Nelson 1971). Ruminants require a constant replenishment of Co for B12 synthesis with plasma and liver B12 concentrations dependent on Co intake

(Stemme et al. 2003). Trace quantities of Co are therefore required from plants these animals graze on with a minimum Co uptake of 0.20 mg kg^{-1} per day recommended (Schwarz, Kirchgessner & Stangl 2000). The Co content of plants is influenced by soil Co concentrations hence Co deficient grazing pastures are treated with Co fortified fertilisers to ensure adequate dietary Co intake. Horses are non-ruminants but possess bacteria in the hind gut that are capable of B12 synthesis (Coenen & Vervuert 2005). Horses can also absorb B12 following ingestion of dietary cobalamin, or its synthetic derivatives, principally cyanocobalamin. No deficiency or toxicity signs have been reported for B12 in horses (Manthe & Youngs 2013). However, with hard work, tissue requirements for B12 may rise in response to the increased rate of energy metabolism (Frape 1988).

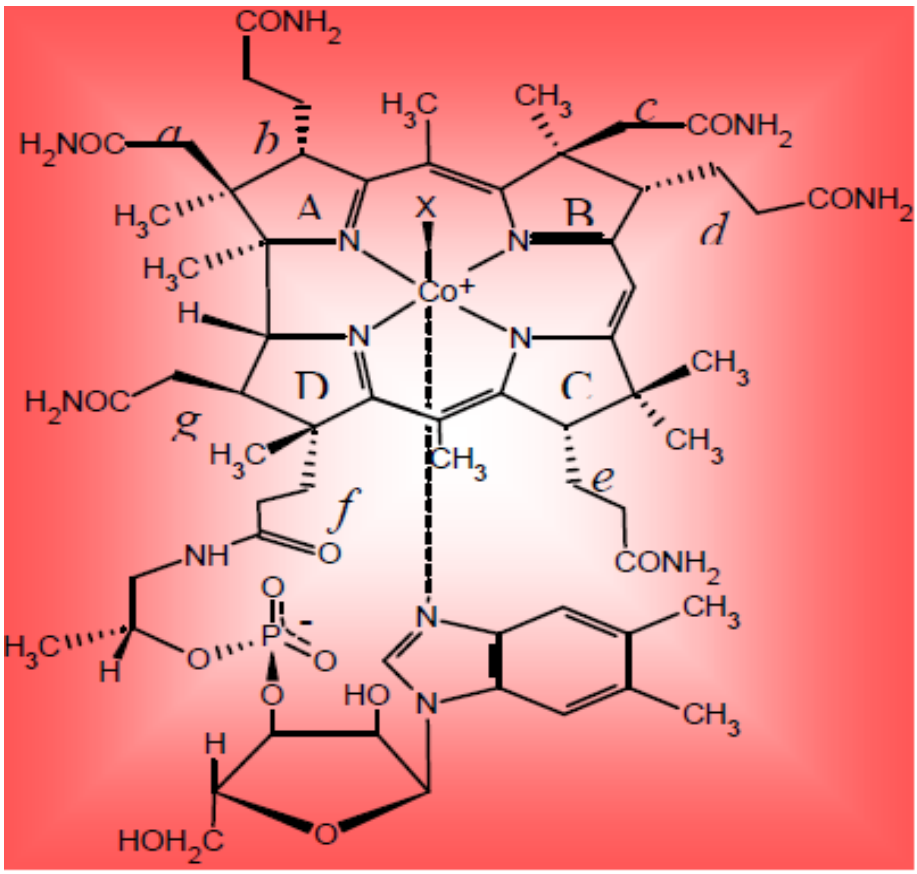

Figure 1.1 Structural formula of cobalamin. Vitamin B12 (cyanocobalamin) has the CN group in the upper ligand (X = CN). Methylcobalamin (X = CH_3-) and adenosylcobalamin (X = 5'-deoxyadenosyl-) are the biologically active cofactor forms of cobalamin. Figure adapted from (Randaccio et al. 2010).

Vitamin B12 functions as the co-factor for the enzymes methylmalonyl-CoA mutase and methionine synthase to orchestrate numerous biochemical pathways (Takahashi-Iñiguez et al. 2012). Vitamin B12 has the lowest required dietary intake for an essential nutrient yet has profound effects on red blood cell manufacture; the metabolism of proteins, carbohydrates, and fats; degradation of the amino acids valine, isoleucine, methionine, and threonine, odd-chain fatty acids, and cholesterol to produce succinylCoA, an intermediate product of the tricarboxylic acid cycle; the generation of the essential amino acid methionine; and the formation of methylmalonic acid. A unique and complex absorption, delivery and activation system has been described for B12 (Yamada 2013). Cobalt is only an essential trace element in mammals as a component of B12. Other less common metalloproteins that bind Co directly have been described. For example, methionine aminopeptidase isolated from porcine liver can be stimulated by Co^{2+} ions but can also be activated by other divalent cations such as Mg^{2+}, i.e. it does not exclusively require Co for activity (Yamada 2013).

1.5 Cobalt toxicity

As noted by Paracelsus 'All things are poisons, for there is nothing without poisonous qualities. It is only the dose which makes a thing poison.' This quote is true of Co where Co is not considered a poisonous substance, though if given in high enough doses adverse effects have been reported. In early experiments investigating the erythropoietic effects of Co (Kleinberg 1934), rabbits weighing 2.0–2.4 kg receiving single subcutaneous injection of 60, 120 or 240 mg Co died within 24 h. Those rabbits receiving subcutaneous injections of 10 and 20 mg Co showed no adverse symptoms while the animal receiving 40 mg Co exhibited slight lethargy for 1 day. Cumulative Co toxicity was also demonstrated in this study, with rabbits receiving daily injections of 10 mg Co as cobalt chloride dying at around 21 days.

Cobalt toxicity in humans is mainly of concern in 2 situations. The first is industrial exposure of workers in metallurgical industries (Martin et al. 2009; Simonsen, Harbak & Bennekou 2012), while the second major area is in orthopaedic patients who have received metal-on-metal hip replacements (Daniel 2007). Occupational exposure is

mainly through the lungs. In the general population Co exposure is through the diet. Goitre and reduced thyroid activity have been described with excessive Co exposure. Exposure to Co alone produces an allergic contact dermatitis and occupational asthma (Barceloux & Barceloux 1999). Several patients were reported to develop reversible hypothyroidism and goitre coincident with their Co treatment. Similarly, hearing loss, visual impairment, and polyneuropathy have been described in patients receiving cobalt chloride treatment for anaemia. Thus, polycythaemia, hypothyroidism, neuropathy, and cardiomyopathy are possible effects that might be consequent to exposure to high doses of Co from metal-on-metal hip implants (Brent & Devlin 2013).

Cobalt concentrations corresponding to adverse health effects remain poorly defined. Cobalt release from metal-on-metal hip implants was extensively studied following the recall of a faulty Depuy ASR prosthesis (Medicines and Healthcare products Regulatory Agency 2010). Premature wear from the cobalt-chromium alloy used in the manufacture of this device resulted in the release of chromium and Co ions into the surrounding tissue and bloodstream. Serum and blood Co concentration are normally less than 1 $\mu g\ L^{-1}$. The Medicines and Healthcare products Regulatory Agency in the United Kingdom recommended that patients with blood Co concentrations greater than 7 $\mu g\ L^{-1}$ and a metal-on-metal prosthesis may require further investigation, but this Co concentration was based on identifying premature wear rather than toxicology outcome. Other associations between Co concentration and clinical outcome are based on single case studies preventing conclusive toxicological findings. For example, an elevated serum Co of 288 $\mu g\ L^{-1}$ from failed implants was proposed as a cause of cardiomyopathy in this patient (Allen et al. 2014). Ethical concerns limit the effectiveness of controlled investigations with upper limits on concentrations of Co administered. In a study conducted to assess the effects of Co ingested at a rate of 1 mg per day, human volunteers were found to attain peak Co whole blood concentrations ranging between 9.4 and 117 $\mu g\ L^{-1}$ that were not associated with clinically significant changes in basic hematologic and clinical variables (Tvermoes et al. 2014).

Epidemiological studies linking Co exposure to toxic outcomes remain dubious. In 1966, the syndrome “beer drinkers’ cardiomyopathy” was first described in Quebec City,

Canada. This syndrome was attributed to cobalt chloride added to beer by local breweries where Co doses of 1 to 1.5 mg L^{-1} were used as a method of reducing foam formation from dishwasher soap residues. The syndrome was characterised by pericardial effusion, elevated haemoglobin concentrations and congestive heart failure (Kesteloot et al. 1968). Though frequently cited when describing Co toxicity, Co was most likely not responsible for the symptoms described. The doses of Co ingested were considerably less than those described to treat refractory anaemia suggesting other causative factors associated with chronic alcohol abuse such as inadequate protein, thiamine, and zinc intake or alcohol-induced heart damage (Barceloux & Barceloux 1999). Studies on workers occupationally exposed to Co also found no association with Co and cardiomyopathy (Lantin et al. 2013).

A comprehensive review of animal toxicology and epidemiology literature suggested the following relationship between blood Co and health effects (Finley et al. 2012):

- Concentrations of 300 µg L^{-1} and less have not been associated with adverse responses of any type in humans.
- Concentrations of 300 µg L^{-1} and higher were associated with certain haematological and reversible endocrine responses, including polycythaemia and reduced iodide uptake.
- Concentrations of 700–800 µg L^{-1} and higher may pose a risk of more serious neurological, reproductive, or cardiac effects.

Chronic cobalt toxicity has not been reported in horses. In a study designed to test the effect of high doses of cobalt chloride, 5 Standardbred mares weighing 460–530 kg were administered 5 doses of cobalt chloride (4, 2, 1, 0.5, or 0.25 mg kg^{-1}) as an IV bolus (infused over 1 min) once weekly for 5 weeks (Burns et al. 2018). Mares receiving 4, 2, or 1 mg kg^{-1} doses developed tachycardia immediately after dosing though cardiovascular parameters had returned to baseline by 1–2 h post-administration. These results indicated that horse welfare could be compromised by injecting more than 1 mg Co kg^{-1}.

1.6 Cobalt as a treatment for anaemia

The erythropoietic effect of Co was first described in trials on rats over 90 years ago (Waltner & Waltner 1929). At that time, it was well known that iron could correct anaemia, returning haemoglobin concentrations to normal while not increasing haemoglobin beyond normal levels as iron replacement continued. Conversely, manganese had been described as capable of inducing polycythaemia prompting suspicion that other elements could also contribute to the elevated haemoglobin levels observed in German miners (Waltner & Waltner 1929). When 0.5–2 % pulverised metallic Co was mixed with rat feed, or subcutaneous injections ranging from 0.01 to 0.1g cobalt chloride or cobalt nitrate were administered to rats, increased red cell and haemoglobin concentrations were noted with a 20 % increase in these parameters reported 24 h after cobalt nitrate injection (Waltner & Waltner 1929).

Experiments conducted in other mammalian species, including dogs (Mascherpa 1930), also demonstrated erythrocytosis in response to Co administration. When investigating Co as a means of correcting nutritional anaemia, it was found that Co alone was of no benefit but instead a combination of iron and copper supplements were required to restore normal haemoglobin levels (Orten, Underhill & Lewis 1932). It was noted that red cell and haemoglobin levels returned to normal levels, only and not beyond, when iron and copper were used to correct anaemia. Polycythaemia was however observed when rats on a basal diet containing iron (0.5 mg) and copper (0.025 mg) also received a mixture of Co (0.5 mg), manganese (1.0 mg), nickel (1.0 mg), and zinc (0.5 mg) (Orten et al. 1932). This led the authors to conclude that one or a combination of these additional metals was required to induce polycythaemia. Haematocrit measurements, in addition to haemoglobin and red cells counts, enabled these authors to confirm that the volume of red cells had also increased. Increasing haematocrit in response to erythropoietic agents are considered a good indicator of changes in red cell mass. The application of this haematological parameter is described further in Chapter 5.4.3.

When Co was administered in combination with manganese, researchers demonstrated that the erythropoietic action of Co could be attained at lower levels with fewer side effects (Kleinberg 1934). This study also demonstrated that chronic Co administration,

rather than a single bolus dose of Co, was necessary to stimulate erythropoiesis. Rabbits that had received a single subcutaneous injection of 10, 20 or 40 mg of cobalt chloride exhibited no increase in daily reticulocyte or red cell counts in the 2 weeks following the injection. In contrast, when 10 mg cobalt chloride was injected daily, marked gradual increases in daily reticulocyte and red cell counts were observed until the injections ceased after 16 days. Initially it was unclear if the cause of the increased red cell mass following chronic Co administration was due to a protective effect of Co causing a decrease in red cell destruction or if the Co stimulated red cell production. Elevated percentages of circulating reticulocytes in juvenile rats receiving an oral supplement containing 0.5 mg Co, and in adult rats weighing from 250 to 330 g receiving daily subcutaneous injections of 0.5 mg Co as cobalt chloride, indicated the latter (Orten 1935).

1.6.1 Erythropoeitin

It is now understood that the erythropoietic action of Co was through its indirect upregulation of erythropoietin (Epo) production (Fisher 1998). Epo is a cytokine protein and the primary and most likely only mediator of hypoxic induction of erythropoiesis. The kidney is responsible for approximately 80 % of Epo production (Bunn 2013). Epo acts on progenitor cells in the bone marrow to stimulate the production of red blood cells (Figure 1.2). Epo has a plasma half-life of around 7 to 8 h and in mammals circulates to maintain erythropoiesis at a low basal level, replacing senescent red blood cells at a rate of 2.3 x 10^6 cells sec^{-1} in human adults (Hodges et al. 2007). In humans, the rate of red cell production can be increased up to eightfold in response to boosted Epo production to compensate for blood loss and other conditions that decrease the oxygenation of arterial blood or tissues. After Epo has initiated erythropoiesis the average transit time from proerythroblast to emergence of the reticulocyte into the circulation is 5 to 7 days with a further 1 to 2 days for reticulocytes to mature into red cells (Narla & Ebert 2018).

Given Epo response duration, the practice of controlling Co misuse by collecting urine samples on race day is ineffective. When a horse is presented to race, ample time has elapsed to allow urinary Co levels to return to normal following Co administered with the intent of upregulating Epo production. It takes up to 3 days for Epo administration to increase red cell output, a further 4 days for HiF1α to trigger increased Epo output (Figure

1.2), and possibly several further days for Co to assist in the 'survival' of HiF1α. All told it could be estimated that the time from Co administration to an increase in the number of circulating red cells would be in the vicinity of several weeks. This estimation is consistent with publications where (Davis & Fields 1958) reported a response after 7–22 days of continuous Co supplementation while (Knych et al. 2014) found nothing happened in horses after 10 days following a single administration of Co without looking any further. If racing authorities were seriously trying to detect Co 'doping' then urine testing should only be conducted about a fortnight before the race. There is no evidence-based information that Co administration on the day of a race, or elevated Co in the blood on that day, enhances performance. An alternative means of assessing prior Co administration would be to measure Co in red blood cells once an appropriate method of testing had been validated and verified.

When administered to gain an unfair racing advantage, the expected effect of Epo in horses is an increase in red blood cell mass, providing improved oxygen carrying capacity of blood and thereby enhancing the horse's aerobic exercise performance. In unfit horses, it was shown that recombinant human Epo enhanced aerobic capacity without either altering anaerobic power, or improving exercise performance (McKeever et al. 2006). Any perceived benefit of increased red cell mass in horses is limited. In contrast to humans, horses can sequester erythrocytes in their spleen, which acts as a reservoir, storing up to 30 % of the total red blood cells when resting. During exercise, the spleen can contract to release up to an extra 12 litres of blood, thereby increasing the blood oxygen carrying capacity. As such, the actual effect of Epo on performance in horses remains unclear. In the context of performance enhancing drug monitoring, the 150-day half-life of red blood cells in the horse allows the putative benefit of Epo to develop over several weeks without the risk of Epo administration being detected. Notably, a threshold has been set for Co on the basis that it could induce Epo production, yet no threshold exists for Epo administration despite recombinant human Epo injections having been proven to increase red cell production in horses.

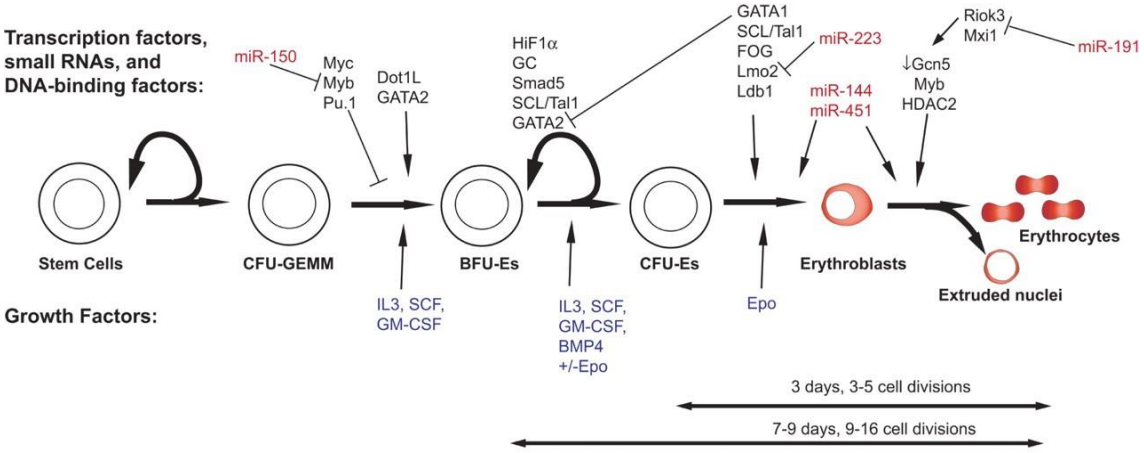

Figure 1.2. Overview of erythropoiesis regulation at numerous levels by multiple factors including Epo. The duration from Epo stimulation to mature erythrocyte release is shown. Epo response duration highlights the limitations of using race day urine collections to investigate Epo upregulation by Co. Figure adapted from (Hattangadi et al. 2011).

1.6.2 Hypoxia-inducible factor

The hypoxic induction of Epo is largely dependent upon the action of hypoxia-inducible factor (HIF). This transcription factor can be activated in nearly all cells in response to hypoxia. Once activated, HIF binds to a consensus sequence to promote *Epo* mRNA production. Numerous other genes are also regulated by HIF including those involved in angiogenesis, intermediary metabolism, and iron homeostasis. Two subunits, HIF-1 and HIF-α, combine to form functional HIF. In adequately oxygenated cells, HIF-α is unstable because it forms a complex with the protein von Hippel-Lindau (VHL) enabling it to undergo ubiquitination (Maxwell et al. 1999). Ubiquitin is a regulatory protein found in most tissues of eukaryotic organisms. It acts as a tag that signals the protein transport machinery to ferry the tagged protein to the proteasome for degradation. In oxygen stressed cells, VHL binding to HIF-α is inhibited. This prevents ubiquitin tagging and subsequent HIF-α degradation enabling accumulation of the HIF-α subunit. HIF-α is then free to enter the nucleus and combine with HIF-1, forming a stable HIF heterodimer capable of promoting Epo production (Bunn 2013).

Ultimately cells respond to changes in intracellular oxygenation through the action of prolyl hydroxylase (PHD). Two highly conserved sequences encoding the oxygen-dependent domain of HIF-α present proline residues at specific recognition sites for the oxygen- and iron-dependent enzyme PHD. This enzyme catalyses the conversion of proline to hydroxyproline, creating a post-translational modification changing the structure of the HIF-α protein to permit binding of VHL. The regulatory oxygen sensing function of PHD is possible because PHD has an active centre where oxygen, an iron atom and molecule of α-keto-glutarate interact to confer PHD catalytic capability.

It has been proposed that Co acts to mimic hypoxia by displacing iron from the PHD active site as both iron chelation and Co fortification have been shown to inhibit PHD activity (Maxwell et al. 1999). An alternative theory for the action of Co centres around the observation that the enzymatic activity of PHD depends on iron being present in the Fe^{2+} state (Fong & Takeda 2008). Ascorbate protects iron from reactive oxygen species that would otherwise reduce active Fe^{2+} to the inactive Fe^{3+} state (Figure 1.3). High extracellular concentrations of Co and nickel have been shown to prevent the intracellular transfer of ascorbate thereby allowing HIF-α to progress to the nucleus. Competitive inhibition of iron uptake has been demonstrated where Co blocks the cellular uptake of iron (Kuiper et al. 2014).

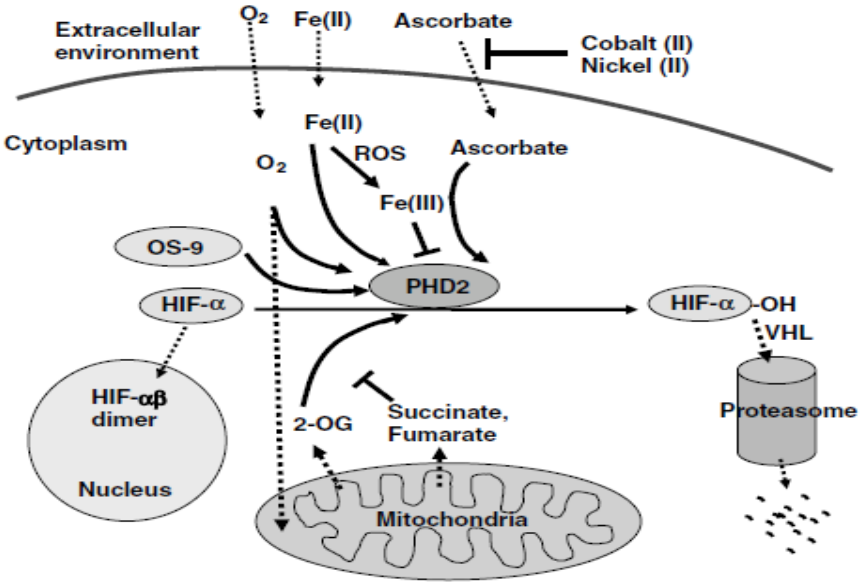

Figure 1.3. Schematic illustration of regulatory mechanisms of PHD hydroxylase activities. Figure adapted from (Fong & Takeda 2008).

In order to induce polycythaemia, oral daily administration of elemental Co are required at concentrations in the range of 0.125–1.0 mg or from the continued parenteral administration of doses of Co salts providing 0.125 mg of elemental Co or more per day (Fisher 1998). Very high concentrations of Co must therefore be used to effect erythropoiesis. Adverse health effects from these sustained high concentrations have been described before haematological changes were noted (Paustenbach et al. 2013). No polycythaemia was reported in a patient with a serum Co concentration of 288 μg L^{-1} due to excessive metal-on-metal hip implant wear (Allen et al. 2014).

1.7 Policy on the use of cobalt in sports

In horses, Co regulation was first instigated for harness racing in New South Wales on 16 December 2013 (Harness Racing New South Wales 2013b). This initial regulatory measure was limited to urine samples where a threshold of 200 μg L^{-1} was applied. The urine Co threshold was amended from 200 to 100 μg L^{-1} on 1 September 2016 as per Rule AR.178C. (1) (1) of the Australian Rules of Racing (Racing Australia 2017), where it was stated that Co was permitted when present at below a mass concentration of 100 μg L^{-1} in urine or 25 μg L^{-1} in plasma. This amendment brought the threshold in line with the International Racing Guidelines where on the 25 January 2016, a Co threshold of 0.1 μg Co mL^{-1} in urine was stated (International Federation of Horseracing Authorities 2016). The International threshold was amended on 22 August 2017 to 0.1 μg Co mL^{-1} in urine or 0.025 μg total Co mL^{-1} (free and protein bound) in plasma. This amendment also included the note that Racing Authorities should provide an advisory regarding the use of Co containing supplements (International Federation of Horseracing Authorities 2017). These threshold levels currently stand (Racing Australia 2019).

Two other entities have sought to regulate Co use. Australian greyhound racing have limited Co testing to urine and enforced a Co threshold of 100 μg L^{-1} in all States from 1 September 2015, with the exception of NSW, where the threshold was implemented on 1 October 2015 (Greyhound Racing New South Wales 2015). International athletics

through the World Anti-Doping Agency have cited Co as a prohibited substance though no regulatory criteria have been applied (World Anti-Doping Agency 2020).

Even though IFHA signatories have committed to an agreement where Co is considered a banned substance above a certain level, there remains no uniform level of penalty for exceeding the threshold. HRNSW originally classified Co a class 1 prohibited substance, grouping Co with substances considered to have the highest potential to affect performance while generally accepted to have no medical use in the racing horse. Class 1 infringements were issued with a 5 year disqualification for a first offence and 10 year disqualification for a second offence (Harness Racing New South Wales 2013a). Racing Victoria currently impose a 3 year minimum disqualification for Co administration (Ractliffe 2020). Additionally, there remains no apparent consistency in the outcome of appeals for Co related offenses. For example, South African regulators dismissed all Co charges on the basis that the methodology used to determine Co did not distinguish between B12 and inorganic Co (National Horseracing Authority of Southern Africa 2017), while Australian regulators were of the opinion that the threshold included B12 supplementation. In cases where Co was shown to have been unknowingly administered, appeals have had vastly different outcomes. An Irish committee comprising high court judges found an elevated Co reading was the result of a salt lick the horse had been exposed to on race day (Forrista 2018), with the trainers €1,000 fine waived. In stark contrast, following an administrative tribunal hearing, Australian trainer Rachel Scott had an original 15 month disqualification reduced to a $6000 fine and 3-month suspension when her horse was also found to have exceeded the threshold following salt lick ingestion (Queensland Civil and Administrative Tribunal 2018). The outcome of an appeal by the Queensland Racing Integrity Commission remains pending. Having prepared expert reports in both cases, I can attest to the similarity in circumstances leading up the respective horses exceeding the urinary Co threshold.

Though quick to act in the introduction of a threshold, Australian regulators have been slow to act on the weight of scientific evidence now highlighting deficiencies in the Co rule. Publications arising from this thesis, and elsewhere, have questioned the reliability of the current approach to regulate Co misuse. At a minimum, penalties should be

uniformly applied so the severity of infringement and penalty are proportional. In response to the lack of perceived benefit of Co administration, the United Kingdom and New Zealand are now considering fines instead of suspension (Ractliffe 2019b).

1.8 Establishment of international threshold values for urine and plasma cobalt

Criteria for establishing a threshold for ubiquitous naturally occurring substances have been described (Toutain 2010), and require the analysis of a large number of representative samples from the target population combined with some administration / food trials. In January 2016, when IFHA set a urinary Co threshold of 100 μg L^{-1} on race day (International Federation of Horseracing Authorities 2016), only 2 studies had been published investigating Co administration (Ho et al. 2015; Knych et al. 2014). These studies were very limited in their examination of factors that could influence Co excretion with Brewer et al. (2016) describing their introduction as premature (Brewer et al. 2016). Acceptance of the urinary threshold was based largely on the work of Ho et al. (2015) after they conducted an administration trial where groups of 1–3 horses of unspecified prior Co exposure were dosed with various commercial Co-containing supplements for 3 consecutive days (Popot et al. 2017). These authors also conducted a statistical analysis on post-race urine and plasma samples from the Hong Kong racing population. They measured non-protein bound plasma Co and proposed a plasma threshold of 2 μg L^{-1} (Ho et al. 2015). That proposal was not adopted, and a critique of the conclusions drawn is presented in Chapter 2. The IFHA set a plasma Co threshold (International Federation of Horseracing Authorities 2017) in the light of work by Knych et al. (2015), who administered a single IV bolus containing 49 mg Co as cobalt chloride to 16 horses which had not received any vitamin or mineral supplementation for 12 months. These authors reported a Co gamma half-life of 4.4 to 10.5 days following the single dose (Knych et al. 2014).

Deficiencies in the process used to establish the urinary Co threshold have been noted (Fenger & Sacopulos 2015). Chapter 2 provides a review of the papers published by Ho (Ho et al. 2015; Ho et al. 2016) in deriving these thresholds. The population studied by Ho et al. (2015) was a group of 109 horses in the Emirates. This population races under strict security and yet 6 horses were considered Co outliers requiring elimination from

the population before the balance of the group would fit the proposed threshold. Similarly, Hibbert (2014) presented results of an Australian study at the 20th International Conference of Racing Analysts and Veterinarians. He examined a group of post-race samples and identified a "natural break" in the data, ultimately requiring the researchers to eliminate 17 horses from a population of 80 to make the proposed threshold fit. 'The elimination of 20 % of the population without further investigation into alternative explanations, such as feed or hay sources is inappropriate for establishing a threshold. Thresholds for naturally occurring substances must be carefully considered, and elimination of data must be done only where considerable investigation is performed' (Fenger & Sacopulos 2015).

Failure to adequately investigate factors for individuals exceeding the threshold prompted the experimental work presented in Chapter 3. The threshold in its current form provided a crude assessment of Co status and did not encompass real world scenarios, an observation supported anecdotally by the large numbers of trainers who succumbed to the Co rule (Dorries 2019). In setting the threshold, no allowance was made to distinguish between horses on a diet of negligible Co (majority of horses) from those provided with Co fortified feeds given at manufacturer recommended rates. Horses were therefore being compared to a threshold skewed by "normal horses" on feeds with very low available Co. The urinary Co threshold was based on 10,238 post-race samples collected in several countries (Popot et al. 2017). As this threshold was set from a population of horses where nothing was known about prior Co exposure; it was consequently invalid to apply the results to horses supplemented within the rules of racing. To define suitable thresholds for the latter would require statistical evaluation of a cohort of racehorses where the supplementation was known. The more variables addressed in such a study, such as gender, age, duration of supplementation, hydration status, B12 administration, etc., the more robust the population statistics and associated threshold.

1.9 Study significance

Cobalt regulation was instigated to bolster the integrity of the racing industry and protect animal wellbeing. With the Australian racing industry contributing approximately $1.4 billion to the Australian Gross Domestic Product in 2017–18, significant efforts are

made to ensure racing operations are appropriately controlled. Further income for the economy is generated by breeding, horse sales, prize money and wagering. Figures from Racing Australia suggest there are approximately 159,000 individuals directly involved in thoroughbred racing nationally. Greyhound racing includes around 30,000 registered participants with Greyhound Racing Australasia indicating that 7,000 people are directly employed in this industry (Australian Industry and Skills Committee 2020). A concerted effort should therefore be made to ensure Co regulation is correctly implemented.

In terms of maintaining integrity, the objective of setting a Co threshold was to ensure that no horses were racing with an unfair advantage. The implication for a horse with a urinary Co concentration above the threshold was that Co was deliberately administered for nefarious gain. With more than 350 trainers charged for Co offences across three horse racing codes since 2014 (Dorries 2019) it would be easy to reach the conclusion that Co "doping" was widespread. This conclusion is not supported by the science. Racing Australia noted that 'in the period 1 September 2016 to September 2019, 42,477 urine tests nationally have produced an average result of 5.1 $\mu g\ L^{-1}$ and only 59 positive samples' (Ractliffe 2019a). The decline in numbers of positives does not prove that Co doping was rife with Co deliberately administered to enhance athletic performance. It more likely shows a decline in inadvertent administration, with some feedstuff and supplement manufacturers now marketing their products as 'Co-free'. Since Co infringements persist, questions remain over the threshold validity, especially as there is no evidence to justify the risk of receiving a penalty over a substance that is easy to detect with no evidence to support a performance enhancing benefit.

The role of the IFHA is to identify and promote best practice in the administration of horseracing worldwide but it has no regulatory powers of enforcement (International Federation of Horseracing Authorities 2017). As a result, penalties handed down by racing jurisdictions for breaches of the IFHA Co threshold vary considerably. The Unites States based Association of Racing Commissioners International, Inc. published guidelines stating: for Co concentrations of less than 25 $\mu g\ L^{-1}$ of blood serum or plasma no penalty is recommended; for Co concentrations of 25 $\mu g\ L^{-1}$ or greater but less than 50 $\mu g\ L^{-1}$ of blood plasma or serum the recommended penalty is a written warning, the

placement of the horse on the Veterinarians List with removal from list only after a blood test confirms that the concentration is below 25 μg L^{-1} of blood plasma or serum; for Co concentrations of 50 μg L^{-1} or greater in blood plasma or serum have, as a trainers first offence, a recommended penalty of a maximum 60 day suspension and maximum $1000 fine (Association of Racing Commissioners International 2016). At the other end of the penalty scale, up until March 2015, the Australian racing jurisdiction HRNSW disqualified 10 trainers where the shortest disqualification was 30 months and ranged up to eight years (Bartley 2015).

Neale Scott, a former harness racing trainer and driver, has collated Co infringement notices to highlight the staggering impact of the Co threshold in Australia. Between December 2013 and December 2019 more than 270 trainers of horses or greyhounds were charged for Co offences under the rules of racing. Of these, about 220 were disqualified or suspended, 15 were fined and a further 20 are still to have their fate determined. To date, the total duration of disqualification and suspension adds up to almost 300 years. No other trace element, vitamin or drug has ever had such a profound social or economic impact in the Australian racing industry (N. Scott 2019, pers. comm., 2 December).

The harsh penalties handed down to trainers in Australia can be financially ruinous and career ending. For large stables, simply the tarnished label of being a drug cheat can have significant repercussions on reputation and revenue. With much at stake and no proven benefit, it would be expected that an appropriately set Co threshold would rarely be breached. However, numerous trainers from a range of backgrounds and experience have found themselves defending a Co charge. A Co infringement halted the training career of Peter Moody, known internationally for training unbeaten champion sprinter Black Caviar to 25 consecutive group 1 wins (AAP/ABC 2016). The 2019 Melbourne Cup would have had a much different outcome had winning trainer Danny O'Brien been unsuccessful in his bid to overturn a Co infringement charge (Dorries 2019). Ballydoyle Racing Stable in Country Tipperary Ireland, acknowledged by many racing experts to be one of the finest training establishments in the world, was required to defend a Co charge (Forrista 2018). Numerous other trainers locally and abroad have also found themselves fighting Co charges. A common theme throughout these cases was the denial of any

knowledge of excessive Co administration and deliberate attempt to break the prohibited substances rules of racing. In reviewing several of these cases and preparing defence submissions where requested, three main factors for exceeding the Co threshold were identified, namely, excessive dehydration, vitamin B12 administration, and the inadvertent use of a Co containing substance. The following cases provide examples of the significance of the research conducted in this thesis to elucidate the fate of Co in horses. Only the aspects of these cases available to the public have been discussed to avoid breaching any confidentiality arrangements.

1.9.1 Darrel Graham case

This case highlights the importance of measuring urine B12 and creatinine and identifies a significant deficiency in the accuracy of information provided on the influence that B12 administration can have on urine Co concentration. Darrel Graham, a Queensland based veteran harness trainer of more than 30 years, was 'totally bewildered' (Dorries 2016) when he had his licence disqualified after a urine sample collected from his horse *Mafuta Vautin*, prior to a race meeting at Albion Park on 30 May 2015, was found to have a Co concentration of 342 μg L^{-1} (Craddock 2015). In appealing this disqualification, I was engaged by Graham's solicitor to conduct further testing on the horse's urine sample and provide expert testimony on my findings. Results of my findings and submissions to the administrative tribunal follow.

With techniques I developed and describe in Chapters 3 and 4, I determined that the urine had a total Co concentration of 429 μg L^{-1}, creatinine 5.4 g L^{-1}, B12 8068 μg L^{-1}. The concentration of Co present in B12 was therefore 351 μg L^{-1} making the concentration of inorganic Co in this sample 78 μg L^{-1}. From these findings it was apparent that B12 administration was the main cause of this sample exceeding the threshold. The urine creatinine measurement confirmed that this sample was unreliable for a quantitative comparison to the threshold value. The significance of measuring urine creatinine is described in Chapter 6 and was presented to the tribunal investigating Graham's disqualification (Queensland Civil and Administrative Tribunal 2019) as being a requisite when measuring analyte concentrations in random urine collections for the two main reasons described in the following paragraphs.

Firstly, creatinine can be used to adjust for variations in an individual's hydration status. With decreasing hydration, the kidney excretes less water causing the concentration of solutes in urine, including creatinine, to increase. Conversely, with increasing hydration the kidney excretes more water. This causes a decrease in the urine concentration of solutes as the urine becomes more dilute. Creatinine is a waste product of muscle metabolism that is formed at a reasonably constant rate and excreted in urine. Creatinine can therefore be used to adjust for variations in hydration status, assuming a given urine analyte is excreted in a similar fashion to creatinine. By dividing the measured urinary concentration of an analyte by the urinary creatinine concentration it is possible to reduce the effect of hydration when using urine for quantitative measurements.

Secondly, and more importantly, in the case of *Mafuta Vautin*, urine creatinine is used to assess the suitability of a urine sample for quantitatively measuring a substance. Urine samples with an extremely high creatinine indicate dehydration. In cases of dehydration, the kidney's secretion, excretion, and/or reabsorption of the target chemical can be altered. As such, World Health Organisation guidelines for occupational monitoring set exclusionary guidelines for urinary creatinine concentrations (Aitio 1996). That is, urine samples produced during extreme dehydration are not suitable for quantitative analysis. Given the functional similarity of the kidneys in mammals, it would be reasonable to expect the same exclusionary criteria to be applied to urine samples used to quantitatively assess Co excretion in horses. With a creatinine concentration of creatinine 5.4 g L^{-1}, *Mafuta Vautin's* urine sample was nearly double the 3.0 g L^{-1} upper limit of creatinine considered acceptable for quantitative assessment (Aitio 1996).

It is not from an analytical perspective that Co is unable to be accurately measured in urine. The inaccuracy lies in the kidney's ability to uniformly transfer and/or reabsorb Co when producing urine during periods of extreme dehydration (Barr et al. 2005). I am not aware of any research being done to establish if dehydration causes the kidneys to excrete or reabsorb more Co, and B12 as renal function changes to maximise water retention. Increased urinary excretion of Co because of dehydration could be a contributing factor resulting in around 1 in every 330 horses tested still exceeding the Co threshold.

One source of confusion surrounding Co regulation can be attributed to inaccurate advice provided by experts. When interviewed over his pending disqualification, Graham lamented '*I just don't understand it. One professor seems to have one view about cobalt, yet another professor has a different view.*' (Dorries 2016). For example, Prof. Paul Mills, Professor of Veterinary Pharmacology at the University of Queensland Gatton Campus, provided expert opinion to a Queensland Racing Integrity Commission investigation regarding an elevated urine Co following B12 administration noting that '*It is unlikely that vitamin B12 contributed substantially to the cobalt levels reported since Cyanocobalamin (vitamin B12) contains negligible cobalt (0.00434 mg cobalt per mg of cyanocobalamin) and can practically be ignored for significantly contributing to cobalt concentrations in the urine.*' (Ashby 2018). Contrary to Professor Mills' opinion, B12 does significantly contribute to the Co concentration in urine when the analytical technique used, inductively coupled plasma – mass spectrometry (ICP-MS), is taken into consideration. Even when allowing for Professor Mills' erroneous conversion factor, out by a factor of 10, he still should have noted that the horse was receiving a significant amount of Co, albeit as a component of cyanocobalamin. The Tripart administered to *Mafuta Vautin* contained cyanocobalamin at a concentration of 500 µg mL^{-1}. The horse was administered 25 mL Tripart (see note) and hence received 12500 µg cyanocobalamin. This was equivalent to 544 µg Co. Note: Tripart is a registered equine supplement marketed as being able to optimise muscle function and recovery, and minimise tying-up or cramping. It was stated to contain potassium aspartate 20 mg mL^{-1}, magnesium aspartate 20 mg mL^{-1}, nicotinamide 60 mg mL^{-1}, L-arginine HCl 100 mg mL^{-1}, L-lysine HCl 50 mg mL^{-1}, selenium (as sodium selenate) 1 mg mL^{-1} and cyanocobalamin 500 µg mL^{-1}.

In an ethics approved experiment conducted in March 2016 with veterinarian Dr Derek Major (Chapter 4), I investigated equine vitamin B12 excretion, with urine collected at 2 hourly intervals following a single 10000 µg cyanocobalamin IV injection, equivalent to 435 µg Co. At 2 h the urine was found to contain 116 µg L^{-1} Co as a component of cyanocobalamin. This concentration dropped rapidly to 39 µg L^{-1} at 4 h, 7 µg L^{-1} at 6 h and 2 µg L^{-1} at 8 h. Assuming *Mafuta Vautin* and the test horse had a similar response to cyanocobalamin injection, the following estimates of cyanocobalamin excretion can be

calculated. *Mafuta Vautin* was administered 25 % more cyanocobalamin than our test horse. Allowing for this difference by increasing the Co from our test horse by 25 %, at 2 h *Mafuta Vautin's* urine could be expected to contain 145 µg L^{-1} Co as a component of cyanocobalamin. The urinary creatinine concentration of our test horse was 1.54 g L^{-1} whereas *Mafuta Vautin's* urinary creatinine concentration was 3.5 times higher at 5.38 g L^{-1}. Allowing for this difference in hydration by increasing the Co from our test horse 3.5 times, at 2 h *Mafuta Vautin's* urine could be expected to contain 507 µg Co L^{-1} as a component of cyanocobalamin. Therefore, directly comparing these 2 horses it is entirely feasible that 2 h after Tripart administration *Mafuta Vautin's* urine could contain 507 µg Co L^{-1} due (mainly) to the presence of cyanocobalamin.

In a healthy, well hydrated horse, I expect an insignificant amount of cyanocobalamin to remain in the urine 12 h after Tripart administration. However, *Mafuta Vautin* had been administered Tripart as an intramuscular injection in the previous days. Prolonged urinary excretion of exogenous compounds following IM compared to IV injection was likely. Also accumulation from previous Tripart doses should be considered. Additionally, *Mafuta Vautin* was notably dehydrated, as evident with a urine creatinine of 5.38 g L^{-1} (reference interval 1.56–2.33 g L^{-1}) (Robert et al. 2010). *Mafuta Vautin* would be excreting a small volume of concentrated urine to retain as much water as possible. In Graham's account of *Mafuta Vautin's* condition prior to urine collection, he noted the horse to be sweating. Water would therefore be lost through sweat that would have otherwise been used to produce urine to flush accumulated cyanocobalamin from the bladder. *Mafuta Vautin* may not have emptied his bladder in the 24 h period from when Tripart was administered and the pre-race urine sample was collected. A horse has a bladder with a volume of 4.5 L. In cases of dehydration, 24 h urine production can decrease to ~3 L (Rumbaugh, Carlson & Harrold 1982), so there is no need for a horse to empty its bladder during this period.

Using these figures, 544 µg Co from the Tripart injection in 3 L of urine would provide a urine Co concentration of 181 µg L^{-1}. Our analysis found a urine Co concentration of 351 µg L^{-1}, nearly twice as high. Therefore, the volume of urine excreted over 24 h was either around 1.5 L or the 25 mL Tripart injections given on previous days (24/5/20105

and 27/5/2015) were still being excreted. A combination of both decreased 24 h urine volume and accumulated Co from previous Tripart injections present a plausible scenario to support Mr Graham's administration claims. In conclusion, the total Co levels measured in *Mafuta Vautin's* urine sample collected on 30/5/2015 at 6:43 pm could have been reached following the 25 mL injection of Tripart as claimed to have been administered by Mr Graham at approximately 5:00 pm on 29/5/2015.

1.9.2 Rachel Scott case

Cobalt accumulation, as demonstrated by the research published in Chapter 5, was brought to light in the administrative tribunal hearing of Rachel Scott that was presided over by Member Gordon (Queensland Civil and Administrative Tribunal 2018). I was engaged by Scott's solicitor to conduct further testing on her horse's urine sample, write experts reports on these findings and other tribunal tendered documents, contribute to an expert's conclave, and testify at the tribunal hearing. This case was a good example of the genuine impact that the research conducted in this thesis can have on an individual's livelihood. The reasons for Member Gordon's decision directly relate to my study's significance and typify the complexity surrounding Co administration. The section of Member Gordon's report (Queensland Civil and Administrative Tribunal 2018) pertaining to Co accumulation has been reproduced below.

'*[10] Ms Scott herself decided the feeding regime for the horse and either mixed it herself or relied on others to do so. What was fed to the horse appears from Ms Scott's evidence, which I accept. The horse was given:-*

(a) a mix of corn, oats, Lucerne chaff and wheaten chaff morning and night;

(b) one cup of sunflower oil daily morning and night;

(c) one to two scoops of Olsson's 007 Mineral Block crushed and mixed in each feed daily; since each scoop was between 25 and 35g, the amount offered to the horse could have been between 25g and 70g of Olsson's a day.

[11] This feeding regime was given to the horse for the 12 months leading up to the race and the taking of the urine sample.

[12] In addition to the above, from time to time the horse would be given supplements intravenously. Two days before the race and the taking of the urine sample, the feeding

regime was stopped. On that day, the horse was given an intravenous drip of 20ml of Vitamin C, 20ml of VAM, 20ml of Vitamin B complex and 20ml of Amino Forte in 1 litre of Darrow's solution.

[13] The horse was probably deprived of water from about midday of the day of the race. Later that day, shortly after 4.30pm, the urine sample was taken. The test result returned 280 µg/L of Co in the urine, and the result from the reserve sample was 284 µg/L.

[14] As the matter approached the hearing, a further test was carried out on the sample which allowed for average specific gravity. This was done at the request of those acting for Ms Scott – they considered this to be relevant bearing in mind the horse had been deprived of water prior to the urine sample being collected and so substances in its urine could have been more concentrated. Adjusting for average specific gravity produced a reading of 219 µg/L.

[15] At the time of the feeding regime, every kilogram of Olsson's 007 Mineral Block contained 400 mg of Co. These means that every 30 g of Olsson's contained some 12 mg of Co. On the feeding regime as described therefore, the horse could have been fed up to 28 mg of Co a day. It is to be noted in passing that this product no longer contains Co.

[16] VAM contains 150 µg/mL of Co and so the 20 mL of VAM given intravenously two days before the race would have given the horse 3 mg of Co on that day. I heard that an intravenous dose of Co would pass directly into the system and would immediately be distributed around the body. Thus it is a much more effective administration of a substance than if it had been taken orally.

The cause of the elevated cobalt

[17] The main evidence on this issue was from two experts, Professor Colin Chapman for Ms Scott and Professor Paul Mills for QRIC. They are both experts in the field of veterinary pharmacology. They gave their opinions in a number of reports, in a joint report in an experts' conclave, and also at the hearing. From this evidence I can quite readily find as a fact what caused the elevated Co reading from the sample taken on 2 April 2016.

[18] Here, the daily administration of Olsson's 007 Mineral Block caused Co to build up in the horse's system and saturate it. On 31 March 2016, the level of Co in the horse was much higher than normal. On that day the administration of VAM boosted the Co level

even higher so that two days later the amount of Co in the urine was over the permitted level.

[19] Both experts formed their opinions based on the feeding history explained by Ms Scott. Professor Chapman was of the opinion that the overwhelming likelihood from that history was a build-up of high levels of Co and the Co boost from the VAM. Although Professor Mills thought that there could be another explanation for the elevated Co reading, he accepted that high levels of Co administered to a horse could build up in the horse's system. He did not dispute that the VAM would have been an added boost.

[20] Professor Chapman's opinion was supported by a third expert called on Ms Scott's behalf, Mr Ross Wenzel, a scientist who works in this field. He had conducted some recent experiments which confirmed that Co fed to a horse at high levels could build up and take time to be excreted.

[21] Although Professor Mills was of the opinion that Co in a saturated system would have been excreted from the horse very quickly, and for this reason doubted the view that the regular Olsson's and the VAM was responsible for the reading two days later on 2 April 2016, this seems to be belied by the test results on the samples taken from the horse in the weeks after 2 April, which showed the Co remaining in the system for some time. It must be accepted that the post 2 April 2016 test results must be regarded with some circumspection because although Olsson's was not given to the horse after 20 April 2016 it may have continued to be given VAM. However, Mr Wenzel's test results show that excretion of Co is slow after a high level of saturation and this tends to confirm the view about this taken by Professor Chapman.

Blameworthiness in the light of this cause

[22] It is said on Ms Scott's behalf that in the light of the cause of the elevated Co readings as I have found, Ms Scott applied a legitimate feeding and supplement regime and unwittingly and not recklessly exceeded the Co permitted level.

[23] In support of this it was pointed out that:-

(a) Olsson's 007 Mineral Block has a statement in capital letters:

'THIS PRODUCT DOES NOT CONTAIN RESTRICTED ANIMAL MATERIAL'

The description of the product says that it is 'an essential mineral and trace element supplement providing race, show and working horses' extra vigour, stamina, muscle and bone strength'.

(b) There was no warning that the Co in this product could build up in the horse's system.
(c) The amount of Olsson's administered to the horse was no more than the manufacturer's recommendations.
(d) Olsson's and VAM were equine mineral and vitamin supplements which were readily available, and which were purchased over the counter from reputable manufacturers and suppliers on the racecourse.
(e) There was no administration of Co other than in normal feed and supplements.
(f) There had been no warning that there was a danger this horse was close to the Co permitted level as a result of a build-up of Co in the system. In particular, one test carried out by the Controlling Body two weeks before the race showed that the horse had a urine reading of 85.9 µg/L which was much higher than normal, and Ms Scott was not informed of this. Had she been informed of this, she could have held back on the supplements.' (Queensland Civil and Administrative Tribunal 2018).

The final point cited above reflects poorly on the approach taken to regulate Co misuse. No attempt was made to notify the trainer of a higher than normal Co giving the impression that regulators were simply waiting to pounce on the unsuspecting rather than taking proactive steps. The process of allowing additional sample testing to enable appropriate scientific investigation was met with harsh resistance. Newsworthy in its own right, The Courier-Mail noted that: '*The Queensland Civil and Administrative Tribunal handed down a most notable decision last week in allowing a licensee charged over an elevated Co level to seek an independent analysis of the urine sample. More specifically, being allowed to interrogate the sample to determine the respective levels of organic and inorganic Co. QCAT Member Olding ruled in favour of licensed harness trainer Rachel Scott that she can seek analysis of the urine sample of her horse, Nolonga Your Choice, by Ross Wenzel, Senior Scientist, Trace Elements Laboratory at NSW's Royal North Shore Hospital for the purpose of identifying "the respective proportions of the element Co held in samples in inorganic and organic form."*'(Exelby 2018).

The tribunal hearing of Ms Scott was conducted in August 2018. Ideally, the approach taken to detect and prosecute Co offences will change in line with the objectives outlined in The Queensland Racing Integrity Commission Annual Report 2018-19 by Queensland

Racing Integrity Commissioner, Mr Ross Barnett. In this report a mandate was presented 'to move away from a traditional primary focus on enforcement driven strategies to achieve greater voluntary compliance through a more proactive and preventative approach' (Queensland Racing Integrity Commission 2019). This report also notes the acquisition of enhanced scientific testing capability with 'a second new instrument dedicated to testing samples for Co and arsenic as well as enabling speciation of both metals to assist Stewards in assessing evidence and prosecuting breaches' (Queensland Racing Integrity Commission 2019). The recognition that further testing is required to speciate Co shows the acceptance work published in Chapter 4.

1.9.3 Peter Moody case

The case of Peter Moody represents one of inadvertent use of a Co containing substance (Curnow 2015). Moody, one of Australia's highest profile trainers, resigned entirely from training following the anguish caused after being found to have run a horse with a urinary Co concentration over the threshold (AAP/ABC 2016). His scathing evaluation of the racing administrator's approach to Co regulation was published in The Sydney Morning Herald (Ractliffe 2019b):

'*Former trainer Peter Moody, who walked away from training following a cobalt suspension, said the risk of litigation would stop racing authorities from changing their stance on cobalt irregularities.*
"It was a sad indictment on racing, the cobalt issue, and it's one that's still going on today, unfortunately and unfairly for the participants that are caught up in it," Moody said on the You Cannot Be Serious podcast.
"It's still a scourge on racing. We think the wastage and the way horses are being handled post-race career is disgusting. You want to have a look at the intricacies of the drug rules and regulations in racing and the pain and suffering that it's causing great people.
"There was a bloke out there called Harry Richardson in Toowoomba in Queensland, the other day he got [nine] months and he's a bloke who slows down on a freeway so he won't squash a bug on his windscreen and they charge him for giving horses this drug.
"It's a nonsense drug but unfortunately it will never change while the likes of me and Danny O'Brien are alive because they're scared of litigation."' (Ractliffe 2019b).

Moody's appraisal of fear of litigation seems a plausible reason for administrators' reluctance to embrace mounting scientific evidence on the futility of the current approach to regulation. Hopefully, the research conducted in this study will highlight the need for an amended approach to Co regulation.

1.9.4 Danny O'Brien case

The experience of Danny O'Brien, like many other trainers charged with Co offences, typifies the frustration and disappointment felt by many. A November 2019 interview of O'Brien with Racenet's Ben Dorries (Dorries 2019) has been reproduced below as it provides further insight into the significance of my study.

'*Melbourne Cup-winning trainer Danny O'Brien says it is time for racing authorities to finally admit they got it wrong with cobalt and has declared the whole ugly saga is "self-inflicted brand damage on a massive scale."*

In a week where Racing Australia refused to change their position on cobalt, O'Brien says authorities must dig themselves out of the cobalt hole which has seen more than 350 trainers charged for cobalt offences across three codes since 2014.

O'Brien, who went through hell and back, and a mountain of legal fees before it was finally proved he had no knowledge of Co being administered to his horses, says enough is enough.

He says the sheer number of Co positives – and the lack of any "nefarious conduct" – shows racing needs to turn back time and admit it got it wrong with Co.

The Australian Trainers' Association, in a letter to Racing Australia, points out it is now accepted that legally sourced and administered commercial products, such as vitamin B12 injectables and even salt blocks, can cause a build-up of Co and result in legal thresholds being exceeded.

"The thing you need to look at with cobalt is why are there so many positives and why isn't there any nefarious conduct around it," O'Brien told Racenet.

"The horses generally aren't supported in betting and they generally don't run any different – it doesn't point towards any sort of nefarious behaviour.

"These cobalt positives are happening in all parts of Australia – in all codes.

"Even the silliest person in Australia can see there is something amiss here.

"There is too many people getting cobalt positives - and coupling that with the silly penalties they give them - that it just beggars belief.

"Eventually they (racing authorities) are going to have to admit they got it wrong."

In recent times in Victoria, Archie Alexander, Mitch Freedman and Steve Pateman are among trainers who have been advised of cobalt positives, but they have not been charged.

O'Brien says the tide has turned with the Co debate and now it is generally accepted that virtually all of the trainers facing Co positives have no idea how it got into the system of their horses.

"Every person now is one step removed from someone who has had a cobalt positive - it's not just the big fanfare that was announced with me and Peter Moody and Mark Kavanagh, now there is hundreds more of them," O'Brien says.

"It is self-inflicted brand damage on a massive scale."

Provided there is no intent to administer Co, O'Brien says it must now be treated the same way as plenty of other substances where trainers are penalised with fines rather than the bans often currently handed down for Co.

"Unless they find anything that establishes nefarious conduct, it's just got to be a prohibited substance just like bute or Lasix and a $500 fine," O'Brien says.' (Dorries 2019).

1.10 Thesis objectives, hypotheses, and hypothesis testing

Research objectives **–** to investigate the basis for considering Co capable of enhancing athletic performance in racehorses and to test the validity of the approach taken by IFHA to regulate Co use.

Hypothesis **–** two hypotheses were investigated. Firstly, that evidence to consider Co a performance enhancing drug is inadequate. Secondly, that current screening methods to identify excess Co exposure do not adequately differentiate between deliberate and innocuous Co use.

These hypotheses were tested **–** by literature review and experimentation through conducting administration trials in horses under controlled conditions.

Research plan to test the hypotheses:

- Review publications to track the imperative to limit Co use and establish the basis for setting control measures (Chapters 1 and 2).
- Investigate potential sources of Co and other factors that could influence Co excretion (Chapter 3).
- Develop a method to determine cyanocobalamin concentrations in equine urine then use this method to ascertain if legitimate doses of B12 administration can result in significant increases in urinary Co concentrations (Chapter 4).
- Conduct controlled cobalt administration trials ascertain appropriate samples types for controlling Co use (Chapter 5).
- Investigate the effect of hydration status on urinary Co concentration (Chapter 6).

1.11 Conclusion

Though Co has been reported to promote red cell production in high doses for close to a century, it was only in the last 6 years that it has been banned in racing and athletics. Cobalt has long been available as an inexpensive, easily attainable, and previously unregulated element. In this time, evidence of enhanced performance was unreported, suggesting that Co may not be the potent performance enhancing drug worthy of current regulatory efforts to control its use. Empirical evidence on Co metabolism in horses is rare (Coenen & Vervuert 2005) and little had changed since this observation and the decision to implement a Co threshold was enacted. The rule was conceived with undue haste and in advance of scientific knowledge and opinion (Mobasheri & Proudman 2015).

1. There is no evidence that Co is performance enhancing.
2. There is no evidence that Co increases red cell production in the horse.
3. There is no evidence that elevation in normal red cell levels in horses increases racing performance.
4. As the scientific evidence mounts that Co does not improve performance the emphasis has shifted to the toxicity and welfare aspects of extreme Co dosage.
5. Urinary Co levels of around 1000 $\mu g\ L^{-1}$ are readily achieved after legitimate vitamin and mineral supplementation.

6. The effect of many variables including racing training, stress, disease, and dehydration is untested and unknown.
7. The effect of long-term usage of legitimate vitamin and mineral supplementation on urinary Co levels is untested and unknown.
8. There is a high level of variation in urinary excretion times between horses in a population.
9. The perceived welfare issues with illicit Co usage could have been managed by other means.
10. Measurement of "raw" urine Co level is an inappropriate way to monitor Co administration.
11. There is no significant relationship between urine and blood Co levels.
12. The statistics presented were sound but were dependent on assumptions that were arbitrary and never validated.
13. The advice to the industry was confused, inconsistent and incorrect.

The research conducted in this thesis has been vindicated with broad interest in the racing fraternity and legal firms seeking to defend trainers found to have horses in breach of the Co threshold. Over the course of conducting this research I have been contacted by numerous individuals and solicitors from around Australia and overseas to provide advice, write expert reports, contribute to legal conclaves, and provide testimony under oath at various tribunals. I have presented parts of this thesis at local and international scientific meetings including - *11th International Society of Trace Element Research in Humans Conference,* Dubrovnik, Croatia, 19th - 22nd October 2015, Oral presentation – "Cobalt – A banned substance?"; *2016 AACB NSW/ACT Branch 2016 Regional meeting,* Dubbo, NSW, Australia, 1st October 2016*,* Invited speaker – "Cobalt: The next chapter for an uncertain villain."; *16th International Symposium on Trace Elements in Man and Animals,* Saint-Petersburg, Russia, 26th - 29th June 2017, Oral presentation – "Determination of cobalt and cyanocobalamin in urine by HPLC-ICP-MS".

The paper published and presented in Chapter 4 has had over 400 reads as notified by ResearchGate. Subsequent publications (Karakka Kal et al. 2020; Knoop et al. 2019) have cited this research. In response to the article published on Co accumulation (Chapter 5),

I was interviewed to feature in an article for an American magazine (Oke 2019). Outcomes of this research have appeared sporadically in mainstream media as I have become increasingly involved in presenting expert opinion to administrative tribunal's dealing with appeals of Co related infringements. The most recent media coverage (18 November 2019) was in The Australian newspaper where it was noted –

'*Ross Wenzel, who works in the trace elements laboratory at the Royal North Shore Hospital, said "Cobalt does not actually have any documented performance-enhancing effects and does not appear to stimulate the production of red blood cells in racehorses."*

"The misuse of cobalt is ill-conceived," he said.

"Excess cobalt is neither useful nor necessary. Public education is needed on this topic."

The comments - and Wenzel's thesis - are certain to be seized upon by several trainers and legal representatives involved in positive cobalt cases.

"The rationale for supplementing horses with cobalt pertains to a presumptive increase in red blood cell production in response to cobalt indirectly inducing hypoxia," Wenzel said.

"In turn, more oxygen will be delivered to exercising muscles to maximise energy production and enhance performance. Research has failed to support this presumption. There is no evidence to suggest that cobalt will stimulate a hypoxic response in excess of that naturally achieved in training."

Several Victorian trainers, notably Melbourne Cup winning Danny O'Brien and Mark Kavanagh, have been embroiled in cobalt cases.

Wenzel said: "With the range of unnecessary cobalt-containing supplements on the market, the need to develop a testing regime capable of differentiating cobalt misuse from regular supplementary intake is apparent,"'(Schlink 2019).

2. COMMENTARY ON RESEARCH PAPER 'CONTROLLING THE MISUSE OF COBALT IN HORSES'

2.1 Summary

Many of the decisions shaping racing authorities' reaction to Co regulation were in response to a research paper titled 'Controlling the misuse of cobalt in horses' which was published in the journal Drug Testing and Analysis (Ho et al. 2015). This paper was adopted as a pivotal reference in the controversial campaign by racing authorities to regulate and control illicit Co administration to racing horses. However, the data and conclusions presented by Emmie Ho and her colleagues could only be considered preliminary. Ho et al. 2015 made the statement: '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 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.' Issues such as dehydration (including sub-clinical dehydration) and low serum albumin (Apple et al. 2002; Lippi, Montagnana & Guidi 2006) may be major factors that can influence the observed levels of Co in horses but were not explored.

Veterinarian Dr Derek Major and I were of the view that numerous assertions made in this paper were open to challenge. Furthermore, we questioned aspects of the research methods and laboratory testing for Co. Consequently we published a commentary (Major & Wenzel 2016) detailing our concerns. This Chapter presents our commentary as published and responses to the authors subsequent reply (Ho et al. 2016).

2.2 Paper commentary

The opening sentence in the abstract, repeated in the first paragraph of this research article (Ho et al. 2015), is the statement 'Cobalt is a well-established chemical inducer of hypoxia-like responses.' This statement is unsupported except by citation (Holly 1955) advising of the use of Co to treat anaemic pregnant women, and those with renal disease or cancer in 1955. This therapy was abandoned in the 1970s. In fact, the statement would appear to be a transcription from a Lippi et al. abstract (Lippi, Franchini & Guidi 2005), which states: 'Cobalt chloride is a well-established chemical inducer of hypoxia-like responses such as erythropoiesis.' Lippi's statement is likewise unreferenced. For a time, Lippi was prolific in the cobalt literature (Lippi, Franchini & Guidi 2005; Lippi, Franchini & Guidi 2006; Lippi & Guidi 2004; Lippi, Montagnana & Guidi 2006). Notably, none of the preceding references are to original research – they are titled, respectively: 'Occasional piece', 'Editorial', and 'Hypothesis'.

Furthermore, these references ultimately rest on earlier work, e.g., on rabbits (Wintrobe et al. 1947), and on human volunteers (Davis & Fields 1958), which are open to challenge with modern scientific methodology and measurement. Consequently, over-reliance on these sources would appear injudicious.

It could easily be inferred from Ho et al. (2015) that Co induces erythropoiesis in the horse, but this conclusion appears to be derived from extreme extrapolation. Under 'Introduction' it is stated that: 'The main mediator hypoxia inducible factor 1α (HIF1α) activates genetic sequences, including those of the erythropoietin (EPO) gene, which promotes efficient adaptation to hypoxia' (Holly 1955). The authors jump straight to the phrase 'Apart from the haemopoietic effects…' apparently assuming the reference cited confirms haemopoiesis in the horse. This was not the case with the reference referred to making no mention of horses but instead being a review article in a journal of cell biology for *in vitro* studies of HIF 1 (Déry, Michaud & Richard 2005).

The paper then draws on the research of Davis and Fields (Davis & Fields 1958). While this was no doubt quality research in its time, this paper itself is open to question:

• Red cell parameters were measured manually with relatively crude instrumentation.

• No control subjects were included.

• No rigorous statistical analysis was applied.

• The rapid return to 'normal' in 9–15 days is inconsistent with current understanding of red cell biology.

• The reported effects resulted from daily administration for 7–22 days of what are now considered toxic Co levels.

The authors then vindicate their conclusions with the bold statement: 'Indeed, the activity of an International EPO Unit (IU) was originally referenced against the biological effect of 5 μM cobalt chloride (Wintrobe et al. 1947)'. This paper from the National Institute for Medical Research in London, reports findings when establishing a reference material for erythropoietin (Cotes & Bangham 1966). It makes only a fleeting reference to the 'so called "cobalt unit"', and in turn cites earlier work on starved rats (Fried et al. 1957; Hodgson et al. 1958).

The paper by Ho et al. (2015) next lists the proposed toxicity of large doses of Co salts. While the motivation of racing authorities in controlling any violation of animal welfare is unquestioned, it must be recognized that toxic effects can be demonstrated for almost any substance, including sodium chloride and water. Support is again mustered from Lippi et al. (their references [14–16]), these being (Lippi, Franchini & Guidi 2005; Lippi, Franchini & Guidi 2006; Lippi & Guidi 2004; Lippi, Montagnana & Guidi 2006) in making the statement: '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.' Notable here, is the inclusion of the words 'and effective in boosting endogenous erythropoietin production'. These do not appear in the Lippi manuscripts, which actually state: 'Cobalt is easily purchasable, inexpensive ….', and 'However, we cannot exclude the possibility that it may become an attractive alternative to traditional performance enhancing drugs.' It is of note that as of 2014, more than 10 years after Lippi's speculation, WADA have not seen fit to introduce

a Co urinary threshold (Krug et al. 2014). The only published work in horses is for a single administration of 109 milligrams of cobalt chloride to each of 16 horses, and NO increase in red cells or erythropoietin was observed (Knych et al. 2014). The following line – 'Due to the ability of cobalt to act as an erythropoietic agent in equine sports, a method to control cobalt misuse is needed' – must likewise be challenged.

The second main area of concern to these correspondents is the Hong Kong laboratory's method of Co determination. The authors state that the plasma was deproteinated by precipitation with trichloroacetic acid and centrifugation. This is inappropriate as in humans and laboratory animals, following chronic administration of Co, a large proportion is redistributed into body tissues and bound to plasma albumin and red cells (Simonsen, Harbak & Bennekou 2012).

I repeated the procedure published by Ho et al. (2015) using samples of equine blood collected into lithium heparin tubes using similar ICP-MS conditions; although, using a different manufacturer's instrument, in my case a Varian 820MS (Varian Inc, Australia). Samples were run in accordance with my laboratory's ISO 15189 accredited method for the analysis of Co in biological samples. Using this method, 50 µL of plasma was added to 1 mL of sample diluent containing 1 % (v/v) high purity grade nitric acid, 0.01 % Triton X100 (w/v), 0.01 % (w/v) ethylenediaminetetraacetic acid and 50 µg L^{-1} gallium as internal standard. The protein free and native plasma samples shown in Table 2.1 were analysed under the same conditions in the same analytical run to eliminate any sample storage or between run variability. Results are shown in Table 2.1. They indicate that the sample preparation method of Ho et al. (2015) would grossly underestimate the concentration of Co in plasma with most of the Co present removed during protein precipitation.

Table 2.1. Comparison of plasma cobalt concentrations before and after protein precipitation.

Analysis method	**Cobalt concentration ($\mu g\ L^{-1}$)**
Direct homogenization and ICP-MS assay	539
Protein precipitation and centrifugation using the method of Ho et al. (2015) followed by ICP-MS assay	12.2

There are additional concerns about the adequacy of the reported administration trial:

• Only 1–3 horses were used for each substance.

• The baseline parameters were not tabulated but clearly the horses had not been subject to long-term Co supplementation.

• The injected doses of Hemo-15 (1 mg daily for 3 days) are not representative of total Co exposure under current racing training regimes.

• No consideration has been given to combined Co exposure such as commonly occurs with prepared feeds, oral tonics, and injectable vitamin preparations.

• The long-term effect of continued use of Co supplements was not studied, although this is the norm in racetrack husbandry. Cobalt is known to have a terminal half-life of around 7 days (Knych et al. 2014) so accumulation is inevitable.

The authors do, however, qualify their report by advising that more work is required over a wide range of locations.

We find it curious that Ho et al. (2015) excluded the data for 6 of their 109 samples due to presumed ‘remnants of earlier treatments with cobalt-containing supplements’. I have ascertained (D. Sykes 2006, pers. comm.) that these excesses were from legitimate long-term oral Co supplementation. In my opinion their exclusion as ‘outliers’ is unwarranted. Lastly, some of the inferences drawn by Ho et al. (2015) from the literature (Ho et al. 2015) are tenuous extrapolations from established science and demand a review.

2.3 Authors' response

The authors disagreed with this criticism, referring to our commentary as subjective (Ho et al. 2016). They stated that the objective of their paper was to present a scientific approach to the equine sports industry for the testing and possible regulation of Co as it was already known to have been abused with profound toxic and welfare implications. No references were provided to support the claim of known Co abuse with profound toxic consequences, nor am I aware of any such cases at that time or since. The only study demonstrating welfare concerns was one where Co doses of 1 mg kg^{-1}, and above, were reported to immediately cause tachycardia in horses, with symptoms returning to baseline within 2 h post-administration (Burns et al. 2018).

Rather than accept that protein precipitation prior to determining Co was an inappropriate method, the authors defended their approach stating that their analysis was applicable provided that all laboratories used this technique. The authors dismissed our evidence that protein precipitation prior to analysis would underestimate Co, preferring instead to question the accuracy of our Co measurement (Ho et al. 2016). Then, for reasons unclear to the defence of their method, the authors reanalysed their samples for total Co and presented the results as a graph of both total and free Co (Figure 2.1). From these graphs it was apparent that with decreasing total Co concentration the proportional difference between total and free Co increased.

It was disconcerting that the authors defended a method where different proportions between total and free Co concentrations were reported. Percent differences for the various samples were 17–53 % in the Co administrated cohort and 40–89 % in the pre-treatment group. In explaining this variation the authors note that the 'ratio is unlikely to be fixed, and can be expected to vary depending on the individual horse, the total amount of cobalt in the body, and the form(s) of cobalt present' (Ho et al. 2016). Surely this varying ratio should be taken as evidence that the protein precipitation step made the proposed method unsuitable for the regulation of Co in racing horses. Our commentary has been vindicated with subsequent plasma thresholds being set based on measurement of 'total cobalt (free and protein bound)' (International Federation of Horseracing Authorities 2017).

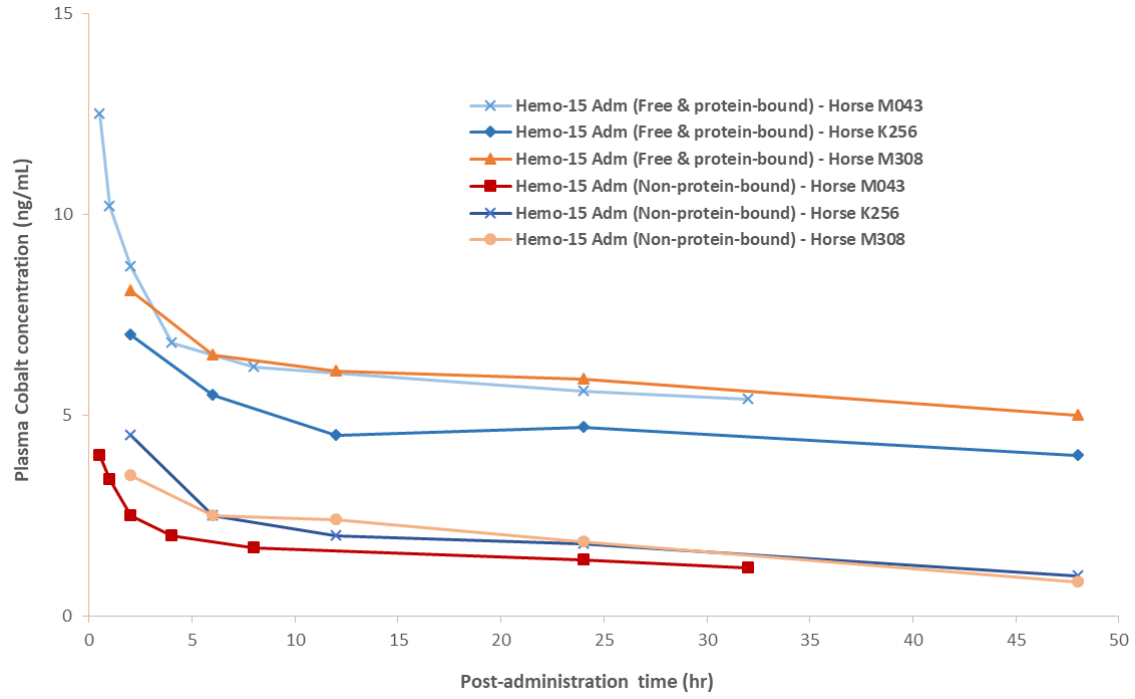

Figure 2.1 Plasma cobalt following IV administration of Hemo-15 to horses. Figure reproduced from (Ho et al. 2016).

In further criticism of our commentary, Ho et al. (2016) avoid the point for the testing of Co in the first place, stating that 'no cobalt deficiency has been reported in the horse that would require long-term supplementation with a significant amount of cobalt'. It is only through long-term supplementation with high doses of Co that the erythropoietic effects of Co would be realised. If the objective of setting a threshold was to detect administration of Co for nefarious gain, then it would be these horses, not those treated with a single supplementation episode that should be targeted.

2.4 Limitations of the statistical approach presented in the paper titled 'Interlaboratory trial for the measurement of total cobalt in equine urine and plasma by ICP-MS'

Another seminal paper in the Co literature relating to racehorses, titled 'Interlaboratory trial for the measurement of total cobalt in equine urine and plasma by ICP-MS', was also published in the journal Drug Testing and Analysis (Popot et al. 2017). In this paper, when describing the statistical analysis and criteria by which the data were assessed, Popot and her colleagues, including Emmie Ho, state that: 'According to previous studies, for both plasma and urine, percentage deviations from the calculated means were performed first. Secondly, if necessary, robust statistics based on the observed median were applied. For the cobalt urine ring test, percentage deviations from the calculated means were performed. Data with deviation higher than 20% were considered as non-compliant. For the cobalt plasma ring test, statistics were conducted, in the first instance, with the assessment of the percentage deviations from the calculated mean as for the urine cobalt ring test. Robust statistics based on the observed median was performed afterwards to assess the data, considering both % deviation higher than 20% and absolute Z-score higher than 2.5 as non-satisfactory. Only data resulting from robust statistics are presented.'

The validity of this statistical approach is flawed. When considering the Royal Society of Chemistry Technical Brief titled 'Robust statistics: a method of coping with outliers', it is stated that using robust statistics raises the contentious question of when it is justifiable to exclude outliers (Analytical Methods Committee 2001). No attempt was made in these papers to justify the exclusion of outliers (Ho et al. 2015; Popot et al. 2017) with Ho and her colleagues contributing 7,462 urine samples to the total of 10,238 analysed (73%), a significant number of which were declared "non-compliant" and were rejected. Importantly, there were no reported attempts made to determine why there were these rejected "outliers". Essentially the authors did not explore, as they should have done, the potential issues of vitamin B12 being included in Co measurements, the possibility of urine concentration variations contributing to the outliers and the role that Co accumulation may have played.

2.5 Conclusions

A critical appraisal was presented on the publications from Ho et al. and Popot et al. (Ho et al. 2015; Ho et al. 2016; Popot et al. 2017) as the advice they provided to IFHA was instrumental in establishing the current urinary Co threshold. The flaws identified in these papers establish a basis whereby trainers found to have breached the regulatory threshold may have done so unwittingly. The publications of Ho et al. can only be considered detrimental to racing's attempts to prevent Co misuse. The author's intention was to present a scientific approach for Co regulation. Instead, poor scientific investigation led to urine being presented as an appropriate medium for regulating Co misuse.

As participants in a study undertaken to assess the ability of racing laboratories to measure Co in urine and plasma, they also contributed to a delay in the acceptance of plasma Co determinations as a viable means of assessing Co status. The results of this study were presented at the 20th International Conference of Racing Analysts and Veterinarians (Popot et al. 2014). Essentially, five laboratories in different countries participated in a sample exchange of 5 urine samples containing 5–500 μL^{-1} Co and 5 plasma samples containing 0.5–20 μL^{-1} Co. All laboratories used ICP-MS as the analytical technique. The main method difference between laboratories was the concentration of nitric acid used when diluting the samples. Urine diluent nitric acid concentrations of 0.3 %, 1 %, 1 %, 3.25 % and 5 %, and plasma diluent nitric acid concentrations of 0.5 %, 1 %, 1 %, 3.25 % and 5 % were described. With nitric acid concentrations over 1 % (v/v) proteins precipitate out of solution, and as demonstrated in Table 2.1, result in lower Co recovery. Urine samples have a low protein content compared to plasma and are therefore not prone to the same degree of Co loss with increasing nitric acid levels. Given the range of nitric acid concentrations between methods, it was not unexpected that the study concluded the methods used to measure urine were suitable while further work was required to obtain the same degree of agreement in plasma. Consequently, urinary Co determinations were initially established for Co regulation while plasma Co determinations were considered unreliable.

The adverse effects of championing urine measurements as an appropriate method to detect Co misuse have been profound. In the years following the introduction of Co

thresholds, those jurisdictions that have relied on plasma Co have not had the same spate of Co non-compliances attributed to those jurisdictions where urine was preferred. The current plasma threshold of 25 μg L^{-1} would appear to adequately differentiate between horses that have had a recent benign exposure to Co from those that were systematically being exposed to excessive doses of Co. However, the latest research concluded that a threshold of 25 μg L^{-1} in plasma may still lead to false conviction while raising the threshold to 71 μg L^{-1} would not jeopardise the integrity of racing, as at this plasma concentration there would be no effect on performance or animal welfare (McKeever et al. 2020).

A thorough literature review should have revealed limitations in proposing a urinary threshold. For example, Toutain in the 2010 edition of *Comparative and Veterinary Pharmacology* stated that 'Currently, most controls are performed using urine but blood (plasma) should be seriously considered as a better matrix for medication control' (Toutain 2010). He cautions that urine concentrations may be influenced by many factors such as urine volume and pH for ionisable drugs. The main consideration for measuring drugs in urine rather than plasma was an analytical issue because most drug concentrations are higher in urine than plasma (Toutain 2010). As adequate sensitivity was afforded the method used to determine Co this would not be a factor when deciding on sample matrix. Additionally, a literature review pointing to Co erythrogenic activity by indirectly stimulating Epo production would direct an astute author to consider long term measures of Co exposure. As red blood cells in a horse have a half-life of 140 days the putative benefit of Epo appears several weeks to months after urinary Co levels return to normal.

3. PILOT STUDIES

3.1 Summary

In this Chapter, to ensure credible Co results were presented, a process of validation and verification was undertaken on the ICP-MS methods used. When validating and verifying methodology, it is expected that rigorous performance evaluation criteria are met. These criteria are typically defined in accordance with international standards. The actual approach used here to validate and verify the ICP-MS Co measurement techniques meet these objectives by following guidelines for accreditation to ISO 15189:2012 for medical testing (National Association of Testing Authorities 2018). These techniques were used to measure the quantity of Co in various feeds and supplements, then to investigate factors that could affect urinary Co excretion. Factors were investigated as a series of pilot studies conducted over several phases. Results of these studies have been refined to present those areas where interesting outcomes were noted. From these pilot studies it was found that vitamin B12 administration, Co accumulation and hydration status warranted further investigation and are the subjects of Chapters 4, 5 and 6, respectively.

3.2 Introduction

The establishment of a Co threshold in racing horses left trainers and veterinarians confused about quantities of Co that could legitimately be given to a horse before exceeding the threshold. The statistical processes used to define the threshold were based on uncontrolled population studies of horses. No details were obtained on prior sources of Co exposure and no explanations were offered for the wide, frequent variation in urinary Co levels between horses. Consequently, there was no information on horse husbandry practices to avoid exceeding this threshold. Published equine Co administration trials had been conducted (Ho et al. 2015; Knych et al. 2014), but these did not address the influence on Co excretion from many of the variables typically faced by racing horses. For instance, the impact of repeated Co dosing over long periods was not investigated.

By not initially adhering to a process of rigorous scientific evaluations before implementing a Co threshold, regulators have been exposed to constant legal challenges.

The statistician who proposed that 50 µg L^{-1} was to be considered the upper level of a 'normal' population of equine urine samples (Adamson 2014) is also cited by the National Association of Testing Authorities (NATA) as an advocate for the validation and verification of quantitative and qualitative test methods (National Association of Testing Authorities 2018). In this NATA document it is clearly stated that several components should be considered when undertaking method validation or method verification. Of particular importance are the issues of matrix variation, where it is noted that matrix variation is, in many sectors, one of the most important but least acknowledged sources of error in analytical measurements. Hence, it may be important to consider the variability of the matrix due to the physiological nature of the sample. Urine is a highly variable sample matrix. It is puzzling, therefore, that NATA's very clear and specific advice was not followed in relation to sample matrix and many other issues when the Co threshold was developed and adopted. Importantly, the approaches used to develop and adopt the world's first Co threshold in NSW have never been published in a peer-reviewed journal nor subjected to subsequent validation and verification processes.

With inadequate information available to describe Co metabolism, I was contacted by Dr Derek Major, a senior veterinarian at a major equine clinic, to help fill the knowledge gap surrounding Co supplementation so that appropriate advice could be provided to clients. My previous experience in measuring Co was to examine Co release from individuals with Co containing metal-on-metal hip implants. Using that analytical experience and Derek's veterinary expertise, we devised a series of Co administration pilot studies. The objectives included: validation of analytical processes to measure Co; and broad scoping of 1) factors that would influence Co excretion in urine; 2) practices for legitimate Co supplementation; and 3) deficiencies in the application of a urinary Co threshold.

3.3 Study outline

To ensure appropriate analytical techniques were being applied to measure Co, the first component of the study was a method validation process. Aqueous, rather than matrix matched calibrators, were used to determine Co in blood, serum, and urine. These samples were prepared in alkaline and acidic diluents and assessed for performance precision.

Administration rates were guided by informal surveys of current training practices and indicated that it was common for horses in racing work to receive 10–50 mg total Co per week in feeds, supplements, and injections. Standard doses of registered vitamin and mineral preparations such as Hemoplex®, VAM® and Aminoplex were often used daily or on alternate days, with IV infusions once or twice per week. A typical infusion contained ~4 mg of Co as cobalt chloride, sulfate, gluconate, or as other compounds.

The pilot studies were sectioned into several discrete experiments to establish the influence on urinary Co excretion from the following factors:

- Cobalt source – Horses were administered Co from a variety of sources such as the registered horse supplement Hemoplex®, vitamin B12 and a variety of natural and fortified feedstuffs.
- Dose – Various dosage rates were trialled.
- Route of administration – Cobalt was supplied orally and by intravenous (IV) or intramuscular (IM) injections.
- Accumulation – Samples were collected after single and prolonged Co administration to determine if Co would accumulate in horses.
- Exercise – Cobalt concentrations in rested and exercised horses were measured.
- Sample storage – Factors that could influence Co stability in urine were assessed. Urine creatinine, pH, centrifugation, storage temperature and free flow versus collection via catheter were studied.
- Analysis conditions – Several approaches have been described for measuring Co in biological fluids (Lu et al. 2015). The effect of pH on sample diluent and gases to decrease ICP-MS interferences were investigated.
- Cobalt displacement – An IV injection of an iron containing supplement was administered to horses previously exposed to Co to determine if an increase in urinary Co excretion would result with iron displacing Co from plasma proteins.

When measuring the concentration of endogenous substances in urine, it is common practice to correct for variations in hydration status using creatinine or specific gravity. Creatinine was therefore measured in each urine sample to normalise the total Co

concentrations. Urine specific gravity was also periodically measured and investigated as an alternative parameter of urine concentration.

In addition, the pilot study evaluated the reliability of urine to accurately monitor Co exposure. This was done by comparing urine Co concentrations with corresponding Co measurements in plasma and whole blood samples. Urine was traditionally the matrix of choice for drug screening purposes as it offered relative ease of collection, a relatively clean sample matrix and often higher concentrations of drugs and drug metabolites than obtained from blood samples. These factors were of great importance, but with changing technology the need for a clean sample matrix with a high analyte concentration has diminished (McKinney 2009).

3.4 Experiment design

Validation of the Co method involved preparing aqueous Co calibrations solutions with additional elements to approximate concentrations present in urine, plasma, and whole blood. Using this approach, matrix matched calibrators were not required enabling all sample matrices to be determined within the same analytical run. Samples were prepared in both alkaline and acidic diluents. ICP-MS conditions were assessed by using alternate gases to minimise interferences when taking measurements in different diluents, i.e. hydrogen gas was used for the alkaline diluent and helium for the acidic diluent.

The study involved administration of registered cobalt-containing supplements according to manufacturers' recommended doses, to groups of 5–6 horses maintained under standard conditions of husbandry. The groups of horses were selected from a herd of mixed breed horses, mostly Standardbreds, between the ages of 3 and 10 years old. The horses were part of a herd of mares previously used for artificial breeding procedures during the season and remained in the herd for the coming season. Additional sampling was carried out on horses in racing training.

The trial comprised several discrete experiments. Where possible, several experiments were run concurrently for maximum efficiency. For each experiment, the relevant

variables were identified and controlled, with blood and urine sampling conducted at predetermined intervals. The various pilot studies are listed below: -

i. Sample storage
ii. Feeds and Supplements
iii. Hemoplex®
iv. Sample Matrix Correlation
v. Accumulation
vi. Exercise
vii. Cyanocobalamin
viii. Pharmacokinetic Study
ix. Cobalt Displacement
x. IM Injection
xi. Oral Administration
xii. Cobalt Salt

The study was conducted in 3 phases (Table 3.1). The first phase replicated the intensive supplementation regimes reported in some racing stables by administration of 4 mg Co equivalent doses in registered vitamin preparations. Concurrent urine and blood samples were taken to investigate Co levels in the 24 h following administration. Horses were managed the same way during the study and were all paddocked together. When investigating the effect of water restriction, water was withheld only to the extent of normal racehorse training husbandry, as outlined in the ethics application. The object was not to cause clinical dehydration, but to induce urinary concentration. In nature, paddocked horses may only drink once or twice a day and can store significant water reserves in their gastrointestinal content. Most of the variability seen in urine concentration may largely be a function of individual horse behaviour and when the horse last drank prior to restricting water access.

Table 3.1. Outline of the horses used and treatment protocols for the 3 phases conducted in the Co administration pilot studies.

1st phase of administration pilot study	
Horses	**Treatment**

A, B	Hemoplex® at a frequency and dose consistent with an intensive supplementation regime for 4 weeks by IV injection. Horses sampled before and after treadmill exercise, with and without water restriction.
C, D, E, F	4 mg of Co as Hemoplex® by IV injection prior to each sampling.
G, H, I, J, K, L	Horses maintained on a common racing feed and supplement diet and administered a typical pre-race vitamin and mineral infusion weekly. The infusion was composed of 10 mL Hemoplex®, 10 mL of Folic B12®, 10 mL Pre-Ferrin and 20 mL of Foliphos®.
2nd phase of administration pilot study	
Horses	**Treatment**
C, D, M, N	Blood samples collected at time 0 (prior to the start of 4 mg Co IV infusion as Hemoplex®) then at 5, 10, 15, 30, 45 and 60 min. Further blood samples were collected at 2, 3, 4, 5, 6, 8, 12, 18, 24, 36 and 48 h from administration. The horses M and N used in this study were harness racing horses in training that had previously produced urine samples greater than the regulatory Co threshold.
3rd phase of administration pilot study	
Horses	**Treatment**
A, B	Control group, no treatment.
C, D	25 mg Co IV as $CoCl_2$ weekly on Tuesday.
E, F	50 mg Co IV as $CoCl_2$ weekly on Tuesday.
G, H	25 mg Co IV as $CoCl_2$ twice weekly, on Tuesday and Friday, i.e. 50 mg weekly.
A, C, E, G	Iron supplement, Pre-Ferrin, administered as 10 mL IV injection 39 days after the last Co treatment. Urine and blood samples collected at time 0 (prior to the start of Pre-Ferrin injection), then after 2, 5, 9 and 26 h.
E, F	Disposition of Co salt investigated by administering VAM® (Co as cobalt chloride) to horse E and cobalt sulfate to horse F, as 10 mL IV injections, 43 days after the last Co treatment. Urine and blood samples collected at time 0 (prior to the start of Co injection) then at 2, 4, 9 and 25 h.

In the second phase, a pharmacokinetic study was conducted on 4 mares to investigate the plasma clearance of Co following an IV injection. Sampling time intervals were based on a previously described method (Knych et al. 2014), where the plasma elimination of 4 mg of IV Co as Hemoplex® was examined.

In the third phase, mares previously exposed to Co, were administered cobalt chloride and cobalt sulfate to determine what influence Co salt would have blood and urinary Co concentrations. An IV injection of an iron supplement was also administered to 4 mares that had previously been maintained on 7 weeks' Co supplementation. Urine and plasma Co concentrations were measured to investigate the possibility of competitive binding of another divalent cation displacing Co bound to albumin and/or other plasma proteins.

3.5 Materials and methods

3.5.1 Testing site

The administration trials were conducted at Agnes Banks Equine Clinic (ABEC), 5 Price Lane, Agnes Banks NSW 2753. ABEC is an accredited hospital with the NSW Veterinary Practitioners Board. Ethics approvals (Appendix 9.1) were granted for these studies by the NSW Department of Primary Industries. They included –

- TRIM 15/216 (3) #1, titled "A study on urinary and plasma levels of Co following administration of standard registered Co supplements", dated 10th July 2015, valid for 3 years from this date.
- TRIM 16/1381(4), titled "A study on urinary, plasma and faecal levels of Co following administration of standard registered Co supplements, compared with administration of the cobalt salt in isolation", dated 23rd August 2016, valid from 15 August 2016 to 15 August 2017.

3.5.2 Laboratory conditions

Feedstuff digests, sample processing, pH measurements and ICP-MS analyses were conducted in the trace elements laboratory located at Royal North Shore Hospital, Pacific Highway, St Leonards, NSW. This laboratory was purpose built for trace element determinations and typically receives samples of blood, serum, plasma, and urine sample collected from human patients for the clinical investigation of trace element deficiencies or excesses. Biological monitoring to assess workplace exposures to toxic elements are also undertaken. Trace element determinations in tissue samples and miscellaneous fluids are performed where clinically indicated. The facility is NATA accredited to ISO/IEC

17025 for medical testing. Procedures and protocols are established for secure sample storage and analysis with swipe card access to laboratory areas.

To ensure suitable operating conditions for trace element determinations, the laboratory operates under positive pressure with a supply of HEPA filtered, temperature-controlled air. Minimal metal fittings are used within the laboratory and cryogenic argon and chilled cooling water are reticulated to the laboratory. A perchloric acid rated constant air velocity fume hood is located within the laboratory for tissue digests. Also located within the laboratory are a clean sample preparation area, calibrated pipettes, vortex, mixer, Hamilton Microlab 600 diluter and Sartorius Research R200D 5 figure analytical balance with vibration resistant table.

3.5.3 Instrumentation

Urine creatinine measurements were performed in the NSW Health Pathology Clinical Biochemistry Laboratory, Royal North Shore Hospital, St Leonards, NSW, using the Jaffe reaction method (Abbott Laboratories 2006) on an Architect c9000 System (Abbott Laboratories, Abbott Park, IL, USA). Sample aliquots were transferred to 5 mL screw cap polypropylene tubes (Thermo-Plas, Adelaide, SA, Australia) and centrifuged at 1250 g rcf for 5 min at room temperature to remove any sample debris immediately prior to analysis. Urine specific gravity measurements were made at the time of collection using a hand-held refractometer (Brix refractometer, Australian Scientific, Kotara, NSW, Australia).

Trace element analysis was by inductively coupled plasma - quadrupole mass spectrometry (ICP-MS) (Varian 820MS) with SPS-5 autosampler running the ICP-MS Expert instrument software (Varian 2007). Cooling water at 22°C was recirculated from an Aqua Cooler Chiller Model No. R150 A1 – P2 (Aqua Cooler Pty Ltd, Meadowbrook, QLD, Australia). Argon was supplied from a cryogenic system reticulated to the ICP-MS. High purity hydrogen and helium was supplied from high-pressure cylinders located in the laboratory. The exhaust system was fitted with a back-draft damper and operated continuously to prevent environmental air from reaching the instrument. Samples were nebulised with a micro-concentric glass nebuliser (MCN) model number MCN100

(Teledyne CETAC, Omaha, NE, USA). A double pass glass spray chamber, quartz ICP torch and platinum sampler and skimmer cones were used.

The Varian 820MS uses interlaced induction coils to generate an argon plasma which is used as the ion source (Date & Gray 1983; Houk 1986). The argon plasma temperature range is 6000–10000 K° and is maintained by a high-power, radio frequency current at 1.2–1.4 kW. In the initial stage of the sampling process ions are removed from the plasma through a small hole at the tip of a water-cooled platinum cone. Interfering molecular ions are controlled using a collision reaction interface (CRI). In this technique, hydrogen or helium gas is injected directly into the sampled plasma as it passes through the tips of the ICP-MS interface cones (Figure 3.1). Collisions between the introduced gas molecules and the plasma ions change the kinetic energy of the ions and can induce reactions to form neutral species. These collisions and reactions occur with potentially interfering ions at a greater rate than the analyte ions. Interfering ions are therefore selectively removed from the plasma before they enter the ion optics.

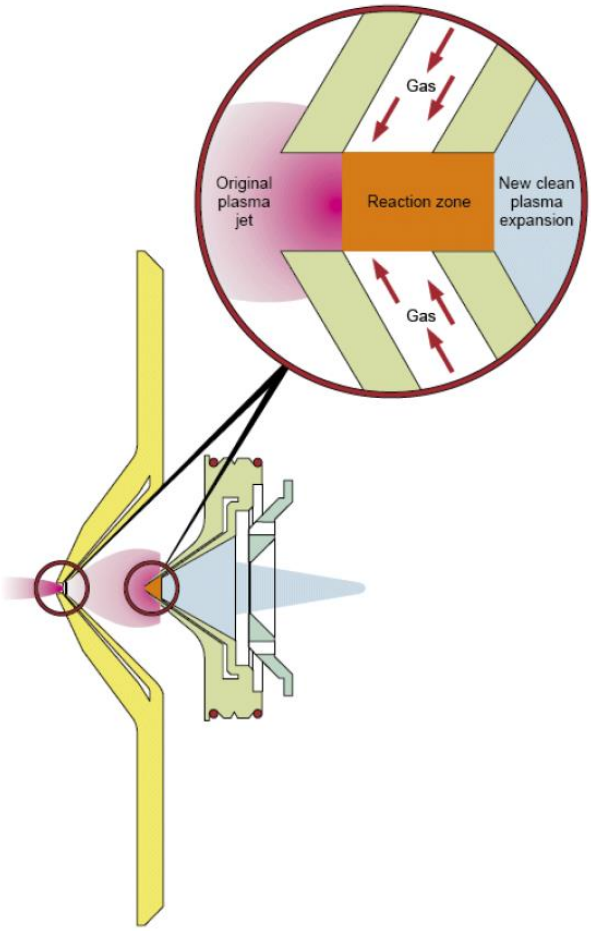

Figure 3.1. Schematic diagram of the collision reaction interface. Figure adapted from (Kalinitchenko, Wang & Sturman 2008).

The vacuum is maintained by differential pumping with the pressure reduced in three stages. The first stage produces a beam of particles in a high vacuum system from an atmospheric pressure source. This is achieved by a pair of coaxial cones separated by a fixed distance, with the intervening space kept at a pressure of ~2 torr. This allows most of the gas entering through the hole in the sampler cone to be removed while passing a beam of gas and ions from the ICP into the second stage of the vacuum system. The plasma entering the interface undergoes extremely rapid expansion and its temperature falls very quickly. There is no time for significant recombination of ions and electrons. Consequently, the composition of the sampled beam is representative of the ICP.

The second stage of the vacuum system is kept at a pressure of around 5 x 10^{-5} torr by a turbo-molecular pump. At this pressure, electrostatic ion lenses can guide ions. Neutral gases diffuse from the beam and are removed by the vacuum pump. The final stage of the

vacuum system contains the quadrupole mass analyser and the detector. It is maintained at a pressure of around 5 x 10^{-6} torr by a second turbo-molecular pump.

Ions emerging from the back of the skimmer cone are captured and focused into the mass analyser using an ion lens assembly (Figure 3.2). The first ion optics element is the extraction lens. Ions captured by the extraction lens are directed through the hole in the gate valve that separates the first and second stage of the vacuum system. They are then focused into the mass analyser by the main lens stack that is mounted directly in front of the quadrupole.

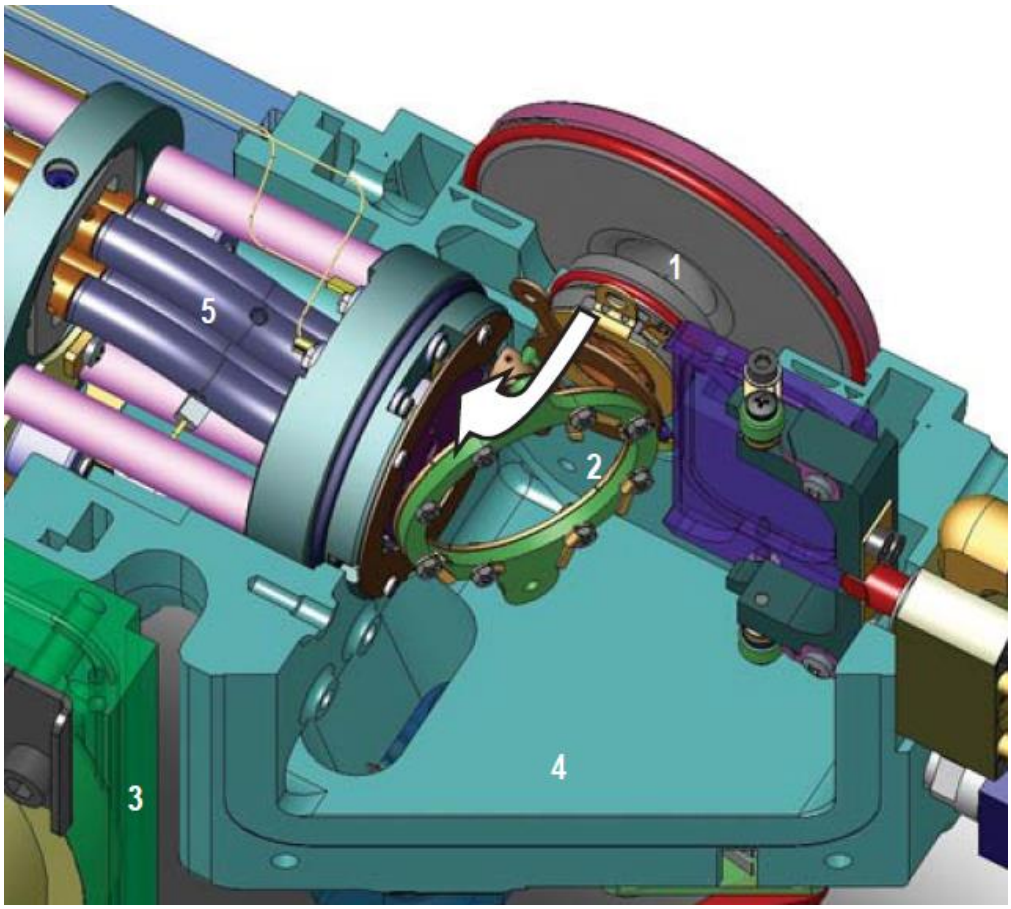

Figure 3.2. Schematic diagram of the 90° reflecting ion optics system of the Varian 820MS. Ions enter through the interface region via the skimmer cone (1) before being reflected (arrow) and focused by the ion mirror (2). The turbomolecular pump (3) is swung away from its operating position (4). A set of curved fringe rods (5) create a double off-axis system. Figure adapted from (Elliott, Knowles & Kalinitchenko 2004).

The quadrupole mass filter separates ions according to their mass-to-charge ratio, scanning from 0–240 AMU every 100 milliseconds. It consists of a set of four conductive rods mounted in insulating supports so that the rods are parallel, and their axes lie on the corners of a square. The geometry of the assembly is correct to within a few millionths of a metre. Opposite pairs of rods are electrically connected. Radio frequency and DC potentials are applied to each pair of rods with opposite pairs of rods given an equal potential of opposite sign. This generates a complex electromagnetic field in the space between the rods that controls the trajectories of ions entering the mass spectrometer. The RF and DC potentials on the quadrupole rods are adjusted very rapidly in a step-wise fashion allowing the sequential passage of ions with progressively larger mass/charge ratios. Ions to be analysed enter at one end at the array of rods and those having the selected mass-to-charge ratio emerge at the other end. For a given combination of RF and DC potentials, only ions of a specific mass-to-charge ratio have stable paths through the quadrupole enabling them to pass through to the detector. Ions with other mass-to-charge ratios have unstable trajectories that cause them to collide with the quadrupole rods.

Detection is by a discrete dynode electron multiplier. Each ion arriving at the detector collides with an electrode maintained at a high negative potential causing the electrode to emit a pulse of electrons. These electrons are then accelerated towards another dynode surface where they release more electrons thereby amplifying the signal. This process continues along 20 individual dynodes to generate a measurable pulse of current. Signal intensity is measured as counts sec^{-1} (c s^{-1}). The more ions reaching the detector the higher the c s^{-1} recorded.

3.5.4 Feedstuffs and supplements

The following registered supplements were used in the administration trials: -

- Hemoplex® (Troy Laboratories, Glendenning, NSW, Australia)
- Folic B12® (Troy Laboratories, Glendenning, NSW, Australia)
- Pre-Ferrin (Ceva Animal Health Pty Ltd, Austinmer, NSW, Australia)
- Tripart (Ceva Animal Health Pty Ltd, Austinmer, NSW, Australia)

In addition to these, the Co concentrations of several other registered supplements and feedstuffs typically provided to racing horses were measured (Figures 3.3 and 3.4), e.g.:

- Vitamin B12 (Ceva Animal Health Pty Ltd, Austinmer, NSW, Australia)
- L-carnitine (Ceva Animal Health Pty Ltd, Austinmer, NSW, Australia)
- VAM® (Ceva Animal Health Pty Ltd, Austinmer, NSW, Australia)
- COpHOS B (Ceva Animal Health Pty Ltd, Austinmer, NSW, Australia)
- Stamazene (Carbine Chemicals, Altona North, VIC, Australia)
- Feratone (Foran Equine, Cherry Orchard Industrial Estate, Dublin, Ireland)
- Carbalene (Carbine Chemicals, Altona North, VIC, Australia)
- Mitavite® Athlete Plus® (Mitavite, Somersby, NSW, Australia)
- Salkavite® (Ranvet, Banksmeadow, NSW, Australia)
- EMag®500 (Swancorp, Rocklea, QLD, Australia)
- Oats (Hawkesbury Stockfeeds, Richmond, NSW, Australia)
- Feramo® H (Equine Solutions, Townsville, QLD, Australia)
- Yellow Chaff (Hawkesbury Stockfeeds, Richmond, NSW, Australia)
- Hygain® TrackTorque® (Hy Gain Feeds Pty Ltd, Officer, VIC, Australia)
- CopRice Cool Conditioner (CopRice, Leeton, NSW, Australia)
- Mitavite® Breeda® (Mitavite, Somersby, NSW, Australia)
- BLUD® (Virbac Australia, Milperra, NSW, Australia)
- Green Chaff (Hawkesbury Stockfeeds, Richmond, NSW, Australia)

Figure 3.3. Injectable solutions measured for total cobalt content.

Figure 3.4. Feedstuffs measured for total cobalt content.

3.5.5 Reagents and materials

Solutions were stored at room temperature and were stable for at least 1 year unless otherwise stated. Pipettes used had a relative accuracy and precision of better than ± 2 %. All reagents were of analytical grade or better. No glassware was used in the preparation or storage of reagents. All plasticware used was assessed to ensure suitability for trace element determinations. A Millipore Milli-Q Gradient water purification system (Millipore Australia, North Ryde, NSW, Australia) provided reagent grade water (resistivity greater than 18.2 MOhm.cm@25°C) that was used throughout. Reagents and materials were sourced from Australian suppliers unless otherwise noted, as follows: -

3.5.5.1 Concentrated nitric acid, A.C.S.-grade 70 % (w/w) (Sigma-Aldrich, Castle Hill, NSW).

3.5.5.2 Concentrated nitric acid, 65 % (w/w). Merck Suprapur Cat. No. 1.00441.0250. Lot # ZU530141 306 (Merck, Kilsyth, VIC).

3.5.5.3 Nitric acid cleaning solution, 2 % (v/v). Add 20 mL concentrated nitric acid (3.5.5.1) to 1 L water.

3.5.5.4 Concentrated hydrochloric acid, A.C.S.-grade 37 % (w/w), 1.15 g mL^{-1} (Sigma-Aldrich, Castle Hill, NSW).

3.5.5.5 Concentrated hydrochloric acid, 36 % (w/w). Cat. No. Tracepur 1.15186.0000. Batch KH9901186 002. U.N. No. 1789 (Merck, Kilsyth, VIC).

3.5.5.6 5 mL polypropylene threaded tube, unlabelled natural cap separate, product code # P7512TUU (Techno Plas, Adelaide, SA). Tubes were filled with 2 % (v/v) nitric acid (3.5.5.3) and stored for at least 24 h then rinsed in reagent grade water immediately prior to use.

3.5.5.7 10 mL polypropylene threaded tube, unlabelled natural cap separate, product code # P10316UU (Techno Plas, Adelaide, SA). Tubes were filled with 2 % (v/v) nitric acid (3.5.5.3) and stored for at least 24 h then rinsed in reagent grade water immediately prior to use.

3.5.5.8 70 mL polypropylene screw cap, flat bottom container, clean environment manufactured, product code # P5744UU (Techno Plas, Adelaide, SA).

3.5.5.9 Aqua regia (AR) was prepared in 70 mL polypropylene container (3.5.5.8) by diluting 1 part concentrated hydrochloric acid (3.5.5.4) to 4 parts concentrated nitric acid (3.5.5.1). Used within 15 minutes of preparation.

3.5.5.10 Triton X100 (Tx100). Octyl Phenol Ethoxylate. Non-ionic surfactant. SG at 20°C 1.062. pH (1 % solution) 6.8. Fronine Cat. No. TRITX500. Batch 3497 (Thermo Fisher Scientific, Scoresby, VIC).

3.5.5.11 Ethylenediaminetetraacetic acid, tetrasodium salt (EDTA). $[CH_2.N(CH_2.COOH).CH_2.COONa]_2.2H_2O$. Minimum assay 99.4 %. Univar Prod. No. 663-100G. Batch No. 70747012 (Bacto Laboratories, Mt Pritchard, NSW).

3.5.5.12 Ethanol (ethyl alcohol), 99.7 to 100 % v/v. AnalaR Prod. No. 10107. Batch 015054 (Thermo Fisher Scientific, Scoresby, VIC).

3.5.5.13 Ammonia solution, 35 % (w/w). Fisher Chemical. Lot No. 1208338. Reorder code A/3240/PB17 (Thermo Fisher Scientific, Scoresby, VIC).

3.5.5.14 Tetramethylammonium hydroxide solution (TMAH), 25 % $(CH_3)_4N.OH$ in water. Acros Organics Cat. No. 420520010. Lot No. A0300977 (Thermo Fisher Scientific, Scoresby, VIC).

3.5.5.15 Ammonium chloride, 99.5+ %, A.C.S. reagent. Sigma-Aldrich Cat. No. 213330-500G. Batch No. 02125CJ (Sigma-Aldrich, Castle Hill, NSW).

3.5.5.16 Ammonium chloride solution, 273 g L^{-1}. Weigh 13.65 g of ammonium chloride (3.5.5.15) into a 70 mL container (3.5.5.8). Dissolve in water to the 50 mL graduation.

3.5.5.17 Tx100/EDTA solution, 1 % (w/v) Tx100 / 1 % (w/v) EDTA, 1 % NH_3. Weigh 0.5 g Tx100 (3.5.5.10) and 0.5 g EDTA (3.5.5.11) into a 70 mL container (3.5.5.8) then add ~ 30 mL water and 500 µL NH_3 (3.5.5.13) to dissolve the EDTA. Add water to the 50 mL graduation. Solution was stable for 6 months.

3.5.5.18 Multi-element misa std # 1 rare earth metals – 100 mg L^{-1} Sc, Y, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Yb, Lu, Th, U. Em Science Cat. No. ICPM0231-1. Lot # A8015030 (Choice Analytical, Thornleigh, NSW).

3.5.5.19 Single element stock standard – 1000 mg L^{-1} Ga. Spec Pure. Lot No. 2-05448 (Thermo Fisher Scientific, Scoresby, VIC).

3.5.5.20 Internal standard intermediate – 50 mg L^{-1} Sc, Ga, Y, La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Yb, Lu, Th, U. In a 10 mL tube (3.5.5.7) add 4.5 mL water, 5.0 mL 100 mg L^{-1} multi-element stock standard (3.5.5.18) and 500 µL 1000 mg L^{-1} Ga stock standard (3.5.5.19). Solution was stable for 1 year.

3.5.5.21 Multi-element stock standard – 100 mg L^{-1} Sb, As, Be, Cd, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Mo, Ni, P, Se, Sr, Tl, Sn, Ti, V, Zn. ACR. Lot No. DF1822 (Australian Chemical Reagents, Moorooka, QLD).

3.5.5.22 ICP Multi-Element Standard XXI for MS 10 mg L^{-1}. Merck. Lot No: OC492641 (Thermo Fisher Scientific, Scoresby, VIC).

3.5.5.23 Single element stock standard – 1000 mg L^{-1} Cu. Fronine Cat. No. 0679/100mL (Fronine, Riverstone, NSW).

3.5.5.24 Single element stock standard - 1000 mg L^{-1} Zn. Fronine Cat. No. 0777/100mL (Fronine, Riverstone, NSW).

3.5.5.25 Single element stock standard - 1000 mg L^{-1} Fe. Fronine Cat. No. 0701/100mL (Fronine, Riverstone, NSW).

3.5.5.26 Single element stock standard - 10000 mg L^{-1} Na. JM Australia. Lot No. 603474J (Choice Analytical, Thornleigh, NSW).

3.5.5.27 Single element stock standard - 10000 mg L^{-1} K. JM Australia. Lot No. 603474M (Choice Analytical, Thornleigh, NSW).

3.5.5.28 Single element stock standard - 1000 mg L^{-1} Ca. ACR. Lot No. FG1996 (Australian Chemical Reagents, Moorooka, QLD).

3.5.5.29 Single element stock standard - 1000 mg L^{-1} Mg. ACR. Lot No. FF1910A (Australian Chemical Reagents, Moorooka, QLD).

3.5.5.30 Calibrator diluent – 10 % (v/v) HCl. In a 10 mL tube (3.5.5.7) add 9.0 mL water and 1.0 mL concentrated HCl (3.5.5.5).

3.5.5.31 Multi-element working standard intermediate – 4 mg L^{-1} Sb, As, Be, Cd, Ca, Cr, Co, Fe, Pb, Li, Mg, Mn, Mo, Ni, P, Se, Sr, Tl, Sn, Ti, V / 24 mg L^{-1} Cu / 56 mg L^{-1} Zn / 80 mg L^{-1} Fe. To a 10 mL tube (3.5.5.7) add 1200 µL calibrator diluent (3.5.5.30), 650 µL water, 50 µL 1000 mg L^{-1} Cu stock standard (3.5.5.23), 200 µL 1000 mg L^{-1} Zn stock standard (3.5.5.24), 200 µL 1000 mg L^{-1} Fe stock standard (3.5.5.25), 100 µL 100 mg L^{-1} Sb, As, Be, Cd, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Mo, Ni, P, Se, Sr, Tl, Sn, Ti, V, Zn ACR stock standard (3.5.5.21) and 100 µL 10 µg mL^{-1} Merck multi-element standard XXI (3.5.5.22). Working standard was stable for 6 months at 4–8°C.

3.5.5.32 Sample diluent (Method 1 acidic diluent) – 1 % (v/v) HNO_3, 0.01 % (w/v) Tx100, 0.01 % (w/v) EDTA, 50 µg L^{-1} internal standard. To a 70 mL container (3.5.5.8) add ~ 40 mL water, 500 µL 70 % HNO_3 (3.5.5.1), 500 µL 1 % Tx100/EDTA (3.5.5.17) and 50 µL 50 mg L^{-1} internal standard intermediate (3.5.5.20). Add water to the 50 mL graduation. Diluent was stable for 24 h.

3.5.5.33 Sample diluent (Method 2 alkaline diluent – 1 % (v/v) NH_3, 2 % (v/v) ethanol, 0.01 % (w/v) Tx100/EDTA, 2.73 g L^{-1} NH_3Cl, 10 µg L^{-1} internal standard. To a 500 mL plastic container add ~ 400 mL water, 5 mL 35 % NH_3 (3.5.5.13), 10 mL ethanol (3.5.5.12), 5 mL 1 % Tx100/EDTA (3.5.5.17) and 100 µL 50 mg L^{-1} internal standard intermediate (3.5.5.20). Add water to the 500 mL graduation. Diluent was stable for 2 months.

3.5.5.34 Tx100 intermediate rinse solution – 7.6 % (w/v) Tx100. Weigh 9.5 g Tx100 (3.5.5.10) into a 125 mL container. Add water to the 125 mL graduation.

3.5.5.35 Rinse solution - 0.1 % (v/v) ammonia, 0.01 % (w/v) Tx100. To an 8 L carboy add ~ 7 L water, 7.6 mL ammonia solution (3.5.5.13) and 10 mL 7.6 % Tx100 (3.5.5.34). Add water to the 7.6 L graduation.

3.5.5.36 Liquid argon (Ace Cryogenic, Kings Park, NSW).

3.5.5.37 Ultra-high purity helium. BOC Size G cylinder. Cat. No. 220G. U.N. No. 1046 (BOC, North Ryde, NSW).

3.5.5.38 Ultra-high purity hydrogen. BOC Size E cylinder. Cat. No. 240E. U.N. No. 1049 (BOC, North Ryde, NSW).

3.5.5.39 Multi-element stock standard – 10 µg mL^{-1} Ba, Be, Ce, Co, In, Pb, Mg, Th. Inorganic Venture Inc. Tune Solution VAR-TS-MS, Lot No. K-MEB46105 (Astral Scientific, Taren Point, NSW).

3.5.5.40 Tune solution – 10 µg L^{-1} Ba, Be, Ce, Co, In, Pb, Mg, Tl, Th. In a 50 mL container add 50 µL 10 µg mL^{-1} multi-element stock standard (3.5.5.39) to ~ 40 mL water. Add 500 µL concentrated nitric acid (3.5.5.1) and water to the 50 mL graduation. Solution was stable for 2 months.

3.5.6 Sample collection and processing

Samples were collected and processed as described unless otherwise noted in the relevant section. Blood was collected from the jugular vein (Figure 3.5) using the Vacutainer® collection system (Becton Dickenson, NSW, Australia) and a 20-guage needle (BD Cat. # 360214), to aspirate blood into 10 mL heparinised tubes (BD green LiHep Cat. # 367526) and 2 mL EDTA tubes (BD lavender K3EDTA Cat. # 367836). The lithium heparin tube from each pair of matched blood samples was immediately centrifuged at 1250 g rcf for 5 min at room temperature with the plasma aspirated and placed into a fresh heparinised vacutainer tube for plasma assay. The EDTA tube was mixed thoroughly by gentle inversion at the time of collection and kept as collected for whole blood analysis. Care was taken to ensure all samples were free from haemolysis. A subset from each batch of heparinised and EDTA tubes was tested and found to contain < 0.1 µg Co L^{-1}, making these tubes suitable for sample collection and storage of aspirated plasma.

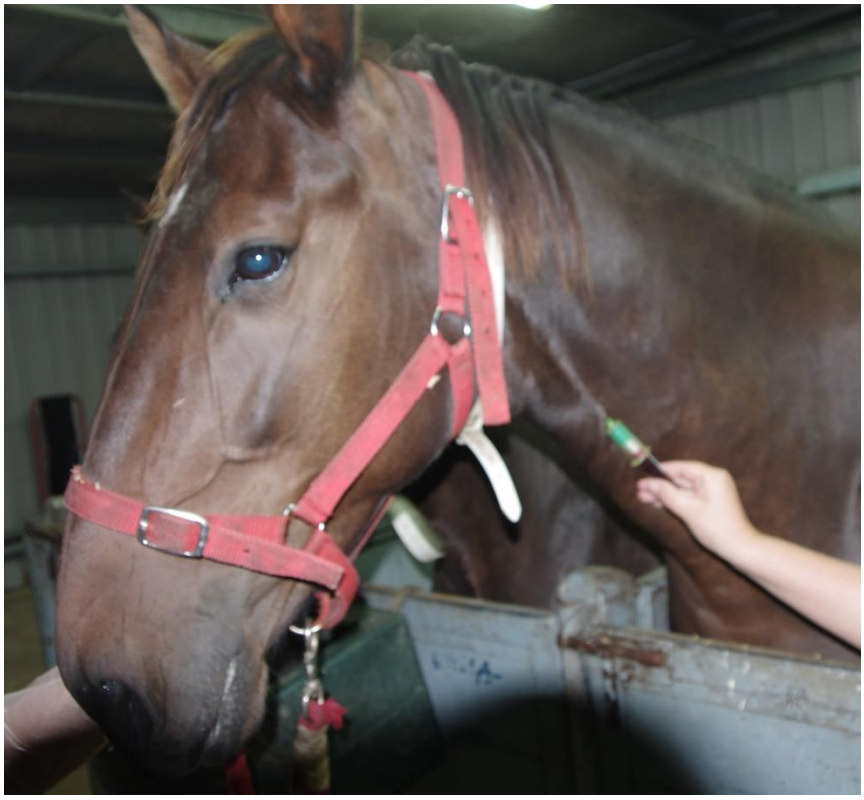

Figure 3.5. Jugular vein blood collection.

A skilled equine veterinarian directly aspirated urine by digital passage of an Infusette® catheter through the urethral sphincter (Figures 3.6 & 3.7). Urine was placed in 70 mL polypropylene sample containers (3.5.5.8) that had been batch tested to ensure they contained less than 0.1 μg L^{-1} Co. 24 h urine collections were performed by connecting a Foley catheter via extension tubing to a sealed collection bag that was tied to a horse rug (Figure 3.8).

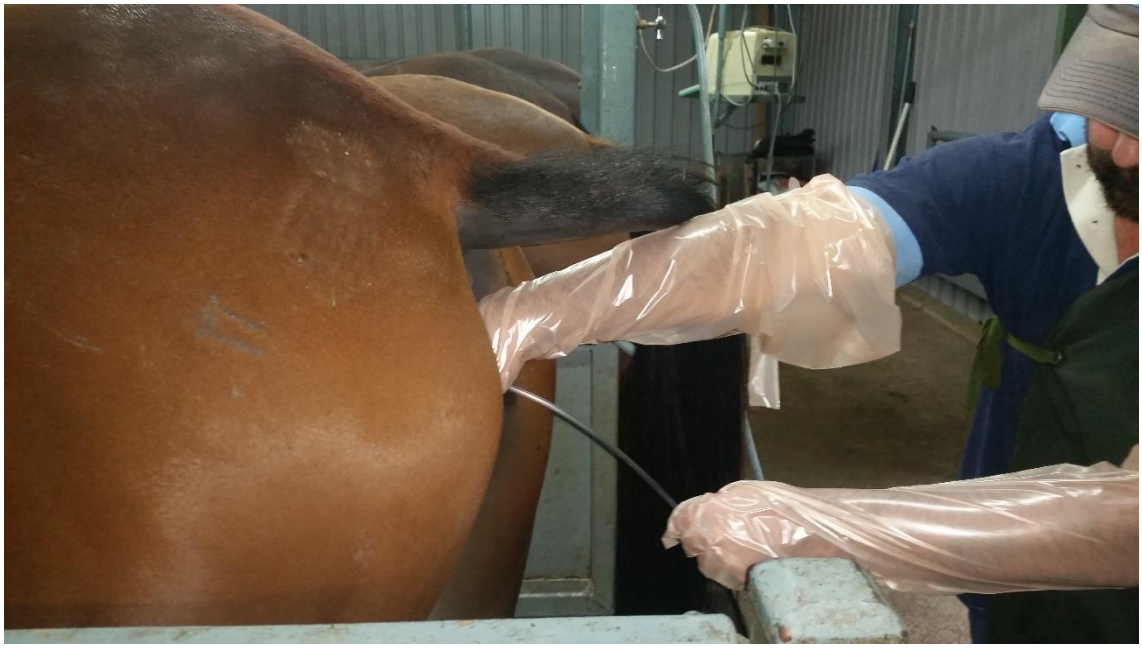

Figure 3.6. Insertion of Infusette® catheter through the urethral sphincter.

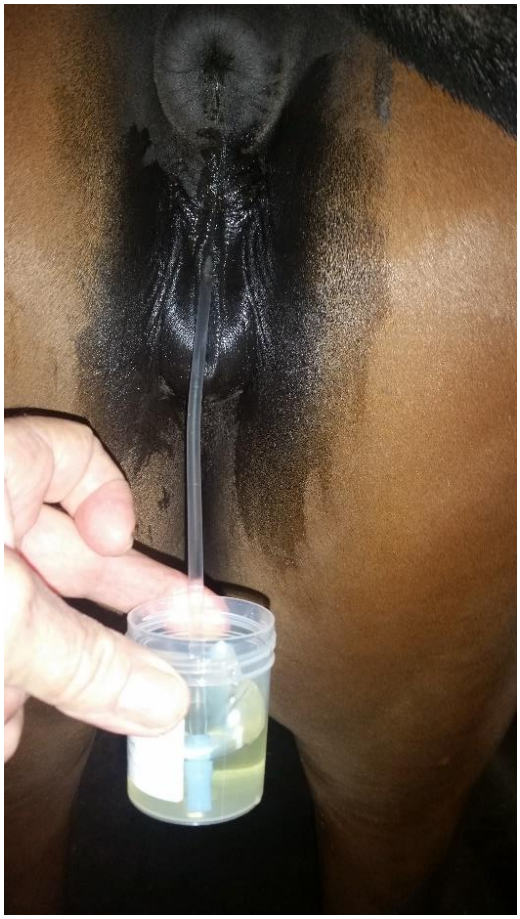

Figure 3.7. Urine collection in 70 mL polypropylene sample container (3.5.5.8) via Infusette® catheter.

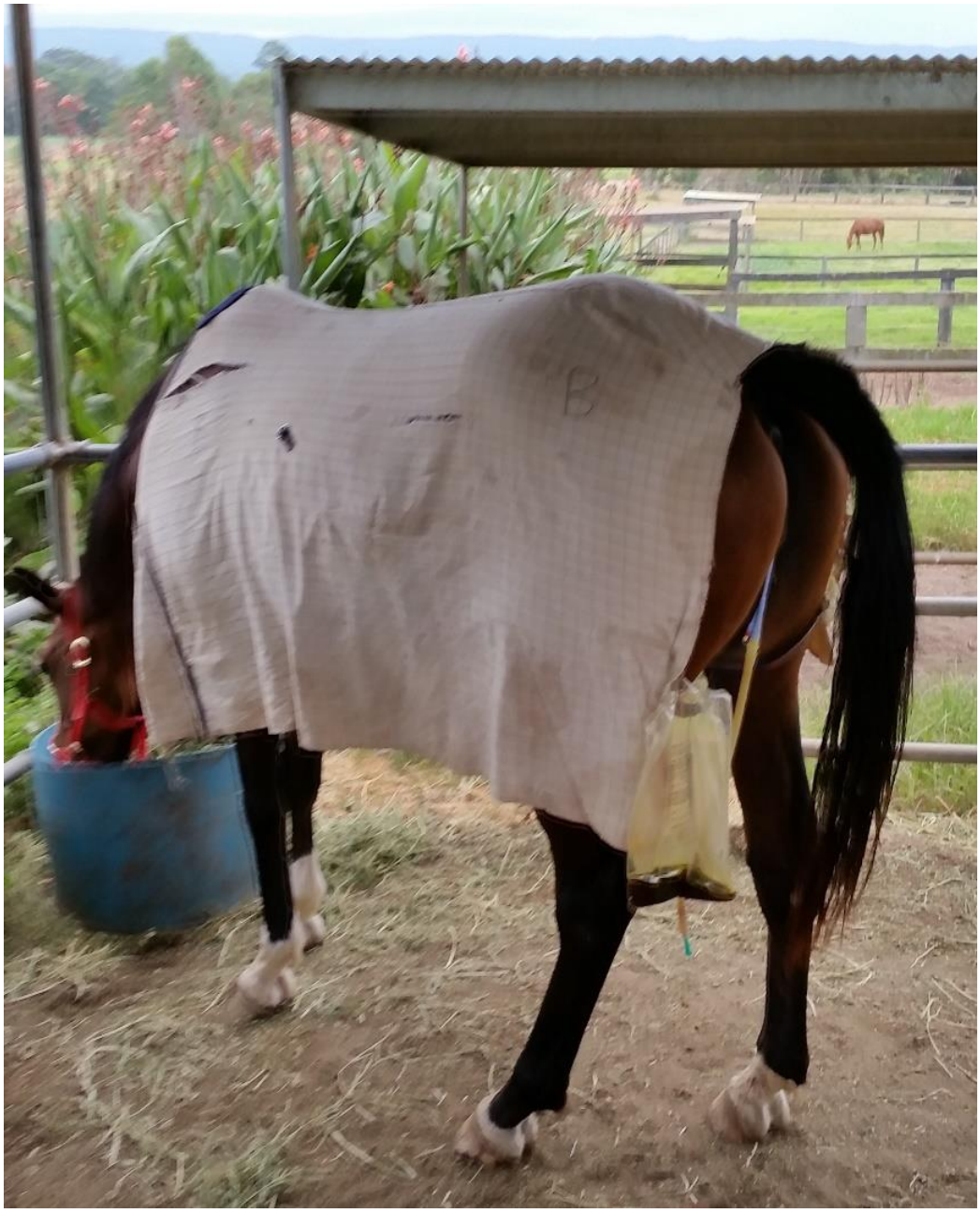

Figure 3.8. 24-hour urine collections were performed by connecting a Foley catheter via extension tubing to a sealed collection bag that was tied to a horse rug.

Manure was collected regularly from each horse that was isolated to a small holding yard. Care was taken to minimise environmental contamination throughout the collection process. The manure from each horse was pooled in separate buckets over 84 h. The total manure collected was then weighed and mixed with a measured volume of water using a paint stirrer (Figure 3.9).

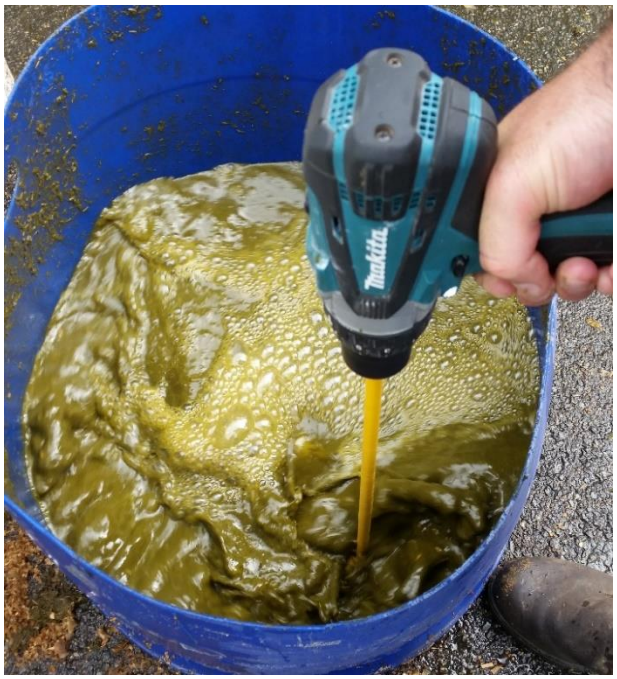

Figure 3.9. Total manure collected over 84 hours mixed with a measured volume of water using a paint stirrer.

Solid samples were weighed into 10 mL tubes (3.5.5.7). Sample digestion steps were performed in an acid rated laminar flow safety cabinet free of metal fixtures. The addition of AR (3.5.5.9) to samples was at a minimum rate of 100 µL AR to every 0.001 g sample. For digestion, sample tubes were loosely capped and placed in a dry block heater (Thermoline Scientific, Wetherill Park, NSW, Australia) at 90°C for 30 min. Digestates where allowed to cool to room temperature before being made to a final volume of 10 mL with reagent water. pH determinations were made using a Mettler Toledo SevenEasy pH meter (Sigma-Aldrich, Castle Hill, NSW, Australia).

3.5.7 Quality assurance

Determinations were performed using equipment and methods accredited to ISO 15189:2012 (International Organization for Standardization 2012) for medical testing as certified by the National Association of Testing Authorities, Australia / Royal College of Pathologists of Australasia, laboratory accreditation number 1981. External quality assurance for Co was through enrolment in the Quebec Multi-Element Quality Assurance Scheme (Institut national de santé publique) and the RCPA QAP Trace Elements Program (Royal College of Pathologists of Australasia Quality Assurance Programs Pty Limited). Additional steps taken to assure data quality included the analysis of Standard Reference Materials (SRMs), reagent blanks and ‘spike’ recoveries.

Two levels of internal quality control were run for each sample matrix and included in each analytical run so that they were analysed before and after the test samples. Control samples included Seronorm Trace Elements Urine L1, Lot 1011644, Exp 2018-01, Seronorm Trace Elements Urine L2, Lot 1011645, Exp 2018-01, Seronorm Trace Elements Serum L1, Lot 1309438, Exp 2020-10, Seronorm Trace Elements Serum L2, Lot 1309416, Exp 2020-10, Seronorm Trace Elements Whole Blood L1, Lot 1406263, Exp 2019-07 and Seronorm Trace Elements Whole Blood L2, Lot 1406264, Exp 2019-07 (SERO, Billingstad, Norway). Standard reference material (SRM) SRM 1643d Trace Elements in Natural Water, SRM 1640a Trace Elements in Natural Water and SRM 1577b Bovine Liver were used as controls for the measurement of Co in supplements and feedstuffs (National Institute of Standards and Technology, Gaithersburg, Maryland, United States).

3.5.8 ICP-MS analysis

3.5.8.1 ICP-MS operation

The Varian 820MS apparatus (Section 3.5.3) was maintained and operated to manufacturer's specifications (Varian 2004). A solution of 0.1 % (v/v) ammonia and 0.01 % (w/v) Tx100 (3.5.5.35) was used for the SPS3 probe wash and switching valve rinse. Instrument tuning, torch alignment and mass calibration were performed using the tune solution (3.5.5.40). A peristaltic pump was used to deliver sample to the nebuliser and to drain waste from the spray chamber. Typical instrument operating parameters are shown in Table 4.1. Internal standard (3.3.5.20) was added to the sample diluent to compensate for variations in sample delivery and rates of ionisation. The internal standard method of correction was by interpolation.

3.5.8.2 Calibration

When using ICP-MS to determine trace element concentrations in biological fluids, calibrators are often prepared in the sample matrix to be analysed. This is done to limit the impact of spectroscopic interferences, reduce the effect of ionisation enhancement and prevent mass biasing whereby lighter elements are preferentially displaced from the ion beam with increasing ion density. Matrix matched calibrations require prior

knowledge of the concentration of elements in the base matrix material and they can only be used for the same matrix samples for which they were specifically prepared. It is also difficult to define a representative urine sample matrix as normal changes in an individual's hydration status have a considerable bearing on urine consistency.

To avoid the need for matrix matched calibrations, in addition to those elements that were of analytical interest, stock calibrators were prepared with matrix elements, namely sodium, potassium, calcium, and magnesium (Table 3.2). The alkaline sample diluent also contained 0.9 g L^{-1} chloride. Internal standard was added to the sample diluents to correct for any mass biases that were not adequately compensated for using this calibration strategy. The alkaline sample diluent also contained 2 % (v/v) ethanol (3.5.5.12) to ensure all elements were afforded the same level of ionisation enhancement. As an easily ionised element in an argon plasma, Co should not be subjected to ionisation enhancement in a high carbon matrix. This theory was confirmed with comparable Co concentrations found across sample matrices when using the acidic diluent, with no additional carbon, and the alkaline sample diluent with ethanol added (Table 3.3). By not relying on matrix matched calibrators, it was possible to perform measurements on urine, blood, plasma, and sample digests within the same analytical run. Not only did this enhance run efficiency, but it reduced the impact of the variability that occurs with each new calibration on the accuracy of comparisons of Co distribution in urine and blood samples.

Table 3.2. Calibration solutions preparation prepared in 5 mL tubes (3.5.5.6) using calibrator diluent (3.5.5.30). Calibrators stable for 2 months at 4–8°C.

	Concentration (µg L^{-1})			**Diluent (µL)**	**H_2O (µL)**	**Standard**
	Co	Fe	Cu			
C1	0.033	0.69	0.23	320	2880	800 µL C2
C2	0.17	3.47	1.2	320	2880	800 µL C3
C3	0.83	17.3	5.8	320	2880	800 µL C4
C4	4.13	86.7	29	240	2160	1600 µL C5
C5	10.3	217	72	255	2295	1700 µL C6
C6	25.8	542	181	0	0	800 µL working stock (3.5.5.31) 1200 µL 10000 mg Na L^{-1} (3.5.5.26) 1000 µL 10000 mg K L^{-1} (3.5.5.27) 500 µL 1000 mg Ca L^{-1} (3.5.5.28) 500 µL 1000 mg Mg L^{-1} (3.5.5.29)

3.5.8.3 Sample measurement

Sample measurements were conducted according to the following steps: -

a) After starting the ICP-MS and allowing 30 min for warm-up, tune solution (3.5.5.40) was aspirated and Be, In, Pb, Ce/CeO ratio and Ba/Ba^{2+} ratio were monitored to optimise torch position, peak resolution, mass calibration, detector voltage, plasma parameters and ion optics. Optimum tuning was achieved when the sensitivity of Be, In and Pb was maximal while Ce/CeO and Ba/Ba^{2+} ratios were minimal (Vaughan & Horlick 1986).

b) All samples reached room temperature and were thoroughly mixed before analysis. Calibrators (Table 3.2), controls (Section 3.5.7), reagent blanks and test samples were diluted 31-fold in 5 mL tubes (3.5.5.6) with 50 µL test solution mixed with 1000 µL sample diluent (3.5.5.32 or 3.5.5.33). Reagent blanks were included throughout a run and prepared as 50 µL water in place of sample to ensure reagents were free from significant contaminants. Solutions were ready for analysis after being vortex mixed then loaded onto the SPS3 autosampler according to the method sequence.

c) Analyte concentrations were interpolated from calibration curves generated by the instrument software. Results were automatically corrected for signal drift using the internal standards. Samples with concentrations exceeding the calibration range were repeated after dilution in water then sample diluent. Samples

immediately following an over-range sample were repeated to ensure they were free from carryover. Concentrations were reported in mass units with dilution factors applied by the software.

d) The following performance criteria were followed with each batch of samples analysed to ensure adequate instrument operation and reliable result quality: -

i. Initial Rinse - Sample diluent was run as the initial 2 samples in the run sequence to ensure the system was clean. If a significant fall in c s^{-1} between rinses was observed, the run was stopped, and the sample introduction path cleaned.

ii. Internal Standard - Once the ICP-MS stabilised, % RSD on internal standard replicates should be < 3 %. If this was not achieved, plasma conditions were further optimising or, if this failed to improve the % RSD, instrument maintenance was initiated. Samples with an internal standard % RSD > 5 % were rerun. Samples showing a sudden change in internal standard ratio > 30 % were repeated.

iii. Calibration – The calibration was considered acceptable if a linear calibration with $R^2 > 0.999$ and calculated standard concentration % error < 10 % was obtained.

iv. Post Calibration Rinse - Sample diluent was run as the first 2 samples after the calibration as an indication of between sample carry-over. The concentration of these samples was less than the element limit of quantitation or the run was stopped and the sample introduction path cleaned.

v. Preparation Blank - Used to correct for calibration inaccuracy at low concentrations. The preparation blank was less than the first calibration point and automatically applied to subsequent measurements by the software.

vi. Sample Blank - These samples were placed throughout a run to ensure assay detection limits were maintained. The run was repeated if blank concentrations exceeded the limit of quantitation.

vii. Within Sample Replicates - The % RSD was flagged by the instrument software as unacceptable when % RSD was high enough to significantly change an analytes concentration. Flagged samples were rerun.

viii. Quality Assurance Material – Controls were included in each analytical run so that at least two levels of matrix matched control were measured before and

after each batch of test samples. Seronorm^{TM} quality control material was used to control serum, blood, and urine assays. National Institute of Standards & Technology (NIST) Standard Reference Material (SRM) 1577b Bovine Liver and NIST waters 1640a or 1643d were determined to control the feedstuff, supplement, and manure assays. Expected performance limits for the control materials were defined from prior statistical evaluation. Analytical runs where controls failed to meet expected performance criteria were considered unacceptable and repeated after troubleshooting the assay.

3.6 Results and discussion

3.6.1 Method validation

Two analytical approaches for Co determinations in blood, serum and urine were evaluated for performance precision to National Committee for Clinical Laboratory Standards (Carey et al. 2006; Tholen et al. 2004). In Method 1, samples were prepared in a diluent containing nitric acid (3.5.5.32) with ICP-MS analysis performed using helium as the CRI gas. In Method 2, samples were prepared in a diluent containing ammonia (3.5.5.33) with ICP-MS analysis performed using hydrogen as the CRI gas. Method 1 provided the more precise data and lower mean Co concentrations were noted across the quality control material (Table 3.3). With only slight differences between the two methods, either could be reliably used to determine Co concentrations in blood, serum, and urine. Cobalt stability and other analysis properties such as sample washout times were not adversely affected by choice of sample diluent for the various sample matrices. The results also show that both helium and hydrogen are effective CRI gases for minimising spectroscopic interferences for Co (predominantly $^{43}Ca^{16}O^{+}$, $^{42}Ca^{16}O^{1}H^{+}$, $^{24}Mg^{35}Cl^{+}$, $^{36}Ar^{23}Na^{+}$, $^{40}Ar^{18}O^{1}H^{+}$) from the sample matrix and argon plasma. Given the slightly better performance data for Method 1, this method was used for the Co analyses in this study.

Table 3.3. Summary of performance precision data (Appendix 9.2). Cobalt concentrations reported in µg L^{-1}. Method 1 samples prepared in acidic diluent (3.5.5.32) with helium as CRI gas. Method 2 samples prepared in alkaline diluent (3.5.5.33) with hydrogen as CRI gas.

	Serum		**Blood**		**Urine**	
Seronorm Lot No.	1309438	1309416	1702821	1702825	1403080	1403081
Method 1 - acidic diluent with helium as CRI gas						
Grand Mean (µg L^{-1})	**1.03**	**2.86**	**0.22**	**4.72**	**0.81**	**10.16**
ME = Total WR Variance (S2r)	0.0029	0.0107	0.0009	0.0244	0.0044	0.0216
Total WR SD (Sr)	0.0538	0.1035	0.0296	0.1561	0.0665	0.1471
Variance of Daily Means (B2)	0.0021	0.0119	0.0012	0.0407	0.0018	0.0376
SD of Daily Means (B)	0.0456	0.1093	0.0343	0.2016	0.0428	0.1939
ST	0.0594	0.1315	0.0402	0.2299	0.0636	0.2200
MD	0.0042	0.0239	0.0024	0.0813	0.0037	0.0752
T	37	33	32	30	39	29
Repeatability (Sr)	**0.0538**	**0.1035**	**0.0296**	**0.1561**	**0.0665**	**0.1471**
CV	**5.2 %**	**3.6 %**	**13.7 %**	**3.3 %**	**8.3 %**	**1.4 %**
Method 2 - alkaline diluent with hydrogen as CRI gas						
Grand Mean (µg L^{-1})	**1.13**	**3.05**	**0.30**	**5.07**	**0.86**	**10.65**
ME = Total WR Variance (S2r)	0.0062	0.0149	0.0024	0.0419	0.0051	0.0578
Total WR SD (Sr)	0.0788	0.1222	0.0494	0.2048	0.0711	0.2405
Variance of Daily Means (B2)	0.0037	0.0097	0.0008	0.0348	0.0027	0.0809
SD of Daily Means (B)	0.0608	0.0986	0.0278	0.1865	0.0523	0.2844
ST	0.0825	0.1311	0.0446	0.2362	0.0725	0.3313
MD	0.0074	0.0194	0.0016	0.0696	0.0055	0.1617
T	39	38	38	36	39	31
Repeatability (Sr)	**0.0788**	**0.1222**	**0.0494**	**0.2048**	**0.0711**	**0.2405**
CV	**7.0 %**	**4.0 %**	**16.4 %**	**4.0 %**	**8.3 %**	**2.3 %**

3.6.2 Sample storage

The aim of this study component was to investigate storage changes in urine that could compromise the accuracy of Co determinations. Horses typically produce alkaline urine that can have a cloudy appearance due to presence of mucus and/or amorphous phosphates or other crystals. Trace metal solutions are most stable when stored at a low pH where an abundance of free hydrogen ions prevent the metals from binding to negatively charged elements, compounds, or surfaces. Of concern when measuring Co in an alkaline urine was that Co could form insoluble salts. These Co salts could produce urine where Co was no longer distributed homogeneously throughout the sample. If urines were centrifuged or not mixed prior to taking an aliquot for analysis, the Co concentration could be underestimated. If urines were inadequately mixed prior to taking an aliquot for analysis, then the Co concentration could be either over or under-estimated depending on the quantity of sediment aliquoted. Sample diluent pH could also affect result accuracy with variable Co salt solubility i.e. higher recovery of Co would be expected in an acidic diluent as the Co is released into solution.

The stability of Co in urine was assessed using a sample that had an initial Co concentration of 161 µg L^{-1} measured 2 days after collection. Sample had been kept refrigerated since collection in a 70 mL container (3.5.5.6). Before analysis it was brought to room temperature and mixed thoroughly. Returned sample to refrigerator for another 3 days then poured off a 6 mL aliquot into a 10 mL tube (3.5.5.6). Tube was immediately centrifuged at 1250 g rcf for 5 min leaving a clear supernatant from which a 50 µL aliquot was found to contain 142 µg Co L^{-1}, i.e. result for sample not allowed to reach room temperature. When thoroughly mixed and retested the sample was found to contain 163 µg Co L^{-1}. The sample was then acidified to contain 1 % (v/v) nitric acid with the addition of 60 µL concentrated nitric acid (3.5.5.2), mixed thoroughly and found to contain 158 µg Co L^{-1}. A decrease in Co concentration was noted when the precipitate that had formed at 4°C was removed by centrifugation.

Further storage conditions were investigated on the samples by pouring off two 6 mL aliquots in 10 mL tubes (3.5.5.6). One aliquot was acidified to contain 1 % (v/v) nitric acid with the addition of 60 µL concentrated nitric acid (3.5.5.2). Both aliquots were then

stored at room temperature for 11 days with Co concentrations measured after 4 and 11 days. Measurements were made on these samples after they have been mixed thoroughly to resuspend any sediment and on the sample supernatant after centrifugation at 1250 g rcf for 5 min. Cobalt concentrations were as follows: -

Day 4	**Day 11**
No acid (mixed) 161 µg L^{-1}	No acid (mixed) 153 µg L^{-1}
No acid (centrifuged) 155 µg L^{-1}	No acid (centrifuged) 156 µg L^{-1}
1 % HNO_3 (mixed) 155 µg L^{-1}	1 % HNO_3 (mixed) 153 µg L^{-1}
1 % HNO_3 (centrifuged) 164 µg L^{-1}	1 % HNO_3 (centrifuged) 161 µg L^{-1}

From this investigation it was concluded Co was stable in urine with minimal storage changes noted after 11 days at room temperature. Urine should however be thoroughly mixed before analysis if not stored at room temperature.

To investigate the disposition of Co in urine sediment, 6 mL of the sediment remaining in the 24 h urine collection bag was transferred to a 10 mL tube (3.5.5.6). Initially this sediment was mixed thoroughly and analysis of a 50 µL aliquot had a Co concentration of 175 µg L^{-1}. The sediment was then centrifuged at 1250 g rcf for 5 min leaving a clear supernatant from which a 50 µL aliquot was found to contain 94 µg Co L^{-1}. The sediment was then acidified with 200 µL concentrated nitric acid (3.5.5.2), mixed thoroughly, and once again centrifuged at 1250 g rcf for 5 min leaving a clear supernatant from which a 50 µL aliquot was found to contain 190 µg Co L^{-1}.

From these results it was clear that insoluble Co salts can form in urine. It was also evident that altering urine pH can bring these salts back into solution. The 24 h collection was chosen for investigation as this sample represented a worst-case scenario for sediment accumulation. Sediment formation in free flow random urine samples was generally not as noticeable as it was in the 24 h collections. However, urine samples with a high creatinine tended to have more sediment present, giving them an appearance more like a 24 h urine sample, than dilute samples with a lower creatinine. As a result of these findings, sample analysis protocol for equine urines were to ensure that samples were

thoroughly mixed immediately prior to taking an aliquot for analysis. Improved Co solubility at low pH support the use of an acidic diluent for sample preparation. Overall, with variable stability of Co in urine, blood and plasma Co measurements should be favoured over urine when assessing Co status. Though not investigated here, consideration should also be given to the possibility of delayed Co excretion through incomplete bladder emptying. Insoluble Co salts could remain in the bladder, particularly during periods of relative dehydration, only to be flushed out with a full bladder later. Current rules of racing mandate a "clear day rule" where trainers are not permitted to administer IV supplements for a 24 h period from 12.00 am to 11.59 pm before a race (Racing Australia 2019). Any factors causing delayed Co excretion over the clear day could result in a urinary Co level exceeding the threshold on race day.

3.6.3 Cobalt content of feedstuffs and supplements

The Co content of many feedstuffs and the potential for intestinal absorption of Co was largely unknown. An inspection of nutritional supplements available at ABEC identified several registered commercial preparations containing Co (Figure 3.3). To establish the quantity of Co that horses typically receive, the Co content of various feedstuffs and supplements were measured. Representative sub-samples of solid feedstuffs and supplements were weighed and digested in duplicate. Samples with a non-homogenous appearance were prepared in triplicate. Feedstuffs that were composed of several individual components, such as mixed grains, were separated into their base materials for analysis. Estimates of total Co concentration were made by averaging the Co measured in these individual components. The heating block was limited to 45 digestion positions, so digests on solid samples were performed in 2 batches (Appendix 9.3). Liquid supplements required no additional processing other than dilution in water if they exceeded the calibration range. They were added directly to the sample diluent (3.5.5.32).

Results of analysis on supplements and feedstuffs (Section 3.5.4) are summarised in Table 3.4. Additionally, magnesium, manganese, iron, nickel, copper, and zinc were simultaneously measured to ensure sub-sample homogeneity and gauge the accuracy of nutritional content stated for the commercial supplements. As divalent cations, these

elements have the potential to reduce Co bioavailability though competitive inhibition of intestinal divalent metal transporter 1 (Espinoza et al. 2011).

Table 3.4. Cobalt concentrations in feedstuffs and supplements summarised from results presented in Appendix 9.3. BLUD sachet results are the average of measurements made in separate batches due to lack of homogeneity in this feed additive. Where provided, manufacturer stated concentrations are shown. The Co content of supplements noted to contain B12 were calculated by multiplying the B12 concentration by 0.04348 and adding this value to the Co content if available.

Sample	Co	Mg	Mn	Fe	Ni	Cu	Zn	Se
	---- (µg g^{-1}) ----							
Tracktorque	0.50	1675	149	155	2.3	101	176	-
Yellow chaff	0.05	1081	87	51	0.3	1.7	4.7	-
Green chaff	0.16	2974	29	118	1.1	7.1	31	-
BLUD sachet (measured)	0.14	0.76	4	2601	0.6	14	1	-
BLUD sachet (stated)	*0.23*	-	-	*3521*	-	*70*	-	-
Feramo H with chromium	5.04	3866	2931	3812	3.5	696	3354	-
Oats	0.06	1543	63	36	3.3	4.8	23	-
Mitavite Breeda	0.36	4742	168	198	2.0	62	172	-
Cool conditioner	0.39	4884	195	181	1.2	167	215	-
Mitavite Athlete Plus	0.97	4358	370	212	3.9	177	563	-
Salkavite	1.47	16599	232	122	8.1	0.50	46	-
Carbalene	0.002	-	-	-	-	-	-	-
E Mag 500	15	-	-	-	-	-	-	-
Feratone (measured)	20	-	-	3574	-	96	-	-
Feratone (stated)	*46*	-	-	*5535*	-	*1538*	-	-
Stamazene	0.002	-	-	-	-	-	-	-
Tripart (measured)	23	1545	-	-	-	-	-	1043
Tripart (stated)	*21*	*1609*	-	-	-	-	-	*955*
VAM (measured)	86	-	-	2515	-	29	-	-
VAM (stated)	*91*	-	-	*2972*	-	*26*	-	-
COpHOS B (measured)	2.0	-	-	-	-	-	-	-
COpHOS B (stated)	*2.1*	-	-	-	-	-	-	-
L-Carnitine	0.010	-	-	-	-	-	-	-
Vitamin B12 (measured)	86	-	-	-	-	-	-	-
Vitamin B12 (stated)	*44*	-	-	-	-	-	-	-

The feedstuffs were found to have relatively low Co concentrations. Given the considerably higher concentrations of other divalent cations found in these feedstuffs, not all the Co present would be expected to be absorbed through competition for transport protein binding sites. Cobalt concentrations in the injectable supplements were considerably higher than those found in the feedstuffs. Even though much smaller volumes of these are used, bypassing the intestinal tract it would result in higher Co concentrations in the circulation.

There was good correlation between the stated and measured concentrations in all the commercial supplements except for Feratone and Folic B12®. The Co, iron, and copper contents the Feratone were notable different to the stated levels with the Co content less than half that expected. The B12 supplement was found to contain twice the Co concentration calculated for the stated B12 level.

3.6.4 Cobalt excretion

In this study, the fate of Co following IV injection was investigated by measuring urinary and faecal Co excretion. These were considered that main routes of excretion though losses through sweat have been reported (Genuis et al. 2011). It was anticipated that most Co administered via IV injection would remain as free Co ions. These ions would be rapidly excreted in the urine while an undefined quantity of Co would bind to transport proteins and albumin (Bal et al. 2013) to potentially be taken up by the hepatic reticulo-endothelial system for biliary excretion. Once in the bile this Co would be available for elimination in the faeces. Given the large intestinal mass of horses (Figure 3.10), it was expected that the faecal excretion of Co would be highly variable. Some Co could be reabsorbed and enter the blood stream for urinary excretion. Ultimately it would be expected that some IV injected Co would be available to the intestinal microbial flora to facilitate B12 synthesis. Without faecal Co excretion, the inclusion of Co in injectable supplements would be unwarranted given that Co is only considered an essential trace element as a component of B12.

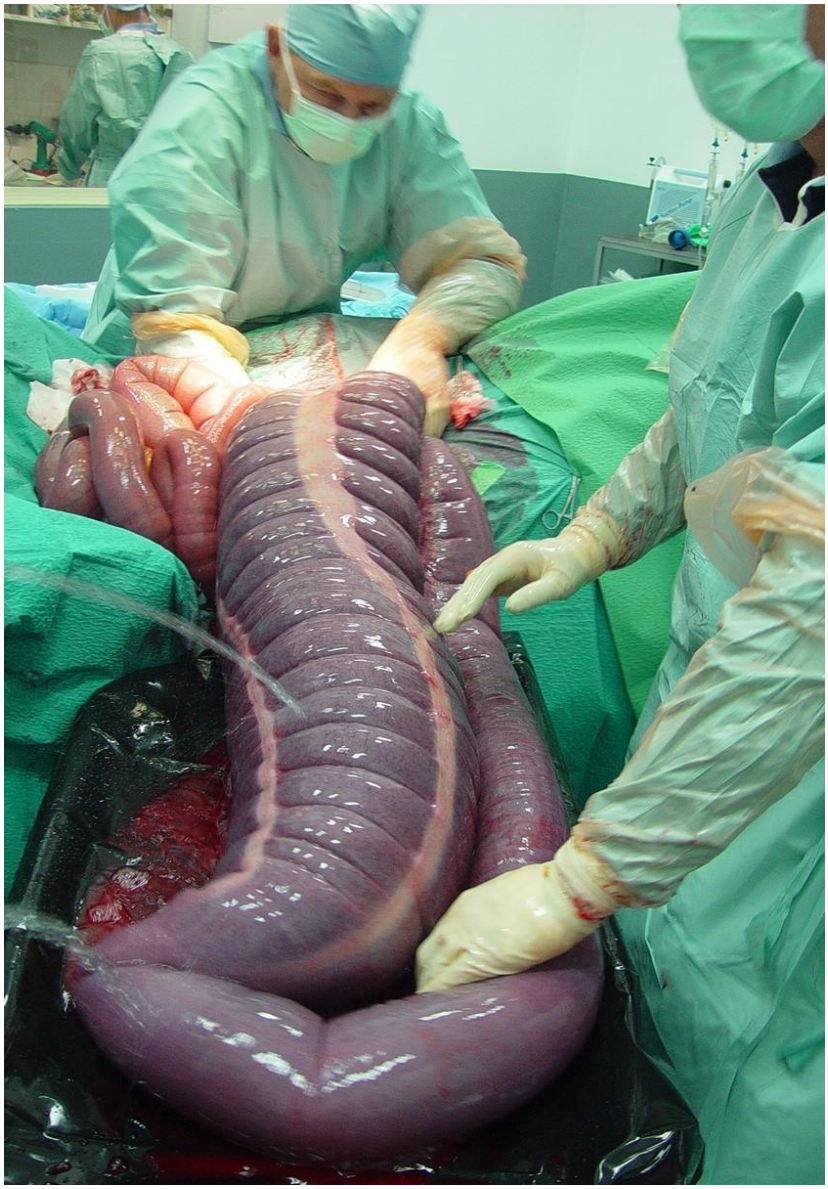

Figure 3.10. Dr Derek Major performing abdominal surgery on a horse at ABEC.

Two horses were used in this study with each horse following the same treatment protocol. Cobalt excretion was investigated after IV injection of the registered supplement VAM® (Ceva Animal Health Pty Ltd, Austinmer, NSW, Australia). In addition to several other nutrients, this supplement contained 150 µg mL^{-1} cyanocobalamin (equivalent to 6.52 mg Co L^{-1}), 240 µg cobalt sulfate mL^{-1} (equivalent to 91.25 mg Co L^{-1}), 27.87 mg copper L^{-1} as copper sulfate and 1501 mg iron L^{-1} as ferric ammonium citrate. Copper and iron were also included in the excretion study to determine if they exhibited a similar excretion pattern to Co. A time 0, the two mares A and B were administered a 40 mL IV

injection of VAM® exposing each mare to a total of 3.91 mg Co, 1.11 mg copper and 60 mg iron. Urine was collected over 24 h with the collection bag emptied every 2-4 h (Infusette® catheter attached to a collection bag tied to a horse coat as shown in Figure 3.3). This urine collection design was not without fault as the collection bag on mare A was dislodged after 4 h so complete urine collection data was only available for mare B.

A pre-treatment manure sample was collected from each horse to establish a baseline concentration of elements attained through normal dietary intake. These samples were prepared for analysis by mixing 1-part manure to 4-parts water i.e. a dilution rate of 5. Measurements from these samples were taken as background manure concentrations and subtracted from all subsequent post-treatment measurements. The tap water used throughout was as delivered through the municipal water supply. Materials used in the collection and processing of manure were assessed for contamination with water tested after the shovel used for manure collection was soaked in 150 L of clean water. Similarly, water was tested from the container used for sample mixing after the mechanical paint stirrer was taken through the mixing process in 150 L clean water. Negligible processing contamination was noted with background contamination concentrations measured in the shovel and container washings typical of the municipal water supply. The pre-treatment manure sample was processed in the same fashion as the post-treatment manure samples. Any sample processing contamination was therefore equally applied to the pre and post administration samples so additional calculations to accommodate background contamination were not necessary. Throughout the study, both mares consumed 20 L water per 24 h. Total weights of manure collected over 84 h were 33.0 kg and 29.5 kg from mares A and B, respectively. Pooled manure samples were suspended in 150 L water each and homogenised with a mechanical paint stirrer (Figure 3.5). Triplicate subsamples were transferred from this mixture to 70 mL samples pots (3.5.5.8).

Sample preparation in the laboratory was by two different techniques to establish both the water-soluble and total manure fractions of Co, copper, and iron. The water-soluble fraction was prepared by centrifugation of manure subsamples at 1250 g rcf for 5 min, leaving a clear supernatant that was suitable for ICP-MS analysis. Total manure element

concentrations were determined by weighing approximately 0.5 g manure into 10 mL tubes (3.5.5.7) and adding 1 mL aqua regia (3.5.5.9). Samples were heated in a dry heating block for 30 min at 90°C before being allowed to cool to room temperature and made to a final volume of 10 mL with water. A limitation of aggressive acid digestion was the potential to include those elements bound to indigestible materials that would not normally be available to the horse through the natural digestive process. These could amount to considerable concentrations in manure as horses naturally consume soil when grazing that passes through the alimentary tract largely undigested. To address this concern, the background manure sample collected prior to VAM® administration was also subjected to the same aggressive acid digest under the assumption that the same proportion of elements would be released. The concentration of elements measured in the post-administration acid digested manure were therefore subtracted from those measured in the acid digested pre-administration manure. Using this approach element bioavailability was superfluous. The differences in element concentration of manure collected pre and post VAM® administration were considered the most accurate means of assessing gastrointestinal tract excretion. Consequently, the acid digested manure fraction was used when calculating excretion totals while the water extractable component of manure provided a proxy for the bioavailable fraction of elements that could be readily reabsorbed with water in the large intestines.

Results of analysis are summarised in Table 3.5. Total urinary excretion of elements from horse A can only be used as an estimate with urine collected from this horse over 4 h rather than the 24 h collection period applied to horse B. Even with the reduced collection time, at least 50 % of the Co administered to horse A and 59 % of the Co administered to horse B had been excreted after 84 h. These results were consistent with rat studies where over 50 % of combined faecal and urinary Co excretion was noted to have occurred 4 days after IV administration (Gregus & Klaassen 1986). When comparing route of excretion, Co was predominantly excreted in urine while copper and iron were primarily excreted in faeces. These findings support the critical roles of copper and iron as essential elements required for normal homeostasis. The copper specific transport protein caeruloplasmin and iron transport protein transferrin ensure these elements are conserved and effectively transported to cells. In contrast, excess Co was readily able to pass through

the proximal tubules of the kidney for urinary excretion. These observations were also supported in rats studies where it was found that faeces was the predominant route of excretion for copper and iron while urine was the predominant route of excretion for Co (Gregus & Klaassen 1986). Similar findings were seen in humans with urinary concentrations of Co higher than those found in bile though Co was noted to be present in bile at considerable concentrations (Ishihara & Matsushiro 1986).

Table 3.5. Excretion of cobalt, copper and iron in urine and manure after administration of 40 mL VAM® (see Appendix 9.4 for calculations and raw data). Excreted totals calculated by adding concentrations measured in acid digested manure and urine.

Horse	**Cobalt (mg)**	**Copper (mg)**	**Iron (mg)**
Concentration administered	*3.91*	*1.11*	*60*
Urine Horse A	1.80	0.07	0.37
Manure Horse A (acid digest)	0.153	1.31	230
Manure Horse A (water soluble)	0.036	0.268	5.60
Horse A total excreted	**1.95**	**1.38**	**230**
Urine Horse B	2.15	0.19	9.16
Manure Horse B (acid digest)	0.152	1.36	252
Manure Horse B (water soluble)	0.026	0.270	4.20
Horse B total excreted	**2.30**	**1.55**	**261**

Urinary excretion patterns for Co, copper and iron were essentially the same at each timed collection point for both horses (Table A4-9.5). One notable exception was iron where in the first 2 h after the VAM® injection providing 60 mg iron, horse A excreted 0.144 mg iron while horse B excreted 6.65 mg iron. This result was taken to indicate that horse B was iron replete with binding sites for iron saturated. The excess iron from the VAM® therefore remained as free ions, passing through the glomerulus and resisting reabsorption via divalent metal transferase 1 in the distal tubules of the nephron (Abouhamed et al. 2006).

The demonstrated excretion of Co in manure supports the inclusion of Co in injectable supplements as a means of facilitating B12 synthesis by intestinal microbial flora. It was however shown to be an inefficient means of delivering Co with a considerable proportion rapidly excreted in urine. Relatively large doses are therefore required with rat studies

showing marked dose dependent increases in bile Co concentrations that were presumed to be due saturation of metal binding sites (Gregus & Klaassen 1986).

Though not investigated here, an interesting addition would be the biliary excretion of Co derived from B12. VAM® contained a mixture of Co and B12, but with only 7 % of the total Co in this supplement bound to B12 the concentration was insufficient to affect the study outcomes. This multi-component supplement was studied because it was considered typical of injectable supplements. A different excretion pattern may have been observed if Co or B12 were administered as individual compounds or in combination with other elements in different proportions. With competitive interaction between metals for binding sites on albumin (Bal et al. 2013) and other plasma proteins the conclusions drawn from this study are limited. Further complexity arises when considering metal transferase affinity, for example divalent metal transferase 1 shows a higher affinity for iron than Co and other metal ions (Illing et al. 2012).

3.6.5 Hemoplex®

This pilot study was designed to examine the excretion pattern of Co in Co naïve and Co supplemented horses. Mares A and B were given IV injections of 40 mL Hemoplex® at time 0 and then 54 and 123 h later. Mare C received no previous parenteral Co supplementation. Mare R was given 10 mL injections of Hemoplex® at time 0 and at 54 h. All mares received 40 mL injections of Hemoplex® at 144 and 312 h. Hemoplex® contained 0.7 mg mL^{-1} cobalt gluconate and 150 µg mL^{-1} B12. Hence, a 40 mL injection of Hemoplex® provided 3.9 mg Co. Blood and urine samples were collected periodically thereafter and results of these analyses are tabulated in Appendix 9.5.

When plotting the results of this study, the creatinine adjusted urinary Co results were preferred over the uncorrected urine Co concentrations to reduce variability associated with changes in hydration status. After each injection, a spike in urinary Co excretion was observed (Figure 3.11). Despite receiving the same pre-treatment, the Hemoplex® injection at 144 h resulted in mare B excreting considerably more Co than mare A. Urine Co levels rapidly returned to near baseline levels after each Hemoplex® injection. Similar

Co excretion patterns were seen for all the mares following the Hemoplex® injection at 312 h.

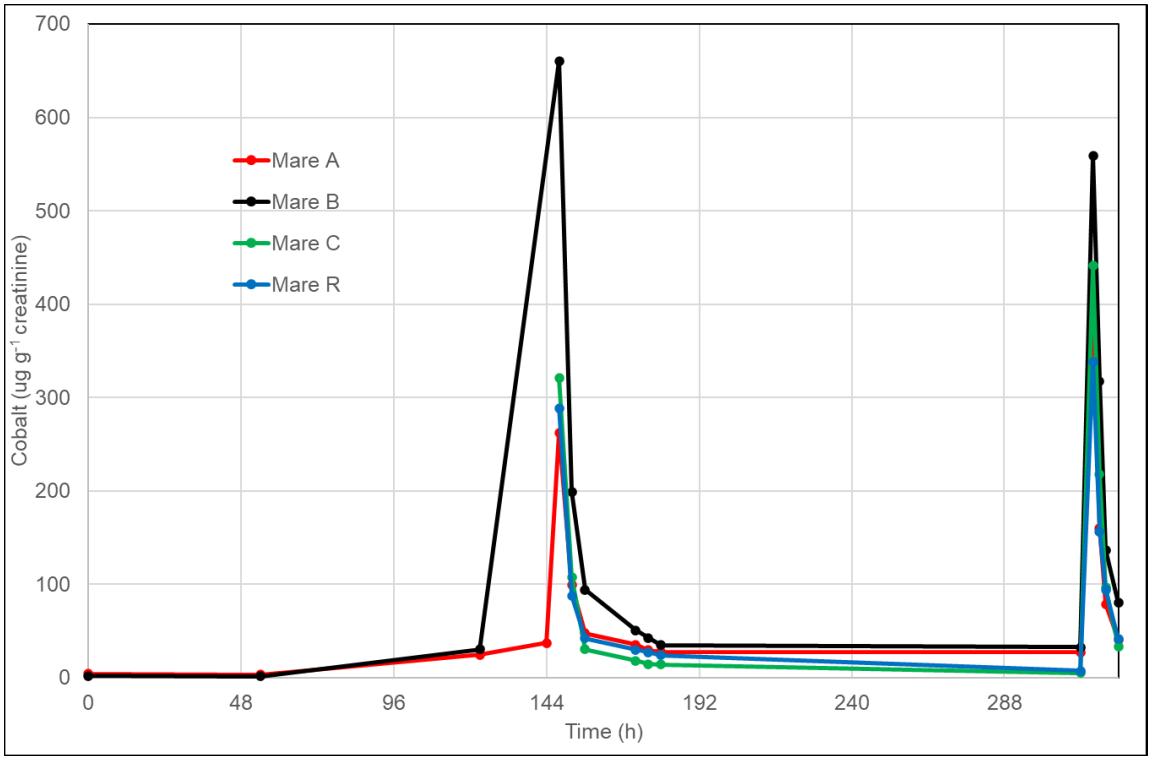

Figure 3.11 Creatinine adjusted urinary Co concentrations following 40 mL injections of Hemoplex® in mares A, B, C and R.

Blood and plasma Co concentrations showed less variability than urinary Co excretion with mares A and B following similar excretion patterns (Figure 3.12). In these samples, Co levels were proportional to the doses given. As the horses receiving the highest initial Co loading, mares A and B had the higher plasma Co concentrations. Mare R, with a lower prior Co exposure had lower Co levels than mares A and B. With no prior Co supplementation, mare C had the lowest overall plasma Co concentrations. Compared to urinary Co levels, the blood and plasma Co levels remained elevated for longer after Hemoplex® injection.

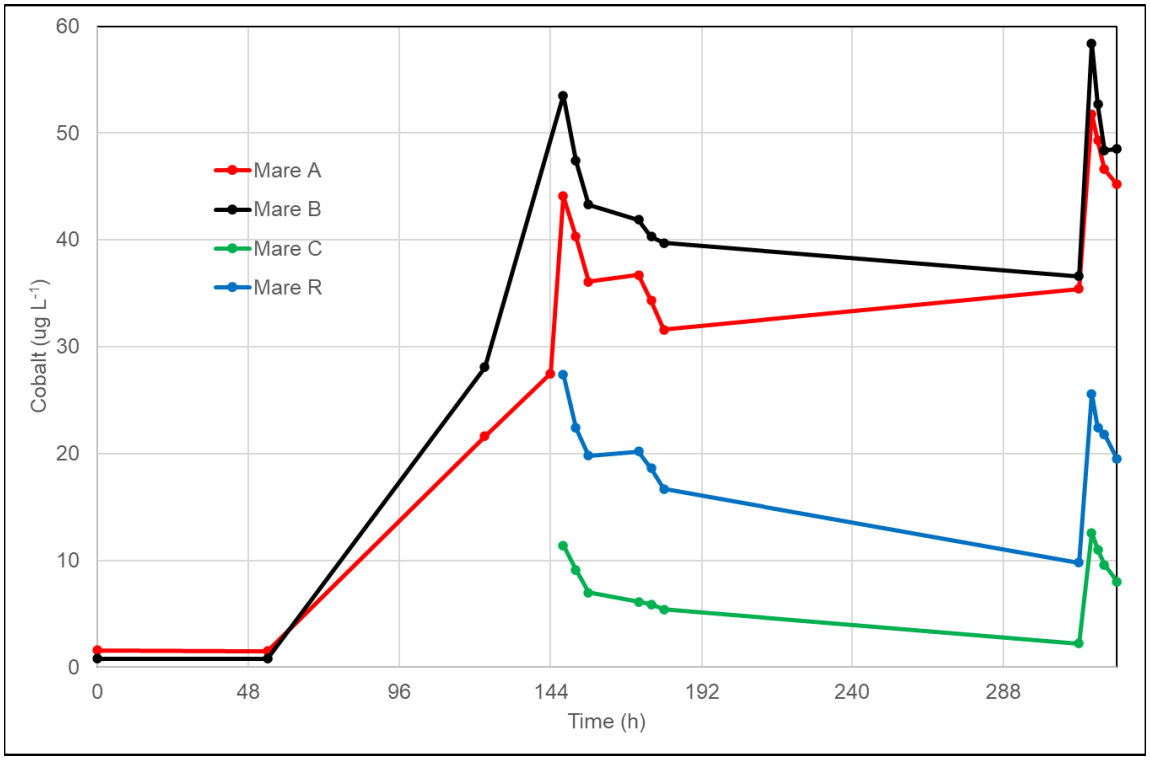

Figure 3.12 Plasma Co concentrations following 40 mL injections of Hemoplex® in mares A, B, C and R.

Urinary Co excretion was too variable to conclude from this limited study that prior Co exposure would result in higher urinary Co excretion or prolongation of urinary Co elimination. However, the markedly elevated urinary Co levels obtained from mare B suggested that this could be the case. Following the Hemoplex® injection at 312 h, relatively higher increases in plasma Co levels were noted and indicate altered Co metabolism following prior Co exposure.

3.6.6 Sample matrix correlation

Regulatory thresholds have been set for both urine and plasma Co levels. The aim of this investigation was to establish if these sample matrices were linearly correlated and therefore equally able to detect cases of Co abuse. Collection points in the pilot studies provided 280 paired urine and blood samples (Appendix 9.5). Urine samples had measurements made for both Co and creatinine, while the blood collections were used for Co measurements on the whole blood and plasma fraction. Plots of the relationship

between these parameters were used to draw conclusions on appropriate approaches to assess Co exposure. Degree of correlation was assessed by linear regression with an $R^2 < 0.2$ indicating poor correlation and an $R^2 > 0.8$ considered adequate correlation.

A plot of urine and plasma Co concentrations shows poor correlation between these sample matrices with $R^2 = 0.12$ (Figure 3.13). Correlation was improved when only plotting points with a urinary Co concentration < 100 µg L^{-1} ($R^2 = 0.60$) but this editing seemed inappropriate given that the regulatory threshold was set for those samples with Co concentrations > 100 µg L^{-1}. A marginal improvement in correlation was observed when plotting creatinine adjusted Co against plasma Co ($R^2 = 0.14$). Plasma and blood Co concentrations were highly correlated $R^2 = 0.99$ (Figure 3.14). This level of correlation suggests that either blood or plasma Co measurements would be suitable for assessing Co status.

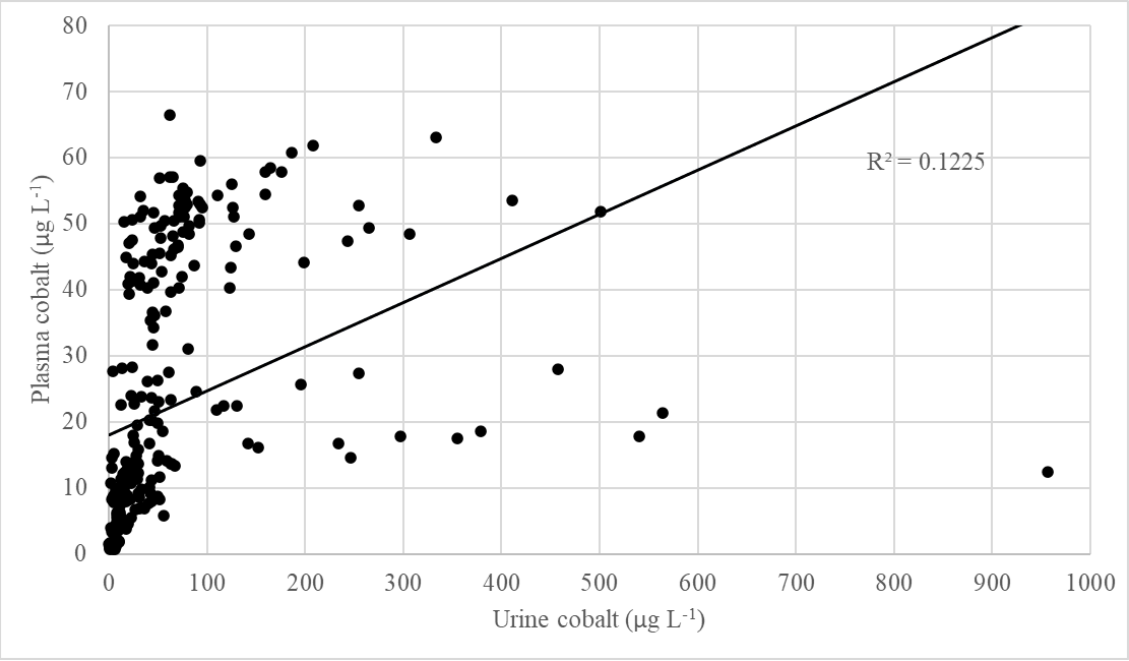

Figure 3.13. Urine and plasma Co concentrations for pilot study samples (n = 280).

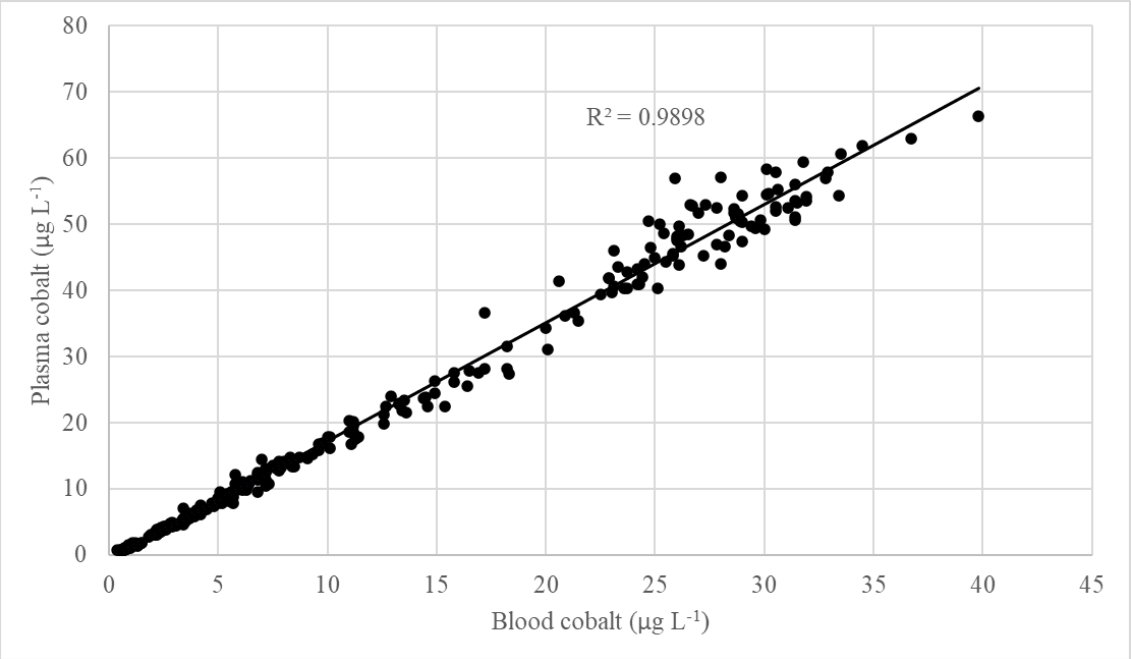

Figure 3.14. Blood and plasma Co concentrations for pilot study samples (n = 280).

From this exercise it was clear that urine and plasma or blood Co levels were not linearly related, especially at Co concentrations above the regulatory threshold. These findings were in contrast to a study on renal clearance of Co from patients with metal-on-metal hip devices (Daniel et al. 2010). In that study, Co was shown to be conserved by the kidneys at low levels with urinary Co concentrations less than the corresponding plasma Co concentrations. As plasma Co concentrations increased following metal-on-metal prosthesis wear, the kidneys were able to concentrate Co in urine so that higher urinary Co levels were observed than in the corresponding plasma samples. An average 2.8-fold increase in renal Co clearance of 3.7 mL min^{-1} was observed for patients with metal-on-metal bearings compared to the control group of patients without a metal-on-metal prosthesis where the renal clearance was 1.3 mL min^{-1}. The efficiency of renal Co excretion was noted to progressively increase at a rate of 9 % for every µg 24 h^{-1} increase in Co release. Renal efficiency was calculated as urine Co concentration (µg L^{-1}) divided by plasma Co concentration (µg L^{-1}). Median renal efficiency was found to be 0.9 (interquartile range, 0.7 to 1.6) in the preoperative control group compared to 3.2 (interquartile range, 1.7 to 5.1) in the patients with metal-on-metal prostheses. The study

demonstrated that the efficiency of renal Co clearance progressively increased with no threshold beyond which the renal capacity for Co ion excretion was overwhelmed.

The same conclusions of progressive increase in Co excretion and not exceeding renal capacity could not be drawn using the samples collected from the pilot study horses. Renal efficiencies ranged from 0.1-77.1 (Appendix 9.5). No correlation was observed between renal efficiency and urine samples with a Co concentration < 80 µg L^{-1} (R^2 = 0.0017). The discrepancy in Co excretion rates between studies probably reflects the nature of Co exposure. Cobalt administered to horses was given as a bolus dose compared to the gradual release of Co associated with metal-on-metal prosthesis wear.

In summary, the lack of correlation between urine and plasma or blood Co levels was due to factors other than variations in hydration status as creatinine adjustment of the urinary Co concentration failed to significantly improve correlation. A previous study conducted on patients with metal-on-metal hip implants was able to demonstrate a relationship between urine and plasma Co concentrations (Daniel et al. 2010). However, these patients were exposed to a gradual release of Co ions with urinary Co measurements performed in timed urine collections in contrast to the pilot study horses where acute Co exposure was measured in spot urine. Spot urine samples did not allow sufficient time for Co levels to equilibrate between urine and plasma and could therefore not be expected to uniformly detect cases of excessive Co administration. The disparity between urine and plasma or blood Co levels was investigated further in Chapters 5 and 6 with the intent of establishing which matrix was the most appropriate for use as a regulatory sample.

3.6.7 Cobalt accumulation

The aim of this experiment was to monitor the accumulation of Co in plasma and blood after repeated administration of injectable Co. Mares A and B were injected with Hemoplex® over 32 days at a rate that was estimated to represent typical high-level Co exposure that could be attained using legitimate Co supplements (Appendix 9.5). Blood was collected immediately prior to injection at the times shown in Table 3.6. Samples assessed were therefore trough levels taken after the previous Co administration.

Table 3.6 Plasma and blood Co concentrations from samples collected immediately prior to IV injection of Hemoplex®.

Date / Time	Hemoplex	Mare A			Mare B		
		Plasma Co	Blood Co	Ratio (plasma /blood)	Plasma Co	Blood Co	Ratio (plasma /blood)
	(mL)	---- (µg L^{-1}) ----			---- (µg L^{-1}) ----		
19/02 8:00	40	1.6	1.2	1.3	0.8	0.6	1.3
21/02 14:00	40	1.5	1.1	1.4	0.8	0.7	1.1
24/02 11:00	40	21.6	13.6	1.6	28.1	18.2	1.5
25/02 8:00	40	27.5	15.8	1.7	31.1	20.1	1.5
4/03 8:00	40	35.4	21.5	1.6	36.6	21.3	1.7
5/03 8:00	40	-	-	-	-	-	-
5/03 17:00	20	60.7	33.5	1.8	66.4	39.8	1.7
9/03 9:00	40	50.5	28.9	1.7	54.3	33.4	1.6
10/03 6:00	40	-	-	-	-	-	-
12/03 8:00	40	41.8	22.9	1.8	40.9	24.2	1.7
18/03 18:00	40	47.5	26.0	1.8	49.7	26.1	1.9
19/03 18:00	40	39.4	22.5	1.8	40.7	23.1	1.8
22/03 21:00	40	-	-	-	-	-	-
23/03 18:00	-	41.0	24.3	1.7	47.9	26.0	1.8
7/04 8:00	-	22.5	12.7	1.8	27.6	16.9	1.6

Both mares had similar Co concentrations at the various sampling points (Figure 3.15). Significant Co accumulation was apparent with Co levels rising rapidly to a peak of 66 µg L^{-1} in plasma and 40 µg L^{-1} in blood. Cobalt administration was stopped on 23rd March and a gradual decline in Co concentration followed. At the last measurement point, 15 days after the cessation of Co administration, Co concentrations in blood and plasma remained markedly greater than the initial baseline levels.

Cobalt has been found to bind tightly to haemoglobin inside red blood cells (Simonsen et al. 2011) and therefore presents a potentially significant site of Co accumulation. Evidence of intracellular Co binding was investigated by calculating the ratio of Co concentration in plasma to whole blood at each collection point (Figure 3.15). Once injected, Co will initially appear in the plasma fraction of whole blood but can then distribute throughout the body, including the intracellular region of red blood cells. Plasma Co levels will be higher than those measured in whole blood following this acute Co exposure. Over time, the proportion of Co in plasma is expected to decrease as

intracellular transport mechanisms shift Co inside red blood cells (Simonsen, Harbak & Bennekou 2011) until eventually the Co concentration of whole blood exceeds that of plasma. The net movement of Co from plasma to red cells would be demonstrated by decreasing plasma to whole blood Co ratios.

In normal equine whole blood, red blood cells are the main cellular component occupying from 34 to 46% of the blood volume, white blood cells and platelets occupy an insignificant proportion constituting less than 2% of the whole blood volume, while the remaining portion is the plasma fraction (Southwood 2013). From these haematological parameters it can be calculated that, within normal biological variability, the ratio of plasma to whole blood ranges from 1.2 (54% / 46%) to 1.9 (66% / 34%). Accordingly, in a normal horse, the Co ratio in plasma to whole blood can range from 1.2 to 1.9 while Co remains in the plasma fraction. To conclude that Co was accumulating in red blood cells, a ratio of less than 1.2 would be required. As none of the measurement points met this criteria, there was no evidence that, over the duration of this pilot study, Co had accumulated in red blood cells.

This pilot study raised questions around factors that could influence rates of Co accumulation such as varying Co dose and frequency of Co administration that were investigated further in Chapter 5. It also highlighted the need to measure haematocrit to calculate red cell Co concentrations rather than relying on plasma to whole blood Co ratios. Normal biological variation in blood haematocrit made ratio measurements insensitive to changes in Co partitioning between plasma and red cells.

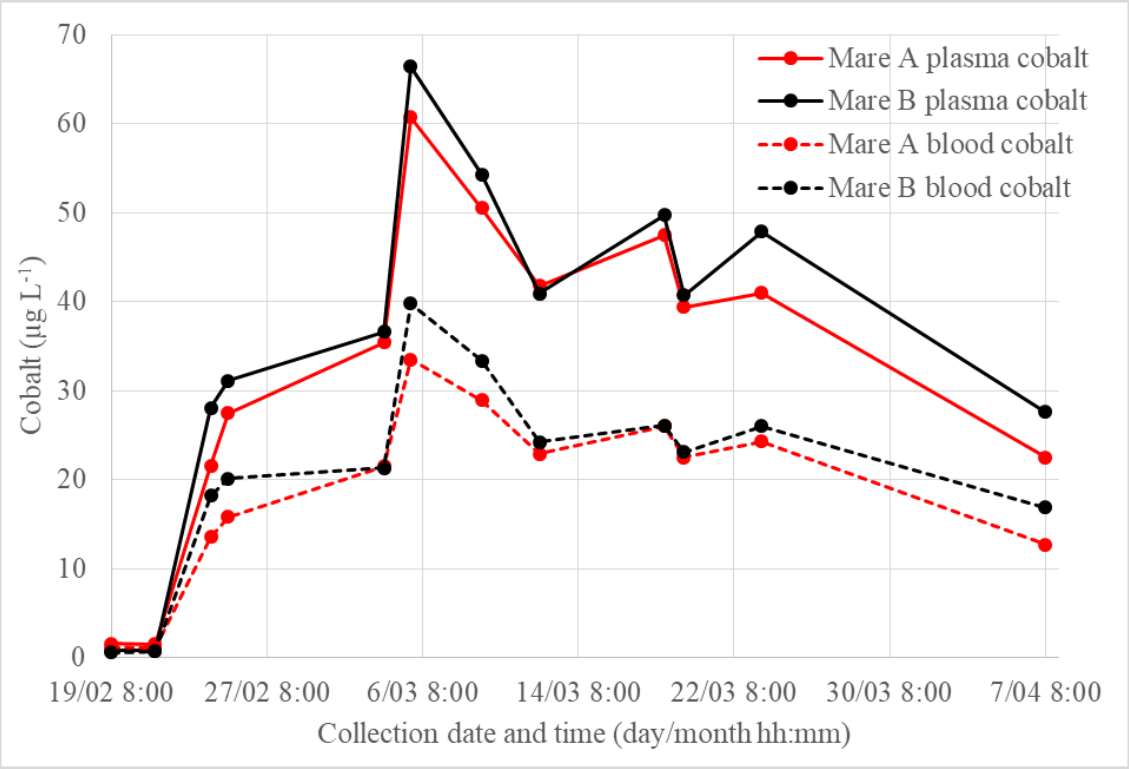

Figure 3.15 Trough plasma and blood Co concentrations from samples collected prior to IV injection of Hemoplex®. Cobalt administration ceased 23rd March.

3.6.8 Exercise

This study was undertaken to investigate the effect of exercise on Co concentrations in urine and blood. A mare in training for harness racing (mare Q), was used in this study. The breeding mares that had been employed for many of the other administration trials were not suitable for track work. A disadvantage of using a race fit horse was that some samples could not be collected to avoid stressing a horse that was not used to the intensive sampling that the breeding mares were accustomed too. Cobalt was administrated to mare Q with the IV injection of 10 mL Hemoplex® at the times shown in Table 3.7. Urine, plasma and blood Co concentrations were measured before and after exercise. Mare Q was moderately exercised through hobbled track work at 1630 19/8, 1400 22/8 and 1400 25/8. Urine pH measurements were made to determine if pH was a factor influencing urinary Co excretion.

Table 3.7 Urine, plasma and blood Co concentrations and urine pH measurements on samples collected from mare Q after IV administration of 10 mL Hemoplex® on the dates shown. Time denotes the duration elapsed since Hemoplex® administration. Exercise was through hobbled track work at 1630 19/8, 1400 22/8 and 1400 25/8. Results for samples collected on exercise days are shown in bold type. NR = No result where sample was unable to be collected from an uncooperative mare.

Date	Time	Urine cobalt	Urine creatinine	Urine cobalt	pH	Plasma cobalt	Blood cobalt
	(h:min)	(µg L^{-1})	(g L^{-1})	(µg g^{-1} Cr.)		---- (µg L^{-1}) ----	
13/08 14:00	-	10.6	1.59	6.6	-	7.0	5.4
13/08 14:00	0:00	IV injection of Hemoplex®					
13/08 17:00	3:00	1713.6	2.04	841.6	-	20.5	15.6
13/08 19:00	5:00	938.1	2.36	396.8	-	14.7	8.8
13/08 18:00	0:00	IV injection of Hemoplex®					
14/08 08:00	14:00	NR	NR	NR	-	11.4	9.4
14/08 12:00	18:00	37.5	2.43	15.4	-	9.9	5.7
14/08 17:00	23:00	26.7	1.87	14.3	-	9.9	5.6
14/08 18:00	0:00	IV injection of Hemoplex®					
15/08 08:00	14:00	28.3	1.39	20.3	-	10.6	7.1
15/08 12:00	18:00	36.2	1.89	19.2	-	10.2	6.4
15/08 17:00	23:00	21.6	1.62	13.4	-	9.6	5.8
15/08 18:00	0:00	IV injection of Hemoplex®					
16/08 08:00	14:00	17.2	0.68	25.3	-	11.6	7.9
16/08 19:00	25:00	19.5	1.38	14.1	-	11.1	7.0
16/08 19:00	0:00	IV injection of Hemoplex®					
17/08 08:00	13:00	23.7	0.62	38.1	-	11.8	7.8
17/08 15:00	20:00	19.9	0.81	24.4	-	11.9	7.7
17/08 19:00	24:00	16.1	0.96	16.7	-	12.5	7.1
17/08 18:00	0:00	IV injection of Hemoplex®					
18/08 09:00	15:00	39.9	1.31	30.4	7.94	12.2	8.5
18/08 14:00	20:00	6.5	0.29	22.1	7.23	11.8	7.7
18/08 17:00	23:00	NR	NR	NR	NR	12.5	7.5
18/08 18:00	0:00	IV injection of Hemoplex®					
19/08 10:30	**16:30**	**11.3**	**0.42**	**27.0**	**8.12**	**12.6**	**7.4**
19/08 14:00	**20:00**	**21.9**	**1.09**	**20.2**	**8.02**	**12.1**	**8.4**
19/08 17:00	**23:00**	**30.5**	**1.55**	**19.7**	**7.18**	**12.2**	**7.8**
19/08 18:00	0:00	IV injection of Hemoplex®					
20/08 10:00	16:00	35.9	1.06	33.8	7.53	12.6	8.7
20/08 15:40	21:40	27.6	1.27	21.8	7.40	12.8	7.6
20/08 18:00	0:00	IV injection of Hemoplex®					
21/08 09:30	15:30	29.1	1.03	28.3	7.69	12.2	8.9
21/08 13:00	19:00	21.7	0.97	22.3	7.64	12.8	7.8
21/08 17:00	23:00	18.6	1.00	18.7	7.49	11.9	8.4
21/08 18:00	0:00	IV injection of Hemoplex®					

22/08 08:00	**14:00**	**9.3**	**0.31**	**30.4**	**8.02**	**14.1**	**9.0**
22/08 15:00	**21:00**	**15.9**	**0.78**	**20.4**	**7.11**	**14.3**	**8.4**
22/08 17:00	**23:00**	**NR**	**NR**	**NR**	**NR**	**NR**	**NR**
22/08 18:00	0:00	IV injection of Hemoplex®					
23/08 09:00	15:00	26.4	1.36	19.4	8.14	12.2	8.5
23/08 14:00	20:00	16.5	0.95	17.4	7.37	12.3	7.9
23/08 17:00	23:00	18.1	1.13	16.0	7.60	11.7	7.2
23/08 18:00	0:00	IV injection of Hemoplex®					
24/08 08:00	14:00	20.5	0.94	21.8	7.86	12.8	7.9
24/08 13:00	19:00	20.3	1.17	17.4	7.84	12.7	8.0
24/08 17:00	23:00	15.1	0.89	16.9	7.76	12.9	8.0
24/08 18:00	0:00	IV injection of Hemoplex®					
25/08 08:00	**14:00**	**48.0**	**1.39**	**34.5**	**8.34**	**13.3**	**9.2**
25/08 15:00	**21:00**	**30.6**	**1.50**	**20.3**	**7.97**	**13.1**	**8.8**
25/08 17:00	**23:00**	**38.2**	**1.96**	**19.5**	**7.40**	**12.4**	**8.3**

From this study it was noted that plasma and blood Co levels remained relatively constant. No conclusions could be drawn from urine pH with pH changes differing by less than 1 pH unit. In the unexercised horse, urine Co levels generally decreased over time while the urinary Co concentration generally increased following exercise. These trends could be explained by changes in the water content of urine following exercise. In order to produce sweat to cool the body after exercise, the horse retained water for sweating and consequently created a more concentrated urine. The higher urinary creatinine concentrations following exercise provide evidence of increasing urine concentration. When adjusting urine Co for creatinine, all post Co administration collection points demonstrate decreasing urinary Co excretion. The effect of even moderate exercise on urine Co concentration was significant. The need for correcting for varying degrees of hydration has been discussed further in Chapter 6.

3.6.9 Cyanocobalamin

This pilot study was conducted to examine the excretion pattern of B12 relative to inorganic Co administered as a Co salt. Three mares were selected (R, E and F) with mare R having been pre-treated with Co injections over the preceding 2 weeks. Mares R and F received 1 mg Co as B12 from a 23 mL injection of a 1 mg mL^{-1} solution of Nature Vet Vitamin B12 while mare E received 1 mg Co as cobalt gluconate from a 10 mL injection of Hemoplex®. Urine and blood samples were collected immediately before the inorganic

Co or B12 injections at time 0 then at 2, 4, 6, 9 and 12 h post injection (Appendix 9.5). Results of analysis are summarised in Table 3.8.

Table 3.8 Urine and blood Co concentrations after IV administration of 1 mg Co as cobalt gluconate (mare E) and 1 mg Co as B12 (mares F and R).

Time	Mare	Sample	Urine cobalt	Creatinine	Urine cobalt	Plasma cobalt	Blood cobalt
(h)	-	-	($\mu g\ L^{-1}$)	($g\ L^{-1}$)	($\mu g\ g^{-1}$ Cr.)	($\mu g\ L^{-1}$)	($\mu g\ L^{-1}$)
0	E	23/3/6/E	2.5	0.2	11.6	8.3	5.1
2	E	23/3/8/E	142.1	0.4	330.6	16.7	9.7
4	E	23/3/10/E	64.4	0.7	98.2	13.7	7.8
6	E	23/3/12/E	30.2	0.6	52.3	12.2	6.8
9	E	23/3/15/E	17.9	0.6	28.8	10.6	6.4
12	E	23/3/18/E	12.0	0.6	21.2	10.2	5.9
0	F	23/3/6/F	7.7	0.6	12.4	5.4	3.4
2	F	23/3/8/F	956.4	1.8	535.1	12.4	7.2
4	F	23/3/10/F	52.1	0.7	73.1	8.3	5.2
6	F	23/3/12/F	43.7	0.9	47.7	7.9	4.7
9	F	23/3/15/F	36.4	1.4	26.2	6.8	4.0
12	F	23/3/18/F	10.1	0.6	17.9	6.2	4.2
0	R	23/3/6/R	22.6	0.9	24.4	24.0	12.9
2	R	23/3/8/R	457.4	1.1	421.2	27.9	16.5
4	R	23/3/10/R	88.7	0.8	117.0	24.5	14.9
6	R	23/3/12/R	63.4	0.8	76.8	23.4	13.5
9	R	23/3/15/R	50.2	1.2	42.3	23.0	13.3
12	R	23/3/18/R	25.8	0.8	31.2	22.7	13.3

No difference in excretion rate was evident for Co when administered as a Co salt or as a component of B12 (Figure 3.16). Plasma and blood Co levels remained relatively constant while a sharp increase in urinary Co excretion was noted after 2 h with a decline to baseline levels after 12 h. Similar B12 excretion patterns were seen between the mare previously exposed to Co (mare R) and the mare with no prior Co exposure (mare F). With a marked increase in urinary Co concentration following B12 administration, it was clear that analytical methods were required to differentiate between B12 and inorganic Co in urine. The research presented in Chapter 4 addresses this need and confirms that urinary B12 excretion occurs without Co being released from the B12 molecule.

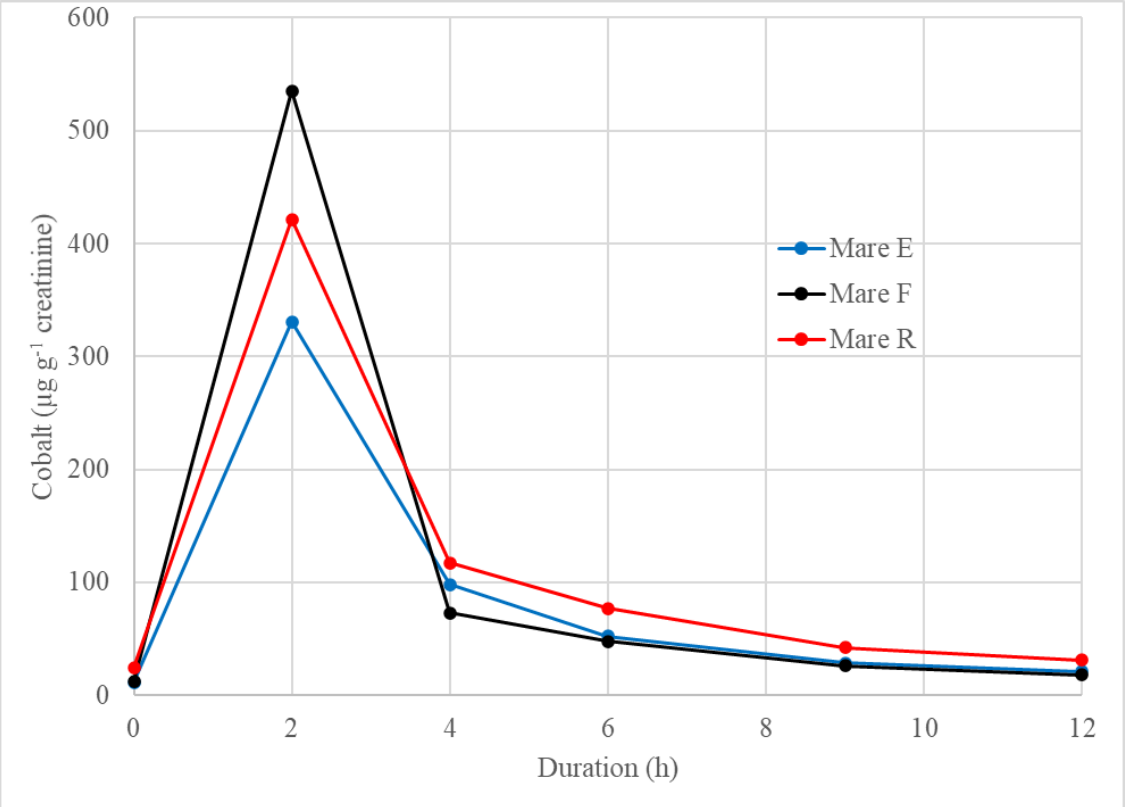

Figure 3.16 Creatinine adjusted urinary Co concentrations following the injection at time 0 of 1 mg Co as cobalt gluconate to mare E and 1 mg Co as B12 to mares F and R.

3.6.10 Pharmacokinetic study

Initial studies to define the rate of elimination of Co were based on a single IV injection of 49 mg Co as $CoCl_2$ to 16 exercised Thoroughbred horses (Knych et al. 2014). The Co doses used were considerably greater than those achieved when using registered supplements at the recommended rate. The aim of this pilot study was to investigate rates of Co excretion from horses administered a Co containing supplement at a realistic dose. Replicating the sampling rates of Knych et al. 2015, blood samples were collected prior to the start of the cobalt injection at time 0 then at 5, 10, 15, 30, 45 min, and 1, 2, 3, 4, 5, 6, 8, 12, 18, 24, 36, 48 h after the Co injection. Horses C and D were control horses while horses M and N were harness racing horses previously found to have breached the Co threshold. All horses received 10 mL Hemoplex® (1 mg Co as cobalt gluconate) at time zero. Results of analysis are shown in Table 3.9.

Table 3.9 Results of pharmacokinetic study following the IV injection of 10 mL Hemoplex®. Horses C and D were control horses while horses M and N were harness racing horses previously found to have breached the Co threshold.

Collection	Horse C	Horse D	Horse M	Horse N
(min)	Plasma cobalt concentration (µg L^{-1})			
0	4.4	2.7	4.5	2.7
5	20.2	15.8	14.3	18.2
10	17.0	13.8	13.8	17.5
15	13.9	12.8	13.5	16.2
30	12.5	11.5	13.2	10.6
45	11.0	10.1	12.0	10.2
60	10.5	9.5	11.1	9.7
120	9.2	7.6	9.7	8.1
180	8.0	6.9	9.1	7.8
240	7.4	6.3	8.8	7.5
300	7.5	5.8	8.0	6.8
360	7.2	6.0	8.3	6.9
480	7.5	5.6	7.8	7.0
720	6.8	4.6	6.9	6.6
1080	5.4	4.4	6.4	5.4
1440	5.8	3.9	6.3	5.6
2160	5.0	4.1	6.1	5.0
2880	4.7	3.9	5.7	4.4

Different rates of Co excretion were observed for the 4 horses (Figure 3.17). This difference was not linked to either the race fit horses M and N or the relatively sedentary breeding horses C and D. Natural biological variability was attributed to the varying rates of Co metabolism. This variability highlights the need to examine a large population of appropriately treated horses to ensure a regulatory threshold incorporate individual rates of Co metabolism.

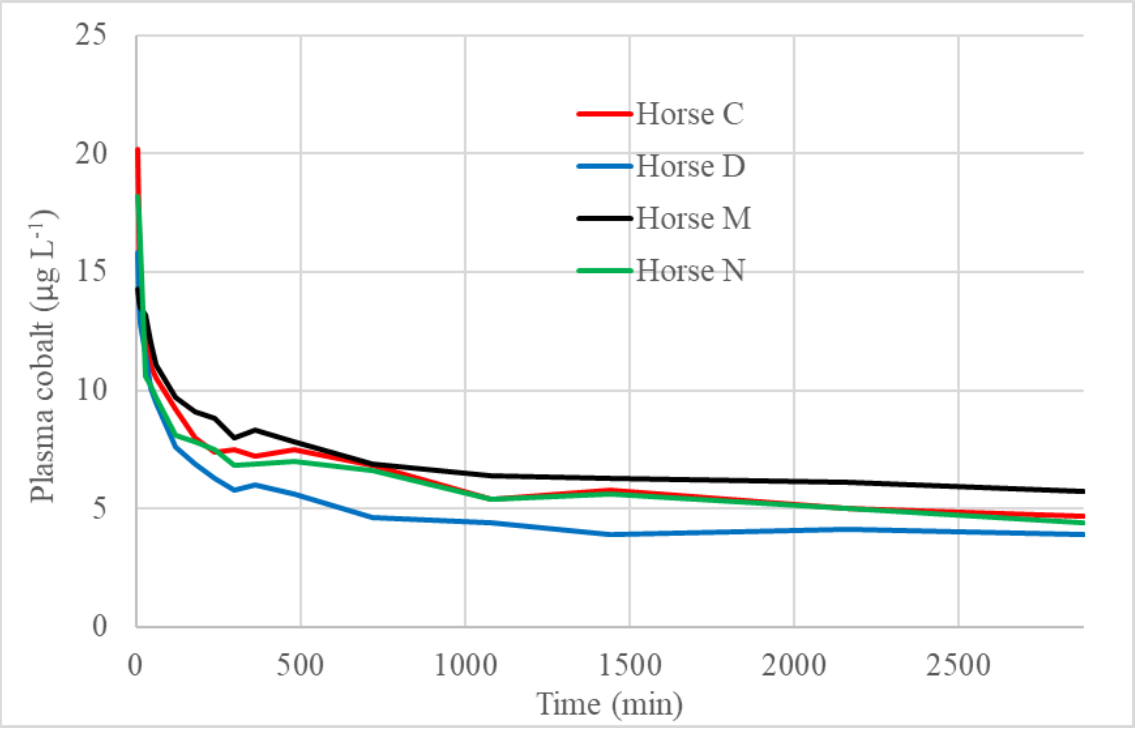

Figure 3.17. Results from pharmacokinetic study following the IV injection of 10 mL Hemoplex®. Horses C and D were control horses while horses M and N were harness racing horses previously found to have breached the Co threshold.

3.6.11 Cobalt displacement

The aim of this study was to investigate the possibility that competitive binding by an alternative divalent cation could result in increased urinary Co excretion. Results of analysis are in Appendix 9.6. A spike in urinary Co excretion (Figure 3.18) was observed 2 h after Pre-Ferrin administration. Before concluding that the Co was displaced by iron in the Pre-Ferrin, I measured both the Co and iron concentrations in this supplement. The Pre-Ferrin (Batch No. B15070) was noted to be an iron only supplement containing 22 g L^{-1} iron. ICP-MS analysis confirmed the iron concentration with a measurement of 21.2 g L^{-1}. However, this supplement was also found to contain 2410 µg L^{-1} Co. A 10 mL injection of Pre-Ferrin would have therefore provided 24 µg Co for urinary excretion, sufficient Co to explain the increased urinary Co output 2 h after Pre-Ferrin injection. Creatinine adjusted urinary Co concentrations 2 h after Pre-Ferrin injection for horses A, C, E and G were 2.6, 3.8, 3.6 and 7.0 µg Co g^{-1} creatinine, respectively. The highest

urinary Co output was from the horse that had previously received the highest single weekly doses of Co. As this horse had the most Co available for displacement, this finding could indicate that Co displacement had occurred. If this was not the case, then all horses would be expected to excrete the same amount of Co. Alternatively, this result may have occurred because less binding sites were available for Co in the horse with the highest Co loading. The additional Co supplied in the Pre-Ferrin was therefore more readily excreted.

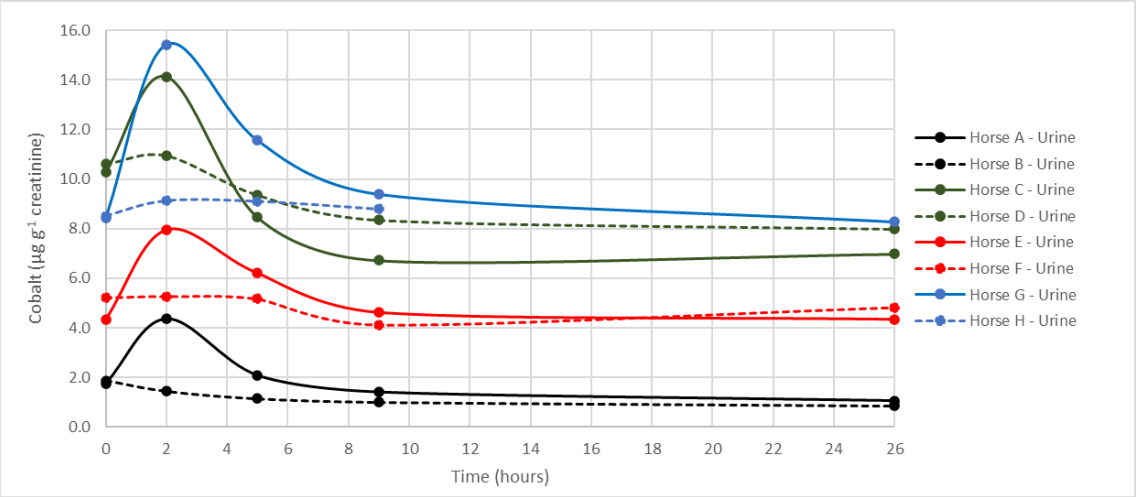

Figure 3.18 Urinary Co concentrations following the IV administration of 10 mL Pre-Ferrin.

Iron administration resulted in minimal variations in plasma and blood Co concentrations (Figures 3.19 and 3.20). It was however interesting to note the different plasma and blood Co concentrations that horses C and D had attained after 7 weeks Co treatment compared to the similar Co concentrations obtained by the other paired treatment groups of horses E and F and horses G and H. This difference points to biological variability in Co metabolism in these horses.

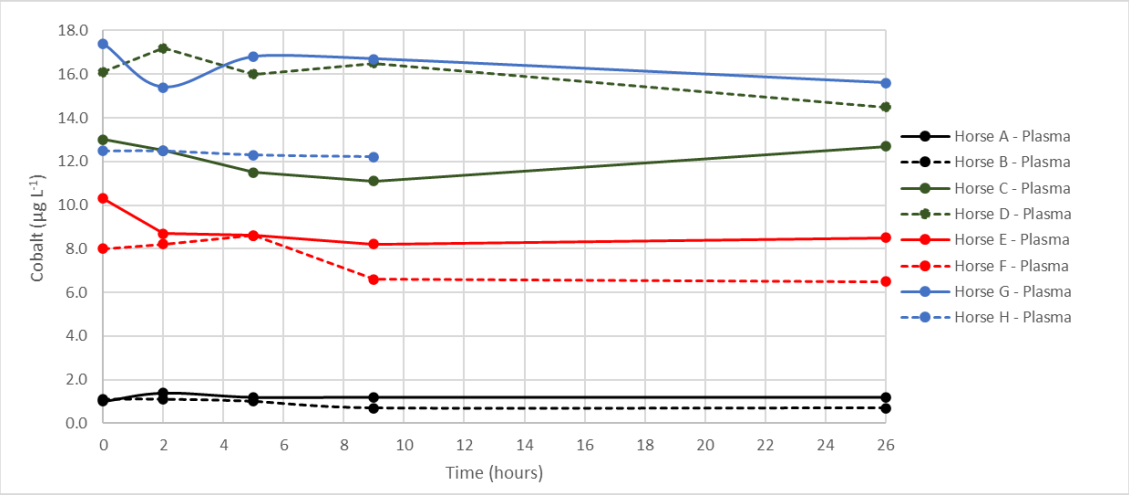

Figure 3.19 Plasma Co concentrations following the IV administration of 10 mL Pre-Ferrin.

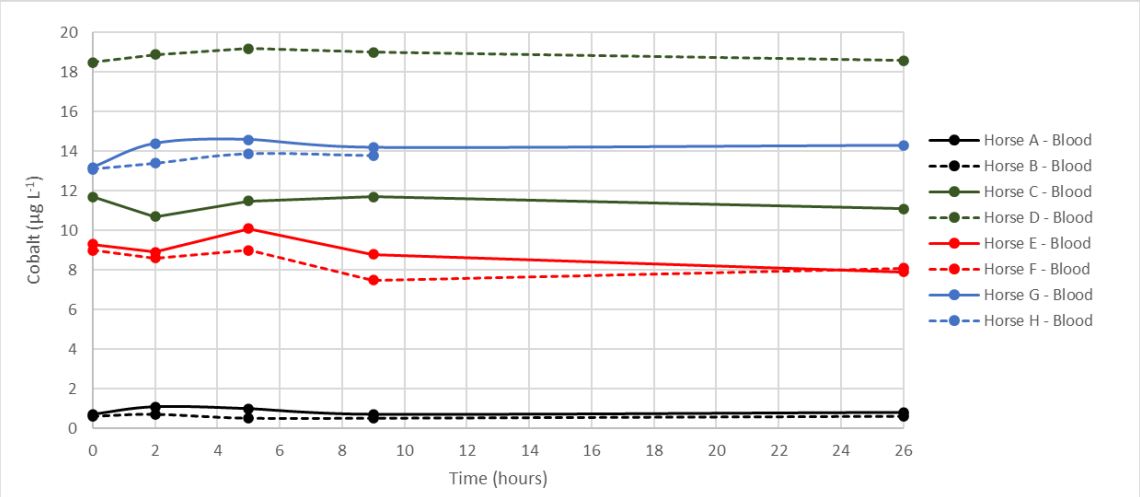

Figure 3.20 Blood Co concentrations following the IV administration of 10 mL Pre-Ferrin.

With Co contamination in the supplement, it was not possible to conclude from this study that Co displacement would result in increased urinary Co excretion. Not investigated in this pilot study was the effect on urinary Co excretion from chelating agents or other cations that may have a higher affinity for protein binding than Co. The fact that significant quantities of Co were found in an iron supplement highlights the challenges faced by trainers in their attempts to maintain optimum nutrient supply without breaching the regulatory Co threshold.

3.6.12 Intramuscular administration

Supplements may be administered by IV or IM injection, though in horse racing, IV injections are generally preferred to avoid muscle discomfort. Occasionally IM injections are required or may be given accidentally when a vein has been missed. The aim of this experiment was to investigate the rate of urinary Co excretion following an IM injection of a Co containing supplement. Tripart was the supplement used for this study as it was specifically formulated for IM or subcutaneous injections. It contained 500 µg mL^{-1} B12, equivalent to 21.7 µg mL^{-1} Co. Administered at the manufacturer's recommended rate of 5 mL per 100 kg body weight, the 25 mL IM injection of Tripart given to the test mare provided 0.54 mg Co as B12. Urine and blood samples were collected at time 0 then at 2, 4, 6, 8 and 24 h post injection. The results from the analysis of these samples are presented in Table 3.10.

Table 3.10 Results of urine and blood analysis following the IM injection of 25 mL Tripart.

Collection time	Urine cobalt	Urine creatinine	Urine cobalt	Plasma cobalt	Blood cobalt
(h)	(µg L^{-1})	(g L^{-1})	(µg g^{-1} creatinine)	---- (µg L^{-1}) ----	
0	7.1	2.49	2.9	1.8	1.1
2	78.4	1.84	42.5	3.2	2.0
4	100.9	1.04	97.0	3.6	2.2
6	117.3	1.54	76.2	3.3	1.9
8	132.3	1.88	70.5	2.9	1.7
24	6.8	1.67	4.1	2.1	1.1

Minimal change was observed in plasma and blood Co concentrations. Urinary Co concentrations were shown to increase markedly before returning to baseline levels after 24 h (Figure 3.21). The uncorrected urine Co levels increased across the 2, 4, 6 and 8 h collections points while the creatinine adjusted urine Co levels gradually increase to 4 h before declining. As shown in the previous pilot studies, creatinine adjustment improves result reliability. The rise and fall of Co levels in the creatinine adjusted urine Co measurements matched that of the plasma Co. Peak creatinine adjusted urinary Co excretion occurred at 4 h following IM Co injection. This excretion peak occurred 2 h later than that observed following IV Co administration (Figures 3.16 and 3.18). Delayed

urinary excretion of Co was to be expected with Co required to pass from the muscle to the circulatory system before being available for renal filtration.

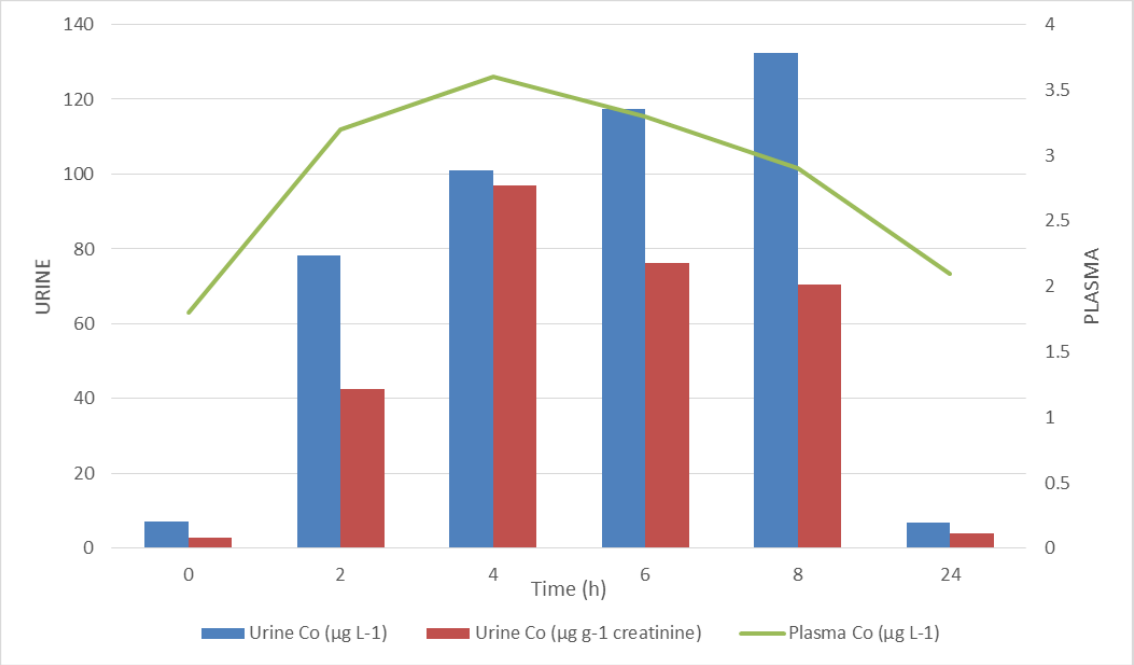

Figure 3.21 Urine and plasma Co concentrations following the IM injection of 25 mL Tripart.

3.6.13 Oral administration

The effect of orally administered Co on urine and blood Co concentrations was investigated. Initially the horse "*Boston*" was administered 25 mg Co as $CoCl_2$ in aqueous solution in the morning feed. Samples were collected at time 0 then at 1, 2 and 4 h post ingestion. This experiment demonstrated that 25 mg Co ingestion resulted in a significant increase in urinary Co excretion. The study was then refined to assess peak urinary Co excretion by extending the collection duration to 12 h with blood and urine collection points at time 0 then at 1, 2, 4, 6, 9 and 12 h post ingestion. The influence of dose concentration on Co absorption and excretion was also investigated with the horse designated "*Mare I*" receiving 10 mg Co as $CoCl_2$ and the horse designated "*Mare J*" receiving 25 mg Co as $CoCl_2$. The results of these experiments are shown in Table 3.11 and Figure 3.22.

Table 3.11 Results of analysis following the administration of 10 mg Co as $CoCl_2$ to *Mare I* and 25 mg Co as $CoCl_2$ to horses *Boston* and *Mare J*. Collection time was the duration elapsed since Co ingestion at time 0. Urine Co concentrations are the mean result of measurements performed in triplicate except for Mare J at the 2 h collection point where the result was the average of duplicate measurements due to insufficient sample volume. Insuff. = Insufficient sample collected for this determination. NT = Not tested as slight haemolysis due to delayed separation of plasma from red cells may have compromised result accuracy.

Collection time	Horse	Urine cobalt	Urine creatinine	Urine cobalt	Plasma cobalt	Blood cobalt
(h)		(µg L^{-1})	(g L^{-1})	(µg g^{-1} creatinine)	----(µg L^{-1})----	
0	Boston	0.6	0.6	1.0	1.3	0.9
1	Boston	3.2	2.4	1.3	1.1	0.7
2	Boston	20.5	2.5	8.1	1.3	0.8
4	Boston	82.1	1.9	44.3	1.7	0.9
0	Mare I	2.4	0.8	2.8	NT	0.9
2	Mare I	9.4	2.4	3.9	NT	0.9
4	Mare I	7.0	1.8	4.0	NT	0.9
6	Mare I	9.5	2.3	4.4	NT	0.9
9	Mare I	14.5	3.2	4.5	NT	2.0
12	Mare I	13.3	2.5	5.3	NT	1.2
0	Mare J	5.3	1.8	3.1	NT	1.2
2	Mare J	91.2	Insuff.	-	NT	2.5
4	Mare J	216.0	2.4	88.4	NT	2.6
6	Mare J	115.2	2.3	50.5	NT	2.4
9	Mare J	82.5	3.0	27.9	NT	1.0
12	Mare J	56.6	3.0	18.7	NT	2.2

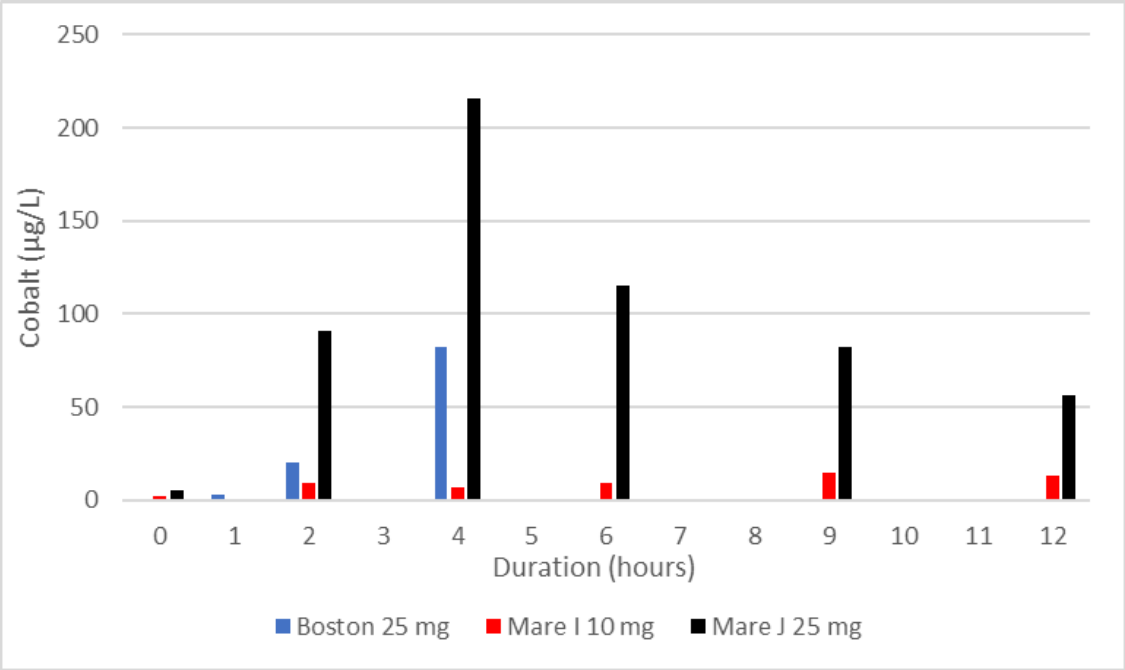

Figure 3.22 Urinary cobalt concentrations measured after the ingestion of 10 mg Co as cobalt chloride (*Mare I*) and 25 mg Co as cobalt chloride (*Boston* and *Mare J*).

When compared to the regulatory plasma threshold of 25 µg L^{-1}, no appreciable changes in plasma or blood Co concentrations were noted for these horses (Table 3.11). Markedly higher blood Co concentrations would be expected before haemopoietic benefit could be obtained. For example, a study was conducted on patients orally administered 25 mg Co as $CoCl_2$ over 4 weeks in aqueous solution twice daily with the dose increased to 50 mg Co for a further 4 weeks (Bowie & Hurley 1975). After 8 weeks, it was concluded that there was a useful rise in haematocrit in most patients with toxic symptoms noted to be negligible. These patients had also been dialysed for 6 h three times a week with the pre-dialysis serum Co concentrations measured. After 1 week, pre-dialysis serum Co concentrations ranged from 0–940 µg L^{-1} and 220–2100 µg L^{-1} after 8 weeks.

The results plotted in Figure 3.22 demonstrate the unpredictable nature of urinary Co excretion following Co ingestion. Horses on the same Co dose showed a 2-fold difference in urinary Co concentration 4 h after $CoCl_2$ ingestion. As expected, lower urinary Co excretion was observed following the ingestion of 10 mg Co when compared to the horses given 25 mg Co. This reduced Co excretion was however not proportionally less. Though

fed 2.5 times less Co, 4 h after ingestion this horse was excreting more than 10 times less Co. This pilot study was not expanded further as too many variables were considered necessary to address. With an immense digestive tract (Figure 3.10), many factors could be envisaged to influence Co absorption within one horse, let alone the variability that would need to be considered across several horses. In additional to the standard variables such as age and gender, factors affecting rates of oral absorption of Co would need to be investigated. These would mainly revolve around Co bioavailability where diets high in phytates would be expected to bind Co making it less available. In contrast the acidic pH of feeds derived for silage stocks could increase the bioavailability of Co.

It was evident from this pilot study that the ingestion of a single meal containing Co could lead to a horse exceeding the urinary Co threshold. This opens the possibility to inadvertently exceeding the threshold following the consumption of apparently innocuous substances. Minimal changes in blood Co concentration were observed for *Mare J* while urinary Co output exceeded the threshold after 4 h and could have done so over the full 12 h depending on hydration status. When used as a therapy for anaemia, chronic oral administration of high doses of Co with concomitant elevations in plasma were required before erythropoietic benefit was attained. Increased red blood production has not been reported following Co ingestion from a single meal containing sufficient Co to cause a transient spike in urinary Co excretion. Regulatory reliance should be place upon plasma Co levels to avoid the prospect of false doping allegations.

3.6.14 Cobalt salt

Cobalt salts such cobalt chloride, cobalt sulfate and cobalt gluconate have been used in the preparation of Co containing supplements. As these salts readily dissociate in aqueous solutions to provide free Co ions it would be expected that they distribute in urine and blood at the same rate. To test this hypothesis 1.0 mg Co as cobalt chloride was administered to *Mare E* as a 10 mL IV injection of the registered supplement VAM® and 1.0 mg Co as cobalt sulfate was administered as a 10 mL IV injection to *Mare F*. The cobalt sulfate was prepared as a stock solution by dissolving ≥ 99 % pure cobalt (II) sulfate heptahydrate ($CoCl_2.7H_2O$) (Sigma-Aldrich, Castle Hill, NSW, Australia) in sterile water for injection (Troy Laboratories, Glendenning, NSW, Australia). Prior to

injection, this stock solution was further diluted to 10 µg L^{-1} to provide an injection volume of 10 mL. The final dilution was passed through a 0.45 µm filter (Merck Millipore, Bayswater, VIC, Australia) to ensure bacterial sterility.

Previous Co exposure was standardised by treating these horses with 50 mg Co as $CoCl_2$ weekly for 6 weeks 30 days before the administration trial. The elevated blood Co concentrations at time 0 confirm this. Blood and urine samples were collected at time 0 then at 2, 4, 9 and 25 h post injection. Results of analysis are shown in Table 3.12. The type of Co salt injected had no notable influence on urine Co concentration (Figure 3.23) or blood cobalt concentration (Figure 3.24). This outcome was as expected from salts that are highly soluble in aqueous solution.

Table 3.12 Results of analysis following the administration of 1.0 mg Co as $CoCl_2$ to *Mare E* and 1.0 mg Co as $CoSO_4$ to *Mare F*. Collection time was the duration elapsed since Co ingestion at time 0. Change from baseline was calculated by subtracting Co concentration at time 0 from subsequent Co measurements.

Collection time	Horse	Urine cobalt	Urine creatinine	Urine cobalt	Blood cobalt	Change from baseline
(hours)		(µg L^{-1})	(g L^{-1})	(µg g^{-1} creatinine)	---- (µg L^{-1}) ----	
0	Mare E	13.0	3.1	4.2	8.8	
2	Mare E	697.7	3.9	180.3	12.0	3.2
4	Mare E	229.4	3.0	76.0	11.4	2.6
9	Mare E	65.4	2.7	24.2	10.7	1.9
25	Mare E	21.2	2.7	7.8	9.3	0.5
0	Mare F	9.4	2.0	4.7	7.3	
2	Mare F	670.1	2.8	242.8	11.9	4.6
4	Mare F	199.3	2.8	71.3	10.1	2.8
9	Mare F	47.8	1.5	32.0	9.9	2.6
25	Mare F	15.7	1.6	9.6	8.9	1.6

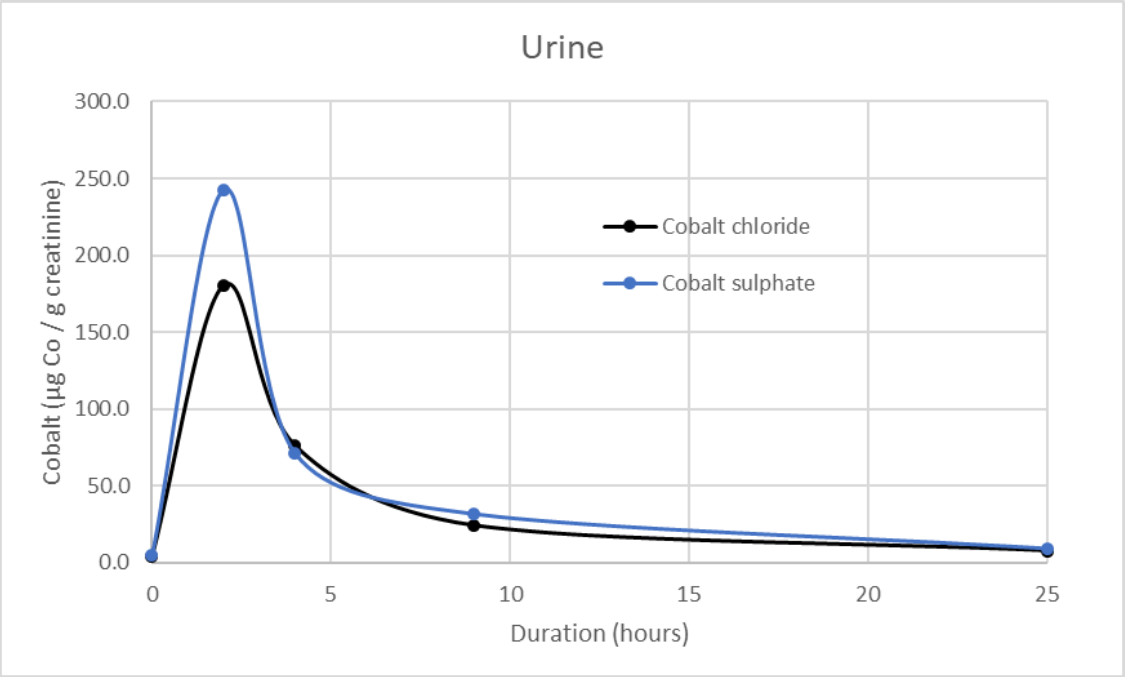

Figure 3.23 Urinary Co concentrations following the administration of 1.0 mg Co as $CoCl_2$ to *Mare E* and 1.0 mg Co as $CoSO_4$ to *Mare F*.

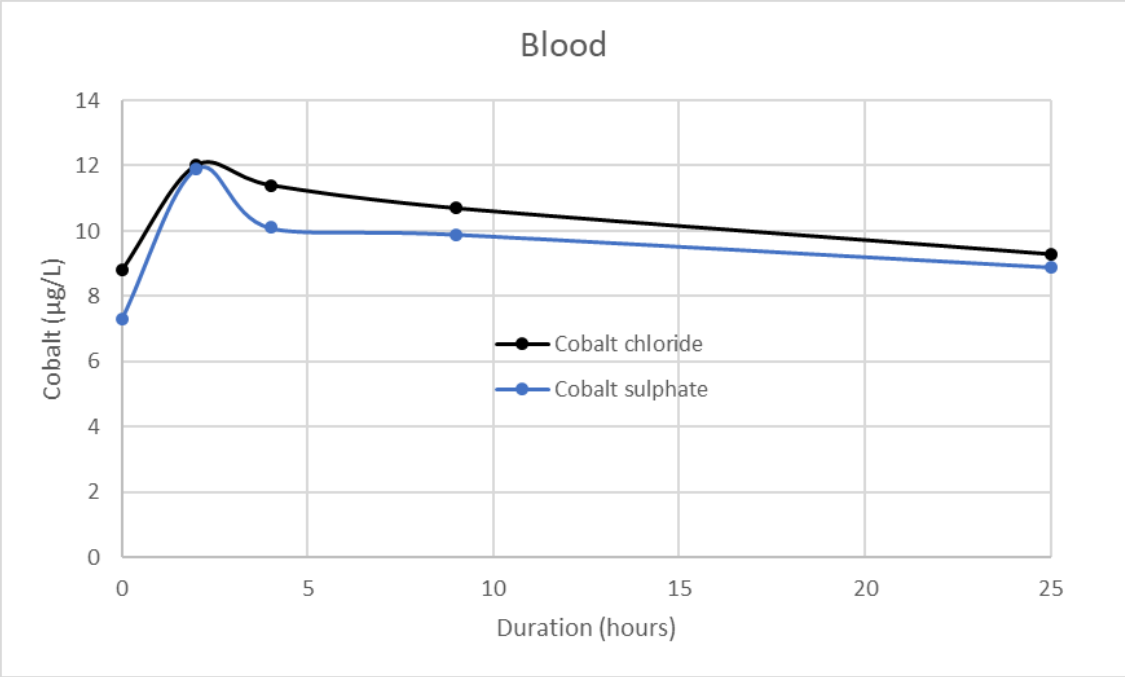

Figure 3.24 Urinary Co concentrations following the administration of 1.0 mg Co as $CoCl_2$ to *Mare E* and 1.0 mg Co as $CoSO_4$ to *Mare F*.

3.6.15 Comparison of creatinine and specific gravity

As urinary creatinine and specific gravity determinations are both used to assess hydration status, a strong correlation between these measurements would be expected. A moderate degree of correlation (R^2 = 0.72) was found when applying a logarithmic trend line (Figure 3.25) to creatinine and specific gravity measurements made on the pilot study urine samples (Appendix 9.5). Further investigation into factors preventing a higher degree of correlation between these measures of hydration was warranted and have been addressed in Chapter 6.

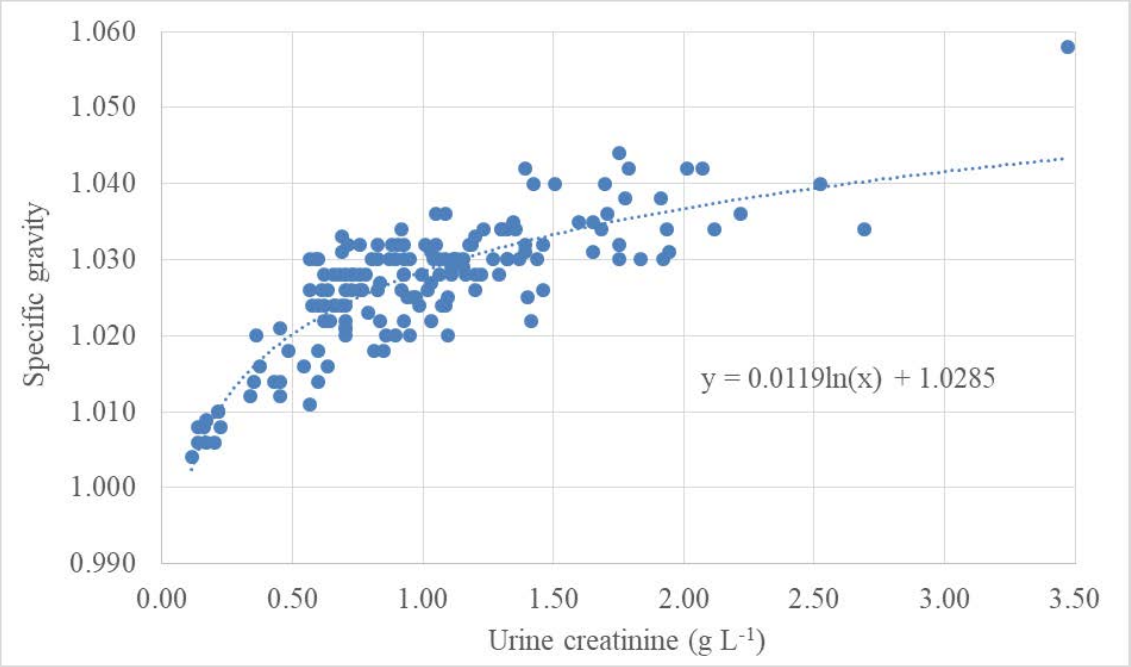

Figure 3.25 Correlation between creatinine and specific gravity determined in 162 urine samples from the pilot study (Appendix 9.5). Specific gravity (y) = 0.0119 ln creatinine (x) + 1.0285; R^2 = 0.72.

3.7 Study Limitations

As the main objective of these pilot studies was to replicate events that can influence urinary Co excretion, the following limitations in study design are noted: -

- The study sampled and treated horses over a limited period so assessment of the effects of long-term administration could not be undertaken.

- The horses were composed, in the main, of paddock-resting, young, Standardbred mares. Although some treadmill and lunging exercises were offered on occasion, the horses were not subjected to the stress of racing or sustained exertion.
- The small sample size of generally 6 mares could not completely replicate a racing population.
- The horses were not transported or subjected to the racing environment.
- The investigations were conducted under a limited range of climatic conditions.
- A limited number of feedstuffs, supplements and grazing conditions were studied.
- pH has been shown to alter the distribution of ionisable drugs rendering the relationship between plasma and urine concentrations imprecise (Toutain 2010). The urine pH measured in pilot study (3.6.8) showed too little variation to assess the effect of urine pH on Co excretion (Wood 1988). To create the conditions necessary to alter urine pH would require changes to the ethics approval granted for the pilot studies. Given the lack of correlation between urine and plasma already demonstrated in these pilot studies, modifications to the ethics approval appeared redundant so were not pursued further.
- The roles of chelators and other divalent cations on distal tubule homeostasis (Barbier et al. 2005) with respect to urinary Co retention and excretion were not investigated.

3.8 Conclusion

These pilot studies have shown that: -

- Cobalt concentrations can be accurately determined in a variety of sample matrices by ICP-MS from samples prepared in either an alkaline or acidic media.
- The urinary Co threshold of 100 µg L^{-1} was regularly exceeded by routine supplementation for up to 8 h after administration.
- Cobalt accumulated in plasma and blood when chronically administered.
- The urine level of Co post-administration was affected by exercise.
- There was marked variability in Co excretion between individuals after administration of identical doses of Co at identical times.
- There was no correlation ($R^2 = 0.12$) between blood and urine Co levels.

- A wide range of creatinine concentrations were found in urine samples that could be used to correct for variations in hydration status.
- The value of the Co displacement study was lessened by Co contamination in the iron supplement. A higher urinary Co excretion in the horse that was previously on the highest weekly dose of Co nonetheless suggested that Co can be displaced from plasma proteins by another divalent cation then excreted in urine.
- Urine Co excretion was delayed when given as an IM injection compared to an IV injection.
- When Co was administered orally, no correlation between urine and blood Co levels were observed. Following Co ingestion, urinary Co concentrations were markedly elevated while blood Co concentrations were essentially unchanged.
- Urine and blood Co concentrations responded similarly following the IV injection of cobalt chloride or cobalt sulfate.

4. DEVELOPMENT OF A METHOD TO DISTINGUISH ORGANIC FROM INORGANIC COBALT

4.1 Summary

To function effectively as a quantitative analytical technique, ICP-MS requires an ion source of singly charged positive ions of the elements to be measured. This is achieved by introducing the sample solution into a high temperature, electron dense, argon plasma where molecules are broken down into their component elements. That is, no information is provided on the chemical form of an element or molecule structure. ICP-MS results are therefore the total concentration of a given element in solution.

Regulating authorities in the racing industry have restricted the administration of potentially performance enhancing Co salts to horses. There are severe penalties for trainers presenting horses with elevated urine Co concentrations, and compliance is ensured via measurement of total urinary Co at thresholds of 100 μg L^{-1}. When Co is present as part of the cobalamin molecule it is not considered performance enhancing. This paper demonstrates that a significant proportion of a commercially available vitamin B12 injection can be excreted intact by a horse. Given the serious consequences from performance enhancing drug offences, we therefore conclude that presumptive Co positives identified by urine total Co measurements require further analysis to differentiate inorganic Co from that present as a constituent of vitamin B12. A liquid chromatography - inductively coupled plasma - mass spectrometry (LC-ICP-MS) method is presented to separate urinary Co into inorganic and organic species.

4.2 Introduction

It has been postulated that Co may enhance athletic performance by indirectly upregulating the synthesis of erythropoietin (EPO) (Lippi, Franchini & Guidi 2005). Since 2015, Co has been listed as a banned substance for athletes by the World Anti-Doping Authority (WADA) (World Anti-Doping Agency 2015b). As yet, threshold values have not been established for athletes (Thevis et al. 2016). The International Federation of Horseracing Authorities (IFHA) have set a threshold level for total Co in equine urine of 100 μg L^{-1} (International Federation of Horseracing Authorities 2017).

Free cobalt ions are required to influence EPO production (Jelkmann 2012); consequently, naturally occurring Co containing molecules such as vitamin B12, may be present and contribute to the total measured concentration of Co without improving athletic performance (Lukaski 2004). Accordingly, a supplementary note released by WADA in reference to Section S.2.1 of the WADA 2014 Prohibited List stated – 'Note that cyanocobalamin (vitamin B12) is not prohibited' (World Anti-Doping Agency 2015a).

I present an adaptation of a technique for measuring cyanocobalamin in urine using liquid chromatography coupled to ICP-MS (Kesava Raju et al. 2013). This method is used to investigate appropriate means to monitor Co exposure. The efficacy of LC-ICP-MS for isolating and quantitatively determining cobalamin is demonstrated in equine urine.

The objective in this Chapter was to develop a reference method to determine vitamin B12 concentration in urine and apply this method to horse urine to answer the questions: a) could B12 be used as a performance enhancing drug if metabolised to provide free Co ions; and, b) could typical courses of B12 supplementation significantly increase the measured concentration of Co in urine.

4.3 Materials and methods

4.3.1 Background

This study was undertaken to discover whether treatment with injectable vitamin B12 solutions could result in breaches of the IFHA total urinary Co threshold of 100 $\mu g\ L^{-1}$. Using minimal animal experimentation in accordance with the ARRIVE guidelines, an initial feasibility study was conducted on a single horse. Definitive findings on this horse made it unnecessary to expand our study with data from additional horses.

4.3.2 Animal

The study was performed on a physically healthy, disease free, 8-year-old Standardbred mare selected from a herd of mares maintained at the Agnes Banks Equine Clinic (ABEC) (Agnes Banks, NSW, Australia). These mares are used for breeding procedures during the season and remain in the herd for the coming season. The horse did not receive any medication, or vitamin and mineral supplements, for at least one month prior to

cyanocobalamin administration. Food and water were available *ad libitum* throughout the duration of the study. Feed stuffs given to this horse were analysed by ICP-MS to ensure they contained low levels of total Co (less than 0.2 µg Co g^{-1} feed stuff). A baseline total Co measurement immediately prior to B12 injection showed this horse's urine to contain of 3.9 µg Co g^{-1} creatinine. The horse was kept in a holding yard at ABEC. The study was approved by the Animal Care and Ethics Committee of the Secretary NSW Trade & Investment (Appendix 9.1) and carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines.

4.3.3 Cobalt administration

A 1 mg mL^{-1} injectable solution of cyanocobalamin was purchased as Nature Vet Vitamin B12 Injection (100 mL) from Ceva Animal Health Pty Ltd (Glenorie, NSW, Australia). Injection was via a 21-guage needle and 10 mL syringe (Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia). The needle was inserted aseptically into the external jugular vein with a total of 10 mL cyanocobalamin solution injected as a bolus dispensed in less than 10 s. This procedure was conducted by a veterinarian at ABEC.

4.3.4 Sample collection

Urine samples were collected at 0, 2, 4, 6, 8 and 12 h after the cyanocobalamin injection. A mare infusion catheter (Infusette™) was inserted into the horse's bladder at each of the times noted to enable urine to be collected into a 50 mL polypropylene sample container (Thermo-Plas, Adelaide, SA, Australia). Urine samples were protected from light and kept refrigerated until analysis, which occurred within one week of collection.

4.3.5 Determination of creatinine concentration

Each urine sample was normalised for hydration against creatinine (Boeniger, Lowry & Rosenberg 1993; Sauve et al. 2015; WADA Laboratory Expert Group 2018; Warrack et al. 2009). 4 mL aliquots of the primary urine sample were transferred into 5 mL screw cap polypropylene tubes (Thermo-Plas, Adelaide, SA, Australia). These samples were centrifuged at 1250 g rcf for 5 min at room temperature to remove any particulates. Creatinine measurements were performed in the NSW Health Pathology Clinical Biochemistry Laboratory, Royal North Shore Hospital, St Leonards, NSW, Australia using the Jaffe reaction method (Abbott Laboratories 2006) on an Architect c9000 System

(Abbott Laboratories, Abbott Park, IL, USA).

4.3.6 Determination of total cobalt concentrations by ICP-MS

Measurements were performed using the facilities and instrumentation described in Section 3.5.3. ICP-MS analysis was conducted with a Varian 820MS, equipped with a patented interference management system known as a Collision Reaction Interface (CRI). Unlike conventional cell technologies, in CRI a collision or reaction gas is injected directly into the plasma through an orifice at the tip of the skimmer cone. By enabling collisions and chemical reactions in a region where the plasma density is high, a clean plasma expansion is created so that an interference free ion beam can be generated (Salazar et al. 2011). The spectroscopic interferences for Co from the sample matrix and plasma, predominantly $^{43}Ca^{16}O^{+}$, $^{42}Ca^{16}O^{1}H^{+}$, $^{24}Mg^{35}Cl^{+}$, $^{36}Ar^{23}Na^{+}$, $^{40}Ar^{18}O^{1}H^{+}$ are effectively reduced using the CRI to a point where they make no significant contribution to the cobalt determinations. The ICP-MS conditions are shown in Table 4.1 with mass calibration and tuning performed daily, prior to the analysis of samples. The method for Co determination was accredited to ISO 15189:2012 (International Organization for Standardization 2012) as certified by the National Association of Testing Authorities, Australia / Royal College of Pathologists of Australasia, laboratory accreditation number 1981. External quality assurance for urinary Co was through enrolment in the Quebec Multi-Element Quality Assurance Scheme (Institut national de santé publique) and the RCPA QAP Trace Elements Program (Royal College of Pathologists of Australasia Quality Assurance Programs Pty Limited).

A sample diluent of 0.75 % v/v nitric acid, 0.01 % w/v Triton X-100 and 0.01 % w/v EDTA was prepared using high purity 70 % nitric acid, ethylenediamine tetra-acetic acid (EDTA) and Triton X-100 that were obtained from Sigma Aldrich (Castle Hill, NSW, Australia). 99.999 % pure helium obtained from BOC (North Ryde, NSW, Australia) was used as the CRI gas. Calibration standards at 0.00, 1.13, 5.63, 28.2, 141, 352 and 880 µg L^{-1} were prepared by dilution of a multi-element standard containing 100 mg L^{-1} Co that was purchased from Australian Chemical Reagents (Moorooka, QLD, Australia). A 1000 mg L^{-1} gallium standard also purchased from Australian Chemical Reagents (Moorooka, QLD, Australia) was added to the sample diluent as an internal standard at a concentration of 50 µg L^{-1}. The reagent water (≥ 18.2 MΩ.cm resistivity) used for

dilution, reagent preparation and consumables washing was prepared in-house using a MilliQ Gradient (Merck Millipore, Bayswater, VIC, Australia). All plastic ware, including disposable pipette tips, were soaked in 2 % v/v nitric acid for a minimum of 24 h then thoroughly washed with reagent water immediately prior to use.

Table 4.1. ICP-MS operating parameters for measuring total urine Co concentrations and as a detector for HPLC to measure cobalt in cyanocobalamin.

Parameter	*Setting (Total Co)*	*Setting (HPLC-ICP-MS)*
Mass / Dwell	59, 69 / 10 ms	59 / 1000 ms
Scan mode	Peak hopping	Peak hopping
Spacing	Fine	Coarse
Points per peak	1	1
Scans/Replicate	25	1
Replicates/Sample	7	-
Spray chamber	Glass - double pass	Glass - double pass
Spray chamber cooling	3 C°	3 C°
Devices	Use autosampler	Galaxie controls run
CRI skimmer gas source	Helium	Hydrogen
CRI skimmer gas flow	150 mL min^{-1}	50 mL min^{-1}
Sampler gas source	OFF	OFF
Isotope	Co59, Ga69	Co59
Plasma flow	18.0 L min^{-1}	18.0 L min^{-1}
Auxiliary flow	1.65 L min^{-1}	1.65 L min^{-1}
Sheath gas	0.43 L min^{-1}	0.43 L min^{-1}
Nebuliser flow	0.93 L min^{-1}	0.93 L min^{-1}
Nebuliser	0.4 mL min^{-1} MicroMist EzyFit & EzyLok	0.4 mL min^{-1} MicroMist EzyFit & EzyLok
Sampling depth	7.0 mm	7.0 mm
RF Power	1.30 kW	1.40 kW
Pump rate	2 rpm	10 rpm
Stabilisation	20 s delay	6 s delay
First extraction lens	-23 V	-23 V

Second extraction lens	-168 V	-168 V
Third extraction lens	- 260 V	- 260 V
Corner lens	- 260 V	- 260 V
Mirror lens left	38 V	38 V
Mirror lens right	16 V	16 V
Mirror lens bottom	28 V	28 V
Entrance lens	0 V	0 V
Fringe bias	-4.1 V	-4.1 V
Entrance plate	-33 V	-33 V
Pole bias	0.0 V	0.0 V
Acquisition mode	Steady-state	Time resolved, sampling time 900.00 s
Sample uptake delay	45 s	0 s
Rinse time	10 s	-

Samples were diluted for analysis in 5 mL screw cap polypropylene tubes (Thermo-Plas, Adelaide, SA, Australia). A 1 mL aliquot of sample diluent was added to each sample tube followed by a 50 µL aliquot of calibrator, control or urine sample that had been allowed to reach room temperature and mixed thoroughly. Sample blanks with a 50 µL aliquot of reagent water were also included. Two levels of control urine samples were analysed, Seronorm Trace Elements Urine L1, Lot 1011644, Exp 2018 and Seronorm Trace Elements Urine L2, Lot 1011645, Exp 2018-01 (SERO, Billingstad, Norway). All solutions were vortex mixed before being loaded onto the autosampler and analysed in a single batch. Urine cobalt concentrations were interpolated from the calibration curve and corrected for signal drift using the gallium internal standard.

4.3.7 Determination of B12 by Chemiluminescent Microparticle Intrinsic Factor Assay

Vitamin B12 measurements were performed in the NSW Health Pathology Clinical Biochemistry Laboratory, Royal North Shore Hospital, St Leonards, NSW, Australia using the Abbott Architect B12 assay (Abbott Laboratories 2018) on an Architect c9000 System (Abbott Laboratories, Abbott Park, IL, USA). 4 mL aliquots of the primary urine sample were transferred into 5 mL screw cap polypropylene tubes (Thermo-Plas, Adelaide, SA, Australia). These samples were centrifuged at 1250 g for 5 min at room

temperature to remove any particulate material. Any samples outside the calibration range of the assay were serial diluted ten-fold in isotonic saline (Baxter, Australia) until sufficiently dilute for the assay.

4.3.8 Determination of B12 by HPLC-ICP-MS

Chromatography was performed on a Varian HPLC system that consisted of a Varian Prostar 410 autosampler with a Varian Prostar 230 inert ternary pump (Varian, Mulgrave, VIC, Australia). Detection was by ICP-MS using a Varian 820MS running the ICP-MS Expert instrument software Version v2.1 b107 (Varian, Mulgrave, VIC, Australia). The ICP-MS operating conditions are shown in Table 1 with mass calibration and tuning performed daily prior to the analysis of samples. Galaxie Chromatography Data System software (Version 1.9.3.2) was used to control the HPLC and ICP-MS instrumentation.

The analytical HPLC column used was a Hamilton PRP-1 Reversed Phase (150 x 4.1 mm; 5 µm particle size) (Hamilton Company, Reno, NV, USA) with corresponding PRP-1 guard column also supplied by Hamilton Company. The metallic components of the sample introduction pathway were kept to a minimum with the HPLC autosampler configured with a PEEK Rheodyne injector valve and a 50 µL PEEK sample loop. The HPLC operating conditions are shown in Table 4.2.

Table 4.2. Instrument parameters: Varian Prostar HPLC system.

Parameter	*Setting*
Injection volume	50 µL
Flow	0.5 mL min^{-1}
Equilibration time	0.1 min
Run time	15.0 min
Flush volume	30 µL
Wash after injection	750 µL
Syringe speed	Normal
Column	Hamilton PRP-1, 150 x 4.1 mm; 5 µm particle size
Autosampler	Varian Prostar model 410
Solvent delivery module	Varian Prostar model 230
Loop	50 µL PEEK

The deionised water (≥ 18.2MΩ.cm resistivity) used for mobile phase preparation and sample dilution was prepared in-house using a MilliQ Gradient (Merck Millipore, Bayswater, VIC, Australia). The HPLC reagents were HPLC-grade methanol and 35 % ammonia solution purchased from Thermo Fisher Scientific Australia Pty Ltd (Scoresby, VIC, Australia). They were used to prepare the mobile phase of 30 % v/v methanol with 500µL per litre ammonium (pH 9.0) filtered through a 0.45 µm nylon filter (Merck Millipore, Bayswater, VIC, Australia). 99.999 % purity hydrogen from BOC (North Ryde, NSW, Australia) was used as the CRI gas. With a low hydrogen gas flow rate instead of the high helium gas flow rate that was used for total Co determinations, the role of the CRI gas following chromatograph was not to reduce spectroscopic interferences. Instead it functions to increase sensitivity by concentrating the ion beam. A 1000 mg cyanocobalamin L^{-1} stock standard was prepared using cyanocobalamin obtained from Sigma Aldrich (Castle Hill, NSW, Australia). This stock standard was diluted to provide calibration standards at 0.00, 3.20, 16.0, 80.0, 400, 2000 and 10000 µg L^{-1}. Using peak area, the HPLC Galaxie software was used to generate calibration curves to calculate concentration.

Samples for HPLC-ICP-MS analysis were transferred to 5 mL screw cap polypropylene tubes (Thermo-Plas, Adelaide, SA, Australia). These tubes were centrifuged at 1250 g rcf for 5 min at room temperature to remove particulates. Further sample clean-up was by filtration of the supernatant through luer lock 0.22 µm 13 mm nylon syringe filters (Grace, Rowville, VIC, Australia) attached to 1 mL tuberculin syringes (Terumo, Macquarie Park, NSW, Australia). The filtrate was collected into 2 mL glass HPLC vials (Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia) and then capped before being loaded onto the autosampler ready for analysis.

4.4 Results and discussion

4.4.1 Method validation

When using ICP-MS as a detector for the HPLC column eluent, only the element Co is measured. Therefore, to ensure that the Co containing peak attributed to B12 was indeed cyanocobalamin, several approaches were taken to confirm peak identity and validate the quantitative capability of this method.

In a paper by Hillyer *et al.* 2017, the Immulite® vitamin B_{12} assay kit (IMMULITE 1000 Vitamin B12 2005) was used in their investigation of equine B12 concentrations. This assay kit is suitable for use on the IMMULITE® 1000, a small bench top immunoassay analyser from Siemens Healthineers AG. The intended use of this kit is for the *in vitro* quantitative measurement of vitamin B12 in serum or heparinised plasma. The concentration range for this assay was 100–1200 pg mL^{-1} requiring urine test sample dilutions of between 1:2 and 1:800. The manual sample pre-analysis preparation steps were as per package insert instructions. The automated component of the assay was conducted on the Immulite® 1000 analyser.

This assay is a solid-phase, competitive chemiluminescent enzyme immunoassay. A preliminary 100ºC heat denaturation step in the presence of dithiothreitol and potassium cyanide is required to release B12 from carrier proteins and inactivate antibodies to intrinsic factor. After the heat denaturation step, treated sample and porcine intrinsic factor are simultaneously added to a sample cup containing a polystyrene bead coated with a B12 analogue. B12 in the treated sample competes with the B12 analogue for a limited number of binding sites on the porcine intrinsic factor. After a 30 min incubation at 37ºC, alkaline phosphatase labelled anti-porcine intrinsic factor is added. Unbound enzyme conjugate is removed by a centrifugal wash.

Another immunoassay relying on specific binding of intrinsic factor is the B12 method used on the ARCHITECT® *i* System (Abbott Laboratories 2018). This is a two-step assay using an automated sample pre-treatment step where potassium cyanide and cobinamide dicyanide are added. An aliquot of pre-treated sample is transferred to a reaction vessel where it is combined with porcine intrinsic factor coated paramagnetic microparticles. B12 present in the sample binds to these microparticles. An aliquot of this reaction mixture is transferred to a matrix cell where the B12-intrinsic factor-microparticle complex bind irreversibly to a glass fibre matrix. The matrix cell is washed to remove materials not bound to the microparticles. A B12 acridinium-labelled conjugate is added forming a B12-intrinisc factor-microparticle-conjugate complex. The matrix cell is washed to remove the unbound conjugate. Trigger solutions are then added resulting in a

chemiluminescent reaction. The proportion of light released is inversely related to the concentration of B12 present in the test sample.

Common to both assays, and key to their ability to detect B12, is the inclusion of porcine intrinsic factor, a protein that will only bind to B12. Neither assay has been validated for the determination of B12 in urine. There are no clinical applications for B12 measurement in urine with clinical investigations into B12 irregularities performed on serum or plasma from blood samples. This test is routinely undertaken in clinical biochemistry laboratories using commercially prepared reagents on automated platforms such as the Roche e602, Abbott Architect, Siemens Centaur and Beckman Dx1800. From an analytical perspective, urine presents as a considerably less complex matrix within which to determine B12 concentrations. This is because urine produced by healthy kidneys is free from the myriad of proteins and immunoglobins that must be considered when developing an immunoassay for use with serum samples. As informative B12 results for clinical samples are obtained from serum, manufacturer method validation data is only provided for B12 determinations in serum.

When performing biochemical assays, accredited test methods are ideally used to instil confidence in result accuracy. The accreditation process requires a laboratory to define certain key performance characteristics to ensure that a given method is fit for its intended use. One such characteristic is a requirement to establish analyte stability. With no control over the collection and storage conditions for horse urine it was not possible to meet this method validation criteria. Degradation or loss of analytes through prolonged and/or unfavourable storage conditions will result in a decrease in measured quantity. In this situation, results for urine creatinine, Co and B12 must therefore be considered to represent an “at least quantity”.

As immunoassays for B12 rely on the specific binding of intrinsic factor to B12, degradation of the B12 molecule will significantly impact the ability of these assays to accurately determine B12 concentrations. The HPLC-ICP-MS method presented in this Chapter can be used to verify immunoassay results. As an entirely different analytical technique, the HPLC-ICP-MS method of B12 determination not only shows the presence

of B12 but provides a picture of B12 degradation products. The combination of both analytical techniques uses the power of an accredited method for determining B12 in serum while addressing the issue of B12 stability in urine samples during storage.

4.4.2 Results

The Co concentration of the "Nature Vet" Vitamin B12 solution was confirmed directly by ICP-MS at 44.5 mg L^{-1}, a difference of 2.4 % from the label. This was within the method measurement uncertainty and taken as confirmatory evidence of the accuracy of the product labelling. Further analysis by HPLC-ICP-MS of the diluted Vitamin B12 solution confirmed that the Co was predominantly in the form of cyanocobalamin with a retention time of 12.3 min (Figure 4.1). Comparison of the "Nature Vet" Vitamin B12 solution with the cyanocobalamin standard from Sigma Aldrich gave peaks with identical retention times. No peak splitting was evident when the "Nature Vet" Vitamin B12 and cyanocobalamin standards were mixed at equal concentrations.

Inorganic Co was poorly retained by the column and may be seen near the void volume with retention times between 2 and 3 min (Figures 4.2–4.4). Also eluting within the first 6 min of analysis are those matrix components forming molecular interferants at m/z 59. By relying on chromatographic separation to remove these interferants rather than kinetic energy discrimination from the ICP-MS, method sensitivity is greatly enhanced. Comparison of Figures 4.2 and 4.4 demonstrates that the sample matrix derived molecular interferants are effectively isolated from cyanocobalamin at different retention times. A heavier urine matrix chromatograph is presented in Figure 4.2 than that presented in Figure 4.4 as indicated by urine creatinine concentrations of 1.5 g L^{-1} and 1.0 g L^{-1}, respectively. Peak heights at m/z 59 are therefore greater in Figure 4.2 with the higher concentration of interferant; nonetheless, all interferants eluted well before the cyanocobalamin peak at 12.3 min.

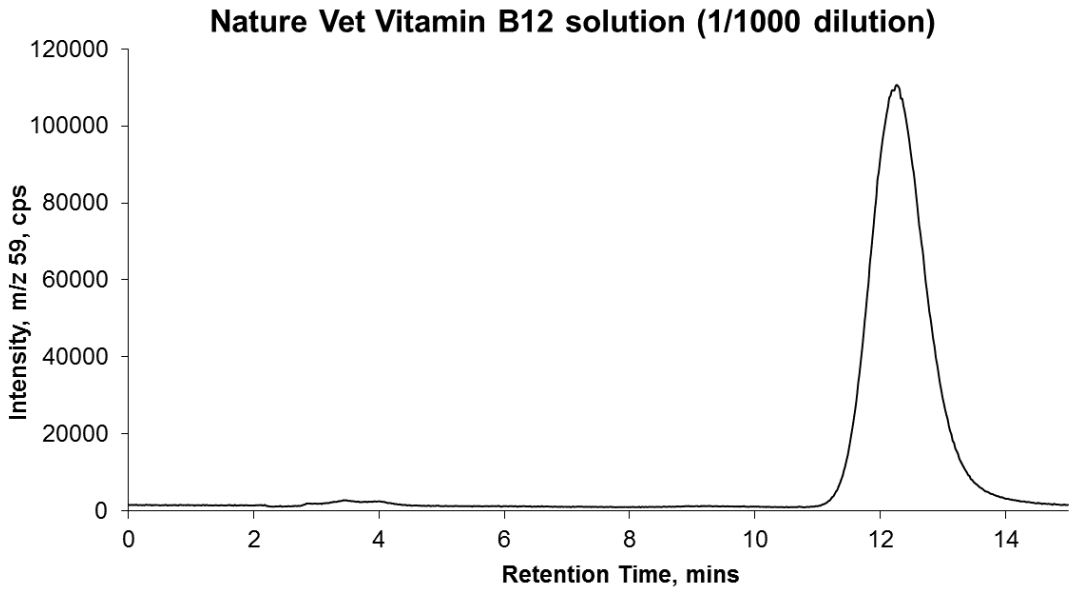

Figure 4.1. HPLC-ICP-MS chromatogram obtained from a 50 µL injection of the Nature Vet Vitamin B12 solution diluted 1000-fold to contain 45 µg L^{-1} cobalt.

The time resolved results of the HPLC-ICP-MS analysis of horse urine show that prior to the IV administration of the Vitamin B12 solution, no cyanocobalamin was detected in the horse urine (Figures 4.2 to 4.4). All Co containing compounds and inorganic forms of Co were eluted from the column within 6 min and molecular interferants at m/z 59. Within 2 h of Vitamin B12 injection, a peak consistent with cyanocobalamin (Figure 4.3) was evident. Cyanocobalamin remained detectable in urine for at least 12 h after the Vitamin B12 injection (Figure 4.4).

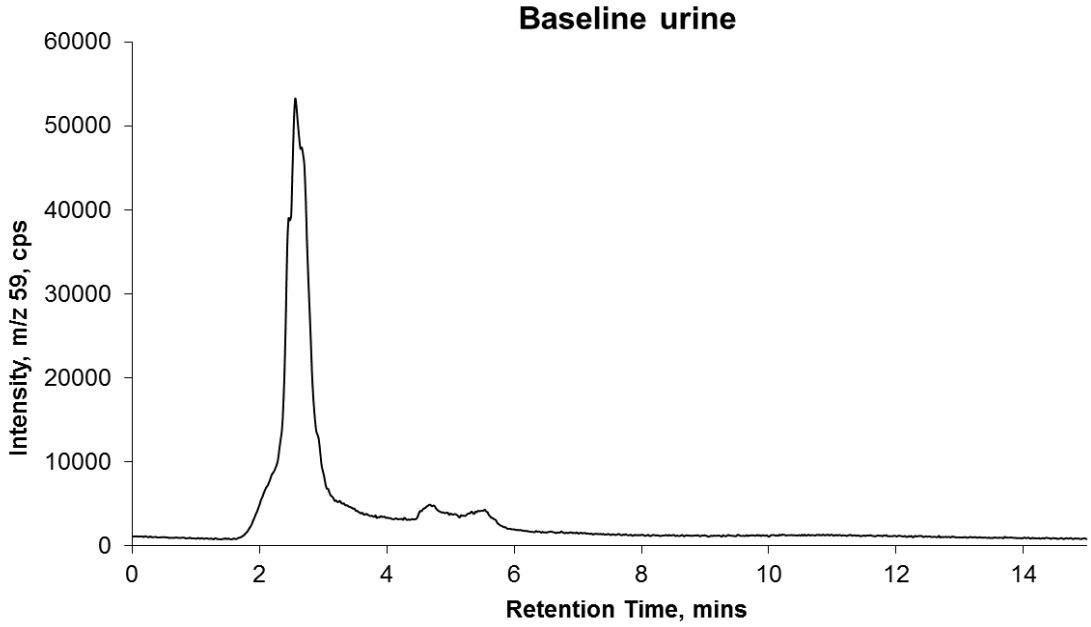

Figure 4.2. HPLC-ICP-MS chromatogram obtained from a 50 µL injection of horse urine collected immediately prior to cyanocobalamin administration.

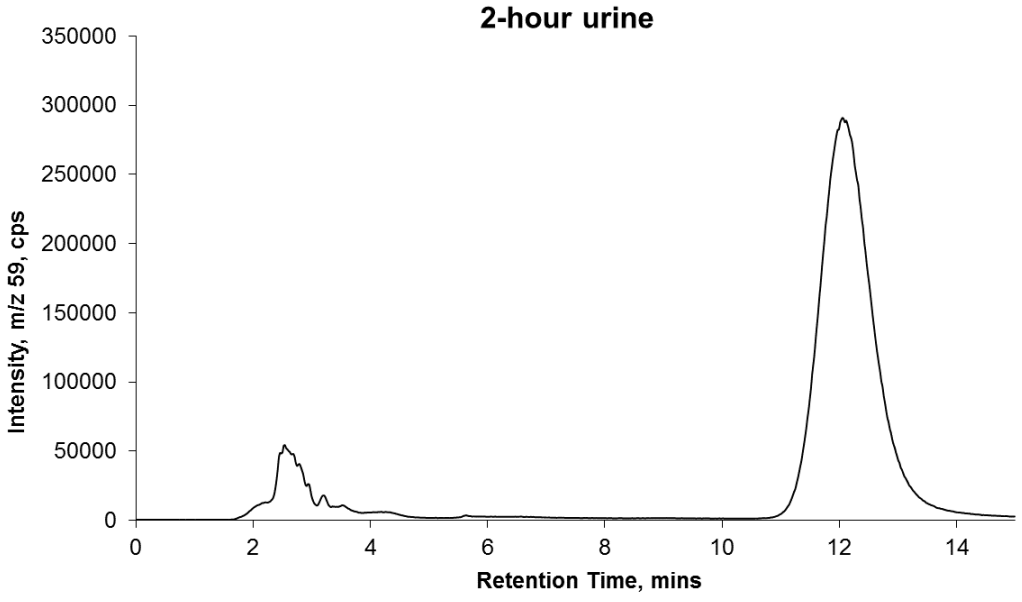

Figure 4.3. HPLC-ICP-MS chromatogram obtained from a 50 µL injection of horse urine collected 2 hours after cyanocobalamin administration.

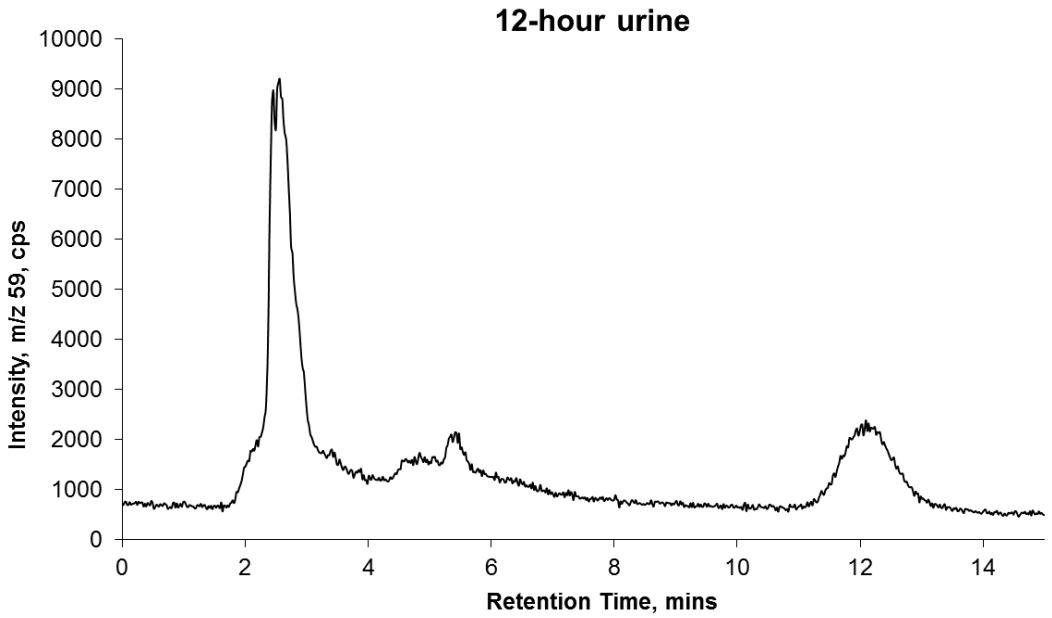

Figure 4.4. HPLC-ICP-MS chromatogram obtained from a 50 µL injection of horse urine collected 12 hours after cyanocobalamin administration.

Total Co, creatinine, cyanocobalamin peak area, cyanocobalamin Co concentration, and the urinary creatinine adjusted results, are summarised in Table 4.3. All Vitamin B12 concentrations are reported as Co for easy comparison. Total urinary Co and calculated cyanocobalamin cobalt concentrations were plotted (Figure 4.5) against sampling time and show that ~70 % of the injected cyanocobalamin was excreted in the urine unchanged.

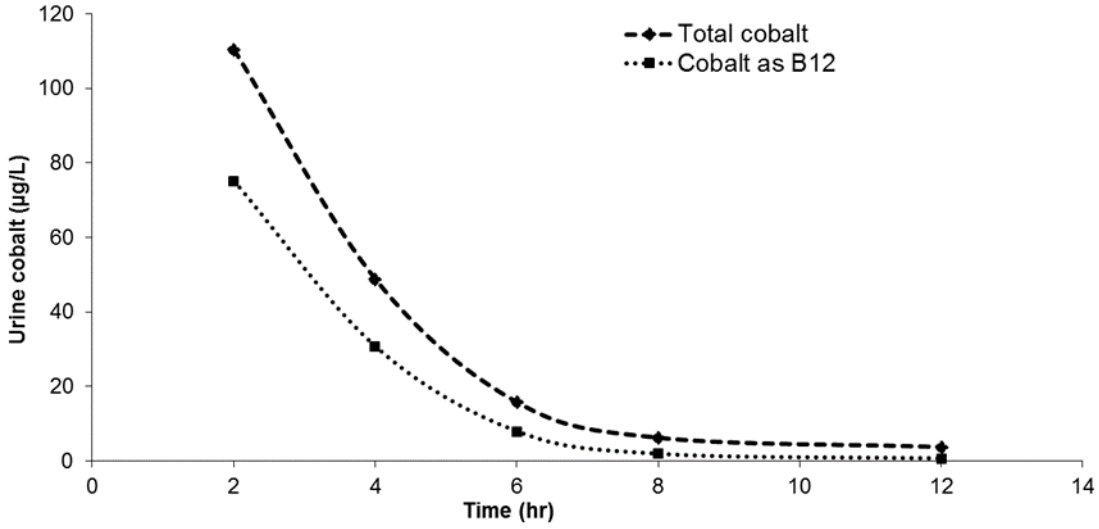

Figure 4.5 Total urinary Co concentration and calculated cyanocobalamin cobalt concentration plotted for the 5 urine collection time points. The 10 mL injection of 1 mg mL^{-1} Vitamin B12 solution was administered at time 0.

Table 4.3. Cobalt, creatinine, and cyanocobalamin results for Nature Vet Vitamin B12 solution and horse urine samples. B12 Co concentrations interpolated from calibration curve of peak areas from stock standard (calibration standards at 0.00, 3.20, 16.0, 80.0, 400, 2000 and 10000 µg L^{-1}). Total Co determined by ICP-MS and creatinine by Jaffe reaction method on automated biochemistry platform. Creatinine adjusted results calculated by dividing B12 or Co result by creatinine concentration.

Sample	Retention time	Peak area	B12 Co	Total Co	Cr.	B12 Co	Total Co
	(min)	(c s^{-1})	(µg L^{-1})	(µg L^{-1})	(g L^{-1})	(µg g^{-1} Cr.)	(µg g^{-1} Cr.)
B12 1/1000	12.26	115707	-	44.5	-	-	-
0-hour urine	-	0	-	6.0	1.53	-	3.9
2-hour urine	12.05	300456	115.6	169.7	1.54	75.1	110.3
4-hour urine	12.07	101974	39.2	62.2	1.28	30.7	48.7
6-hour urine	12.09	17422	6.7	13.3	0.85	7.9	15.7
8-hour urine	12.09	4417	1.7	5.6	0.92	1.9	6.1
12-hour urine	12.14	1580	0.6	3.6	1.00	0.6	3.6

4.4.3 Discussion

Immunological methods are frequently used in clinical laboratories to measure vitamin B12 concentrations but are subject to interferences typical of immunoassays (Ward et al. 2017). More reliable methods using ICP-MS detection of Co in vitamin B12 have been published. For example, a method using size exclusion chromatography coupled with ICP-MS detection (Kerger et al. 2013). This technique could separate cyanocobalamin, molecules of protein bound Co with a mass greater than 50 kDa and small molecular Co free ions including bound forms with a mass of less than 1 kDa. A microfluidic HPLC-MS chip for isolating B12 (Bishop et al. 2017) has also been developed. Both these methods are relatively complex and have been presented as suitable for serum samples. Current IFHA thresholds for Co apply to both blood and urine samples. The method presented here can be easily implemented in laboratories with HPLC-ICP-MS capabilities and used to measure cyanocobalamin concentrations in urine. The simple mobile phase composition of 30 % methanol and 500 μL L^{-1} ammonium at a flow rate of 0.5 mL min^{-1} did not require specialised sample introduction systems to prevent build-up of carbon on the cones. As a method that uses chromatography rather than kinetic energy discrimination to remove molecular interferants, it is readily adaptable across a range of ICP-MS platforms.

There are several chemical forms of cobalamin with variations in the sixth coordination site of cobalamin giving rise to cyanocobalamin, hydroxylcobalamin, or the active coenzymes methylcobalamin or 5-deoxyadenosylcobalamin. The chemical form and route through which vitamin B12 is administered varies. Food additives are typically cyanocobalamin as it is easy to crystallise and not sensitive to air oxidation. Injections are either cyanocobalamin, hydroxocobalamin or methylcobalamin that may be administered intravenously, intramuscularly, or subcutaneously. Therapeutic vitamin B12 supplementation regimes are usually through regular intramuscular injections to ensure sustained exposure for maximum pharmacological benefit. Intramuscular injections are generally preferred over intravenous injections where rapid urinary excretion is reported (APP Pharmaceuticals 2008). Racehorses, however, are typically administered vitamin B12 intravenously by veterinarians. There is an aversion to intramuscular injections close to competition to avoid an injection site reaction. In developing a method for vitamin B12 determinations, the aim was to demonstrate a

technique that was sufficiently sensitive to monitor changes in urinary cyanocobalamin concentration, even after a single intravenous dose of vitamin B12. As shown in Figure 4.4, this was achieved with cyanocobalamin detectable 12 h post intravenous injection.

Once in the liver, cyanocobalamin is converted to its active forms, methylcobalamin and adenosylcobalamin (Jelkmann 2012). The main factors influencing cobalamin excretion are dose quantity and route of administration. Investigations into cobalamin oral absorption found that in subjects given 500 µg cobalamin day^{-1} (equivalent to 22 µg Co), urinary Co concentrations did not exceed 2 µg L^{-1} (Krug et al. 2014). It was noted that IV doses of cyanocobalamin (Jelkmann 2012) in excess of 100 µg are cleared rapidly from the plasma and excreted in the urine. Most pharmacological doses of cyanocobalamin are excreted in urine without metabolic conversion to inorganic Co (Hillyer et al. 2017). Our findings (Table 4.3), support this observation with more than two-thirds of the total urinary Co measured present in cyanocobalamin. Other forms of Co, including inorganic Co, may also be present as indicated in Figure 4.5 where a constant difference between total Co and cyanocobalamin bound Co is observed. The quantity of inorganic Co released from cyanocobalamin is insignificant when considering the levels that are required to induce hypoxia. Levels of inorganic Co associated with increased erythropoietin production are considerably greater than those that have been set by racing authorities that were based on population studies (Ho et al. 2015) and not related to doses associated with hypoxic effect. In a review of animal toxicology and epidemiology literature (Finley et al. 2012), it was found that blood Co concentrations of 300 µg L^{-1} and less, have not been associated with haematological changes. This figure is 12 times greater than the total blood Co actionable threshold of 25 µg L^{-1} (International Federation of Horseracing Authorities 2017). No studies have attempted to define haematological changes as a function of urinary Co concentration. However, by applying the 12-fold difference in actionable threshold to haemopoietic effect for urine samples, it would be expected that urinary Co concentrations would need to exceed 1200 µg L^{-1} before the hypoxic effect of Co was evident.

4.5 Conclusions

This study demonstrates that intravenously administered vitamin B12 was excreted in urine without notable metabolic conversion. As only the inorganic form of Co has been shown to influence EPO production, the measurement of total urinary Co concentrations is a potentially inadequate screen for Co misuse. Presumptive positive findings should therefore be investigated further to eliminate vitamin B12 supplementation as a cause. A suitable technique for this purpose has been presented using a reverse phase HPLC column coupled with ICP-MS detection.

5. COBALT ACCUMULATION IN HORSES FOLLOWING REPEATED ADMINISTRATION OF COBALT CHLORIDE

5.1 Preamble

Prior to setting a threshold for urinary Co concentrations in horses, no studies had been conducted to assess the influence that long term Co exposure could have on urinary Co excretion. This Chapter presents original published research (Wenzel et al. 2019) on monitoring urine Co concentrations in horses chronically exposed to Co. Recognition of the importance and level of international interest in this research was swift. Soon after publication I was contacted by Dr Stacey Oke, a practicing veterinarian and freelance medical writer and editor from the United States, to base an article on our research findings. The addendum to this Chapter cites this article (Oke 2019) that was published in the mainstream magazine "The Horse: Your Guide To Equine Health Care".

5.2 Summary

Anecdotal evidence of abuse of elemental Co in the horse racing industry has compelled authorities to regulate, monitor and legislate against its overuse. The premise of abuse is the belief that Co indirectly induces erythropoiesis, and improves athletic performance through enhanced aerobic capacity. This study was designed to investigate the effects of chronic Co administration and builds on previous work examining acute Co exposure pharmacokinetics (Knych et al. 2014). We repeatedly administered cobalt chloride intravenously to six horses then measured blood haematocrit along with urine, plasma, whole blood, and red cell Co levels. These samples were collected immediately prior to Co administration so that **trough Co levels** were determined throughout. Accumulation of Co was demonstrated with increasing Co concentrations for the various sample matrices. The urine Co threshold was only effective at detecting acute Co exposure. The plasma Co threshold was able to consistently identify chronic high-level Co exposure and potential Co misuse. Red cell Co levels remained elevated for at least 12 weeks after cessation of administration, consistent with the lifespan of the red cell. The threshold values legislated for urine Co do not correlate with those set for plasma. The acute nature of urinary Co excretion provides a relatively small window through which to detect Co

administration. Plasma and red cell Co concentrations can provide a clearer picture of potential Co misuse.

5.3 Introduction

Cobalt is an essential trace element required by the intestinal bacteria of horses for the synthesis of vitamin B12 (cobalamin). In a 500 kg working horse consuming 10–12.5 kg dry mass feed per day, a minimum daily dietary intake of 0.1–0.15 mg Co kg^{-1} of dry feed is recommended with signs of toxicity unlikely to occur up to a daily dietary intake of 20 mg Co kg^{-1} dry feed (Harris et al. 2006). The uptake and distribution of Co has principally been studied in laboratory animals and humans (Simonsen, Harbak & Bennekou 2012). With prolonged exposure, increasing amounts of Co enter the red blood cells via transfer proteins. Once sequestered within red cells, Co remains for the life of the cell, approximately 120 days in humans (Tvermoes et al. 2014) and approximately 150 days in horses (Carter et al. 1974).

A comprehensive review of Co in horses emphasised the need for further work to clarify the pharmacodynamics of long-term Co exposure (Kinobe 2016). This review also noted that there was no evidence of either performance enhancing or toxic effects of Co in horses and emphasised that, with a compartmental excretion pattern and a long elimination half-life, the cumulative effect of repeated Co dosing was unknown.

Most human studies consider whole blood, plasma, or serum Co levels, although some studies have compared all 3 samples. Van der Straeten, in Estey et al., (Estey 2013) noted that blood or plasma was preferred and advised that 24 h elimination samples are more reliable for urine Co measurement. For the biological monitoring of Co in hard metal factory workers, the Co concentrations in whole blood were found to be a reliable indicator of Co exposure in contrast to urinary Co determinations that showed wide daily and weekly fluctuations (Princivalle et al. 2017). It has been stated that ‘Taking account of only the concentration of metal in urine is unsatisfactory and subject to error from differential urinary dilution’ (Daniel et al. 2007). Consequently, when proposing a human athletic regulatory threshold, it was noted that urine levels would need correction for creatinine (Krug et al. 2014). These authors also stated that further studies were required

to elucidate other factors which may elevate Co levels, proposing measurement of Co accumulation in erythrocytes as a method of determining long-term exposure (Krug et al. 2014).

The International Federation of Horseracing Authorities (IFHA) set maximum permissible raceday thresholds of 0.1 µg total Co mL^{-1} in urine and 0.025 µg total Co (free and protein bound) mL^{-1} in plasma (International Federation of Horseracing Authorities 2017). The urine threshold was based largely on the published work of Ho et al. (Ho et al. 2015), who conducted an administration trial where groups of 1-3 horses of unspecified prior Co exposure were dosed with various commercial Co-containing supplements for 3 consecutive days. The plasma level was set in light of a pharmacokinetic study following a single IV administration of Co to horses (Knych et al. 2014). These authors comprehensively documented the pharmacodynamics of a single Co bolus. They reported a gamma half-life of 4.4 to 10.5 days following a single dose, with peak recorded levels in serum of 429 µg L^{-1} 30 min after administration. The highest urine level recorded was 7687 µg Co L^{-1}, with a mean of 3855 ± 1378 µg L^{-1}. The pilot horse exceeded the international urine threshold for 4 days, and the serum level at 7 days was 69 µg Co L^{-1}.

Several factors are known to influence the urinary levels and excretion rate of Co in humans and other animal species. There is competition and displacement from binding sites with other metallic elements such as iron, selenium, calcium and magnesium (Flora & Pachauri 2010; Llobet, Domingo & Corbella 1986). Ischemia-modified albumin has a reduced binding affinity for Co (Apple et al. 2002; Lippi, Montagnana & Guidi 2006). Cobalt is tightly bound to the red cell and is released into the reticuloendothelial system on cell death. Intravascular haemolysis, e.g., caused by racing or transport stress leads to premature death of red cells (Hanzawa & Watanabe 2000; Yaqub, Mshelia & Ayo 2014). The effect of these factors on urinary Co concentrations in horses have not been reported with no studies having been conducted to investigate urinary Co excretion in samples collected pre- and post-race.

The aims of this chapter were to:

1. Expand on the pharmacokinetic study following a single IV administration of Co (Knych et al. 2014), by administering similar doses of cobalt chloride at repeated intervals.
2. Record the disposition of Co in urine, red blood cells and plasma over time, with repeated exposure.
3. Compare results to the current IFHA thresholds in urine and plasma to detect Co misuse.
4. Observe the effect of repeated high parenteral Co dosing on haematocrit.

5.4 Materials and methods

The research was conducted under NSW Department of Primary Industries Animal Care and Ethics Committee Permits TRIM 15/216 and TRIM 16/1381 (Appendix 9.1). Eight mares were selected from a pool of 15 Standardbred mares aged 3–10 years. These mares were chosen on advice from their regular handlers as horses least likely to be stressed by regular blood tests and direct urine aspiration. They had received no known Co supplementation for at least 3 months prior to the trials. Their maintenance diet was approximately 2 kg of oats and 6 kg of lucerne hay per day, fed in two portions. They were kept in half- to one-hectare grassed paddocks and housed in galvanised steel day yards during periods of intensive sampling.

The mares were randomly assigned to 4 treatment groups, with 2 mares in each group:

i. Control group.
i. 25 mg Co intravenously as $CoCl_2$ weekly on Tuesday.
ii. 50 mg Co intravenously as $CoCl_2$ weekly on Tuesday.
iii. 25 mg Co intravenously as $CoCl_2$ twice weekly, on Tuesday and Friday, i.e. 50 mg weekly.

These doses were selected following a previously published pharmacokinetic study (Knych et al. 2014). Concurrent urine and blood samples were collected and measured. These authors comprehensively documented peak levels and excretion over time of a single dose. In our study, mares were sampled **before** each weekly administration, so in

each case “trough” levels were obtained, these being the lowest point in each treatment cycle. The samples obtained from the “twice weekly” group were 4 full days from the last treatment. The remaining samples were 7 days from last treatment.

A stock solution of 10 µg mL^{-1} cobalt chloride was prepared by dissolving reagent grade hydrated cobalt chloride ($CoCl_2.6H_2O$) (Sigma-Aldrich, Castle Hill, NSW, Australia) in sterile water for injection (Troy Laboratories, Glendenning, NSW, Australia). The concentration of Co in the stock solution was measured after preparation and at the completion of the study to ensure solution stability. Prior to injection, this stock solution was further diluted to 5 µg mL^{-1} to provide an injection volume of 5 or 10 mL. The final dilution was passed through a 0.45 µm Millipore filter (Merck Millipore, Bayswater, VIC, Australia) to ensure bacterial sterility.

The control mares were not injected with any solutions. They had blood collected for haematocrit measurement, and periodic blood and urine samples for Co assay. They were maintained, handled, and sampled in identical manner to the treatment groups.

5.4.1 Sample collection

Blood and urine samples were initially collected at time zero then each Tuesday, within 1 h before Co administration, for 10 weeks. Three further collections of blood and urine were made 81, 106 and 127 days after time zero. Blood was collected with the Vacutainer® collection system (Becton Dickenson, NSW, Australia) using a 20-guage needle (BD Cat. # 360214) to aspirate blood into 10 mL heparinised tubes (BD green LiHep Cat. # 367526) and 2 mL EDTA tubes (BD lavender K3EDTA Cat. # 367836). One of each pair of matched blood samples was immediately centrifuged at 1250 g rcf for 5 min at room temperature with the plasma aspirated and placed into a fresh heparinised vacutainer tube for plasma assay. Care was taken to ensure all samples were free from haemolysis. A subset from each batch of heparinised tubes was tested and found to cause contamination of < 0.1 µg Co L^{-1}, making these tubes suitable for sample collection and storage of aspirated plasma.

A skilled equine veterinarian directly aspirated urine by digital passage of an Infusette® catheter through the urethral sphincter. No indwelling catheters were placed. Urine was placed in 50 mL polypropylene sample containers (Thermo-Plas, Adelaide, SA, Australia) that had been batch tested to ensure they caused contamination of < 0.1 μg Co L^{-1}. Samples were protected from light and kept refrigerated until analysis. The final sampling was conducted approximately 7 months after the first administration.

5.4.2 Determination of creatinine concentration

Urine creatinine measurements were performed to ensure horses were adequately hydrated and therefore producing urine at a uniform rate suitable for quantitative determinations. As there is currently no provision for hydration status in the urinary Co threshold, results are reported as total urine Co. When measuring the concentration of endogenous substances in urine, it is common practice to correct for variations in hydration status with creatinine or specific gravity (Boeniger, Lowry & Rosenberg 1993; Sauve et al. 2015; WADA Laboratory Expert Group 2018; Warrack et al. 2009). Therefore, to permit accurate comparison between urine concentrations, Co quantities in urine have also been reported as μg Co g^{-1} of creatinine. Samples for creatinine were obtained by pouring off an aliquot of around 4 mL from the primary urine sample into 5 mL screw cap polypropylene tubes and centrifuging at 1250 g rcf for 5 min at room temperature to remove any particulate material. Creatinine measurements were performed in the NSW Health Pathology Clinical Biochemistry Laboratory, Royal North Shore Hospital, St Leonards, NSW, Australia using the Jaffe reaction method (Abbott Laboratories 2006) on an Architect c9000 System (Abbott Laboratories, Abbott Park, IL, USA).

5.4.3 Determination of haematocrit

Haematocrit is a commonly utilised haematological measurement that defines the ratio of the volume of red blood cells to the total volume of blood as determined by the separation of red blood cells from the plasma. It can be expressed as a ratio (red cell volume over total blood volume and reported with the units L/L) or as a percentage (ratio multiplied by 100). An increase in the number and/or size of red bloods, as occurs in response to

hypoxia, results in an increase in haematocrit. In this study, haematocrit measurements were used as a quantitative indicator of cobalt induced hypoxia and also in the calculation of red cell cobalt concentrations as described in Section 5.4.5,

Haematocrit was measured at the time of sampling using a microhematocrit centrifuge (Boeco H-240 Hematocrit Centrifuge, Wiltshire, United Kingdom). Red cells were resuspended by vortex 6 times on an agitator platform prior to charging glass micro haematocrit tubes and sealing with plasticine. Precision and repeatability of haematocrit was determined by performing "blind" determinations on 16 replicates of 4 blood samples obtained for the study, the correlation obtained using Lin's concordance coefficient analysis was 0.9995.

5.4.4 Determination of cobalt concentration

I measured Co in the NSW Health Pathology Trace Elements Laboratory, Royal North Shore Hospital, St Leonards, NSW, Australia. Analysis was by Inductively Coupled Plasma Quadrupole Mass Spectrometry using a Varian 820MS with SPS autosampler running the ICP-MS Expert instrument software Version v2.1 b107 (Varian, Mulgrave, VIC, Australia). The method for Co determination was accredited to ISO 15189:2012 (International Organization for Standardization 2012) as certified by the National Association of Testing Authorities, Australia / Royal College of Pathologists of Australasia, laboratory accreditation number 1981. External quality assurance for Co was through enrolment in the Quebec Multi-Element Quality Assurance Scheme (Institut national de santé publique) and the RCPAQAP Trace Elements Program (Royal College of Pathologists of Australasia Quality Assurance Programs Pty Limited).

A sample diluent of 0.75 % v/v nitric acid, 0.01 % w/v Triton X-100 and 0.01 w/v EDTA was prepared using A.C.S. grade 70 % nitric acid, ethylenediamine tetra-acetic acid (EDTA) and Triton X-100 that were obtained from Sigma Aldrich (Castle Hill, NSW, Australia). 99.999 % pure helium obtained from BOC (North Ryde, NSW, Australia) was used as the collision reaction interface (CRI) gas to minimise the presence of polyatomic interferences. Aqueous calibration standards at 0.00, 1.13, 5.63, 28.2, 141, 352 and 880 µg L^{-1} were prepared by dilution in 1 % v/v nitric acid, of a multi-element standard

containing 100 mg L^{-1} Co that was purchased from Australian Chemical Reagent (Moorooka, QLD, Australia).

To compensate for changes in sample matrix, a 1000 mg L^{-1} gallium standard, also purchased from Australian Chemical Reagents, was added to the sample diluent as an internal standard at a concentration of 50 µg L^{-1}. The reagent water (≥ 18.2MΩ.cm resistivity) used for dilution, reagent preparation and consumables washing, was prepared in-house using a MilliQ Gradient (Merck Millipore, Bayswater, VIC, Australia). All plastic ware, including disposable pipette tips, were soaked in 2 % v/v nitric acid for a minimum of 24 h then thoroughly washed with reagent water immediately prior to use.

All test samples were analysed in triplicate. Samples were diluted for analysis in 5 mL screw cap polypropylene tubes (Thermo-Plas, Adelaide, SA, Australia). A 1 mL aliquot of sample diluent was added to each sample tube followed by a 50 µL aliquot of calibrator, control or sample that had been allowed to reach room temperature and mixed thoroughly. Whole blood samples were centrifuged at 1250 g rcf for 5 min at room temperature to obtain plasma. Sample blanks with a 50 µL aliquot of reagent water were also included to ensure reagents were free from Co contamination. Two levels of control for each sample matrix were included in each analytical run so that they were analysed before and after the test samples. Control samples included Seronorm Trace Elements Urine L1, Lot 1011644, Exp 2018-01, Seronorm Trace Elements Urine L2, Lot 1011645, Exp 2018-01, Seronorm Trace Elements Serum L1, Lot 1309438, Exp 2020-10, Seronorm Trace Elements Serum L2, Lot 1309416, Exp 2020-10, Seronorm Trace Elements Whole Blood L1, Lot 1406263, Exp 2019-07 and Seronorm Trace Elements Whole Blood L2, Lot 1406264, Exp 2019-07 (SERO, Billingstad, Norway). Method accuracy and imprecision for the various control materials are presented in Table 5.1. All solutions were vortex mixed before being loaded onto the autosampler. Cobalt concentrations were interpolated from the calibration curve and corrected for signal drift using the gallium internal standard.

5.4.5 Statistical analysis and calculations

Statistical analyses were conducted throughout this Chapter by Dr Evelyn Hall, Associate Lecturer in Veterinary Biostatistics, University of Sydney. The selection of appropriate statistical models were at the direction of Dr Hall. All statistical calculations were conducted in Genstat (v17, VSNi). In order to determine if significant differences were introduced with the various treatment regimes, statistical analyses of the results were conducted using a restricted maximum likelihood (REML) model. Least squares based methods can be employed to analyse variance, but the REML approach was considered a superior means of describing our results as it avoids small sample size bias. REML was used to fit our results to a linear mixed model where both the strength of the fixed effects and relative variance of the random effects can be determined. This statistical method works by using a likelihood function calculated from a transformed set of data so that not all the information is required, providing a maximum likelihood estimate that ensures nuisance parameters have so effect (McNeish 2017). The variance component estimation of the initial data is replaced by a set of contrasts calculated from the data. The likelihood function is then calculated from the probability of these contrasts. In this way, small sample size bias introduced by random effects can be avoided with REML producing unbiased estimates of variance and covariance.

Cobalt measurements were performed in triplicate over 3 separate analytical runs. The REML model was run to determine precision of measurement over the 3 runs with no difference between the Co measurements detected. All further analysis was conducted on the average values of these 3 Co measurements. All raw data was assessed for normality using the Anderson-Darling test and transformed as required to ensure that the normality assumptions of the REML model were met. Consequently, total urine Co, creatinine adjusted urine Co, plasma Co and whole blood Co measurements were all square root transformed for normality to enable calculation of predicted mean Co concentrations.

To assess the effects of time and treatment on Co concentration, multiple REML models were run. Outcome variables were total urine Co, creatinine adjusted urine Co, whole blood Co, plasma Co, red cell Co and haematocrit. The fixed effect parameters were treatment and time, whilst the random effect parameter was mare. Post hoc analysis using

least significant differences (LSD) was conducted to determine pairwise comparison differences. Pearson correlations between the different Co measures were obtained (Table 5.2) and correlation data was plotted (Figures 5.7–5.11). For all statistical analyses, a P value of < 0.05 was considered significant. Curves were plotted as predicted means derived from the REML modelling. The included standard errors provide an indication of the variation within and between animals. Significant differences were evident when plotted results were outside the confines of the standard error bars.

Table 5.1. Performance characteristics of cobalt in quality control material. Seronorm™ trace element controls were used as internal controls. External quality assurance data are presented from the 2018 rounds of the Royal College of Pathologists of Australasia Quality Assurance Program (RCPAQAP) and Quebec Multi-Element Quality Assurance Scheme (QMEQAS). Method accuracy is indicated by bias, calculated as the % difference relative to the mean of participant results or Seronorm™ assigned target. A negative bias occurs when results are below the consensus mean while a positive bias occurs when results are greater than the consensus mean. The coefficient of variation (CV) and 'z-score' provide a measurement of method imprecision.

Quality control material	Assigned Co (µg L^{-1})	Bias	Imprecision (% CV)	Imprecision (z-score)
Serum				
Seronorm Lot # 1309438	1.12	-5.9 %	14.0 %	
Seronorm Lot # 1309416	3.05	-7.5 %	10.6 %	
RCPAQAP 2018		2.3 %	2.6 %	
QMEQAS 2018		-0.06 %		-0.48
Blood				
Seronorm Lot # 1406263	0.20	-8.9 %	103 %	
Seronorm Lot # 1406264	5.18	-16.2 %	16.0 %	
RCPAQAP 2018		1.1 %	1.6 %	
QMEQAS 2018		-0.09 %		-0.57
Urine				
Seronorm Lot # 1011644	0.72	1.2 %	19.7 %	
Seronorm Lot # 1011645	10.6	-0.42 %	14.6 %	
RCPAQAP 2018		4.3 %	3.7 %	
QMEQAS 2018		-0.01 %		-0.18

As a horse's overall level of hydration changes, homeostatic balance is maintained by regulating the amount of water excreted in the urine. To allow for variations in the water content and dilution of Co in urine, a creatinine adjustment was performed. Creatinine adjusted urinary Co concentrations were calculated by dividing the total urinary Co concentration by the urine creatinine concentration.

A formula commonly used in clinical biochemistry for calculating red cell concentrations of an analyte was applied in this study to calculate the concentration of cobalt in red cells (Piyathilake, Robinson & Cornwell 2007):

$$\text{Red cell cobalt} = \frac{\text{Whole blood cobalt} - [\text{Plasma cobalt} \times (1 - \text{Haematocrit})]}{\text{Haematocrit}}$$

Red cell, whole blood and plasma Co concentrations were in units of $\mu g\ L^{-1}$ while the haematocrit was measured as a ratio of the volume of red blood cells to the total volume of blood and expressed with the units $L\ L^{-1}$.

5.5 Results

The predicted mean urine Co levels are plotted in Figure 5.1 for urine samples taken at the times described in the sample collection section. With this collection regime, only those horses administered Co biweekly exceeded the regulatory urine threshold of 100 $\mu g\ L^{-1}$. In the 25 mg and 50 mg weekly treatments, Co levels did not exceed the threshold at any time.

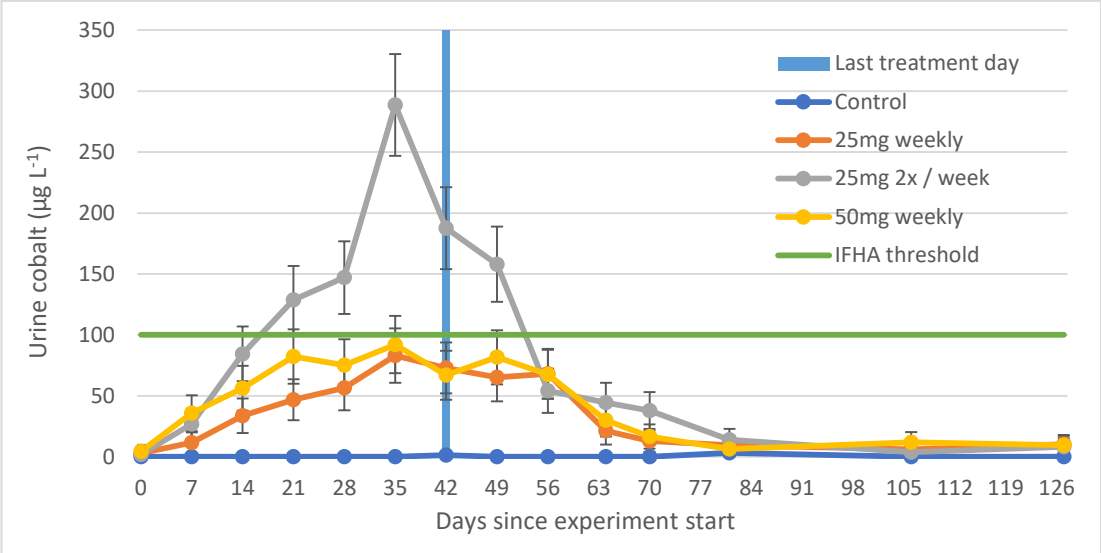

Figure 5.1. Total urinary Co concentrations for the various groups. Last day of treatment at day 42 and IFHA urinary Co threshold of 100 µg L^{-1} are marked on the graph. Each plotted point is the predicted mean value of 6 measurements (triplicate analysis of each sample collected from the 2 horses in the different groups). Error bars depict standard error of the mean.

It would be expected that with higher weekly doses of Co, higher urinary Co concentrations would be observed. This was not the case as shown in Figure 5.1, where the paired treatment groups administered 25 mg and 50 mg doses had urinary Co concentrations that differed by less than the measured uncertainty. This observed anomaly was corrected when allowing for differences in the horse's levels of hydration using the creatinine adjusted urine results (Figure 5.2). To investigate the relationship between urine Co levels before and after creatinine adjustment with blood and plasma Co levels, Pearson correlation analysis was conducted on these sample matrices. Given the established merit of creatinine adjustment to correct for changes in hydration status (Boeniger, Lowry & Rosenberg 1993; Sauve et al. 2015; Warrack et al. 2009), it was expected that correlation between the sample matrices would be greater for the creatinine adjusted measurements. This hypothesis was true for our cohort of 6 horses with higher correlation coefficients (R^2) demonstrating a marked decrease in the difference between urine and both blood and plasma Co concentrations following creatinine adjustment (Table 5.2).

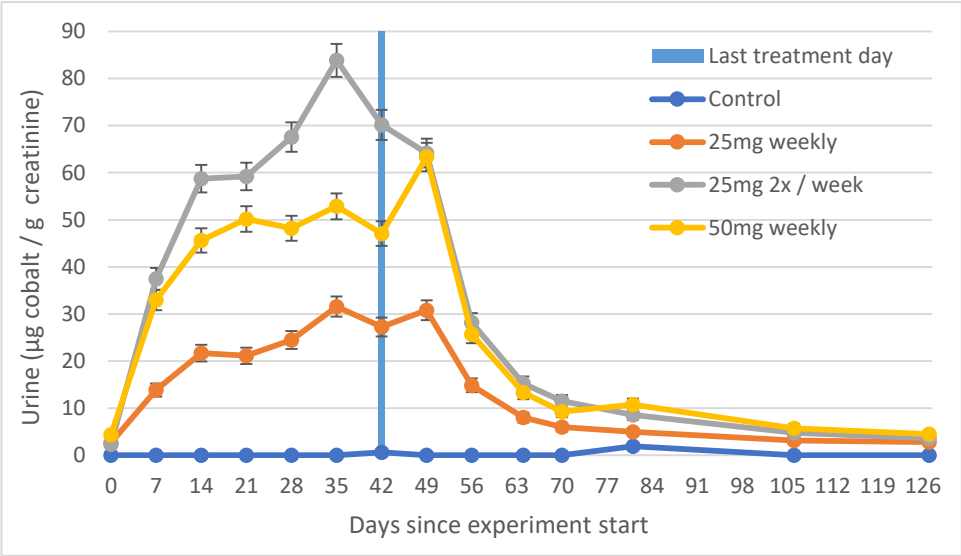

Figure 5.2. Creatinine adjusted urinary cobalt concentrations for the various groups. Last day of treatment at day 42 marked on the graph. Each point is the predicted mean value of 6 measurements (triplicate analysis of each sample collected from the 2 horses in the different groups). Error bars depict standard error of the mean.

Table 5.2. Summary of Pearson correlation analysis of cobalt concentration between different sample matrices (Figures 5.7–5.11). Calculations performed on results from all the collection points for the 6 treatment horses.

Sample Matrix		Correlation	R^2
Total urine Co	Blood Co	0.827	68.4
Creatinine adjusted urine Co	Blood Co	0.933	87.1
Blood Co	Plasma Co	0.979	95.9
Creatinine adjusted urine Co	Plasma Co	0.950	90.3
Total urine Co	Plasma Co	0.855	73.0

Plasma Co levels (Figure 5.3) had a linear rise over the first 6 weeks. By day 7, the 25 mg twice weekly and 50 mg weekly groups breached the regulatory threshold of 25 µg L^{-1} and remained over this level until at least 14 days after cessation. The group receiving 25 mg per week exceeded the 25 µg L^{-1} regulatory threshold by day 14 and remained above this level for at least 14 days after the last dose of Co. Whole blood Co levels

(Figure 5.4) followed a similar pattern of results to the plasma Co levels but remained elevated for a longer time after cessation of Co administration.

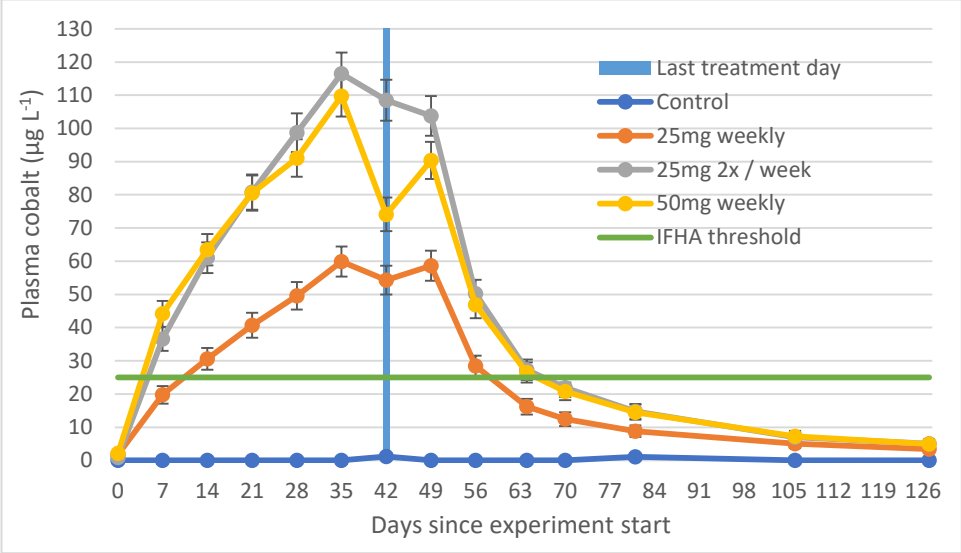

Figure 5.3. Plasma cobalt concentrations for the various groups. Last day of treatment at day 42 and IFHA plasma cobalt threshold of 25 µg L^{-1} are marked on the graph. Each point is the predicted mean value of 6 measurements (triplicate analysis of each sample collected from the 2 horses in the different groups). Error bars depict standard error of the mean.

Predicted mean red cell Co concentrations (Figure 5.5) were calculated from the plasma, whole blood, and haematocrit results. To accurately calculate red cell Co concentrations, an appreciable quantity of Co must be present in the red cells to negate the combined measurement uncertainties from these 3 analytes. This combined uncertainty is shown by large error bars (Figure 5.5). Without a significant quantity of Co in the red cells relative to the plasma, inaccurate and even negative results are possible (Table A7-9.12). Consequently, red cell cobalt concentrations have only been plotted from day 42 once sufficient cobalt had entered the red cells. Measurement uncertainty prevented definitive separation of the various treatment regimes. Graphically it was noted that points plotted for red cell Co concentrations were highest in the group receiving 50 mg Co once per week compared to the group receiving a weekly total of 50 mg but given as two 25 mg doses.

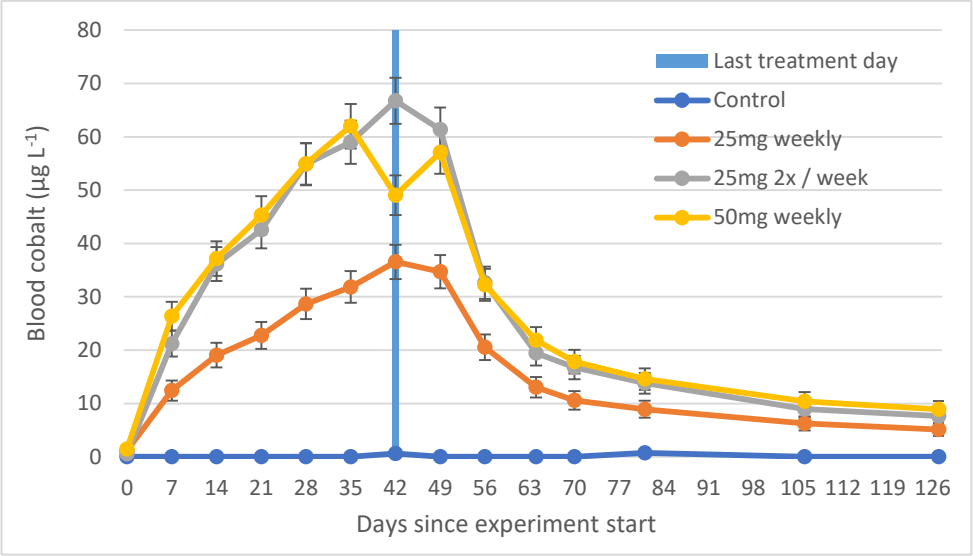

Figure 5.4. Whole blood cobalt concentrations for the various groups. Last day of treatment at day 42 marked on the graph. Each point is the predicted mean value of 6 measurements (triplicate analysis of each sample collected from the 2 horses in the different groups). Error bars depict standard error of the mean.

There was a significant effect of time on haematocrit levels ($P < 0.001$) indicating some variation across the length of the experiment (Figure 5.6). There was no significant difference between treatment groups ($P = 0.771$), showing that regardless of the amount of Co given, the haematocrit levels were not different. Using post hoc LSD analysis, it can be determined that the predicted mean haematocrit level at day 0 of the experiment (40 ± 1.17) was not significantly different from the predicted mean level at day 127 (42.12 ± 1.17).

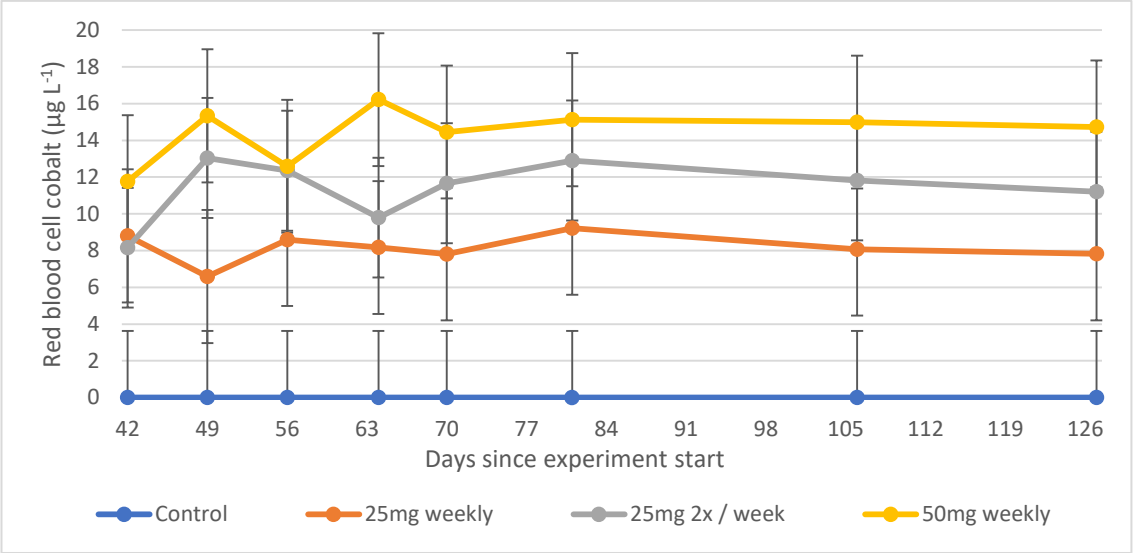

Figure 5.5. Red blood cell cobalt concentrations for the various groups. Red cell cobalt concentrations only plotted from day 42. Prior to this there was insufficient cobalt in the red cells to accurately calculate concentrations. Each point is the predicted mean value of 6 measurements (triplicate analysis of each sample collected from the 2 horses in the different groups). Error bars depict standard error of the mean.

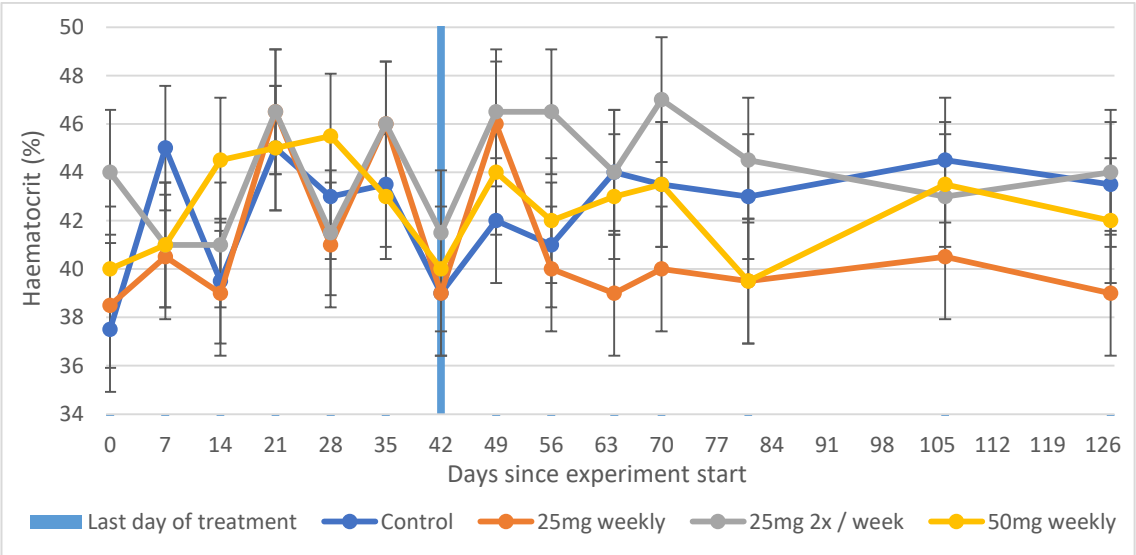

Figure 5.6. Haematocrit for the various groups. Last day of treatment at day 42 marked on the graph. Each point is the predicted mean value of 6 measurements (triplicate analysis of each sample collected from the 2 horses in the different groups). Error bars depict standard error of the mean.

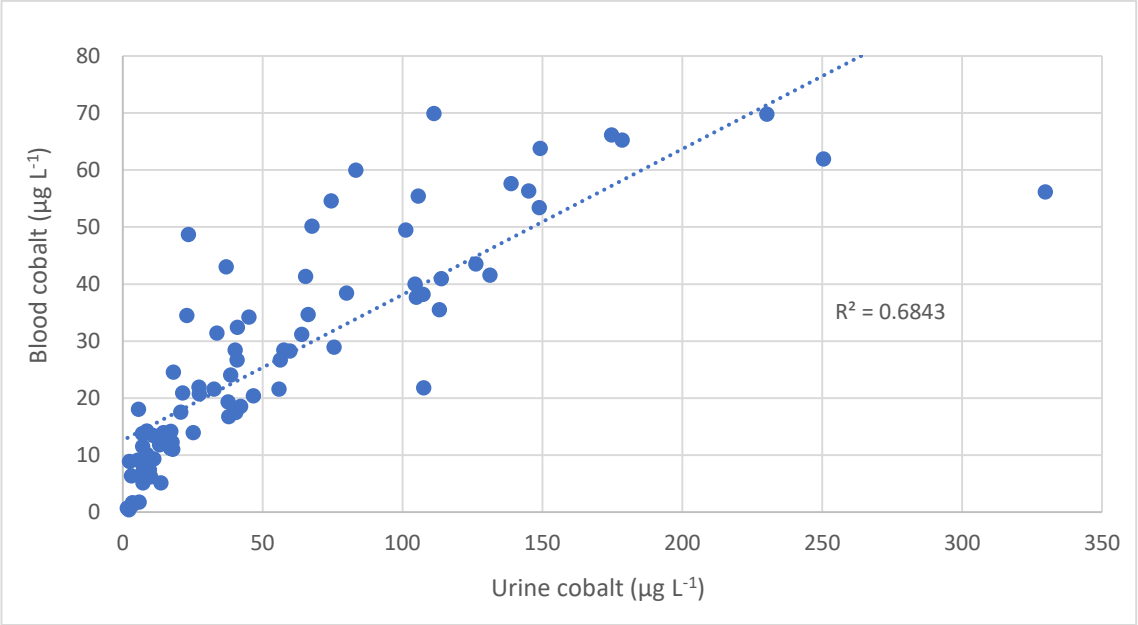

Figure 5.7 Pearson correlation between whole blood cobalt and urine cobalt concentrations for each sample collected from the 2 horses in the different treatment groups.

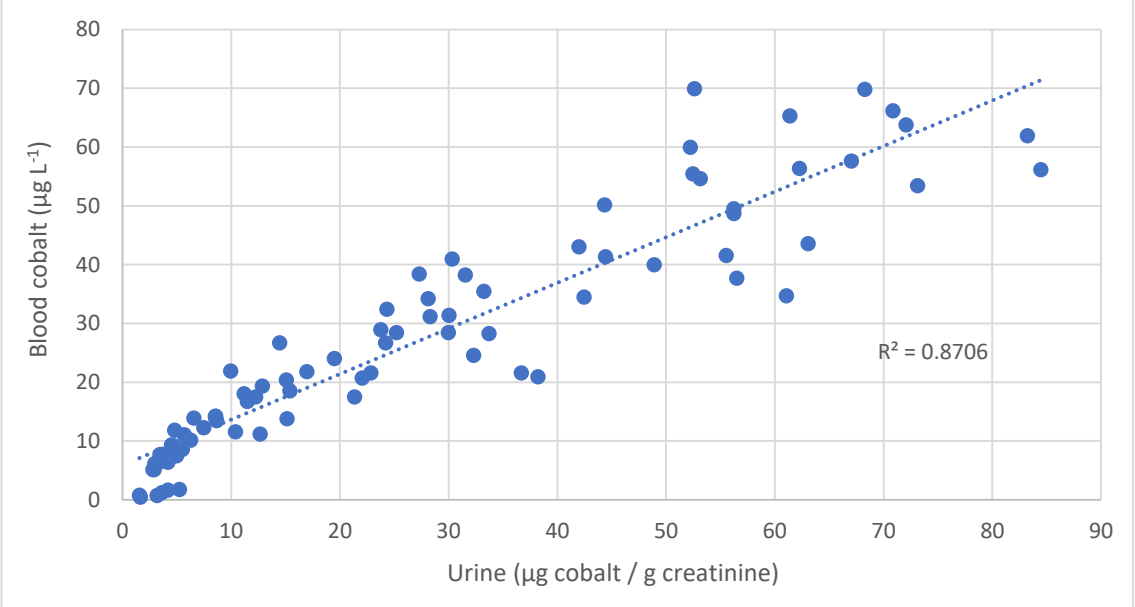

Figure 5.8 Pearson correlation between whole blood cobalt and creatinine adjusted urine cobalt concentrations for each sample collected from the 2 horses in the different treatment groups.

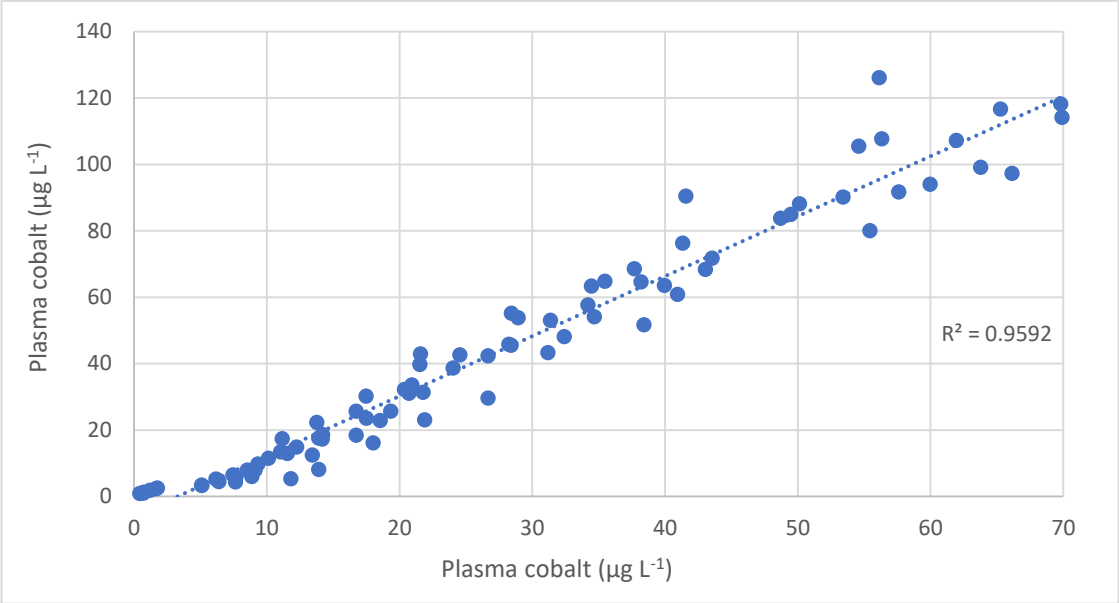

Figure 5.9 Pearson correlation between plasma cobalt and creatinine adjusted urine cobalt concentrations for each sample collected from the 2 horses in the different treatment groups.

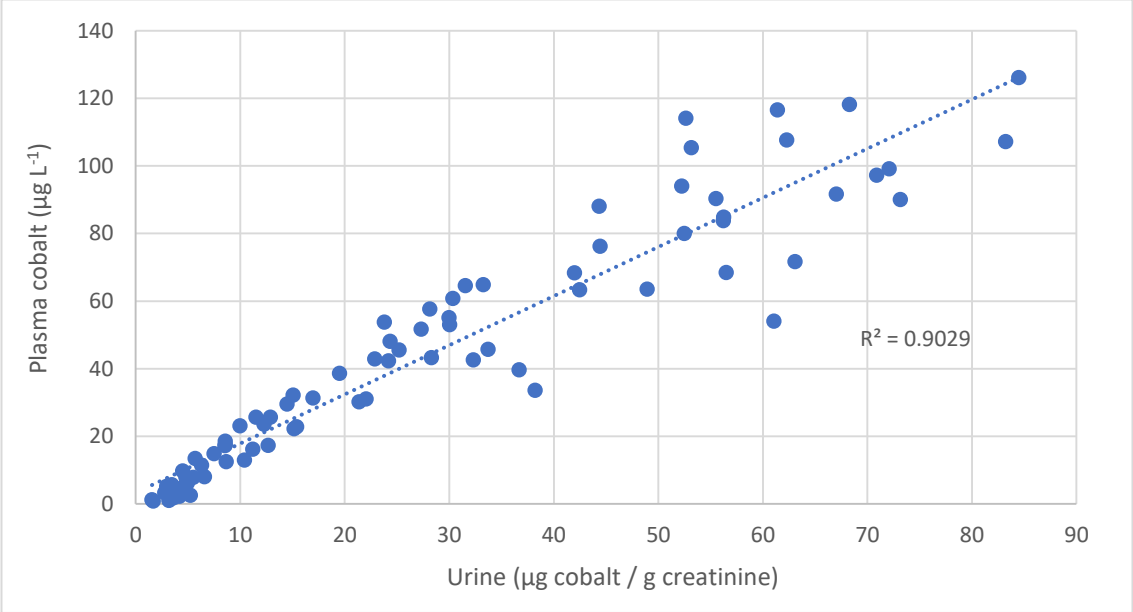

Figure 5.10 Pearson correlation between plasma cobalt and creatinine adjusted urine cobalt concentrations for each sample collected from the 2 horses in the different treatment groups.

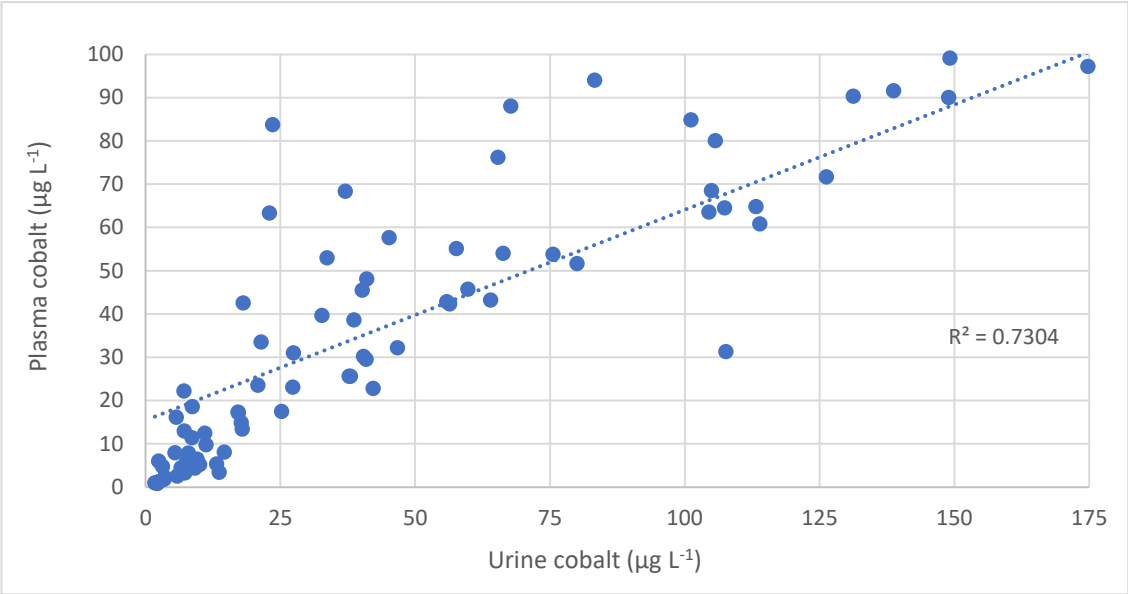

Figure 5.11 Pearson correlation between plasma cobalt and whole blood cobalt concentrations for each sample collected from the 2 horses in the different treatment groups.

5.6 Discussion

To account for differences between animals, each dosage level was administered to 2 horses that were sampled multiple times over 18 weeks. When graphing this data, the measurement uncertainty created a jagged line connecting the time points. By applying a statistical process to calculate the predicted measurement rather than plotting raw data, a smoothed line is generated while maintaining the same error bars to provide a graphical representation of the measurements in an easy to interpret format (Figures 5.1–5.6). The sample size used in this study was enough to demonstrate the cumulative nature of Co and compared favourably with sample numbers used in other studies in this area (Burns et al. 2018; Ho et al. 2015; Knych et al. 2014).

With continued Co administration, we anticipated a gradual increase in measured Co concentrations in the various sample matrices. However, a slight decline in Co concentration was observed at day 42. While all care was taken in the preparation and administration of the Co injections, we cannot exclude incorrect administration of the

injected solution as a cause for this deviation. There was no obvious explanation for this observation.

A prolonged elevation of plasma Co concentrations were noted with the cessation of Co administration (Figure 5.3). This elevation was consistent with the findings of Knych et al. (Knych et al. 2014). When discussing their study findings following an IV administration with a single dose of 49 mg of Co as cobalt chloride or 22 mg Co as cobalt gluconate, they stated that: 'Of particular importance when regulating the use of cobalt in performance horses is that cobalt administration appears to be associated with long-term retention, which may aid in detecting illicit administration. In the presently reported study, the gamma half-life was prolonged following intravenous administration (4.4 to 10.5 days); however, it is important to note that serum concentrations were above the pre-dose concentration were still easily quantifiable in the last sample collected.' I agree with this statement but find the next part of their discussion to be a tenuous extrapolation for the duration of Co retention in horses with the authors stating that: 'The elimination half-life of inorganic cobalt in humans varies greatly from study to study and appears to be dependent upon the duration of sample collection (Leggett 2008). Reports of very prolonged biological half-lives for cobalt in humans are common (Beleznay & Osvay 1994; Letourneau et al. 1972; Newton & Rundo 1971; Smith, Edmonds & Barnaby 1972). In one study, following a single intravenous dose to humans, 40 % of the administered cobalt was excreted during the first 24 h post administration and 70 % within one week (Smith, Edmonds & Barnaby 1972). In that same study, 10 % of the administered dose was still present one-year post cobalt administration (Smith, Edmonds & Barnaby 1972).' These studies have no bearing on Co concentrations in urine or serum as they were conducted on body radioactivity measurements. They are misleading presented when read in the context of this report to give the impression that Co administration could be detected for over a year. There is no evidence to suggest that urine or serum Co measurements could be used to define the percentage retentions of Co that were described when using body radioactivity measurements.

It was clear from this study and the work of others such as Ho et al. (Ho et al. 2015) that low level exposure to Co salts can cause transient peaks in raw urine Co levels sufficient

to breach the rules of racing. This has been demonstrated by oral and parenteral administration. Likewise, Knych et al. (Knych et al. 2014) showed that an acute 49 mg Co administration could breach the international threshold for around 48 h.

The Co levels administered in this study exceeded those commonly used in supplementation regimes. The levels followed on from the single administration trials conducted by Knych et al. (Knych et al. 2014). Although some trainers in the past have documented weekly administration of up to 44 mg per week, this has included oral supplementation. The absorption of oral Co in horses has received little study.

Despite the relatively high chronic Co dosage, only one treatment group (25 mg twice weekly) breached the internationally recognised Co threshold in **urine** (100 μg L^{-1}) at the time of testing. On the other hand, all treatment groups were consistently above the international **plasma** threshold after 2 weeks and remained over 25 μg L^{-1} for at least 2 weeks after Co administration had ceased. These findings indicate that at their current level, urine Co determinations are an ineffective means of detecting potential Co misuse. Data from our study group of 6 horses suggest that to monitor and regulate potential "doping levels" of Co in horses requires measurement of blood and plasma levels.

The benefit of adjusting for variations in urine concentration are shown by the improved correlation coefficients obtained when calculations were performed using creatinine adjusted urine Co results compared to those made using total urine Co measurements (Table 5.2). Kynch et al. (Knych et al. 2014) made no allowances for urine concentration variations in response to hydration status fluctuations. Consequently, from the results presented in Table 5 of their paper, it can been seen that the horse having received a single Co gluconate injection presented with a higher urine total Co concentration in the sample collected 126 h after Co administration compared to the sample collected 96 h post injection. If Co results were adjusted using a commonly applied indicator of urine concentration such as creatinine, specific gravity or osmolality, this apparent anomalous increase in urine Co could be corrected. Any attempts to define prior Co administration based on the pharmacokinetic factors presented by Knych et al. (Knych et al. 2014) are flawed where no allowances have been made to changes in the proportion of water in

urine. For example, differences in total Co concentrations between urine collected from a specific horse as pre- and post-race samples do not provide evidence of dose or duration from prior Co exposure. Rapid changes in urinary Co excretion between pre- and post-race samples may simply reflect the variables that can impact an individual horses' level of hydration. Extreme of hydration are possible over the course of a race meeting. Horses may be tested pre-race after little or no access to drinking water, along with periods of profuse sweating during transport to racetracks, and then tested again post-race (only an hour two later) after coming back to the stables and given unlimited access to drinking water. Changes in hydration will change the proportion of water and therefore relative Co concentration of urine. The importance of assessing urine concentration when determining urinary Co excretion is covered in detail in Chapter 6.

Based on the demonstrated persistence of Co in red blood cells, Co concentrations in this matrix can provide additional information on cobalt supplementation by extending the window to detect Co post administration. The results in Figure 5.5 indicate that Co quantity and not exposure duration had the greatest impact on how much Co was bound within red cells. It is also worth noting that unlike the urine, plasma and whole blood Co levels that dropped once Co administration was ceased, the concentration of Co in red cells remained elevated. The persistently high Co levels in red cells following chronic Co administration was consistent with previous research which indicates that Co is sequestered within the cell for the life of the red cell (Simonsen et al. 2011). From this study's duration we were not able to predict the effects of continued administration beyond 7 weeks, or the eventual return to baseline. Long term high level "administration" such as is proposed but not proven to enhance performance would be reflected in persistently elevated red cell Co.

In our 6 study horses, we have demonstrated that after regular Co dosing over 7 weeks, no change in haematocrit was observed. This finding was consistent with that of Knych et al. (Knych et al. 2014) and Burns et al. (Burns et al. 2018), bringing into question the validity of the proposed "performance enhancing effect" of Co salts, namely stimulation of erythropoiesis.

Given the animal welfare concerns of Co administration coupled with mounting scientific evidence that Co salts are neither useful nor necessary in horses, a continuing public education campaign is required. This approach was supported by Mobasheri (Mobasheri & Proudman 2015) who stated that Co is 'potentially lethal'. The levels used in this study are a small fraction of those used by Burns et al. (Burns et al. 2018) when they reported acute toxic signs (up to 2000 mg of cobalt chloride per horse). In our study the maximum blood Co levels recorded were under 70 μg L^{-1}, a fraction of the human chronic toxic threshold of between 300 and 800 μg L^{-1} proposed by Finley et al. (Finley et al. 2012).

5.6.1 Limitations of this study

Cobalt uptake, distribution, metabolism, and excretion in the racing horse is a complex topic. This study investigated selected aspects of this topic and its limitations include:

- The effects of biological variability on Co accumulation were not assessed within the study design. Only mares were used and there were insufficient horses supplemented to stratify aspects of biological variability. Given the demonstrated accumulation of Co, further studies would be warranted to determine if factors such as gender and age influence the rate of Co uptake and storage.
- The subject herd of 8 horses was maintained at pasture and was not subjected to the stresses of training, transportation, variable conditions of nutrition, hydration, and racing. A larger cohort of horses incorporating these variables would be a valuable addition when assessing current regulatory thresholds.
- Only a single cobalt-containing substance (cobalt chloride) was studied. There is a complex and largely unstudied relationship between the various Co sources to which the racehorse is environmentally and nutritionally exposed, including vitamin B12 and its synthetic analogues.
- The administration was conducted over 7 weeks and could not completely replicate the long-term Co intake of horses in training. Cobalt and B12 are known to be stored in body tissues for months and years.

5.7 Conclusion

This study shows that with regular administration, there is considerable accumulation of Co. The current IFHA Co threshold of 100 µg L^{-1} in urine is a poor predictor of potential Co misuse and has no benefit over plasma Co determinations. The current urine Co threshold failed to identify chronic Co dosing while the plasma Co concentration remained consistently above the IFHA threshold of 25 µg L^{-1}. Plasma or whole blood Co determinations should therefore be used to assess excess Co administration. Red cell Co measurements provide a useful addendum when investigating chronic Co abuse as Co persists in red cells for as long as they remain in circulation.

5.8 Magazine article - *Cobalt Misuse in Horses is 'Ill-Conceived'*

The following article typifies the general interest amongst horse owners in the research presented in this thesis. Shortly after publishing the paper 'Cobalt accumulation in horses following repeated administration of cobalt chloride' (Wenzel et al. 2019), I was contacted by US based freelance medical writer Dr Stacey Oke. This resulted in the following article being published for the mainstream magazine "The Horse: Your Guide To Equine Health Care" (Oke 2019). The article has been reproduced below –

Researcher: Cobalt Misuse in Horses is 'Ill-Conceived'

Blood testing provides a clearer picture of potential cobalt misuse.

Posted by Stacey Oke, DVM, MSc | Nov 15, 2019 |

Since Georg Brandt discovered Co in 1793, people have exploited its virtues for a wide range of uses, from paint pigmentation with its rich blue color to enhancing human athletic performance. Because some racehorse trainers in the United States and Australia have abused Co in horses, an Australian research team recently investigated Co concentrations in urine and blood after chronic Co administration to determine whether current regulatory drug detection thresholds proved useful for this purpose.

"Cobalt is an essential trace element required to synthesize vitamin B12, which plays a vital role in cellular metabolism and DNA synthesis," said Ross Wenzel, GradDip

(ClinBiochem), MAppSc (Thesis) of the Trace Elements Laboratory at the Royal North Shore Hospital, in New South Wales.

An average 500-kg (roughly 1,100-lb) working horse, fed approximately 2 % of its body weight in forage per day, requires 0.1-0.15 mg Co kg^{-1} dry feed daily. These levels typically can be met through diet alone and, because no known reports of Co deficiency exist, equine nutritionists advise a maximum of 25 mg kg^{-1} dry matter intake.

"The rationale for supplementing horses with Co pertains to a presumptive increase in red blood cell production in response to Co indirectly inducing hypoxia," Wenzel said. In turn, more oxygen will be delivered to exercising muscles to maximize energy production and enhance performance."

In other words, some people believe that giving Co indirectly diminishes the amount of oxygen reaching the muscles, causing the body to compensate, producing more red blood cells to deliver more oxygen to the muscles.

"Research has failed to support this presumption," he added. "There is no evidence to suggest that Co will stimulate a hypoxic response in excess of that naturally achieved in training."

"With the range of unnecessary cobalt-containing supplements on the market, the need to develop a testing regime capable of differentiating Co misuse from regular supplementary intake is apparent," said Wenzel.

He explained that his group pursued the study "on the initiative of study co-author and equine veterinarian Derek Major in response to a high rate of racing Co infringements where anecdotally, up to 500 mg of cobalt chloride were being administered intravenously.

To better understand how horses metabolize Co, Wenzel and co-workers administered 25-50 mg cobalt chloride intravenously once to twice weekly for 10 weeks. They assessed both urine and blood Co concentrations routinely during the study period and again 81, 106, and 127 days after its commencement.

“We found marked Co accumulation throughout the study, with increasing cobalt concentrations in collected samples as the study progressed. Cobalt levels in red blood cells remained elevated for at least 12 weeks after intravenous administration, which is consistent with the lifespan of red blood cells,” said Wenzel.

Throughout the study the researchers found that only blood analysis, not urine testing, identified Co levels exceeding the respective international thresholds and, therefore, appeared to be a better means of assessing Co misuse in horses.

“Urine samples collected on race day from horses suspected of cobalt doping may no longer exhibit elevated levels from Co administered in the previous weeks,” he said, explaining that any perceived hypoxic benefit attained from cobalt administration would take several days to be realized, and they were only able to detect very recent exposure to cobalt in urine. “Hence, only those jurisdictions determining blood cobalt levels are effectively screening for nefarious cobalt use,” he said.

He said urine Co determinations are disadvantaged for other reasons: Urine Co concentrations vary markedly with hydration levels, and that urine can potentially be contaminated with environmental sources of Co during collection. He also said that urine “can return elevated cobalt levels from the innocuous cobalt contained in vitamin B12, with excess amounts of this compound excreted in urine”.

“Considering that Co does not actually have any documented performance-enhancing effects and does not appear to stimulate the production of red blood cells in racehorses, the misuse of Co is ill-conceived,” said Wenzel, adding, “Excess cobalt is neither useful nor necessary. Public education is needed on this topic.”

You can find the study, “Cobalt accumulation in horses following repeated administration of cobalt chloride,” in the *Australian Veterinary Journal*, Volume 97, No. 11, November 2019, p 465–472.

6. INFLUENCE OF HYDRATION STATUS ON URINARY COBALT CONCENTRATION

6.1 Preamble

The threshold for urinary Co concentrations in horses was evaluated as an epidemiological study from a population of racehorses. All variables such as age, gender, diet, hydration status, genetic variability, etc. were averaged across the population and can therefore only be compared to other populations where sample numbers are also large enough to average these variables. For example, the urinary Co concentration in a population of racehorses in Australia may be 10 μg L^{-1} compared to a population of racehorses in New Zealand with a urinary Co concentration of 5 μg L^{-1}. From this comparison it could be concluded that the average concentration of Co in horses from New Zealand was half that of horses from Australia. The same comparison could not be made against an individual horse as the variables that had been averaged across these populations are no longer in place. Considering the variability that extremes of hydration can introduce to urine analyte concentrations, compliance to an uncorrected threshold in some cases can be attributed more too good luck than good management.

6.2 Introduction

When comparing an individual to a population, variability can be addressed by recognising the source of variability and applying an appropriate adjustment. For example, if age were found to significantly alter the concentration of an analyte then the population could be stratified into groups based on age. When using urine to assess the concentration of solutes excreted by a healthy individual, a significant physiological variable affecting diurnal changes in urine concentration occurs in relation to the volume of water excreted. With decreasing hydration, the kidney excretes less water causing the concentration of solutes in urine to increase. Conversely, with increasing hydration the kidney excretes more water. This causes a decrease in the urine concentration of solutes as the urine becomes more dilute. The most accurate approach to eliminate the influence of this diurnal variation is to collect all the urine excreted during the day and express results as concentration of analyte excreted per day. Timed urine collections are

considered the “gold standard” procedure for normalisation to account for variations in hydration status and diurnal variation (Heavner et al. 2006). However, 24 h urine collections are onerous to perform in humans and an impractical means of collecting urine from horses.

An alternative means of compensating for variable degrees of hydration is to collect spot or random urine samples and adjust the concentration of analyte in both the population and individual to a unit of measurement that incorporates the proportion of water. Analyte result per unit of specific gravity (SG) or osmolality have been used for this purpose. Another approach relies on adjusting for urine creatinine concentration (Boeniger, Lowry & Rosenberg 1993). Creatinine is an endogenous metabolite excreted in urine at a constant rate therefore can be applied to adjust measurements from individuals for comparison to population derived values. The creatinine adjusted urine Co is determined by measuring the concentration of Co (micrograms Co per litre of urine) and dividing this by the concentration of creatinine (grams creatinine per litre of urine). This calculation is the most widely used method of adjusting for dilution with results reported as weight of analyte (in this case Co) per gram of creatinine (Barr et al. 2005).

The concentration of an analyte in urine when measured in units of mass per volume can vary by a factor of 10 with normal diurnal changes proportional to the amount of water present. At extremes of hydration or dehydration, diurnal variations in concentration by factors of 20 or 30 are possible. In this Chapter:

- The effects of hydration status on urinary Co concentrations in horses are investigated.
- The advantages and disadvantages of the various methods used to correct for hydration status are discussed.
- A method for calculating SG adjusted Co from creatinine adjusted Co is validated.
- Results of creatinine measurements performed on horses found to have breached the Co rule are tabulated to gauge the proportion of horses exceeding the threshold that may have done so through dehydration.

6.3 Materials and methods

6.3.1 Determination of creatinine concentration and specific gravity

This investigation was undertaken to assess the relationship between creatinine and specific gravity in equine urine. Samples collected for the study presented in Chapter 5 were used. These urines had been collected by direct aspiration with an equine Infusette® pipette and placed in 50 mL polypropylene sample containers (Thermo-Plas, Adelaide, SA, Australia). One aliquot was decanted from each container and retained in a 5 mL screw cap polypropylene tube (Thermo-Plas, Adelaide, SA, Australia) for specific gravity determination and subsequently stored at -20°C. Specific gravity was measured on the duplicate aliquot at the time of collection using a hand-held refractometer (Brix refractometer, Australian Scientific, Kotara, NSW, Australia). Samples for creatinine were obtained by pouring off an aliquot of around 4 mL from the primary urine sample container into 5 mL screw cap polypropylene tubes. These samples were centrifuged at 1250 g for 5 min at room temperature to remove any particulate material. Creatinine measurements were performed in the NSW Health Pathology Clinical Biochemistry Laboratory, RNSH, St Leonards, NSW, Australia using the Jaffe reaction method (Abbott Laboratories 2006) on an Architect c9000 System (Abbott Laboratories, Abbott Park, IL, USA). Statistical evaluation of results was carried out by means of variance analysis and regression coefficient *r*. Significance was assumed from $P < 0.05$.

6.3.2 Determination of creatinine in urine samples exceeding the cobalt threshold

Urine samples from horses found to have breached the Co rule that resulted in disciplinary charges being laid against their trainers were tested to confirm the Co concentrations and determine the creatinine concentrations. These samples had been collected by racing stewards from various locations. Collection, storage, and secure transport of samples were as per procedures typically defined in the rules of racing (Racing Animal Welfare and Integrity Board 2012). Samples had initially been sent to racing regulator approved laboratories for Co testing. In response to Civil and Administrative Tribunal orders, samples were securely transported to the trace elements laboratory at RNSH for additional testing including total Co and creatinine determinations. Storage conditions of samples when not located at RNSH were unknown though samples are generally thought to have

been kept frozen for long term storage. Methods of analysis for total Co and creatinine were as described above and in Chapter 3. Creatinine adjusted results were calculated by dividing the total Co concentration measured in μg L^{-1} by the creatinine concentration measured in g L^{-1}.

6.4 Results

Results for creatinine concentrations and SG are plotted in Figure 6.1. A good correlation ($r = 0.90$, $P < 0.001$) was observed between the ln of creatinine and specific gravity. Table 6.1 shows the Co and creatinine results on samples that had been found to exceed the Co threshold after testing at racing regulator approved laboratories. In this table, SG results were calculated from the correlation points plotted in Figure 6.1 using the equation y = 0.0188ln(x) + 1.023 where y = SG and x = creatinine concentration. Creatinine adjusted Co results were calculated by dividing the total Co concentration by the creatinine concentration. Standardised SG adjusted Co concentrations were calculated using the equation (Levine & Fahy 1945) of $C_{st} = C_m \times ((SG_{ref} - 1)/(SG_{meas} - 1))$ where C_{st} is the urinary analyte adjusted to a standardised SG concentration; C_m is the measured analyte value before adjustment; SG_{ref} is the reference value (in this case 1.0337 as the indicated median SG for racehorses) to which analyte concentrations are normalised; SG_{meas} is the SG measured in a given specimen.

6.5 Discussion

6.5.1 Creatinine

Of the three commonly applied techniques to adjust for variations in hydration status, creatinine corrections have found the most widespread use (Cocker et al. 2011; Middleton, Watts & Polya 2019). In mammals, creatinine is a waste product of normal muscle metabolism that is formed at a reasonably constant rate and excreted in urine. Phosphocreatine, an energy-storing molecule in muscle, undergoes spontaneous cyclisation to form creatine and inorganic phosphorous. Creatine then decomposes to creatinine (Borsook & Dubnoff 1947). Creatinine is filtered freely through the glomerulus and not reabsorbed in the renal tubules making creatinine a reliable measure of glomerular

filtration rate. Creatinine can therefore be used to account for fluctuations in urine dilution, assuming a given urine analyte is excreted in a similar fashion to creatinine such as other nonpolar chemicals or metabolites. By dividing the measured urinary concentration of an analyte by the urinary creatinine concentration it is possible to reduce the effect of hydration when using urine for quantitative measurements.

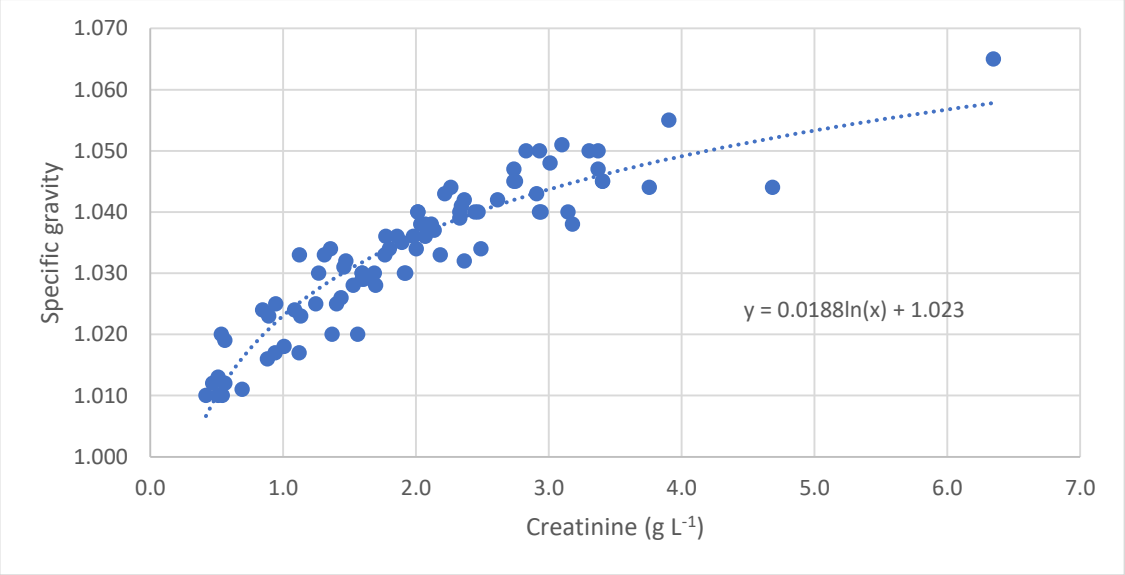

Figure 6.1. Correlation between creatinine and specific gravity determined in 85 urine samples collected during the study presented in Chapter 5. A logarithmic trendline has been calculated from unadjusted urine creatinine (x-axis) and specific gravity (y-axis) results. Specific gravity (y) = 0.0188 ln creatinine (x) + 1.023; $r = 0.90$, $P < 0.001$.

When evaluating equine physiology, creatinine concentrations in body fluids are typically determined by a modified kinetic Jaffe reaction (Toribio 2007). The method used to determine creatinine in these samples was no exception (Abbott Laboratories 2006). Reagent preparations for measuring creatinine are the same across mammalian species. In the Jaffe reaction used here, urine samples are taken to an alkaline pH where creatinine in the sample reacts with picrate to form a creatinine-picrate complex. The rate of increase in absorbance at 500 nm due to the formation of this complex is directly proportional to the concentration of creatinine in the sample.

Table 6.1. Cobalt and creatinine results on 12 urine samples from horses found to have exceeded the Co threshold. Specific gravity (SG) results were calculated from the correlation points plotted in Figure 6.1 using the equation y = 0.0188ln(x) + 1.023 where y = SG and x = creatinine concentration. Creatinine adjusted Co results were calculated by dividing the total Co concentration by the creatinine concentration. Standardised SG adjusted cobalt concentrations were calculated using the equation (Levine & Fahy 1945) of $C_{st} = C_m$ x $((SG_{ref} - 1)/(SG_{meas} - 1))$ where C_{st} is the urinary analyte adjusted to a standardised SG concentration; C_m is the measured analyte value before adjustment; SG_{ref} is the reference value (in this case 1.0337 as the indicated median SG for racehorses) to which analyte concentrations are normalised; SG_{meas} is the SG measured in a given specimen. Variation is the percent difference between the uncorrected raw total Co measurement and the standardised SG adjusted Co concentration.

Sample	Creatinine	SG	Total cobalt	Creatinine adj. cobalt	Standardised SG adj. cobalt	Variation
	(g L^{-1})		(µg L^{-1})	(µg Co g^{-1} creat.)	(µg L^{-1})	
1	3.4	1.046	141	41	103	-27 %
2	3.7	1.048	563	153	399	-29 %
3	0.6	1.013	180	306	466	159 %
4	2.8	1.042	116	41	92	-21 %
5	4.4	1.051	175	39	116	-34 %
6	2.3	1.039	337	145	292	-13 %
7	1.7	1.033	152	88	154	1 %
8	3.7	1.047	189	52	134	-29 %
9	4.4	1.051	183	41	121	-34 %
10	3.7	1.047	107	29	76	-29 %
11	5.8	1.056	125	21	75	-40 %
12	3.8	1.048	112	30	79	-29 %

6.5.2 Osmolality

The "gold standard" for estimating urinary concentration is the measurement of urine osmolality (Chadha, Garg & Alon 2001; Middleton et al. 2016). Osmolality is the measure of total solute concentration which is dependent only on the number of particles in the solution under study. It is not affected by urine temperature or the size or charge of the particles. In the laboratory, osmolality is usually measured by freezing point using a

cryoscopic osmometer. The specimen needs to be centrifuged to remove any particulate matter and a drop of the specimen is then loaded in the instrument which measures its freezing point and converts it directly to the osmolality reading. The SG of urine can be estimated from osmolality by the following equation: mOsm kg^{-1} H_2O = (SG – 1.000) × 40000 (Miller 1983). Osmolality is a more precise indicator of the diuretic state of the racehorse than urine SG (Bosken et al. 2003) but was not used in this study as urine osmolality has been shown to decrease when samples are frozen for storage (Adams et al. 2017).

6.5.3 Specific gravity

Specific gravity adjustments are an attractive alternative to those using creatinine or osmolality as SG measurements can be performed rapidly at the site collection using a refractometer or reagent strips. It takes 45–60 sec for a SG reagent strip dipped in urine to indirectly measure SG from the ionic strength of the sample. A polymer of methyl vinyl ether/maleic anhydride in the reagent strip facilitates a pH change in response to salts in urine (Chadha, Garg & Alon 2001). SG is measured from colour change in a pH indicator of bromothymol blue read from a colour chart or strip reader. SG reagent strips and creatinine determinations have been reported as equally suitable methods to adjust for hydration status (Singh et al. 2015). Portable, hand-held refractometers are easy to operate and measure the proportion of dissolved solids in urine. As the amount of dissolved solute increases, so too does the refractive index of a solution. Refractometers provide a unit less measurement relative to the SG of pure water that has been assigned a value of 1.000. While convenient for use in the field, refractometers often lack the quality monitoring steps mandated for accredited testing methods. Refractometers typically give measurements in increments of 0.001, SG reagent strips in increments of 0.005 and creatinine measurements are typically reported in increments of 0.1 g L^{-1}. SG refractometers can overestimate hydration. Unlike osmolality, which is only affected by the number of particles, refractometry is affected by the number, mass, and chemical structure of the dissolved particles. When compared with urinary osmolality, SG is influenced by proteinuria so that for each 10 g L^{-1} protein the SG increases by 0.003 and SG is influenced by glucosuria so that SG increases by approximately 0.002 per 10 g L^{-1} glucose (Chadha, Garg & Alon 2001).

6.5.4 Sample stability

Urine osmolality and SG have been shown to remain stable at room temperature for 1 day and when refrigerated at 7 °C for up to 7 days but freezing at -20 and -80 °C significantly decreased the values of these hydration markers (Adams et al. 2017). In all but extreme circumstances, urine creatinine is unaffected by storage time and temperature with significant decreases in urine creatinine levels only noted after storage for 30 days at 55 °C (Spierto et al. 1997). Creatinine deterioration has been demonstrated through repeated freezing and thawing cycles (Garde, Hansen & Kristiansen 2003). Stability is a critical factor given the range of collection sites and transport conditions to be considered when collecting samples from horses. Given that samples are frozen after testing to comply with accreditation requirements on the duration of sample storage, creatinine is the most appropriate means to assess hydration status from these specimens.

6.5.5 Validation of hydration correction method

When deciding on the most appropriate means to correct for hydration status, several factors were considered. Complexity of instrumentation dictates that both osmolality and creatinine measurements are performed in a laboratory. Of these two techniques, osmolality is considered the most accurate (Yeh et al. 2015). However, storages changes can affect osmolality and SG making creatinine the preferred laboratory-based method. When comparing creatinine and SG, the ease and versatility of being able to measure SG at the point of collection make this the most attractive method. Both urinary creatinine and SG have been shown to be valid and interchangeable correction standards for dilution (Heavner et al. 2006; Muscat, Liu & Richie 2011) though proponents of creatinine correction should be aware that age, sex, muscle mass and time of the day can influence creatinine excretion (Cocker et al. 2011; Sauve et al. 2015). A case therefore exists for both creatinine and SG measurements with SG determinations made at the point of collection where possible and creatinine determinations used to confirm SG results when required. The confirmatory test must be suitable for samples that have been frozen in line with the long-term storage requirements of samples tested for drug screening.

To validate creatinine adjustment as a confirmatory technique for SG adjustment in equine urine, measurements on SG and creatinine in equine urine were compared to a previously established mathematical formula (Carrieri, Trevisan & Bartolucci 2001). This formula was derived from 534 spot urine samples collected from 385 men and 149 women with a mean creatinine of 1.69 g L^{-1} (range 0.16–4.36 g L^{-1}) and mean SG of 1.022 (range 1.002–1.037). The correlation between creatinine and SG for this group was $y = 0.0098\ln(x) + 1.018$; $r = 0.82$ and $P < 0.001$. The data set for 85 equine urine samples had a mean creatinine of 1.22 g L^{-1} (range 0.42–6.35 g L^{-1}) and mean SG of 1.024 (range 1.010–1.065). The correlation between creatinine and SG for this group was $y = 0.0188\ln(x) + 1.023$; $r = 0.90$ and $P < 0.001$ (Figure 6.1). Both groups displayed a logarithmic distribution with a significant correlation between creatinine and SG.

With the correlation between creatinine and SG confirmed in equine urine, the correlation between creatinine and standardised SG adjusted Co concentrations were investigated. SG adjustments were standardised to a SG of 1.024 using the formula $C_{st} = C_m \times 0.024/SG - 1.0$ where C_{st} is the urinary analyte adjusted to a standardised SG concentration and C_m is the measured value before adjustment (Levine & Fahy 1945). The correlation between Co values adjusted to corresponding values of creatinine and standardised SG determined in 85 urine samples collected during the study presented in Chapter 5 was $y = 1.38x$; $r = 0.97$ and $P < 0.001$ (Figure 6.2). This ratio was less than 7 % different to that found when correlating hypothetical values adjusted to corresponding values of creatinine or SG determined in 534 spot urine samples collected from 385 men and 149 women where $y = 1.48x$; $r = 0.88$ and $P < 0.001$ (Carrieri, Trevisan & Bartolucci 2001). Standardisation to a SG of 1.024 can therefore be considered appropriate in horses. If ratios were to match exactly then a SG adjustment to a standardised SG of 1.0257 would be required. This slightly higher standard SG in horses may indicate that horses excrete relatively more creatinine in urine than do humans. Alternatively, with such a small difference between ratios, the difference may simply indicate an analytical bias in the method used to measure creatinine and SG or a combination of biases from both methods.

It can be concluded that the correction techniques applied to human urine samples to adjust for variations in hydration status can be equally applied to equine urine samples. The linear response obtained when applying these correction methods to Co (Figure 6.2) also confirms that Co is a suitable element for creatinine correction, showing the same rate of glomerular filtration as creatinine with a negligible amount of tubular secretion.

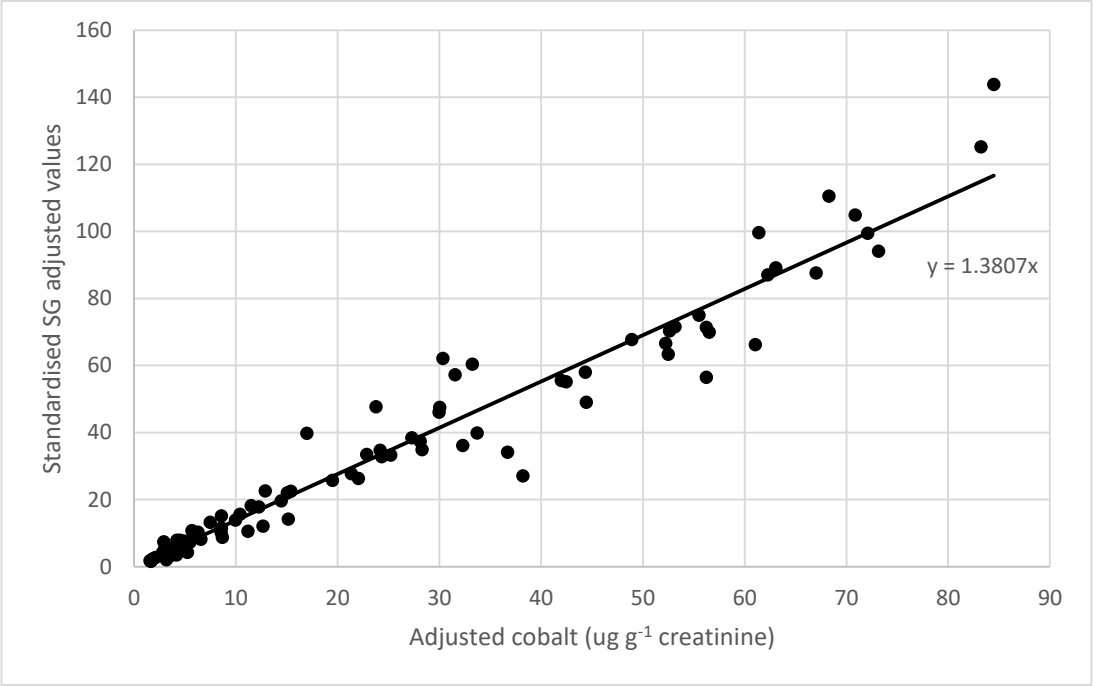

Figure 6.2. Correlation between cobalt values adjusted to corresponding values of creatinine and standardised specific gravity (SG) determined in 85 urine samples collected during the study presented in Chapter 5. y = 1.38x; $r = 0.97$, $P < 0.001$.

6.5.6 Derivation of a hydration corrected urinary cobalt threshold

Urine SG is expected to vary considerably between and within individual horses in response to diurnal changes in overall hydration status. If Co concentrations in individual horses are to be accurately compared to a threshold defined from a population of horses, then the hydration status of this population must also be defined. Ideally this would be calculated from SG measurements taken from the same samples used to define the Co threshold. However, as these measurements were not made, SG measurements from published studies must be referred to. As anticipated for a measurement incorporating a

wide range of hydration states attainable in normal health, considerable variation in SG means have been reported.

From the 85 SG measurements made on the horses studied in Chapter 5, results ranged from 1.010–1.065, mean 1.024 ± 0.012 SD. The SG was shown to vary between 1.005 and 1.055 in urine samples collected from 1669 normal horses in the USA (Cohen et al. 2002). There is no equivalent data from urine collected from horses in Australia. Urine SG reference intervals of 1.025–1.050 (Reed, Bayly & Sellon 2009) and 1.025–1.060 (Knottenbelt 2006) have been reported. In an earlier study to define reference ranges in normal adult horses, urine SG results in 65 horses ranged from 1.003–1.051, mean 1.030 ± 0.011 SD (Edwards, Brownlow & Hutchins 1989). The mean SG of 6–7 endurance horses undertaking various training and riding schedules was 1.030–1.047 and were within the provided SG reference range of 1.020–1.050 (Robert et al. 2010). In a study where 24 h urine samples were collected from 11 horses, a mean SG of 1.028 was found (Rumbaugh, Carlson & Harrold 1982). Diet has also been shown to influence urine SG with concentrated urine having a SG greater than 1.035 as noted in horses fed fibrous diets with a high percentage of hay (Savage 2008). A 60 to 72 h water deprivation test, sufficient to decrease mean body weight by 13.5 %, was conducted on 6 horses with initial urinary SG results ranging from 1.026–1.044. Post water deprivation, maximum urine SG concentrations ranged from 1.042–1.054, mean 1.050 ± 0.004 SD (Brobst & Bayly 1982). This paper noted that with water deprivation sufficient to produce a 12–15 % decrease in body weight, 95 % of healthy horses should have a urinary SG of at least 1.042.

The spread of SG results in response to diet, training and water deprivation highlight the need to refine a mean SG for calculating a SG-adjusted Co threshold in racehorses. From a paper investigating the effect of furosemide on osmolality and SG, 114 untreated thoroughbred racehorses were stratified into 4 groups where a mean and median urinary SG of 1.0337 ± 0.010 SD was obtained (Bosken et al. 2003). In races of 1.6 km or more, 18 winners (first three finishers), had a SG of 1.0346 ± 0.0100 SD while 34 nonwinners had a SG of 1.0308 ± 0.0095 SD. For sprint races of 1.3 km or less, 23 winners were found to have a SG of 1.0360 ± 0.0102 SD while 39 nonwinners had a SG of 1.0345 ± 0.0114 SD. The study found no seasonally significant differences in the 4 groups

of horses, with mean SG differences differing by only 0.0052, the mean urinary SG of 1.0337 from the racehorses that had not been treated with furosemide was considered appropriate to use when calculating a SG adjusted Co threshold. Therefore, standardised SG adjusted Co concentrations suitable for comparison to the threshold can be calculated using the equation (Levine & Fahy 1945) of $C_{st} = C_m \times ((SG_{ref} - 1)/(SG_{meas} - 1))$ where C_{st} is the urinary analyte adjusted to a standardised SG concentration; C_m is the measured analyte value before adjustment; SG_{ref} is the reference value (in this case 1.0337 as the indicated median SG for racehorses) to which analyte concentrations are normalised; SG_{meas} is the SG measured in a given specimen.

To evaluate the effect of using a Co threshold standardised to SG, this calculation was retrospectively applied to Co and creatinine results on 12 urine samples from horses found to have exceeded the Co threshold. These results appear in the second last column of Table 6.1 where it is apparent that several horses have only infringed the population defined Co threshold of 100 µg L^{-1} where urine hydration was greater than the population median. This highlights the importance of applying a standardised SG adjustment to horses producing urine samples that are more concentrated than average. Equally, urine samples produced from over-hydrated horses that would have otherwise been found compliant can be adjusted for urine hydration. The influence that hydration status alone can have on the interpretation of a threshold level are profound. The percentage between the uncorrected raw total Co measurement and the standardised SG adjusted Co concentration in just 12 horses ranged from – 40 % to + 159 % (Table 6.1). Clearly the use of an adjustment to correct for variations in hydrations status would result in a more scientifically sound approach to screening for potential Co misuse.

Of the 3 different approaches used to adjust for urine hydration (Table 6.1), a standardised SG adjusted Co was the most useful given the circumstances whereby the Co threshold was derived. Creatinine adjusted Co measurements cannot be directly compared to the threshold as they are reported in different units. The uncorrected threshold was expressed as a raw concentration in µg L^{-1} while creatinine adjusted results are in units of µg Co g^{-1} creatinine. To be comparable to the threshold, measurements of both Co and creatinine would be required when the threshold was initially established so that both the

threshold and creatinine adjusted results were expressed in units of µg Co g^{-1} creatinine. As creatinine measurement were not made, the median creatinine concentration of racehorses was estimated from the median SG of 1.0337 (Bosken et al. 2003) by applying the correlation equation established from the correlation points plotted in Figure 6.1. Using this equation of y = 0.0188 ln (x) + 1.023 where y = SG and x = creatinine, the median creatinine concentration in racehorses was 1.77 µg L^{-1}. An estimated threshold of 57 µg Co per gram of creatinine can therefore be applied. Of the 12 horses originally found to be over the threshold, 8 would now be below when corrected for creatinine. However, only 4 horses would be below the standardised SG adjusted threshold. This discrepancy highlights the potential to over correct when relying on a simple creatinine adjustment. The standardised SG adjustment, as advocated by WADA (WADA Laboratory Expert Group 2018), provides a more robust correction.

6.5.7 Can urine be used for quantitative determinations at extremes of hydration?

The necessity to determine the relative proportion of water in urine extends beyond providing a means to correct for variations in hydration status. It also provides a statement that the kidney is functioning in a reliable fashion to produce urine that is representative of the blood supply it is filtering. In cases of overhydration or dehydration, the kidney's secretion, excretion, and/or reabsorption of the target chemical can occur in an unpredictable fashion. As such, World Health Organisation guidelines for occupational monitoring (World Health Organization 1996) set exclusionary guidelines based on urine creatinine or SG results to confirm the suitability of a urine sample for quantitatively measuring a substance. These guidelines state that when urine is very dilute (SG < 1.010 or urinary creatinine < 0.3 g L^{-1}) or concentrated (SG > 1.030 or urinary creatinine > 3.0 g L^{-1}) it is unlikely that any correction will give accurate results. Given the comparative function across mammalian kidneys and similar normal hydration levels in equine and human urine (Section 6.5.5), it can be reasonably expected that the same exclusionary criteria be applied to urine samples collected to quantitatively assess cobalt excretion in horses. With the median SG of 1.034 in racehorses (Bosken et al. 2003) already over the recommended SG of 1.030, urine cobalt measurement cannot be expected to reliably provide accurate results on cobalt exposure.

6.6 Conclusion

This Chapter confirms that equine urinary creatinine and SG levels correlate. Validation as a means of correcting for changes in hydration status in equine urine was achieved by demonstrating equivalence to a study involving 385 men and 149 women (Carrieri, Trevisan & Bartolucci 2001). Hydration correction of urinary Co measurements using a SG standardised to a population of racehorses was found to be appropriate. It was consistent with the approach taken by WADA for endogenous threshold substances where normalised SG adjustments are applied to urine with a SG greater than 1.020 (WADA Laboratory Expert Group 2018). Retrospective hydration adjustments applied to racehorses that had been found to have exceeded the threshold identified several where threshold infringements were through excessive dehydration. Overall, urine was considered an unreliable matrix within which to regulate a threshold. The relatively high median urine SG found in racehorses excludes these samples from quantitative measurements due to inconsistent renal output at extremes of dehydration.

7. GENERAL DISCUSSION

7.1 Can cobalt enhance performance in trained athletes?

Section S.2.1 of the WADA 2014 Prohibited List (World Anti-Doping Agency 2014) stated that -

'*S2. PEPTIDE HORMONES, GROWTH FACTORS AND RELATED SUBSTANCES*

The following substances, and other substances with similar chemical structure or similar biological effect(s), are prohibited:

1. Erythropoiesis-Stimulating Agents [e.g. erythropoietin (EPO), darbepoetin (dEPO), hypoxia-inducible factor (HIF) stabilizers, methoxy polyethylene glycol-epoetin beta (CERA), peginesatide (Hematide)]'.

Though not mentioned specifically, cobalt would be classified as a HIF stabiliser (Al Okail 2010). As a prohibited substance it is therefore implied that cobalt can confer an unfair athletic advantage.

By 2015, WADA had updated the HIF stabiliser sub-section, placing it in a separate paragraph and specifically naming cobalt (World Anti-Doping Agency 2015b) -

'*S2. PEPTIDE HORMONES, GROWTH FACTORS, RELATED SUBSTANCES AND MIMETICS*

The following substances, and other substances with similar chemical structure or similar biological effect(s), are prohibited: 1. Erythropoietin-Receptor agonists:

1.1 Erythropoiesis-Stimulating Agents (ESAs) including e.g. darbepoietin (dEPO); erythropoietins (EPO); EPO-Fc; EPO-mimetic peptides (EMP), e.g. CNTO 530 and peginesatide; and methoxy polyethylene glycol-epoetin beta (CERA);

1.2 Non-erythropoietic EPO-Receptor agonists, e.g. ARA-290, asialo EPO and carbamylated EPO;

2. Hypoxia-inducible factor (HIF) stabilizers, e.g. cobalt and FG-4592; and HIF activators, e.g. argon, xenon'.

Six years after cobalt was first implicated by WADA as a performance enhancing drug and placed on the Prohibited List, no regulatory threshold has been introduced. Section

S.2.1 of the current WADA 2020 Prohibited List (World Anti-Doping Agency 2020) has been reformatted with cobalt remaining as a prohibited HIF activating agent -

'*S2. PEPTIDE HORMONES, GROWTH FACTORS, RELATED SUBSTANCES AND MIMETICS*

The following substances, and other substances with similar chemical structure or similar biological effect(s), are prohibited:

1. Erythropoietins (EPO) and agents affecting erythropoiesis, including, but not limited to:

1.1 Erythropoietin-Receptor Agonists, e.g. Darbepoetins (dEPO); Erythropoietins (EPO); EPO based constructs [e.g. EPO-Fc, methoxy polyethylene glycol-epoetin beta (CERA)]; EPO-mimetic agents and their constructs (e.g. CNTO-530, peginesatide).

1.2 Hypoxia-inducible factor (HIF) activating agents, e.g. Cobalt; Daprodustat (GSK1278863); Molidustat (BAY 85-3934); Roxadustat (FG-4592); Vadadustat (AKB-6548); Xenon'.

Prior to citing Co as a prohibited substance, WADA funded a study titled 'Endogenous erythropoietin stimulation by CO-breathing and by stabilizing HIF-1 by oral cobalt application' (World Anti-Doping Agency 2013). The authors of this project, W. Schmidt and D. Schwenke, from the University of Bayreuth, Germany, listed the project aims as - '*1. to monitor the physiological effects of different doses of cobalt and carbon monoxide on plasma-EPO, Hb-mass, and aerobic performance, 2. to prove if the effects of cobalt and CO can be detected by the recently developed Bayesian probabilistic inference techniques, which are the basis for the athletes biological passport, 3. to develop and evaluate direct detection methods for cobalt-misuse, and 4. to evaluate whether the endogenously produced EPO after stimulation by cobalt and CO differs from normal native EPO. In a first study the optimal dosage of cobalt and carbon monoxide on endogenous EPO production will be determined. In a second step, application of either cobalt or CO for 3 weeks will provide data on (1.) performance effects of both drugs, (2.) stimulation of endogenous EPO, (3.) changes of haemoglobin mass, and (4) changes of haematological parameters which will be analyzed by the statistics of the athlete biological passport*'. By 2017, no findings had been released from this study to vindicate cobalt activity in athletes and my attempts to contact the study investigators were

unanswered. Since then, 3 papers co-authored by W. Schmidt and/or D. Schwenke have been published covering aspects of their WADA funded project (Hoffmeister et al. 2018; Hoffmeister et al. 2019; Schmidt et al. 2019). The initial 2 papers were received for review in close succession, 1st December 2017 (Hoffmeister et al. 2019) and 22nd December 2017 (Hoffmeister et al. 2018). In the first paper received for review it was found that oral doses of 5 mg L^{-1} ($p < 0.05$) or 10 mg L^{-1} ($p < 0.001$) Co significantly increased plasma EPO levels. Despite having both blood and urine samples collected throughout the study, only results for urinary Co concentrations are reported. Correlation of results to blood cobalt concentration would appear an essential component in this study as the authors have noted that 'most of the ingested Co is not absorbed' and urine Co concentrations were highly variable.

From this study it was taken that further investigation of the oral 5 mg Co per day dosage was required (Hoffmeister et al. 2018). The study aim was to determine the effects of a small oral dose of Co on aerobic performance with the objective of providing a basis for establishing upper threshold limits of Co in urine. Sixteen healthy volunteers described as young leisure-trained men, received an oral daily dose of 5 mg Co per day for 3 weeks. No significant changes were noted between test and control groups for the parameters of haemoglobin, haematocrit, red cell count, mean erythrocyte volume, mean erythrocyte haemoglobin content, mean haemoglobin concentration, reticulocyte count, immature reticulocyte fraction, red cell distribution width, Epo, VO_{2max}, serum transferrin receptor, ferritin, and C-reactive protein. The only parameter to show a significant difference was the Hbmass using a method evaluated by this paper's corresponding co-author Schmidt (Schmidt & Prommer 2005). The lack of significance in VO_{2max} measurement was attributed to methodological noise and other probable variables in individual responses. A stronger explanation would be expected given that Schmidt had previously stated that 'the highest impact on VO_{2max} is obtained when athletes increase haemoglobin concentration by blood manipulation' (Schmidt & Prommer 2010). The authors speculated that variable intestinal absorption of Co may have contributed to some of their findings. However, no blood Co measurements were performed, or at least reported, on the blood samples that had been collected to support this. Even though the parameters purported to be directly influenced by Co, namely upregulated Epo production resulting

in increased haemoglobin and haematocrit, showed no significant differences to the control group, the authors concluded that the erythropoietic threshold was exceeded with the oral administration of 5 mg Co per day. They also concluded that 'it seems to be feasible to establish upper threshold limits for urine Co concentrations' despite excessive mean standard deviations in the test group with mean values of 305 ± 209 μg L^{-1} found after week 1 and 456 ± 402 μg L^{-1} at the end of week 3.

Results presented in the papers (Hoffmeister et al. 2018; Hoffmeister et al. 2019) were combined to produce an additional synopsis of these oral Co administration trials (Schmidt et al. 2019). With no further experimental data, the authors were now able to conclude that 'WADA has, therefore, to implement reasonable reference limits which we suggest being 14 ng mL^{-1} in urine'. An omitted conflict of interest in this review was the acknowledgment of the WADA funding provided and noted in the previous papers that were the foundation of this review.

A clear discrepancy exists between the erythropoietic effects noted when 10 mg Co was ingested by 7 healthy human male subjects (Hoffmeister et al. 2019) compared to horses, where 7 healthy race fit Standardbreds were administered IV injections of 50 mg Co (McKeever et al. 2020). As no blood Co measurements were reported for the human subjects a direct comparison of actual Co doses cannot be made between the groups. However, it would be expected that horses received a higher dose of Co given the inefficient intestinal absorption described for Co. No changes were observed in the haematological parameters and markers of exercise in horses while significant differences were reported for the same parameters measured in the human subjects. Possible causes for these contrasting finding could include:

- Different response to Co between species. Unlikely given the conserved biological activity noted for EPO production and action in mammals.
- Different presentation of Co to cells when absorbed via the intestine compared to IV administration. Unlikely as only the free Co ion has been reported as responsible as a HIF stabiliser. If there were a difference between administration routes it would be anticipated that a bolus IV injection would expose the cells to a higher dose of Co compared to the more gradual route of intestinal absorption.

- Different physiological response to hypoxia when Co is administered to athletes already exposed to hypoxic conditions through regular training. The human subjects studied were not athletes while the horses studied were race fit. For Co to increase EPO production in athletes it would presumably need to be present at concentrations that exceed the hypoxia naturally attained through training or competition at altitude (Schumacher et al. 2015).

No studies have been conducted in athletes to demonstrate athletic advantage attained through Co misuse. Studies conducted to demonstrate the HIF stabilising capability of Co were either conducted in vivo or with animal models. The haematological improvements from Co administration were not described for athletes but rather anaemic individuals with comorbidities causing their anaemic presentation. The relatively high levels of Co required to induce a hypoxic response in athletes may negate any athletic improvement through gastrointestinal discomfort or other side effects. In can only be assumed that the high level of Co would be unbearable given the poorly tolerated doses need to stimulate EPO production in sedentary individuals.

Ultimately a study on cyclists was able to demonstrate that even though a cohort treated with recombinant human EPO were able to show significant improvement in laboratory tests of maximal exercise, the more relevant submaximal test and road race times were not affected (Heuberger et al. 2017). Horses are even less likely to benefit from factors introduced to increase red cell mass as they naturally possess a splenic reserve providing access to an additional one third to half their red cells when increased aerobic capacity is required (McKeever et al. 2010). In summary, while extreme Co doses have been found to increase red cell production, no studies have shown that Co can enhance performance in trained athletes.

7.2 Should cobalt use be regulated?

With inadequate evidence to foster the prospect that Co can improve athletic performance, the focus on Co regulation should be one of education with regulation done as a means of ensuring individual and animal safety. In this vein the multi-element capability of ICP-MS analysis can be readily expanded to include other elements. The concept of only

vilifying Co appeared flawed from the outset as elemental nickel and manganese (Goldberg, Dunning & Bunn 1988; Goldwasser et al. 1958), either alone or in combination, had also been reported to stimulate erythropoiesis. Cobalt was just one of several other unregulated elements routinely added to supplements such as selenium, iodine, iron, copper or zinc that had been shown to influence the haematopoietic process (Nogueira-Pedro, Hastreiter & Borelli 2018). Any of these elements can be considered toxic when present at high enough levels. Selenium and iodine are good examples of elements where the range between normal and toxic is considerably tighter than that described for Co (Pagan 2000). Excesses of these elements have a much more profound and well documented influence on animal wellbeing than those attributed to Co.

Expanding the regulation of Co to other elements would also identify health limiting nutrient deficiencies that may have arisen from an unbalanced diet. If toxic environmental and potential feed contaminants such as arsenic, lead or mercury where added to the regulated element list, they would conceivably be of a greater benefit in terms of health and welfare than Co. Ultimately a case exists for regulating Co use, but the time and resources allocated to Co could be better spend including other elements that have a significant impact on individual or animal wellbeing.

7.3 Call for review of cobalt testing and penalties in the Australian racing industry

As my work on this thesis progressed, it was becoming increasingly obvious that the rules adopted by racing authorities to limit Co use were incorrect. Not only did Co fail to enhance performance but the choice of threshold and means used to assess exposure were inappropriate. Other noted professionals were of the same opinion. Dismay over the number of trainers being unfairly penalised by the Co rule prompted our decision to collaborate and compose an open letter addressing our concerns.

On Friday, 23 August 2019, this letter was circulated to:

- Mr Barry O'Farrell, CEO, Racing Australia Limited
- Mr Andrew Kelly, CEO, Harness Racing Australia
- CEO, Greyhounds Australia

- Minister for Better Regulation and Innovation NSW
- Minister for Racing and Minister for Multicultural Affairs QLD
- Minister for Recreation, Sport and Racing SA
- Minister for Racing, TAS
- Minister for Racing, VIC
- Minister for Tourism; Racing and Gaming WA

The letter provides a useful summary of the work addressed in this thesis. It has been reproduced below. An abbreviated version (Appendix 9.3) was sent to various media outlets.

CALL FOR REVIEW OF COBALT TESTING AND PENALTIES IN THE AUSTRALIAN RACING INDUSTRY

We are a group of professionals with extensive experience in the field of veterinary medicine, laboratory science and public health studies. In recent years we have observed the introduction of rules pertaining to the use of Co in racing, gallops, harness and greyhounds. We have kept ourselves informed on hearings and judgements, and we have kept abreast of relevant scientific literature; some of us have assisted trainers in defending charges emanating from alleged use of Co in their animals. There has been frequent discussion within the group and we have now reached the point where we are united in the opinion that, from a scientific perspective, the present approach to detecting improper use of Co in racing is seriously flawed and must be revisited as a matter of urgency. We feel we are obliged to make this submission to those who control racing in Australia. We have set out the reasons for our concerns below, but before going further we want to leave no doubt that we endorse the efforts of regulators to identify and punish those who seek to gain an advantage by unfair means – which includes use of performance-enhancing substances. We also understand why the industries responded to allegations of widespread use of Co.

A Current Perspective of Cobalt Regulation in Australian Racing

In 2013 Australian racing regulators publicly expressed their concern that Co was being used in racing animals to gain an unfair advantage. Put simply, there was a view that salts

of Co (particularly cobalt chloride) stimulate red cell production which in turn increases the supply of oxygen to muscles, thereby leading to improved performance – although many professionals would question this assertion. There were also, rightly, concerns that Co toxicity had implications for animal welfare. Authorities began investigating the improper use of Co using advanced laboratory instrumentation, inductively coupled plasma mass spectrometry (ICPMS), to determine the concentration of Co in urine. It was reported that numerous "cobalt positives" were detected, first in harness racing and later in gallops. Further studies provided additional data which were used to determine a "threshold concentration" above which stewards made the interpretation that the animal had been "doped". Routine testing was introduced in various jurisdictions, commencing with Harness Racing New South Wales in 2014. Since then more than 300 instances of alleged Co misuse have been prosecuted by Australian racing authorities, across three codes. In the large majority of cases, prize money was forfeited, and the trainers involved were disqualified, despite almost universal expressions of innocence. Never in the history of racing regulation in Australia has there been such an abundance of "doping" cases. While Racing's reputation has almost certainly been damaged as a result, there is also no doubt that many observers hold the view that innocent parties have been wrongly penalised.

Scientists seeking an explanation for the explosion in cases acknowledge that ICPMS is a highly **sensitive** assay, detecting minute quantities of Co. But importantly it lacks **specificity** because it identifies **atoms** of Co, rather than those **compounds** (e.g. Co chloride) which are the targets of the regulators.

There is universal agreement that the essential vitamin (B12) has a Co atom within its structure and will therefore contribute to a "cobalt positive" result when measured using ICPMS. Vitamin B12 meets none of the criteria for prohibited substances under the rules of racing (or any other professional sport). In this context, tribunals such as QCAT have directed authorities to submit "cobalt positive" samples for supplemental testing designed to discriminate between Co atoms coming from, say, cobalt chloride, and those coming from Vitamin B12. On the basis of results that have been made known to us, we conclude that much of the "cobalt" in some test samples has in fact come from Vitamin B12,

thereby supporting the trainers' assertions that the horses had not been "doped". While the origin of the B12 in the urine is yet to be elucidated, a possible factor is the use by trainers of various B12-containing approved supplements administered as directed by manufacturers.

It is also clear that at least some of the cobalt "infringements" have been caused by feed and environmental exposure to Co, outside the knowledge or control of the trainer. It is stressed that Co is an element ubiquitous in nature, and in trace quantities essential to life. It should be stressed that levels being considered in current cases are very small, and in all probability inconsequential.

The key reasons for our concerns are as follows:

1. THE TEST METHOD

As presently applied, ICPMS (the cornerstone test for Co) cannot differentiate Co atoms coming from target compounds such as cobalt chloride and those from Vitamin B12. There can be no questioning the fact that B12 is produced naturally in the horse and has no biological properties in common with those of cobalt chloride. Quite rightly, regulators' initial concerns for misuse of Co in racing did not extend to Vitamin B12.

2. THE TEST SAMPLE

Reliance on results from a single urine sample is prone to error. Tests on other body fluids such as plasma or whole blood are likely to be more informative. Single, qualitative Co tests on urine are not utilised in human pathology – in the main because the test subjects' level of hydration at the time urine is sampled can have a great influence on the concentration of target analytes. (We understand the operational simplicity of urine sample.)

3. USE OF A THRESHOLD VALUE

We do not support the practice of setting "threshold" regulatory levels for urine Co based on population studies on individuals whose history of legal exposure to veterinary products containing Co and Vitamin B12 is unknown and/or ignored. The official studies

to construct the threshold have never been peer reviewed or published. The populations used were small and insufficiently pedigreed, and the ICPMS values used were not corrected for dehydration or presence of Vitamin B12. The Co threshold so applied is tenuous and does not specifically define the Prohibited Substance of HIFS Cobalt described under Schedule 1 of the ARB Rules.

4. CONFUSION IN THE RACING INDUSTRY REGARDING IMPACT AND TOXICITY OF COBALT SALTS

While we should not need to state that we do not condone any unscrupulous behaviour or animal abuse, it is our firm view that both the potency and potential toxicity of Co salts in the current context have been exaggerated.

We are unanimous in the belief that the current approach to detecting Co abuse in Australian racing animals is misdirected and has the potential to lead to unjust convictions and unfair penalties for participants. We are also concerned that significant reputational damage to industry is a possible consequence. Accordingly, we are urging industry regulators to take the following steps at first opportunity:

1. Implement a moratorium on any action regarding Co irregularities pending clarification of the matters set out above

2. Provide administrative and financial support for a Committee of Inquiry comprising representatives from racing's regulatory bodies and industry stakeholders, as well as veterinarians, scientists with appropriate qualifications and experience, and statisticians (the latter groups to be independent of racing bodies)

The charge to the Committee should be to find a consensus approach to the future regulation of Co in racing animals. Apart from meeting the expectations of regulatory bodies and industry groups, such a consensus must be so robust as to achieve unequivocal endorsement by the wider veterinary and scientific communities.

One of the Terms of Reference should be to determine the role of Vitamin B12 in the testing for improper use of Co and in the interpretation of test results. While we have read the extensive body of peer-reviewed evidence on the topic, we see the need for additional research on the potential for popular supplements to create “false-positives” in tests for Co.

Please contact us for any further explanation, information, and assistance in this matter. Please bring this letter to the attention of your Board.

Yours sincerely,

Dr Andrew Clarke

- Principal Consultant, Equine Connections
- Former Professor of Equine Studies and Head of the Equine Centre School of Veterinary Science, University of Melbourne
- Former President and CEO Equine Research Centre Inc Guelph Ontario Canada
- Former Head of The Equine Research Centre and Professor Department Biomedical Sciences University of Guelph, Ontario Canada
- Former Research Fellow School of Veterinary Science University of Bristol England

Mr David Dawson

- Director, Brisbane Racing Club
- Formerly Chief Scientist Qld Department of Health Pathology Services
- Former Consultant to World Health Organization
- Author/co-author of more than 50 peer-reviewed scientific publications

Dr Derek Major

- Equine Veterinary Consultant
- Co-author of peer-reviewed, published scientific papers on Co administration studies in horses

Mr Neale Scott

- Former Harness Racing Trainer and Driver
- Co-Investigator into the epidemiology of Co positives in Australian racing codes

Mr Ross Tinniswood

- Retired Medical Scientist and former Queensland Health Dept Executive
- Extensive administrative experience in diagnostic laboratory testing, equipment and technologies, and test result interpretation
- A former NATA Laboratory Assessor and former Director of the Brisbane Turf Club

Mr Ross Wenzel

GradDip(ClinBiochem), MAppSc(Thesis)

- Senior Medical Scientist with over 20 years' experience determining trace element concentrations in clinical samples
- Co-author of peer-reviewed, published scientific papers on Co administration studies in horses. Researcher at UTS Centre for Forensic Science

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7.4 Conclusions

There is no justification for considering Co a performance enhancing drug. All the evidence from published studies investigating Co administration to horses, including ours, confirms that there is no increase in red cell production that could be associated with enhanced athletic performance.

From a toxicological perspective, urine Co determinations are of limited value. It was clear from our research findings that urine is an unreliable sample matrix within which to assess Co exposure. When using urine for quantitative measurements, steps must be taken to adjust for variations in hydration status by adopting commonly applied corrections techniques such as creatinine or specific gravity corrections. Any presumptive positive findings should be tested further to exclude the presence of B12. Given the acute nature of urinary Co excretion, the use of this sample matrix cannot be recommended for screening purposes. Conversely, blood Co determinations compared to an appropriately set threshold, could reliable be used to detect potentially harmful levels of Co exposure.

Given that the only benefit of controlling the use of Co is through animal welfare concerns, it is reasonable to expect that other nutritional trace elements to be subjected to the same level of scrutiny. Laboratories currently approved by racing administrators to undertake Co testing already have this capability. As the ICP-MS instrumentation used to determine Co is a multi-element technique, it can be readily adapted to simultaneously measure other trace and toxic elements. Using samples already prepared for Co analysis, the additional time and resource allocation requirements are minimal. Searching for deficiencies or toxic excesses of other elements would provide a much greater return for investment in protecting horse welfare.

At the time of introducing a Co threshold in equine urine little was known about its effects and metabolism. That which was known was either not appropriately investigated or ignored to hasten the process to control potential Co misuse. The limited and in some cases misleading research available when the Co rule was first introduced can only have hampered racing administrator's decision to use set a threshold for Co in urine. To continue down the same path after being enlightened with new research such as that presented in this thesis would seem naive. Changes to the Co rule are required. Failure to do so can only serve to diminish the credibility of racing authorities in their ability to provide a fair and safe racing environment.

7.5 Final recommendations

At the completion of this research the following points are recommended for the regulation of Co in racing:

- Urine should not be used assess Co exposure. Measurements performed in this sample matrix do not meet the objective of protecting racing integrity. As no relationship between urinary Co concentration and adverse health effects have been defined, these measurements cannot be used to ensure that horse well-being has been protected.
- A threshold for Co should not be based on population studies as they do not adequately address inadvertent exposure to a naturally occurring substance. Instead controlled administration trials such as the one performed by McKeever et al. (McKeever et al. 2020) should be referred to.

- Both plasma and whole blood determinations should be performed with a regulatory of threshold limit of 71 μg L^{-1} enforced when either total Co measurement in plasma or whole blood are found to exceed this concentration.
- ICP-MS was found to be an appropriate analytical technique for determining Co concentrations provided care was taken to ensure protein bound Co remained in solution. Both alkaline- and acid-based diluents were found to be acceptable when preparing samples for ICP-MS analysis. When using acidic diluents caution should be taken to prevent protein precipitation, for example concentrations of nitric acid should not exceed 0.75 % v/v.

8. LITERATURE CITED

AAP/ABC 2016, *Peter Moody content to walk away from racehorse training after cobalt suspension*, ABC News, viewed 11 April 2016, <https://www.abc.net.au/news/2016-03-22/peter-moody-to-walk-away-from-racehorse-training-after-cobalt-s/7266488>.

Abbott Laboratories 2006, *Creatinine*, Abbott Laboratories, viewed 21 April 2016, <http://www.ilexmedical.com/files/PDF/Creatinine_ARC_CHEM.pdf>.

Abbott Laboratories 2018, *ARCHITECT B12*, Abbott Laboratories, viewed 10 January 2019, <http://www.ilexmedical.com/files/PDF/B12_ARC.pdf>.

Abouhamed, M., Gburek, J., Liu, W., Torchalski, B., Wilhelm, A., Wolff, N.A., Christensen, E.I., Thévenod, F. & Smith, C.P. 2006, 'Divalent metal transporter 1 in the kidney proximal tubule is expressed in late endosomes/lysosomal membranes: implications for renal handling of protein-metal complexes', *American Journal of Physiology-Renal Physiology*, vol. 290, no. 6, pp. F1525-F33.

Adams, J., Kavouras, S.A., Johnson, E.C., Jansen, L.T., Capitan-Jimenez, C., Robillard, J.I. & Mauromoustakos, A. 2017, 'The effect of storing temperature and duration on urinary hydration markers', *International Journal of Sport Nutrition and Exercise Metabolism*, vol. 27, no. 1, pp. 18-24.

Adamson, J. 2014, 'Day v Harness Racing New South Wales [2014] NSWSC 1402', Supreme Court New South Wales, 14 October 2014, viewed 30 July 2015, <https://jade.io/article/348773>.

Aitio, A. 1996, 'Quality assurance', in A. Wright (ed.), *Biological monitoring of chemical exposure in the workplace: guidelines*, vol. 1, World Health Organization, Geneva, pp. 23-4.

Al Okail, M.S. 2010, 'Cobalt chloride, a chemical inducer of hypoxia-inducible factor-1 α in U251 human glioblastoma cell line', *Journal of Saudi Chemical Society*, vol. 14, no. 2, pp. 197-201.

Allen, L.A., Ambardekar, A.V., Devaraj, K.M., Maleszewski, J.J. & Wolfel, E.E. 2014, 'Clinical problem-solving. Missing elements of the history', *The New England Journal of Medicine*, vol. 370, no. 6, pp. 559-66.

Analytical Methods Committee 2001, *Robust statistics: a method of coping with outliers*, The Royal Society of Chemistry, viewed 14 June 2021, <https://www.rsc.org/images/robust-statistics-technical-brief-6_tcm18-214850.pdf>.

Anderson, S. 2015, *The cobalt threshold - How it was determined*, Racing.com, viewed 18 July 2018, <http://www.racing.com/news/2015-01-19/the-cobalt-threshold>.

APP Pharmaceuticals 2008, *Cyanocobalamin Injection, USP*, APP Pharmaceuticals, Schaumburg, IL 60173, viewed 17 March 2017, <http://editor.fresenius-kabi.us/PIs/US-PH-Cyanocobalamin-Inj-USP-FK-45813E-04-2008-PI.pdf>.

Apple, F.S., Quist, H.E., Otto, A.P., Mathews, W.E. & Murakami, M.M. 2002, 'Release characteristics of cardiac biomarkers and ischemia-modified albumin as measured by the albumin cobalt-binding test after a marathon race', *Clinical Chemistry*, vol. 48, no. 7, pp. 1097-100.

Ashby, K. 2018, *Internal Review 0026-18*, Queensland Racing Integrity Commission, viewed 15 April 2020, <https://www.qric.qld.gov.au/wp-content/uploads/2018/04/INTERNAL-REVIEW-DECISION-NICOLE-HANRAHAN.pdf>.

Association of Racing Commissioners International, Inc. 2016, *Uniform classification guidelines for foreign substances and recommended penalties model rule*, viewed 2 November 2017, <http://tharacing.com/wp-content/uploads/2017/03/Uniform-Classification-Guidelines-Version-13-00.pdf>.

Australian Industry and Skills Committee 2020, *Racing*, AISC, viewed 5 May 2020, <https://nationalindustryinsights.aisc.net.au/industries/racing>.

Bal, W., Sokołowska, M., Kurowska, E. & Faller, P. 2013, 'Binding of transition metal ions to albumin: Sites, affinities and rates', *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1830, no. 12, pp. 5444-55.

Barbier, O., Jacquillet, G., Tauc, M., Cougnon, M. & Poujeol, P. 2005, 'Effect of Heavy Metals on, and Handling by, the Kidney', *Nephron Physiology*, vol. 99, no. 4, pp. p105-p10.

Barceloux, D.G. & Barceloux, D. 1999, 'Cobalt', *Journal of Toxicology: Clinical Toxicology*, vol. 37, no. 2, pp. 201-16.

Barr, D.B., Wilder, L.C., Caudill, S.P., Gonzalez, A.J., Needham, L.L. & Pirkle, J.L. 2005, 'Urinary creatinine concentrations in the U.S. population: Implications for urinary biologic monitoring measurements', *Environmental Health Perspectives*, vol. 113, no. 2, pp. 192-200.

Bartley, P. 2015, 'Trainer Danny O'Brien's horse Bullpit tests positive to cobalt', *The Sydney Morning Herald*, March 18, 2015, viewed 6 July 2018, <https://www.smh.com.au/sport/racing/trainer-danny-obriens-horse-bullpit-tests-positive-to-cobalt-20150317-1m1d61.html>.

Beleznay, E. & Osvay, M. 1994, 'Long-term clearance of accidentally inhaled ^{60}Co aerosols in humans', *Health Physics*, vol. 66, pp. 392-9.

Bishop, D.P., Blanes, L., Wilson, A.B., Wilbanks, T., Killeen, K., Grimm, R., Wenzel, R., Major, D., Macka, M., Clarke, D., Schmid, R., Cole, N. & Doble, P.A. 2017, 'Microfluidic high performance liquid chromatography-chip hyphenation to inductively coupled plasma–mass spectrometry', *Journal of Chromatography A*, vol. 1497, pp. 64-9.

Boeniger, M.F., Lowry, L.K. & Rosenberg, J. 1993, 'Interpretation of urine results used to assess chemical exposure with emphasis on creatinine adjustments: a review', *American Industrial Hygiene Association Journal*, vol. 54, no. 10, pp. 615-27.

Borsook, H. & Dubnoff, J.W. 1947, 'The hydrolysis of phosphocreatine and the origin of urinary creatinine', *Journal of Biological Chemistry*, vol. 168, no. 2, pp. 493-510.

Bosken, J., Tobin, T., Mundy, G.D., Fisher, M., Gantz, M.G. & Banks, R.O. 2003, 'Effect of furosemide on urine specific gravity and osmolality in thoroughbred racehorses', *Veterinary Therapeutics*, vol. 4, no. 3, pp. 292-8.

Bowie, E. & Hurley, P. 1975, 'Cobalt chloride in the treatment of refractory anaemia in patients undergoing long-term haemodialysis', *Australian and New Zealand Journal of Medicine*, vol. 5, no. 4, pp. 306-13.

Brent, J. & Devlin, J.J. 2013, 'Dilemmas about the toxicological consequences of metal-on-metal hip prostheses - What we do and do not know, and what we should do?', *Clinical Toxicology*, vol. 51, no. 4, pp. 195-8.
Brewer, K., Maylin, G., Fenger, C. & Tobin, T. 2016, 'Cobalt use and regulation in horseracing: a review', *Comparative Exercise Physiology*, vol. 12, no. 1, pp. 1-10.
Brobst, D.F. & Bayly, W.M. 1982, 'Responses of horses to a water deprivation test', *Journal of Equine Veterinary Science*, vol. 2, no. 2, pp. 51-6.
Bunn, H.F. 2013, 'Erythropoietin', *Cold Spring Harbor Perspectives in Medicine*, vol. 3, no. 3, p. 1.
Burns, T., Dembek, K., Kamr, A., Dooley, S., Dunbar, L., Aarnes, T., Bednarski, L., O'Brien, C., Lakritz, J. & Byrum, B. 2018, 'Effect of intravenous administration of cobalt chloride to horses on clinical and hemodynamic variables', *Journal of Veterinary Internal Medicine*, vol. 32, no. 1, pp. 441-9.
Carey, R.N., Anderson, F.P., George, H., Hartmann, A.E., Janzen, V.K., Kallner, A., Levine, J.B., Schiffgens, J., Srinivasan, A. & Tholen, D.W. 2006, *User verification of performance for precision and trueness; approved guideline—second edition*, vol. 25, no. 17, Clinical and Laboratory Standards Institute: Wayne, PA.
Carrieri, M., Trevisan, A. & Bartolucci, G. 2001, 'Adjustment to concentration-dilution of spot urine samples: Correlation between specific gravity and creatinine', *International Archives of Occupational and Environmental Health*, vol. 74, pp. 63-7.
Carter, E., Valli, V., McSherry, B., Lumsden, J., Milne, F. & Robinson, G. 1974, 'The kinetics of hematopoiesis in the light horse I. The lifespan of peripheral blood cells in the normal horse', *Canadian Journal of Comparative Medicine*, vol. 38, no. 3, p. 303.
Chadha, V., Garg, U. & Alon, U.S. 2001, 'Measurement of urinary concentration: a critical appraisal of methodologies', *Pediatric Nephrology*, vol. 16, no. 4, pp. 374-82.
Cocker, J., Mason, H., Warren, N. & Cotton, R. 2011, 'Creatinine adjustment of biological monitoring results', *Occupational Medicine*, vol. 61, no. 5, pp. 349-53.
Coenen, M. & Vervuert, I. 2005, 'The physiological role of minerals and vitamins in the growing horse', in V. Julliand & W. Martin-Rosset (eds), *The growing horse: Nutrition and prevention of growth disorders*, vol. 114, Wageningen Academic Pub, p. 192.
Cohen, N.D., Peck, K.E., Smith, S.A. & Ray, A.C. 2002, 'Values of urine specific gravity for Thoroughbred horses treated with furosemide prior to racing compared with untreated horses', *Journal of Veterinary Diagnostic Investigation*, vol. 14, no. 3, pp. 231-5.
Cotes, P.M. & Bangham, D. 1966, 'The international reference preparation of erythropoietin', *Bulletin of the World Health Organization*, vol. 35, no. 5, pp. 751-60.
Craddock, R. 2015, 'Harness trainer Darrell Graham and thoroughbred trainer Peter Moody meet over cobalt findings', *The Courier-Mail*, 27 August, viewed 6 July 2018, <https://www.couriermail.com.au/sport/superracing/qld-racing/harness-

trainer-darrell-graham-and-thoroughbred-trainer-peter-moody-meet-over-cobalt-findings/news-story/0d48a466d63e819a458869539504e096>.
Curnow, D. 2015, *Peter Moody blames hoof powder as cobalt trial begins*, Race Media, viewed 14 June 2020, <https://horsebetting.com.au/peter-moody-blames-hoof-powder-as-cobalt-trial-begins/418/>.
Dabeka, R.W. & Mckenzie, A.D. 1995, 'Survey of lead, cadmium, fluoride, nickel, and cobalt in food composites and estimation of dietary intakes of these elements by Canadians in 1986–1988', *Journal of AOAC International*, vol. 78, no. 4, pp. 897-909.
Daniel, J., Ziaee, H., Pradhan, C., Pynsent, P.B. & McMinn, D.J. 2007, 'Blood and urine metal ion levels in young and active patients after Birmingham hip resurfacing arthroplasty: four-year results of a prospective longitudinal study', *The Journal of Bone and Joint Surgery (British Volume)*, vol. 89-B, no. 2, pp. 169-73.
Daniel, J., Ziaee, H., Pradhan, C., Pynsent, P.B. & McMinn, D.J. 2010, 'Renal clearance of cobalt in relation to the use of metal-on-metal bearings in hip arthroplasty', *The Journal of Bone and Joint Surgery (American Volume)*, vol. 92, no. 4, pp. 840-5.
Daniel, J., Ziaee, H., Pynsent, P.B., McMinn, D.J.W. 2007, 'The validity of serum levels as a surrogate measure of systemic exposure to metal ions in hip replacement', *The Journal of Bone and Joint Surgery (British Volume)*, vol. 89-B, pp. 736-41.
Date, A.R. & Gray, A.L. 1983, 'Development progress in plasma source mass spectrometry', *Analyst*, vol. 108, no. 1283, pp. 159-65.
Davis, J.E. & Fields, J.P. 1958, 'Experimental production of polycythemia in humans by administration of cobalt chloride', *Proceedings of the Society for Experimental Biology and Medicine*, vol. 99, no. 2, pp. 493-5.
Déry, M.-A.C., Michaud, M.D. & Richard, D.E. 2005, 'Hypoxia-inducible factor 1: regulation by hypoxic and non-hypoxic activators', *The International Journal of Biochemistry and Cell Biology*, vol. 37, no. 3, pp. 535-40.
Dorries, B. 2016, 'Trainer Darrel Graham vows to fight 15-month cobalt ban', *The Courier-Mail*, 30 August, viewed 6 July 2018, <https://www.couriermail.com.au/sport/superracing/qld-racing/trainer-darrel-graham-vows-to-fight-15month-cobalt-ban/news-story/c88612c6ae208e89f4c72e068cd7636d>.
Dorries, B. 2019, *Danny O'Brien to racing bosses: Admit you got it wrong with cobalt*, Racenet, viewed 5 February 2020, <https://www.racenet.com.au/news/danny-o-brien-to-racing-bosses--admit-you-got-it-wrong-with-cobalt-20191121>.
Edwards, D.J., Brownlow, M.A. & Hutchins, D.R. 1989, 'Indices of renal function: reference values in normal horses', *Australian Veterinary Journal*, vol. 66, no. 2, pp. 60-3.
Elliott, S., Knowles, M. & Kalinitchenko, I. 2004, 'A new direction in ICP-MS', *Spectroscopy*, vol. 19, no. 1, pp. 30-8.
Espinoza, A., Le Blanc, S., Olivares, M., Pizarro, F., Ruz, M. & Arredondo, M. 2011, 'Iron, copper, and zinc transport: Inhibition of divalent metal transporter 1 (DMT1) and human copper transporter 1 (hCTR1) by shRNA', *Biological Trace Element Research*, vol. 146, pp. 281-6.
Estey, M.P., Diamandis, E. P., Van Der Straeten, C., Tower, S. S., Hart, A. J., Moyer, T. P. 2013, 'Cobalt and chromium measurement in patients with metal hip prostheses.', *Clinical Chemistry*, vol. 59, no. 6, pp. 880-6.

Exelby, N. 2018, 'Monday Mail: The Monstar must buck historical trend to win the Stradbroke', *The Courier-Mail*, 3 June, viewed 6 July 2018, <https://www.couriermail.com.au/sport/superracing/qld-racing/monday-mail-the-monstar-must-buck-historical-trend-to-win-the-stradbroke/news-story/4d2b722448c5bf4429242fd2984bdc0a>.

Fenger, C. & Sacopulos, P. 2015, *What is cobalt?*, Musings on Equine Medicine, viewed 29 June 2015, <http://musingsonequinemedicine.blogspot.com/2015/04/what-is-cobalt.html>.

Finley, B.L., Monnot, A.D., Gaffney, S.H. & Paustenbach, D.J. 2012, 'Dose-response relationships for blood cobalt concentrations and health effects: a review of the literature and application of a biokinetic model', *Journal of Toxicology and Environmental Health, Part B*, vol. 15, no. 8, pp. 493-523.

Fisher, J.W. 1998, 'A quest for erythropoietin over nine decades', *Annual Review of Pharmacology and Toxicology*, vol. 38, no. 1, pp. 1-20.

Flora, S.J.S. & Pachauri, V. 2010, 'Chelation in metal intoxication', *International Journal of Environmental Research and Public Health*, vol. 7, no. 7, pp. 2745-88.

Fong, G. & Takeda, K. 2008, 'Role and regulation of prolyl hydroxylase domain proteins', *Cell Death & Differentiation*, vol. 15, no. 4, pp. 635-41.

Forrista, R. 2018, *Joseph O'Brien winner disqualified after testing positive for cobalt*, Racing Post, viewed 7 January 2019, <https://www.racingpost.com/news/joseph-o-brien-winner-disqualified-after-testing-positive-for-cobalt/358481>.

Frape, D. 1988, 'Dietary requirements and athletic performance of horses', *Equine Veterinary Journal*, vol. 20, no. 3, pp. 163-72.

Fried, W., Plzak, L., Jacobson, L. & Goldwasser, E. 1957, 'Studies on erythropoiesis. III. Factors controlling erythropoietin production', *Proceedings of the Society for Experimental Biology and Medicine*, vol. 94, no. 1, pp. 237-41.

Garde, A., Hansen, Å.M. & Kristiansen, J. 2003, 'Evaluation, including effects of storage and repeated freezing and thawing, of a method for measurement of urinary creatinine', *Scandinavian Journal of Clinical and Laboratory Investigation*, vol. 63, no. 7-8, pp. 521-4.

Genuis, S.J., Birkholz, D., Rodushkin, I. & Beesoon, S. 2011, 'Blood, urine, and sweat (BUS) study: monitoring and elimination of bioaccumulated toxic elements', *Archives of Environmental Contamination and Toxicology*, vol. 61, no. 2, pp. 344-57.

Goldberg, M.A., Dunning, S.P. & Bunn, H.F. 1988, 'Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein', *Science*, vol. 242, no. 4884, pp. 1412-5.

Goldwasser, E., Jacobson, L.O., Fried, W. & Plzak, L.F. 1958, 'Studies on erythropoiesis: V. The effect of cobalt on the production of erythropoietin', *Blood*, vol. 13, no. 1, pp. 55-60.

Gregus, Z. & Klaassen, C.D. 1986, 'Disposition of metals in rats: a comparative study of fecal, urinary, and biliary excretion and tissue distribution of eighteen metals', *Toxicology and Applied Pharmacology*, vol. 85, no. 1, pp. 24-38.

Greyhound Racing New South Wales 2015, *Cobalt use in greyhounds*, GRNSW, viewed 14 June 2020, <https://www.grnsw.com.au/uploads/Cobalt%20Fact%20Sheet.pdf>.

Hamilton, E.I. 1994, 'The geobiochemistry of cobalt', *Science of the Total Environment*, vol. 150, no. 1-3, pp. 7-39.
Hanzawa, K. & Watanabe, S. 2000, 'Changes in osmotic fragility of erythrocytes during exercise in athletic horses', *Journal of Equine Science*, vol. 11, no. 3, pp. 51-61.
Harness Racing New South Wales 2013a, *HRNSW Penalty Guidelines 21.03.2013*, HRNSW, viewed 30 April 2016, <https://www.hrnsw.com.au/uploads/files/hrnsw%20policys/130321%20hrnsw%20penalty%20guideines.pdf>.
Harness Racing New South Wales 2013b, *New local rule - Cobalt Threshold*, Harnesslink, viewed 30th April 2016, <http://www.harnesslink.com/News/HRNSW-Media-Release---NEW-LOCAL-RULE---COBALT-THRESHOLD>.
Harris, P., Coenen, M., Frape, D., Jeffcott, L. & Meyer, H. 2006, 'Equine nutrition and metabolic diseases', *The equine manual*, Saunders Ltd., United States, p. 72.
Hattangadi, S.M., Wong, P., Zhang, L., Flygare, J. & Lodish, H.F. 2011, 'From stem cell to red cell: regulation of erythropoiesis at multiple levels by multiple proteins, RNAs, and chromatin modifications', *Blood, The Journal of the American Society of Hematology*, vol. 118, no. 24, pp. 6258-68.
Heavner, D.L., Morgan, W.T., Sears, S.B., Richardson, J.D., Byrd, G.D. & Ogden, M.W. 2006, 'Effect of creatinine and specific gravity normalization techniques on xenobiotic biomarkers in smokers' spot and 24-h urines', *Journal of Pharmaceutical and Biomedical Analysis*, vol. 40, no. 4, pp. 928-42.
Heuberger, J.A., Rotmans, J.I., Gal, P., Stuurman, F.E., van't Westende, J., Post, T.E., Daniels, J.M., Moerland, M., van Veldhoven, P.L. & de Kam, M.L. 2017, 'Effects of erythropoietin on cycling performance of well trained cyclists: a double-blind, randomised, placebo-controlled trial', *The Lancet Haematology*, vol. 4, no. 8, pp. e374-e86.
Hillyer, L., Ridd, Z., Fenwick, S., Hincks, P. & Paine, S. 2017, 'Pharmacokinetics of inorganic cobalt and a vitamin B12 supplement in the Thoroughbred horse: Differentiating cobalt abuse from supplementation', *Equine Veterinary Journal*.
Ho, E.N., Chan, G.H., Wan, T.S., Curl, P., Riggs, C.M., Hurley, M.J. & Sykes, D. 2015, 'Controlling the misuse of cobalt in horses', *Drug Testing and Analysis*, vol. 7, no. 1, pp. 21-30.
Ho, E.N., Curl, P., Sykes, D. & Wan, T.S. 2016, 'Responses to commentary on paper: "Controlling the misuse of cobalt in horses"', *Drug Testing and Analysis*, vol. 8, no. 8, pp. 882-84.
Hodges, V.M., Rainey, S., Lappin, T.R. & Maxwell, A.P. 2007, 'Pathophysiology of anemia and erythrocytosis', *Critical Reviews in Oncology/Hematology*, vol. 64, no. 2, pp. 139-58.
Hodgson, G., Perreta, M., Yudilevich, D. & Eskuche, I. 1958, 'Assay of "Hemopoietine"' in Starved Animals; Properties of Urinary Hemopoietine', *Proceedings of the Society for Experimental Biology and Medicine*, vol. 99, no. 1, pp. 137-42.
Hoffmeister, T., Schwenke, D., Krug, O., Wachsmuth, N., Geyer, H., Thevis, M., Byrnes, W.C. & Schmidt, W.F. 2018, 'Effects of 3 weeks of oral low-dose Cobalt on hemoglobin mass and aerobic performance', *Frontiers in Physiology*, vol. 9, pp. 1-9.

Hoffmeister, T., Schwenke, D., Wachsmuth, N., Krug, O., Thevis, M., Byrnes, W.C. & Schmidt, W.F. 2019, 'Erythropoietic effects of low-dose cobalt application', *Drug Testing and Analysis*, vol. 11, no. 2, pp. 200-7.

Holly, R.G. 1955, 'Studies on iron and cobalt metabolism', *Journal of the American Medical Association*, vol. 158, no. 15, pp. 1349-52.

Houk, R.S. 1986, 'Mass spectrometry of inductively coupled plasmas', *Analytical Chemistry*, vol. 58, no. 1, pp. 97A-105A.

Illing, A.C., Shawki, A., Cunningham, C.L. & Mackenzie, B. 2012, 'Substrate profile and metal-ion selectivity of human divalent metal-ion transporter-1', *Journal of Biological Chemistry*, vol. 287, no. 36, pp. 30485-96.

IMMULITE 1000 Vitamin B12 2005, *IMMULITE/IMMULITE 1000 Vitamin B12 (PILKVB-9, 2005-11-18)*, viewed 21/04/2018 2018, <http://www.dpcweb.com/package_inserts/immulite/pdfs/Anemia/lkvb-9.pdf>.

International Federation of Horseracing Authorities 2016, *International Agreement on Breeding, Racing and Wagering*, IFHA Executive Council, viewed 30 April 2016, <https://www.ifhaonline.org/resources/2015Agreement.pdf>.

International Federation of Horseracing Authorities 2017, *International Agreement on Breeding, Racing and Wagering*, IFHA Executive Council, viewed 2 November 2017, <http://www.ifhaonline.org/resources/2017Agreement.pdf>.

International Organization for Standardization 2012, *ISO 15189:2012 Medical laboratories -- Requirements for quality and competence*, International Organization for Standardization, viewed 22 April 2016, <http://www.iso.org/iso/catalogue_detail?csnumber=56115>.

Ishihara, N. & Matsushiro, T. 1986, 'Biliary and urinary excretion of metals in humans', *Archives of Environmental Health: An International Journal*, vol. 41, no. 5, pp. 324-30.

Jelkmann, W. 2012, 'The disparate roles of cobalt in erythropoiesis, and doping relevance', *Open Journal of Hematology*, vol. 3, no. 1, p. 1.

Kalinitchenko, I., Wang, X. & Sturman, B. 2008, 'Simple and effective control of spectral overlap interferences in ICP-MS', *Spectroscopy*, pp. 38-46.

Karakka Kal, A.K., Perwad, Z., K Karatt, T., Nalakath, J. & Subhahar, M. 2020, 'Using inductively coupled plasma mass spectrometry to assess essential and performance-enhancing metals in the urine of racehorses', *Journal of Analytical Toxicology*.

Kerger, B.D., Gerads, R., Gurleyuk, H., Thuett, K.A., Finley, B.L. & Paustenbach, D.J. 2013, 'Cobalt speciation assay for human serum, Part I. Method for measuring large and small molecular cobalt and protein-binding capacity using size exclusion chromatography with inductively coupled plasma-mass spectroscopy detection', *Toxicological & Environmental Chemistry*, vol. 95, no. 4, pp. 687-708.

Kesava Raju, C.S., Yu, L.L., Schiel, J.E. & Long, S.E. 2013, 'A simple and sensitive LC-ICP-MS method for the accurate determination of vitamin B12 in fortified breakfast cereals and multivitamin tablets', *Journal of Analytical Atomic Spectrometry*, vol. 28, no. 6, pp. 901-7.

Kesteloot, H., Roelandt, J., Willems, J., Claes, J.H. & Joossens, J.V. 1968, 'An enquiry into the role of cobalt in the heart disease of chronic beer drinkers', *Circulation*, vol. 37, no. 5, pp. 854-64.

Kinobe, R.T. 2016, 'Towards the elimination of excessive cobalt supplementation in racing horses: A pharmacological review', *Research in Veterinary Science*, vol. 104, pp. 106-12.
Kleinberg, W. 1934, 'The hemopoietic effect of cobalt and cobalt-manganese compounds in rabbits', *American Journal of Physiology-Legacy Content*, vol. 108, no. 3, pp. 545-9.
Knoop, A., Görgens, C., Geyer, H. & Thevis, M. 2019, 'Elevated urinary cobalt concentrations identified in routine doping controls can originate from vitamin B12', *Rapid Communications in Mass Spectrometry*.
Knottenbelt, D.C. 2006, 'Chapter 12 - The urinary system', in A.J. Higgins & J.R. Snyder (eds), *The Equine Manual (Second Edition)*, W.B. Saunders, Edinburgh, pp. 659-712.
Knych, H.K., Arthur, R.M., Mitchell, M.M., Holser, I., Poppenga, R., Smith, L.L., Helm, M.N., Sams, R.A. & Gaskill, C.L. 2014, 'Pharmacokinetics and selected pharmacodynamics of cobalt following a single intravenous administration to horses', *Drug Testing and Analysis*, vol. 7, no. 7, pp. 619-25.
Krug, O., Kutscher, D., Piper, T., Geyer, H., Schanzer, W. & Thevis, M. 2014, 'Quantifying cobalt in doping control urine samples - a pilot study', *Drug Testing and Analysis*, vol. 6, no. 11-12, pp. 1186-90.
Kuiper, C., Dachs, G.U., Currie, M.J. & Vissers, M.C. 2014, 'Intracellular ascorbate enhances hypoxia-inducible factor (HIF)-hydroxylase activity and preferentially suppresses the HIF-1 transcriptional response', *Free Radical Biology and Medicine*, vol. 69, pp. 308-17.
Lantin, A.-C., Vermeulen, J., Mallants, A., Vanoverschelde, J.-L., Speybroeck, N., Swennen, B., Hoet, P. & Lison, D. 2013, 'Occupational exposure to cobalt is not associated with incipient signs of dilated cardiomyopathy in a Belgian refinery', *Occupational and Environmental Medicine*, no. 70, pp. 386-92.
Leggett, R.W. 2008, 'The biokinetics of inorganic cobalt in the human body', *Science of The Total Environment*, vol. 389, no. 2–3, pp. 259-69.
Letourneau, E., Jack, G., McCullough, R. & Hollins, J. 1972, 'The metabolism of cobalt by the normal human male: whole body retention and radiation dosimetry', *Health Physics*, vol. 22, no. 5, pp. 451-9.
Levine, L. & Fahy, J. 1945, 'The significance of the specific gravity', *Journal of Industrial Hygiene Toxicology*, vol. 27, pp. 217-23.
Lippi, G., Franchini, M. & Guidi, G. 2005, 'Cobalt chloride administration in athletes: a new perspective in blood doping?', *British Journal of Sports Medicine*, vol. 39, no. 11, pp. 872-3.
Lippi, G., Franchini, M. & Guidi, G.C. 2006, 'Blood doping by cobalt. Should we measure cobalt in athletes?', *Journal of Occupational Medicine and Toxicology*, vol. 1, no. 1, p. 18.
Lippi, G. & Guidi, G. 2004, 'Gene manipulation and improvement of athletic performances: new strategies in blood doping', *British Journal of Sports Medicine*, vol. 38, no. 5, pp. 641-.
Lippi, G., Montagnana, M. & Guidi, G.C. 2006, 'Albumin cobalt binding and ischemia modified albumin generation: an endogenous response to ischemia?', *International Journal of Cardiology*, vol. 108, no. 3, pp. 410-1.

Llobet, J.M., Domingo, J.L. & Corbella, J. 1986, 'Comparison of the effectiveness of several chelators after single administration on the toxicity, excretion and distribution of cobalt', *Archives of Toxicology*, vol. 58, no. 4, pp. 278-81.
Lu, Y., Kippler, M., Harari, F., Grandér, M., Palm, B., Nordqvist, H. & Vahter, M. 2015, 'Alkali dilution of blood samples for high throughput ICP-MS analysis—comparison with acid digestion', *Clinical Biochemistry*, vol. 48, no. 3, pp. 140-7.
Lukaski, H.C. 2004, 'Vitamin and mineral status: effects on physical performance', *Nutrition*, vol. 20, no. 7–8, pp. 632-44.
Major, D. & Wenzel, R. 2016, 'Commentary on Paper: "Controlling the misuse of cobalt in horses"', *Drug Testing and Analysis*, vol. 8, no. 8, pp. 880-81.
Manthe, B.N. & Youngs, C.R. 2013, 'An overview of vitamin requirements of the domestic horse', *Natural Sciences Education*, vol. 42, no. 1, pp. 179-84.
Martin, A., Bois, F.Y., Pierre, F. & Wild, P. 2009, 'Occupational exposure to cobalt: a population toxicokinetic modeling approach validated by field results challenges the Biological Exposure Index for urinary cobalt', *Journal of Occupational and Environmental Hygiene*, vol. 7, no. 1, pp. 54-62.
Mascherpa, G. 1930, 'Le pouvoir hématopoietique du cobalt (The haematopoietic power of cobalt)', *Archives Italiennes de Biologie*, vol. 82, no. 22, pp. 112-20.
Maxwell, P.H., Wiesener, M.S., Chang, G.-W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R. & Ratcliffe, P.J. 1999, 'The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis', *Nature*, vol. 399, no. 6733, pp. 271-5.
McKeever, K., Agans, J., Geiser, S., Lorimer, P. & Maylin, G. 2006, 'Low dose exogenous erythropoietin elicits an ergogenic effect in standardbred horses', *Equine Veterinary Journal*, vol. 38, no. S36, pp. 233-8.
McKeever, K., Malinowski, K., Fenger, C., Duer, W. & Maylin, G. 2020, 'Evaluation of cobalt as a performance enhancing drug (PED) in racehorses', *Comparative Exercise Physiology*, pp. 1-10.
McKeever, K.H., Wickler, S.J., Smith, T.R. & Poole, D.C. 2010, 'Effects of high altitude and exercise on plasma erythropoietin in equids', *Comparative Exercise Physiology*, vol. 7, no. 4, pp. 193-9.
McKinney, A.R. 2009, 'Modern techniques for the determination of anabolic–androgenic steroid doping in the horse', *Bioanalysis*, vol. 1, no. 4, pp. 785-803.
McNeish, D. 2017, 'Small sample methods for multilevel modeling: A colloquial elucidation of REML and the Kenward-Roger correction', *Multivariate Behavioral Research*, vol. 52, no. 5, pp. 661-70.
Medicines and Healthcare products Regulatory Agency 2010, *Medical Device Alert*, MHRA, viewed 5 February 2012, <https://www.medsafe.govt.nz/hot/RecallActionNoticesNew/MetalOnMetalHipImplants/MHRA%20MDA-2010-069.pdf>.
Middleton, D.R., Watts, M.J., Lark, R.M., Milne, C.J. & Polya, D.A. 2016, 'Assessing urinary flow rate, creatinine, osmolality and other hydration adjustment methods for urinary biomonitoring using NHANES arsenic, iodine, lead and cadmium data', *Environmental Health*, vol. 15, no. 1, p. 68.
Middleton, D.R., Watts, M.J. & Polya, D.A. 2019, 'A comparative assessment of dilution correction methods for spot urinary analyte concentrations in a UK population exposed to arsenic in drinking water', *Environment International*, vol. 130, p. 104721.

Miller, R.B. 1983, *Textbook of nephrology*, vol. 2, Williams & Wilkins, Baltimore, MD.
Mobasheri, A. & Proudman, C.J. 2015, 'Cobalt chloride doping in racehorses: Concerns over a potentially lethal practice', *The Veterinary Journal*, vol. 205, no. 3, pp. 335-8.
Mørkeberg, J. 2013, 'Blood manipulation: current challenges from an anti-doping perspective', *Hematology*, vol. 2013, no. 1, pp. 627-31.
Muscat, J.E., Liu, A. & Richie, J.P. 2011, 'A comparison of creatinine vs. specific gravity to correct for urinary dilution of cotinine', *Biomarkers*, vol. 16, no. 3, pp. 206-11.
Narla, A. & Ebert, B.L. 2018, 'Red Cell Production', in E.J. Benz Jr, N. Berliner & F.J. Schiffman (eds), *Anemia*, Cambridge University Press, United Kingdom, pp. 14-22.
National Association of Testing Authorities 2018, *General Accreditation Guidance - Validation and verification of quantitative and qualitative test methods*, viewed 10 October 2019, <https://www.nata.com.au/phocadownload/gen-accreditation-guidance/Validation-and-Verification-of-Quantitative-and-Qualitative-Test-Methods.pdf>.
National Horseracing Authority of Southern Africa 2017, *South African testing for cobalt to recommence after inquiry*, ThoroughbredNEWS, viewed 2 March 2018, <https://www.thoroughbrednews.com.au/News/Story/94540>.
Newton, D. & Rundo, J. 1971, 'The long-term retention of inhaled cobalt-60', *Health Physics*, vol. 21, no. 3, pp. 377-84.
Nogueira-Pedro, A., Hastreiter, A. & Borelli, P. 2018, 'A review of select minerals influencing the haematopoietic process', *Nutrition Research Reviews*, vol. 31, no. 2, pp. 267-80.
Oke, S. 2019, 'Researcher: Cobalt misuse in horses is 'Ill-Conceived'', *The Horse: Your Guide To Equine Health Care*, Nov 15, 2019, <https://thehorse.com/181548/researcher-cobalt-misuse-in-horses-is-ill-conceived/>.
Ontario Racing Commission 2009, *ORC advises on use of cobalt sulfate*, Ontario Racing Commission, viewed 15 October 2019, <https://standardbredcanada.ca/notices/8-26-09/orc-advises-use-cobalt-sulfate.html>.
Orten, J.M. 1935, 'On the mechanism of the hematopoietic action of cobalt', *American Journal of Physiology-Legacy Content*, vol. 114, no. 2, pp. 414-22.
Orten, J.M., Underhill, F.A. & Lewis, R.C. 1932, 'A study of certain metals in the prevention of nutritional anæmia in the., Oct., 1932 rat', *Journal of Biological Chemistry*, vol. 96, pp. 1-9.
Orten, J.M., Underhill, F.A., Mugrage, E.R. & Lewis, R.C. 1932, 'Polycythemia in the rat on a milk-iron-copper diet supplemented by cobalt', *Journal of Biological Chemistry*, vol. 96, no. April, pp. 11-6.
Pagan, J. 2000, 'Micromineral requirements in horses', *World Equine Veterinary*, vol. 5, pp. 15-21.
Paustenbach, D.J., Tvermoes, B.E., Unice, K.M., Finley, B.L. & Kerger, B.D. 2013, 'A review of the health hazards posed by cobalt', *Critical Reviews in Toxicology*, vol. 43, no. 4, pp. 316-62.
Piyathilake, C.J., Robinson, C.B. & Cornwell, P. 2007, 'A practical approach to red blood cell folate analysis', *Analytical Chemistry Insights*, vol. 2, pp. 107-10.

Popot, M.-A., Ho, E.N.M., Wan, T.S.M., Arthur, R.M., Benson, D., Russo, C., Hincks, P., Pearce, C. & Bonnaire, Y. 2014, 'An international collaboration on cobalt for setting up a threshold value', paper presented to the *The 20th International Conference of Racing Analysts and Veterinarians*, Mauritius, 20-27 September 2014.

Popot, M.A., Ho, E.N., Stojiljkovic, N., Bagilet, F., Remy, P., Maciejewski, P., Loup, B., Chan, G.H., Hargrave, S. & Arthur, R.M. 2017, 'Interlaboratory trial for the measurement of total cobalt in equine urine and plasma by ICP-MS', *Drug Testing and Analysis*, vol. 9, no. 9, pp. 1400-6.

Princivalle, A., Iavicoli, I., Cerpelloni, M., Franceschi, A., Manno, M. & Perbellini, L. 2017, 'Biological monitoring of cobalt in hard metal factory workers', *International archives of occupational and environmental health*, vol. 90, no. 2, pp. 243-54.

Queensland Civil and Administrative Tribunal 2018, *Scott v Queensland Racing Integrity Commission (No 2) [2018] QCAT 301*, QCAT, viewed 10 October 2018, <https://archive.sclqld.org.au/qjudgment/2018/QCAT18-301.pdf>.

Queensland Civil and Administrative Tribunal 2019, *Graham v Queensland Racing Integrity Commission [2018] QCAT 198*, QCAT, viewed 31 July 2019, <https://archive.sclqld.org.au/qjudgment/2019/QCAT19-198.pdf>.

Queensland Racing Integrity Commission 2019, *The Queensland Racing Integrity Commission Annual Report 2018-19*, viewed 14 June 2020, <https://www.parliament.qld.gov.au/documents/tableOffice/TabledPapers/2019/5619T1630.pdf>.

Racing Animal Welfare and Integrity Board 2012, *The Collection Procedures*, 4 edn, Queensland Government, pp. 1-46.

Racing Australia 2017, *Australian Rules of Racing*, Racing Australia, viewed 2 February 2018, <https://racingaustralia.horse/uploadimg/Australian_rules_of_Racing/Australian_Rules_of_Racing_01_August_2017.pdf>.

Racing Australia 2019, *Australian Rules of Racing*, Racing Australia, viewed 14 June 2020, <https://www.racingaustralia.horse/uploadimg/Australian_rules_of_Racing/Australian_Rules_of_Racing_01_March_2019.pdf>.

Ractliffe, D. 2019a, 'Cobalt threshold suffice, says Racing Australia', *The Sydney Morning Herald*, 21 November, viewed 3 March 2020, <https://www.smh.com.au/sport/racing/cobalt-threshold-suffice-says-racing-australia-20191121-p53cvp.html>.

Ractliffe, D. 2019b, 'Racing Australia maintains tough stance on cobalt breaches', *The Sydney Morning Herald*, 20 November, viewed 5 March 2020, <https://www.smh.com.au/sport/racing/racing-australia-maintains-tough-stance-on-cobalt-breaches-20191120-p53cdh.html>.

Ractliffe, D. 2020, 'Emerging trainer slapped with cobalt charges', *The Age*, 5 June, viewed 10 June 2020, <https://www.theage.com.au/sport/racing/emerging-trainer-slapped-with-cobalt-charges-20200605-p5500q.html>.

Randaccio, L., Geremia, S., Demitri, N. & Wuerges, J. 2010, 'Vitamin B12: unique metalorganic compounds and the most complex vitamins', *Molecules*, vol. 15, no. 5, pp. 3228-59.

Reed, S., Bayly, W. & Sellon, D. 2009, 'Equine internal medicine. St. Louis, MO', USA: Elsevier Health Sciences, p. 1165.
Robert, C., Goachet, A.G., Fraipont, A., Votion, D.M., van Erck, E. & Leclerc, J.L. 2010, 'Hydration and electrolyte balance in horses during an endurance season', *Equine Veterinary Journal*, vol. 42, pp. 98-104.
Rumbaugh, G., Carlson, G. & Harrold, D. 1982, 'Urinary production in the healthy horse and in horses deprived of feed and water', *American Journal of Veterinary Research*, vol. 43, no. 4, pp. 735-7.
Salazar, R.F., Guerra, M.B., Pereira-Filho, E.R. & Nóbrega, J.A. 2011, 'Performance evaluation of collision–reaction interface and internal standardization in quadrupole ICP-MS measurements', *Talanta*, vol. 86, pp. 241-7.
Sauve, J.F., Levesque, M., Huard, M., Drolet, D., Lavoue, J., Tardif, R. & Truchon, G. 2015, 'Creatinine and specific gravity normalization in biological monitoring of occupational exposures', *Journal of Occupational and Environmental Hygiene*, vol. 12, no. 2, pp. 123-9.
Savage, C.J. 2008, 'Urinary clinical pathologic findings and glomerular filtration rate in the horse', *Veterinary Clinics of North America: Equine Practice*, vol. 24, no. 2, pp. 387-404.
Schlink, L. 2019, 'Horse racing: Vet says cobalt doesn't enhance performance', *The Australian*, 18 November 2019, viewed 2 April 2020, <https://www.theaustralian.com.au/sport/horse-racing/horse-racing-vet-says-cobalt-doesnt-enhance-performance/news-story/910a6aef3ff15f908a1b9b9f133c0a15>.
Schmidt, W. & Prommer, N. 2005, 'The optimised CO-rebreathing method: a new tool to determine total haemoglobin mass routinely', *European Journal of Applied Physiology*, vol. 95, no. 5-6, pp. 486-95.
Schmidt, W. & Prommer, N. 2010, 'Impact of alterations in total hemoglobin mass on $\dot{V}$O2max', *Exercise and Sport Sciences Reviews*, vol. 38, no. 2, pp. 68-75.
Schmidt, W.F.J., Hoffmeister, T., Wachsmuth, N. & Byrnes, W.C. 2019, 'Cobalt Misuse in Sports', *German Journal of Sports Medicine*, vol. 70, no. 5, pp. 129-33.
Schumacher, Y.O., Garvican, L.A., Christian, R., Lobigs, L.M., Qi, J., Fan, R., He, Y., Wang, H., Gore, C.J. & Ma, F. 2015, 'High altitude, prolonged exercise, and the athlete biological passport', *Drug Testing and Analysis*, vol. 7, no. 1, pp. 48-55.
Schwarz, F., Kirchgessner, M. & Stangl, G. 2000, 'Cobalt requirement of beef cattle—feed intake and growth at different levels of cobalt supply', *Journal of Animal Physiology and Animal Nutrition*, vol. 83, no. 3, pp. 121-31.
Scott, N. 2019, 'Impact of cobalt regulation on the Australian racing industry', personal communication, 2 December.
Simonsen, L.O., Brown, A.M., Harbak, H., Kristensen, B.I. & Bennekou, P. 2011, 'Cobalt uptake and binding in human red blood cells', *Blood Cells, Molecules, and Diseases*, vol. 46, no. 4, pp. 266-76.
Simonsen, L.O., Harbak, H. & Bennekou, P. 2011, 'Passive transport pathways for Ca^{2+} and Co^{2+} in human red blood cells. $^{57}Co^{2+}$ as a tracer for Ca^{2+} influx', *Blood Cells, Molecules, and Diseases*, vol. 47, no. 4, pp. 214-25.
Simonsen, L.O., Harbak, H. & Bennekou, P. 2012, 'Cobalt metabolism and toxicology - A brief update', *Science of the Total Environment*, vol. 432, pp. 210-5.
Singh, G.K., Balzer, B.W., Desai, R., Jimenez, M., Steinbeck, K.S. & Handelsman, D.J. 2015, 'Requirement for specific gravity and creatinine adjustments for urinary

steroids and luteinizing hormone concentrations in adolescents', *Annals of Clinical Biochemistry*, vol. 52, no. 6, pp. 665-71.
Smith, T., Edmonds, C. & Barnaby, C. 1972, 'Absorption and retention of cobalt in man by whole-body counting', *Health Physics*, vol. 22, no. 4, pp. 359-67.
Southwood, L.L. 2013, *Practical guide to equine colic*, 1st edn, John Wiley & Sons, Inc., New Jersey.
Spierto, F., Hannon, W., Gunter, E. & Smith, S. 1997, 'Stability of urine creatinine', *Clinica Chimica Acta*, vol. 264, no. 2, pp. 227-32.
Stemme, K., Meyer, U., Lebzien, P., Flachowsky, G. & Scholz, H. 2003, 'Cobalt and vitamin B12 requirement of dairy cows', *Vitamine und Zusatzstoffe in der Ernährung von Mensch und Tier*, vol. 9, pp. 61-7.
Stillions, M., Teeter, S. & Nelson, W. 1971, 'Utilization of dietary vitamin B12 and cobalt by mature horses', *Journal of Animal Science*, vol. 32, no. 2, pp. 252-5.
Takahashi-Iñiguez, T., García-Hernandez, E., Arreguín-Espinosa, R. & Flores, M.E. 2012, 'Role of vitamin B12 on methylmalonyl-CoA mutase activity', *Journal of Zhejiang University. Science. B*, vol. 13, no. 6, pp. 423-37.
The Meadowlands 2014, *The Meadowlands uncovers use of new performance-enhancing drug*, Paulick Report, viewed 10 October 2019, <https://www.paulickreport.com/news/the-biz/the-meadowlands-uncovers-use-of-new-performance-enhancing-drug/>.
Thevis, M., Kuuranne, T., Walpurgis, K., Geyer, H. & Schänzer, W. 2016, 'Annual banned-substance review: analytical approaches in human sports drug testing', *Drug Testing and Analysis*, vol. 8, no. 1, pp. 7-29.
Tholen, D.W., Kallner, A., Kennedy, J.W., Krouwer, J.S. & Meier, K. 2004, *Evaluation of precision performance of quantitative measurement methods; approved guideline—second edition*, vol. 24, no. 25, Clinical and Laboratory Standards Institute: Wayne, PA.
Toribio, R.E. 2007, 'Essentials of equine renal and urinary tract physiology', *Veterinary Clinics of North America: Equine Practice*, vol. 23, no. 3, pp. 533-61.
Toutain, P.-L. 2010, 'Veterinary medicines and competition animals: the question of medication versus doping control', in F. Cunningham, J. Elliott & P. Lees (eds), *Comparative and Veterinary Pharmacology*, Springer, Heidelberg, pp. 315-39.
Tvermoes, B.E., Unice, K.M., Paustenbach, D.J., Finley, B.L., Otani, J.M. & Galbraith, D.A. 2014, 'Effects and blood concentrations of cobalt after ingestion of 1 mg/d by human volunteers for 90 d', *The American Journal of Clinical Nutrition*, vol. 99, no. 3, pp. 632-46.
U.S. Geological Survey 2020, *Cobalt statistics and information*, USGS, viewed 9th June 2020, <https://www.usgs.gov/centers/nmic/cobalt-statistics-and-information>.
Varian 2004, *Varian 820MS operation manual*, Varian Inc., Mulgrave, VIC, Australia.
Varian 2007, *ICP-MS Expert*, ICP-MS Instrument Software, version v2.1 b107, Varian Australia Pty. Ltd., viewed 27/06/2007.
Vaughan, M.A. & Horlick, G. 1986, 'Oxide, hydroxide, and doubly charged analyte species in inductively coupled plasma/mass spectrometry', *Applied Spectroscopy*, vol. 40, no. 4, pp. 434-45.
WADA Laboratory Expert Group 2018, *Decision limits for the confirmatory quantification of threshold substances*, World Anti-Doping Agency, viewed 18

May 2020, <https://www.wada-ama.org/sites/default/files/resources/files/td2018dl_v1_en.pdf>.
Waltner, K. & Waltner, K. 1929, 'Kobalt und blut (Cobalt and blood)', *Klinische Wochenschrift*, vol. 8, no. 7, p. 313.
Ward, G., Simpson, A., Boscato, L. & Hickman, P.E. 2017, 'The investigation of interferences in immunoassay', *Clinical Biochemistry*, vol. 50, no. 18, pp. 1306-11.
Warrack, B.M., Hnatyshyn, S., Ott, K.H., Reily, M.D., Sanders, M., Zhang, H. & Drexler, D.M. 2009, 'Normalization strategies for metabonomic analysis of urine samples', *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 877, no. 5-6, pp. 547-52.
Wenzel, R., Major, D., Hesp, K., Hall, E. & Doble, P. 2019, 'Cobalt accumulation in horses following repeated administration of cobalt chloride', *Australian Veterinary Journal*, vol. 97, no. 11, pp. 465-72.
Wintrobe, M., Grinstein, M., Dubash, J., Humphreys, S., Ashenbrucker, H. & Worth, W. 1947, 'The anemia of infection; the influence of cobalt on the anemia associated with inflammation.', *Blood*, vol. 2, pp. 323-31.
Wood, T.W., Weckman, T.J., Henry, P.A., Chang, S.-L., Hyde, W., Blake, J.W., Tobin, T. 1988, 'Population Parameters of Equine Urine pH and Methods of Acidification', *Proceedings of the 7th International Conference of Racing Analysts and Veterinarians*.
World Anti-Doping Agency 2013, *Endogenous erythropoietin stimulation by CO-breathing and by stabilizing HIF-1 by oral Cobalt application*, Accepted as a WADA funded study 2013, viewed 16 October 2016, <https://wada-main-prod.s3.amazonaws.com/resources/files/13d21ws_pr._schmidt_summary.pdf>.
World Anti-Doping Agency 2014, *The 2014 Prohibited List World Anti-Doping Code*, viewed 13 April 2016, <https://www.wada-ama.org/en/resources>.
World Anti-Doping Agency 2015a, *2015 Prohibited List Summary of Major Modifications and Explanatory Notes*, World Anti-Doping Agency, viewed 18 April 2016, <https://www.wada-ama.org/en/resources>.
World Anti-Doping Agency 2015b, *The 2015 Prohibited List World Anti-Doping Code*, viewed 24 July 2016, <https://www.wada-ama.org/en/resources>.
World Anti-Doping Agency 2020, *The 2020 Prohibited List World Anti-Doping Code*, WADA, viewed 21 May 2020, <https://www.wada-ama.org/sites/default/files/wada_2020_english_prohibited_list_0.pdf>.
World Health Organization 1996, 'Biological monitoring of chemical exposure in the workplace: guidelines'.
Yamada, K. 2013, 'Chapter 9. Cobalt: Its Role in Health and Disease', in A. Sigel, H. Sigel & R.K.O. Sigel (eds), *Interrelations between Essential Metal Ions and Human Diseases*, 1st edn, vol. 13, Springer Netherlands, Dordrecht, pp. 295–320.
Yang, J. & Black, J. 1994, 'Competitive binding of chromium, cobalt and nickel to serum proteins', *Biomaterials*, vol. 15, no. 4, pp. 262-8.
Yaqub, L.S., Mshelia, W.P. & Ayo, J.O. 2014, 'Erythrocyte osmotic fragility and hematological responses of horses administered ascorbic acid and exposed to road transportation', *Journal of Equine Veterinary Science*, vol. 34, no. 11-12, pp. 1324-8.

Yeh, H.-C., Lin, Y.-S., Kuo, C.-C., Weidemann, D., Weaver, V., Fadrowski, J., Neu, A. & Navas-Acien, A. 2015, 'Urine osmolality in the US population: implications for environmental biomonitoring', *Environmental Research*, vol. 136, pp. 482-90.

9. APPENDICES

1. Ethics approvals for animal research

NSW GOVERNMENT | Department of Primary Industries

TRIM 15/216 (3)
Secretary's ACEC Meeting 166, 2 February 2015

24 February 2015

Dr Derek Major
PO Box 419
RICHMOND NSW 2753

Dear Dr Major

I refer to your application for approval of a research proposal entitled:

A STUDY ON URINARY AND PLASMA LEVELS OF COBALT FOLLOWING ADMINISTRATION OF STANDARD REGISTERED COBALT SUPPLEMENTS

The Secretary's Animal Care and Ethics Committee met on 2 February 2015 and considered the documentation for this project.

I am pleased to advise you of approval for your project for a period of three years on the understanding that annual reports on the research are provided in the format supplied. The Committee should be informed immediately if any significant problems occur during the course of the research.

Should the project extend beyond three years from the date of approval a full application for renewal must be submitted. Your Animal Research Authority must be renewed annually.

Do not hesitate to contact me should you have any questions about this advice or your obligations under the Code of Practice and the Animal Research Act.

Yours sincerely

Amanda Paul

AMANDA PAUL
Executive Officer
Secretary's Animal Care and Ethics Committee
Encl: Report Form (General)

ANIMAL WELFARE UNIT – BIOSECURITY NSW
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ABN 72 189 919 072
Animal Ethics Infolink – www.animalethics.org.au
Tel: 02 6391 3682
Fax: 02 6391 3740

ANIMAL RESEARCH AUTHORITY

Issued by the

SECRETARY
NSW TRADE & INVESTMENT

Principal Investigator: **Dr Derek Major**
PO Box 419
RICHMOND NSW 2753

Other Participants: **Veterinarians and nursing staff at Agnes Banks Equine Clinic**

are authorised to conduct the following research

A STUDY ON URINARY AND PLASMA LEVELS OF COBALT FOLLOWING ADMINISTRATION OF STANDARD REGISTERED COBALT SUPPLEMENTS

Being a study on urinary and plasma levels of Cobalt following administration of standard registered Cobalt supplements

Location: **Agnes Banks Equine Clinic, 5 Price Lane, AGNES BANKS, New South Wales**

as approved by and in accordance with the
ANIMAL CARE AND ETHICS COMMITTEE OF THE SECRETARY NSW TRADE & INVESTMENT

Being animal research carried out in accordance with the Code of Practice, for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

This authority remains in force from **2 February 2015** to **2 February 2016** to unless suspended, cancelled or surrendered

SUZANNE ROBINSON **24 February 2015**
SENIOR MANAGER – ANIMAL WELFARE*
ANIMAL WELFARE UNIT
NSW Department of Primary Industries, an office of Trade and Investment
*Delegate of the Secretary of the Department of Trade and Investment, Regional Infrastructure and Services

TRIM File 15/216 (3)
Secretary's Meeting 166, 2 February 2015

ANIMAL CARE AND ETHICS COMMITTEE OF THE SECRETARY NSW TRADE & INVESTMENT

CERTIFICATE OF APPROVAL

Dr Derek Major
PO Box 419
RICHMOND NSW 2753

Is approved to conduct the following research

A STUDY ON URINARY AND PLASMA LEVELS OF COBALT FOLLOWING ADMINISTRATION OF STANDARD REGISTERED COBALT SUPPLEMENTS

as approved by and in accordance with the

ANIMAL CARE AND ETHICS COMMITTEE OF THE SECRETARY NSW TRADE & INVESTMENT

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This approval remains in force from **2 February 2015** to **2 February 2018** unless suspended, cancelled or surrendered

Amanda Paul

AMANDA PAUL
EXECUTIVE OFFICER (Secretary's ACEC) *
ANIMAL WELFARE UNIT

Date: 24 February 2015

NSW Department of Primary Industries, an office of the NSW Trade and Investment

* Delegate of the Secretary of the Department of Trade and Investment, Regional Infrastructure and Services

PAGE 3 OF 3

NSW GOVERNMENT | Department of Primary Industries

TRIM 16/1381 (4)
Secretary's ACEC Meeting 177, 15 August 2016

23 August 2016

Dr Derek Major
PO Box 419
RICHMOND NSW 2753

Dear Dr Major

I refer to your application for approval of a research proposal entitled:

STUDY ON URINARY, PLASMA AND FAECAL LEVELS OF COBALT FOLLOWING ADMINISTRATION OF STANDARD REGISTERED COBALT SUPPLEMENTS, COMPARED WITH ADMINISTRATION OF THE COBALT SALT IN ISOLATION

The Secretary's Animal Care and Ethics Committee met on 15 August 2016 and considered the documentation for this project.

I am pleased to advise you of approval for your project for a period of one year on the understanding that a final report on the research is provided in the format supplied. The Committee should be informed immediately if any significant problems occur during the course of the research.

Should the project extend beyond one year from the date of approval a full application for renewal must be submitted.

Do not hesitate to contact me should you have any questions about this advice or your obligations under the Code of Practice and the Animal Research Act.

Yours sincerely

Amanda Paul

AMANDA PAUL
Executive Officer
Secretary's Animal Care & Ethics Committee
Encl: ARA and Protocol Certificates

Animal Welfare Unit | Locked Bag 21 | Orange NSW 2800 | T: 02 6391 3682 | F: 02 6391 3740
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TRIM 16/1381 (4) Secretary's ACEC Meeting 177 15 AUGUST 2016

ANIMAL RESEARCH AUTHORITY

Issued by the

SECRETARY
NSW INDUSTRY, SKILLS AND REGIONAL DEVELOPMENT

Principal Investigator: **Dr Derek Major**
PO Box 419
RICHMOND NSW 2753

Associate Investigator: **Mr Ross Wenzel**

Other Participants: **Veterinarians and nursing staff at Agnes Banks Equine Clinic**

are authorised to conduct the following research

STUDY ON URINARY, PLASMA AND FAECAL LEVELS OF COBALT FOLLOWING ADMINISTRATION OF STANDARD REGISTERED COBALT SUPPLEMENTS, COMPARED WITH ADMINISTRATION OF THE COBALT SALT IN ISOLATION

Being a study on urinary and plasma levels of Cobalt following administration of standard registered Cobalt supplements

Location: Agnes Banks Equine Clinic, 5 Price Lane, AGNES BANKS, New South Wales

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ANIMAL CARE AND ETHICS COMMITTEE OF THE SECRETARY NSW INDUSTRY, SKILLS AND REGIONAL DEVELOPMENT

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This authority remains in force from **15 August 2016** to **15 August 2017** unless suspended, cancelled or surrendered

SUZANNE ROBINSON **23 August 2016**
DIRECTOR – ANIMAL WELFARE*
ANIMAL WELFARE UNIT
NSW Department of Primary Industries, an office of NSW Department of Industry, Skills & Regional Development
*Delegate of the Secretary of the Department of Industry, Skills & Regional Development

16/1381 (4) Secretary's ACEC 177 15 AUGUST 2016

ANIMAL CARE AND ETHICS COMMITTEE OF

THE SECRETARY
NSW INDUSTRY, SKILLS AND REGIONAL DEVELOPMENT

CERTIFICATE OF APPROVAL

Dr Derek Major
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RICHMOND NSW 2753

Is approved to conduct the following research

STUDY ON URINARY, PLASMA AND FAECAL LEVELS OF COBALT FOLLOWING ADMINISTRATION OF STANDARD REGISTERED COBALT SUPPLEMENTS, COMPARED WITH ADMINISTRATION OF THE COBALT SALT IN ISOLATION

as approved by and in accordance with the

ANIMAL CARE AND ETHICS COMMITTEE OF THE SECRETARY NSW INDUSTRY, SKILLS AND REGIONAL DEVELOPMENT

Being animal research carried out in accordance with the Code of Practice, for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence

This approval remains in force from **15 August 2016** to **15 August 2017** unless suspended, cancelled or surrendered

Amanda Paul

AMANDA PAUL **23 August 2016**
EXECUTIVE OFFICER (Secretary's ACEC)*
ANIMAL WELFARE UNIT
NSW Department of Primary Industries, an office of NSW Department of Industry, Skills & Regional Development
*Delegate of the Secretary of the Department of Industry, Skills & Regional Development

PAGE 3 OF 3

2. Method precision data as described in Section 3.6.1.

Table A2-9.1 Method 1 precision data for samples prepared in an acidic diluent with helium as the CRI gas.

Matrix		**Serum**		**Blood**		**Urine**	
QC	**Seronorm Lot #:**	**1309438**	**1309416**	**1702821**	**1702825**	**1403080**	**1403081**
Units	$\mu g\ L^{-1}$						
Day #	Measurement	Result	Result	Result	Result	Result	Result
1	1st reading	1.0787	2.9551	0.2101	4.4227	0.8533	9.7239
	2nd reading	1.1580	2.9477	0.1283	4.5222	0.7240	9.8407
2	1st reading	0.9563	2.6060	0.2056	4.5745	0.7786	10.3070
	2nd reading	1.0486	2.8083	0.1670	4.5551	0.8344	9.8678
3	1st reading	1.0555	3.1427	0.2363	4.3839	0.9052	10.3202
	2nd reading	0.9809	2.7462	0.2342	4.5286	0.8144	10.3311
4	1st reading	0.9807	2.8521	0.2113	4.9071	0.8993	10.1267
	2nd reading	1.1442	2.9657	0.1638	5.0113	0.8472	9.9591
5	1st reading	1.1374	2.8591	0.2514	5.0865	0.7349	9.7457
	2nd reading	1.0417	2.6728	0.2278	4.8289	0.8100	9.9789
6	1st reading	1.0985	3.0449	0.2458	4.9297	0.7986	10.2997
	2nd reading	0.9563	2.8720	0.2097	4.6206	0.8916	9.9769
7	1st reading	1.0561	2.9178	0.2548	4.9217	0.9146	9.5968
	2nd reading	1.0218	2.7592	0.1585	5.1836	0.7742	9.9285
8	1st reading	0.9621	2.8958	0.2044	4.7875	0.7717	10.2765
	2nd reading	1.0323	2.7297	0.2431	4.6658	0.8550	10.1837
9	1st reading	1.0160	2.9945	0.2183	4.7024	0.7113	10.1783
	2nd reading	1.0180	2.9964	0.1580	4.6030	0.8119	10.1134
10	1st reading	0.9647	2.9924	0.1995	4.6456	0.7998	10.1569
	2nd reading	0.9688	2.8004	0.2450	4.9571	0.8444	10.1480
11	1st reading	0.9293	3.0239	0.1593	4.5720	0.7963	10.2856
	2nd reading	0.9955	3.0465	0.2037	4.6715	0.7691	10.5533
12	1st reading	1.0257	2.8522	0.2663	4.3557	0.7346	10.0238
	2nd reading	1.0542	2.9006	0.2477	4.3185	0.7792	10.1434
13	1st reading	0.9658	2.7242	0.1407	4.6709	0.7179	10.1588
	2nd reading	1.0475	2.8377	0.1475	4.8562	0.7512	10.2805
14	1st reading	1.0061	2.7229	0.2219	4.9216	0.7884	10.4292
	2nd reading	1.1346	2.7055	0.2366	4.6690	0.7146	10.2863
15	1st reading	0.9791	2.8935	0.2869	4.4213	0.8514	10.5046
	2nd reading	0.9646	2.8017	0.3229	4.4592	0.8464	10.2312
16	1st reading	0.9963	2.7040	0.2325	5.1982	0.7584	10.3730
	2nd reading	1.0050	2.6506	0.2254	4.8748	0.7967	10.1424
17	1st reading	1.0347	2.7375	0.2107	4.6682	0.7654	9.9755
	2nd reading	1.0475	2.8355	0.2383	4.9787	0.7587	10.2528
18	1st reading	1.0514	2.8444	0.2078	4.6418	0.8917	10.1787
	2nd reading	1.0710	2.9602	0.2312	4.6975	0.7699	10.0816
19	1st reading	1.0854	2.7141	0.2388	4.6885	0.6838	10.5368
	2nd reading	1.1456	2.8119	0.2103	4.5479	0.9174	10.4213
20	1st reading	0.9848	3.0207	0.2021	4.5865	0.9303	10.1726
	2nd reading	1.0008	3.0789	0.2203	5.0827	0.8158	10.1736
	Grand Mean	1.03	2.86	0.22	4.72	0.81	10.16
	Number of days (I)	20	20	20	20	20	20
	Runs per day	1	1	1	1	1	1

	Number of replicates per run (N)	2	2	2	2	2	2
	ME = Total WR Variance (S2r)	0.0029	0.0107	0.0009	0.0244	0.0044	0.0216
	Total WR SD (Sr)	0.0538	0.1035	0.0296	0.1561	0.0665	0.1471
	Variance of Daily Means (B2)	0.0021	0.0119	0.0012	0.0407	0.0018	0.0376
	SD of Daily Means (B)	0.0456	0.1093	0.0343	0.2016	0.0428	0.1939
	ST	0.0594	0.1315	0.0402	0.2299	0.0636	0.2200
	MD	0.0042	0.0239	0.0024	0.0813	0.0037	0.0752
	T	37	33	32	30	39	29
	Repeatability (Sr)	0.0538	0.1035	0.0296	0.1561	0.0665	0.1471
	CV	5.2 %	3.6 %	13.7 %	3.3 %	8.3 %	1.4 %

Table A2-9.2 Method 2 precision data for samples prepared in an alkaline diluent with hydrogen as CRI gas.

Matrix		**Serum**		**Blood**		**Urine**	
QC	**Seronorm Lot #:**	**1309438**	**1309416**	**1702821**	**1702825**	**1403080**	**1403081**
Units	µg L^{-1}						
Day #	Measurement	Result	Result	Result	Result	Result	Result
1	1st reading	1.0281	2.7440	0.2683	4.6285	0.8977	9.8047
	2nd reading	1.1976	2.9571	0.2775	4.9294	0.7845	10.0224
2	1st reading	1.0191	2.8588	0.3255	4.7149	0.8221	10.7490
	2nd reading	1.2440	2.8548	0.2966	4.9872	0.7595	10.3990
3	1st reading	1.0714	2.9092	0.3372	5.1603	0.8420	10.6717
	2nd reading	1.0175	2.8222	0.2890	4.8521	0.7919	10.5187
4	1st reading	1.1654	3.0913	0.3656	5.0381	0.7757	10.7037
	2nd reading	1.1443	2.9783	0.2571	5.2304	0.8503	10.7758
5	1st reading	1.1758	3.2078	0.3006	4.8137	0.9438	10.6834
	2nd reading	1.1794	3.1464	0.3583	4.6897	0.8390	10.9525
6	1st reading	1.2547	3.1880	0.3330	5.5777	0.9409	10.5939
	2nd reading	1.0629	2.9618	0.2246	5.2756	0.8691	10.6064
7	1st reading	1.1454	3.0652	0.2924	5.2986	0.8271	10.8400
	2nd reading	1.2972	3.0221	0.2662	5.0825	0.8087	10.9018
8	1st reading	1.2291	2.9057	0.3464	5.0208	0.8098	10.5086
	2nd reading	1.0787	3.2087	0.2084	5.1863	0.7689	10.5660
9	1st reading	1.1129	3.0173	0.2461	5.3932	0.8772	10.7562
	2nd reading	1.1596	3.1165	0.2970	5.1200	0.9064	11.1891
10	1st reading	1.1936	3.1163	0.3150	5.4935	0.9025	10.6870
	2nd reading	1.2928	3.1914	0.2945	5.2119	0.8666	10.6252
11	1st reading	1.0436	3.1290	0.2553	4.9794	0.8183	11.1372
	2nd reading	1.0880	3.1925	0.3070	5.0495	0.9347	10.9825
12	1st reading	1.0263	3.2525	0.3899	4.9378	0.8595	11.0243
	2nd reading	1.1305	2.8395	0.3851	4.8579	0.7893	10.6759
13	1st reading	1.0876	3.1042	0.2908	5.2142	0.8971	10.5708
	2nd reading	1.0394	2.9727	0.3351	4.9204	0.9638	11.2721

14	1st reading	1.1697	3.1186	0.3164	5.3253	0.8435	10.1171
	2nd reading	1.1062	3.1135	0.2566	5.1863	0.9071	10.6817
15	1st reading	1.2631	3.0895	0.2497	5.1565	1.1039	10.8372
	2nd reading	1.1563	2.7952	0.3449	4.7862	0.8547	10.5876
16	1st reading	1.0715	3.2610	0.2981	4.7903	0.7807	9.8777
	2nd reading	1.0428	3.0819	0.2193	5.0426	1.0105	10.3734
17	1st reading	1.1532	3.0929	0.3503	4.5923	0.8249	11.0412
	2nd reading	0.9856	3.0053	0.2519	5.2871	0.8124	10.7716
18	1st reading	1.1346	3.0289	0.2965	5.0692	0.9161	10.5022
	2nd reading	1.0352	3.0752	0.3188	5.3939	0.8113	10.9101
19	1st reading	1.1861	3.1639	0.2621	5.0297	0.8393	10.0738
	2nd reading	1.1580	3.0308	0.3553	5.0138	0.7758	10.6579
20	1st reading	1.0616	2.9819	0.3405	5.3823	0.7932	10.7469
	2nd reading	1.0304	3.1539	0.2927	5.0074	0.7838	10.7292
	Grand Mean	1.13	3.05	0.30	5.07	0.86	10.65
	Number of days (I)	20	20	20	20	20	20
	Runs per day	1	1	1	1	1	1
	Number of replicates per run (N)	2	2	2	2	2	2
	ME = Total WR Variance (S2r)	0.0062	0.0149	0.0024	0.0419	0.0051	0.0578
	Total WR SD (Sr)	0.0788	0.1222	0.0494	0.2048	0.0711	0.2405
	Variance of Daily Means (B2)	0.0037	0.0097	0.0008	0.0348	0.0027	0.0809
	SD of Daily Means (B)	0.0608	0.0986	0.0278	0.1865	0.0523	0.2844
	ST	0.0825	0.1311	0.0446	0.2362	0.0725	0.3313
	MD	0.0074	0.0194	0.0016	0.0696	0.0055	0.1617
	T	39	38	38	36	39	31
	Repeatability (Sr)	0.0788	0.1222	0.0494	0.2048	0.0711	0.2405
	CV	7.0 %	4.0 %	16.4 %	4.0 %	8.3 %	2.3 %

3. Results of trace element determinations in feedstuffs and supplements described in Section 3.6.3.

Table A3-9.3 Results of analysis for first batch of digested feedstuffs and supplements. Replicate measurements and dry weights of the sub-samples given. Track torque was composed of 4 different types of grain. They have been analysed in triplicate and designated grain A, B, C and D. The 28.4 g BLUD sachet was labelled to contain 150 µg B12, 100 mg iron and 5 mg copper, equivalent to 0.23 µg g^{-1} cobalt, 3521 µg g^{-1} iron and 70 µg g^{-1} copper.

Sample	Co	Mg	Mn	Fe	Ni	Cu	Zn
	---- (µg g^{-1}) ----						
Reagent blank	0.00	8	0	-2	0.0	0	-2
NIST Liver 1577b 0.00589g	0.28	660	9	193	0.8	169	138
Green chaff 0.03914g	0.09	2940	26	86	0.7	8	28
Green chaff 0.01868g	0.27	3441	37	137	2.0	7	45
Green chaff 0.03182g	0.13	2541	23	131	0.7	7	20
Total	**0.16**						
Track torque 0.13572g - Grain A	0.60	1592	166	61	1.3	30	167
Track torque 0.19614g - Grain A	0.44	1973	194	73	2.1	37	151
Track torque 0.19452g - Grain A	0.69	2072	333	615	1.7	87	174
Track torque 0.05505g - Grain B	0.75	1253	119	73	1.4	568	246
Track torque 0.05297g - Grain B	0.47	1189	155	76	2.5	69	146
Track torque 0.06545g - Grain B	0.45	1219	294	69	2.0	40	218
Track torque 0.05115g - Grain C	0.65	2426	103	543	3.7	68	220
Track torque 0.03746g - Grain C	0.45	2322	87	68	6.3	44	172
Track torque 0.03622g - Grain C	0.38	3156	80	73	6.3	160	195
Track torque 0.11880g - Grain D	0.46	595	113	55	0.3	45	180
Track torque 0.29403g - Grain D	0.30	1244	74	96	0.2	27	114
Track torque 0.15160g - Grain D	0.38	1065	67	57	0.4	36	126
Total	**0.50**						
NIST Liver 1577b 0.00288g	0.27	770	11	220	1.5	197	172
Reagent blank	-0.01	4	0	0	0.0	0	-2
Feramo H with chromium 0.03566g	4.71	4117	2910	3901	3.4	658	3318
Feramo H with chromium 0.02600g	5.45	3633	2915	4052	3.6	769	3304
Feramo H with chromium 0.06400g	4.95	3847	2968	3482	3.5	662	3439
Total	**5.04**						
Oats 0.04657g	0.05	1234	57	33	1.7	5	17
Oats 0.03675g	0.08	2056	62	33	4.5	6	35
Oats 0.05303g	0.05	1340	71	41	3.6	4	16
Total	**0.06**						
Cool conditioner 0.05382g	0.36	5012	193	176	1.2	19	150
Cool conditioner 0.08256g	0.41	4756	197	187	1.2	316	279
Total	**0.39**						
Reagent blank	0.00	24	0	0	0.0	1	-2
NIST SRM 1577b 0.00589g	0.22	664	8	184	0.5	166	129
Mitavite Breeda 0.28696g	0.36	4577	168	190	1.9	64	179

Mitavite Breeda 0.24952g	0.35	4781	168	192	2.0	58	175
Mitavite Breeda 0.25578g	0.37	4869	167	213	2.0	63	162
Total	**0.36**						
Yellow chaff 0.04470g	0.04	946	98	34	0.3	2	5
Yellow chaff 0.05418g	0.05	968	81	50	0.4	1	4
Yellow chaff 0.03662g	0.05	1330	82	68	0.3	2	5
Total	**0.05**						
BLUD sachet 0.06201g	0.15	6	4	2877	0.6	9	2
BLUD sachet 0.06482g	0.12	1	4	2872	0.7	8	1
BLUD sachet 0.05682g	0.15	0	5	3621	0.8	44	1
Total	**0.14**	**3**	**5**	**3123**	**0.7**	**20**	**1**
NIST Liver 1577b 0.00288g	0.28	599	8	353	1.6	191	164
Reagent blank	0.00	1	-1	5	0.1	0	1

Table A3-9.4 Results of analysis for second batch of digested feedstuffs and supplements. Replicate measurements and dry weights of the sub-samples given. Mitavite athlete plus was composed of 3 different types of grain designated grain A, B, C and loose debris and salt designated sample D. The 28.4 g BLUD sachet was labelled to contain 150 µg B12, 100 mg Fe and 5 mg Cu, equivalent to 0.23 µg g^{-1} Co, 3521 µg g^{-1} Fe and 70 µg g^{-1} Cu.

Sample	Co	Mg	Mn	Fe	Ni	Cu	Zn
	---- (µg g^{-1}) ----						
Reagent blank	0.00	-8	0	6	0.0	0	1
NIST Liver 1577b 0.00099g	0.19	509	10	188	0.0	163	128
Mitavite Athlete Plus							
Mitavite 0.12644g - Grain A	0.24	3690	113	80	8.9	46	319
Mitavite 0.07135g - Grain A	0.39	5483	158	78	3.4	146	315
Mitavite 0.18300g - Grain B	1.09	3519	277	205	2.5	80	464
Mitavite 0.09683g - Grain B	0.34	2376	208	69	1.6	141	260
Mitavite 0.10024g - Grain C	0.92	3979	283	149	3.5	416	755
Mitavite 0.09107g - Sample D	1.97	5478	525	452	3.6	274	774
Mitavite 0.04830g - Sample D	1.84	5982	1028	448	3.6	136	1055
Total	**0.97**	**4358**	**370**	**212**	**3.9**	**177**	**563**
E Mag 500 0.04752g	14.58	486393	806	1065	93.4	14	8
E Mag 500 0.01647g	14.32	484628	785	1211	97.4	15	10
Total	**14.45**	**485511**	**796**	**1138**	**95**	**14.5**	**9.0**
Salkavite 0.04473g	1.49	16802	230	123	8.0	1	46
Salkavite 0.02888g	1.45	16396	234	120	8.1	0	46
Total	**1.47**	**16599**	**232**	**122**	**8.1**	**0.5**	**46**
BLUD sachet 0.07061g	0.18	0	3	1838	0.4	6	0
BLUD sachet 0.02419g	0.09	-4	3	1796	0.5	5	1
Total	**0.14**	**-2**	**3**	**1817**	**0.5**	**6**	**1**
NIST Liver 1577b 0.00273g	0.23	599	10	179	0.3	156	123
Reagent blank	0.01	39	0	2	0.1	0	0

Table A3-9.5 Results of analysis for liquid supplements. Where provided, manufacturer stated concentrations are shown. Liquid density was determined by weighing 1 mL of supplement. The Co content of supplements containing both B12 and Co were added to provide the total calculated Co concentration. ICP-MS measurements were performed on supplements diluted by volume. Element concentrations by mass were calculated from the sample density.

Sample	Density	Molar mass	Element	Element proportion	Calculated concentration	Measured concentration	Calculated quantity	Measured quantity
	($g\ mL^{-1}$)	($g\ mol^{-1}$)		(%)	---- ($mg\ L^{-1}$) ----		---- ($\mu g\ g^{-1}$) ----	
Carbalene	1.146	-	Co	-	-	0.0022	-	0.0019
COpHOS B	1.036	-	-	-	-	-	-	-
50 ug mL^{-1} cyanocobalamin	-	1355.38	Co	4.348	2.174	2.117	2.10	2.04
VAM	1.076	-	Total Co	-	97.78	92.43	90.9	85.9
15 mg mL^{-1} ferric ammonium citrate	-	261.98	Fe	21.32	3197	2706	2972	2515
100 mg mL^{-1} nicotinamide	-	-	-	-	-	-	-	-
20 mg mL^{-1} glycine	-	-	-	-	-	-	-	-
100 mg mL^{-1} riboflavine	-	-	-	-	-	-	-	-
150 ug mL^{-1} cyanocobalamin	-	1355.38	Co	4.348	6.52	-	-	-
10 mg mL^{-1} pyridoxine HCl	-	-	-	-	-	-	-	-
15 mg mL^{-1} D-panthenol	-	-	-	-	-	-	-	-
10 ug mL^{-1} biotin	-	-	-	-	-	-	-	-
10 mg mL^{-1} inositol	-	-	-	-	-	-	-	-
240 ug mL^{-1} cobalt sulfate	-	154.996	Co	38.02	91.25	92.43	-	-
70 ug mL^{-1} copper sulfate	-	159.609	Cu	39.81	27.87	30.66	25.9	28.5
10 mg mL^{-1} choline bitartrate	-	-	-	-	-	-	-	-
20 mg mL^{-1} l-lysine	-	-	-	-	-	-	-	-
20 mg mL^{-1} dl-methionine	-	-	-	-	-	-	-	-

Tripart	1.047	-	-	-	-	-	-	-
1 mg mL^{-1} selenium (as Na selenate)	-	-	Se	-	1000	1092	955	1043
20 mg mL^{-1} magnesium aspartate	-	288.494	Mg	8.42	1685	1618	1609	1545
20 mg mL^{-1} potassium aspartate	-	-	-	-	-	-	-	-
60 mg mL^{-1} nicotinamide	-	-	-	-	-	-	-	-
100 mg mL^{-1} arginine-L HCl	-	-	-	-	-	-	-	-
50 mg mL^{-1} lysine-L HCl	-	-	-	-	-	-	-	-
500 ug mL^{-1} cyanocobalamin	-	1355.38	Co	4.348	21.74	23.62	20.8	22.6
L-carnitine	1.034	-	-	-	-	-	-	-
200 mg L^{-1} carnitine	-	-	Co	-	-	0.0099	-	0.0096
Vitamin B12	0.998	-	-	-	-	-	-	-
1000 ug mL^{-1} cyanocobalamin	-	1355.38	Co	4.348	43.48	86.22	43.6	86.4
Stamazene	1.059	-	-	-	-	0.0023	-	0.0022
Feratone	1.084	-	Total Co	-	50.1	21.55	46.3	19.9
6000 mg L^{-1} iron	-	-	Fe	-	6000	3874	5535	3574
50 mg L^{-1} cobalt	-	-	Co	-	50	-	-	-
3333 ug L^{-1} B12	-	1355.38	Co	4.348	0.145	-	-	-
1667 mg L^{-1} copper	-	-	Cu	-	1667	104	1538	96
500 mg L^{-1} folic acid	-	-	-	-	-	-	-	-

4. Results of analysis for excretion study described in Section 3.6.4.

Table A4-9.6 Cobalt, copper and iron concentrations in timed urine collections from horses A and B.

Urine sample	Time since bag emptied (h)	Volume	Creatinine	Creatinine adjustment factor#	Cobalt	Adjusted baseline cobalt	Baseline adjusted cobalt	Cobalt	Total cobalt	Copper	Adjusted baseline copper	Baseline adjusted copper	Copper	Total copper	Iron	Adjusted baseline iron	Baseline adjusted iron	Iron	Total iron
		(L)	(g L^{-1})		-------- (µg L^{-1}) ------			(µg g^{-1} creatinine)	(mg)	-------- (µg L^{-1}) -------			(µg g^{-1} creatinine)	(mg)	---------- (µg L^{-1}) -------			(µg g^{-1} creatinine)	(mg)
10/3/T=0/A	0		2.9	1.000	2.5	2.5	0.0	0.9		25	25	8.6	0		719	719	247.3	0	
10/3/T=2/A	2		1.7	0.591	1039.4	1.5	1037.9	604.5	**1.038**	39	15	22.7	24	**0.024**	569	425	330.9	144	**0.144**
10/3/T=4/A	2		1.7	0.591	766.3	1.5	764.8	445.7	**0.765**	64	15	37.2	49	**0.049**	649	425	377.5	224	**0.224**
Total	**4**								**1.80**					**0.073**					**0.368**
10/3/T=0/B	0		2.3	1.000	1.9	1.9	0.0	0.8		11	11	4.8	0		45	45	19.5	0	
10/3/T=2/B	2	0.525	2.8	1.230	2544.1	2.3	2541.8	896.0	**1.334**	111	14	39.1	97	**0.051**	12713	55	4477.5	12658	**6.645**
10/3/T=4/B	2	1.050	1.4	0.593	323.3	1.1	322.2	236.2	**0.338**	39	7	28.5	32	**0.034**	388	27	283.5	361	**0.379**
T=6/B	2	1.025	1.5	0.632	168.5	1.2	167.3	115.5	**0.171**	45	7	30.8	38	**0.039**	882	28	604.4	854	**0.875**
T=8/B	2	1.250	1.6	0.696	79.5	1.3	78.2	49.5	**0.098**	23	8	14.3	15	**0.019**	560	31	348.6	529	**0.661**
10/3/T=11/B	3	1.200	1.7	0.730	51.6	1.4	50.2	30.6	**0.060**	14	8	8.3	6	**0.007**	103	33	61.1	70	**0.084**
T=14/B	3	1.400	1.8	0.789	39.2	1.5	37.7	21.5	**0.053**	18	9	9.9	9	**0.013**	107	36	58.8	71	**0.100**
T=16/B	2	0.890	2.1	0.892	43.3	1.7	41.6	21.0	**0.037**	28	10	13.6	18	**0.016**	265	40	128.7	225	**0.200**
10/3/T=20/B	4	0.615	2.1	0.922	35.6	1.8	33.8	16.7	**0.021**	21	10	9.9	11	**0.007**	124	41	58.3	83	**0.051**
T=24/B	4	1.600	1.8	0.775	22.1	1.5	20.6	12.4	**0.033**	13	9	7.3	4	**0.007**	135	35	75.5	100	**0.160**
Total	**24**	**9.555**							**2.15**					**0.194**					**9.16**

To accurately calculate element excretion following VAM® injection, allowance was made for the normal or baseline excretion of a given element. The baseline level was taken as urine concentration at time 0. To adjust for variations in hydration status, baseline concentrations were corrected to the creatinine measured at time 0.

Table A4-9.7 Cobalt concentrations in manure collected over 84 hours from Horses A and B.

Liquid portion of manure						**Manure after acid digest**							
	Co	Co	Av. Co (mg)	Co excretion corrected for dilution (mg)	Baseline corrected Co excretion (mg)		Weight	Co	Reagent blank corrected	Co	Background adjusted Co	Co excretion corrected for dilution	Baseline corrected Co excretion
	($\mu g\ L^{-1}$)	---------- (mg) ----------					(g)	($\mu g\ L^{-1}$)		---------- (mg) ----------			
Shovel Washings *	0.11	0.0001				Shovel Washings *		0.11					
Container Washings *	0.06	0.0001				Container Washings *		0.06					
						Reagent blank		0.0143	-0.0045				
A Pre Rx	9.1	0.0091	0.008			A Pre Rx	0.40159	0.0468	0.0280	0.015	0.013		
A Post (Aliquot) 1	40.9	0.0409		0.033		A Post (Aliquot) 1	0.40842	0.3054	0.2866	0.147		0.134	
A Post (Aliquot) 2	44.7	0.0447		0.036		A Post (Aliquot) 2	0.54334	0.4579	0.4391	0.170		0.157	
A Post (Aliquot) 3	46.1	0.0461		0.038	**0.036**	A Post (Aliquot) 3	0.22144	0.2102	0.1914	0.182		0.168	**0.153**
B Pre Rx	10.0	0.0100	0.008			B Pre Rx	0.42260	0.0841	0.0653	0.032	0.027		
B Post (Aliquot) 1	32.9	0.0329		0.025		B Post (Aliquot) 1	0.45471	0.3517	0.3329	0.154		0.127	
B Post (Aliquot) 2	34.6	0.0346		0.026		B Post (Aliquot) 2	0.40673	0.3674	0.3486	0.180		0.153	
B Post (Aliquot) 3	34.0	0.0340		0.026	**0.026**	B Post (Aliquot) 3	0.51815	0.5179	0.4991	0.202		0.176	**0.152**

***** Negligible background contamination in the shovel and container washings with concentrations of these elements typical of the municipal water supply.

Pre-treatment manure diluted 5-fold with 1-part manure added to 4-parts water. # Horse A - total manure 33.0 kg added to 150 L water therefore dilution rate = (150 + 33) / 33 = 5.545. Multiply pre-treatment result by 5 / 5.545 to align dilution rates then subtract from post treatment result to correct for baseline concentration of measured element. # Horse B total manure 29.5 kg added to 150 L water therefore dilution rate = (150 + 29.5) / 29.5 = 6.085. Multiply pre-treatment result by 5 / 6.085 to align dilution rates then subtract from post treatment result to correct for baseline concentration of measured element.

Table A4-9.8 Copper concentrations in manure collected over 84 hours from Horses A and B.

Liquid portion of manure						Manure after acid digest							
	Cu	Cu	Av. Cu	Cu excretion corrected for dilution	Baseline corrected Cu excretion (mg)		Weight	Cu	Reagent blank corrected	Cu	Background adjusted Cu	Cu excretion corrected for dilution	Baseline corrected Cu excretion
	($\mu g\ L^{-1}$)	---------- (mg) ---------					(g)	($\mu g\ L^{-1}$)			---------- (mg) ----------		
Shovel Washings *	97	0.097				Shovel Washings *		97					
Container Washings *	114	0.114				Container Washings *		114					
						Reagent blank		0.0188	0.0000				
A Pre Rx	175	0.175	0.158			A Pre Rx	0.40159	1.8489	1.8301	0.957	0.863		
A Post (Aliquot) 1	408	0.408		0.250		A Post (Aliquot) 1	0.40842	3.5386	3.5198	1.810		0.947	
A Post (Aliquot) 2	426	0.426		0.268		A Post (Aliquot) 2	0.54334	5.2455	5.2267	2.020		1.157	
A Post (Aliquot) 3	443	0.443		0.285	**0.268**	A Post (Aliquot) 3	0.22144	2.8699	2.8511	2.704		1.841	**1.31**
B Pre Rx	220	0.220	0.181			B Pre Rx	0.42260	2.9228	2.9040	1.443	1.186		
B Post (Aliquot) 1	445	0.445		0.264		B Post (Aliquot) 1	0.45471	5.2171	5.1983	2.401		1.215	
B Post (Aliquot) 2	461	0.461		0.280		B Post (Aliquot) 2	0.40673	5.083	5.0642	2.615		1.429	
B Post (Aliquot) 3	445	0.445		0.264	**0.270**	B Post (Aliquot) 3	0.51815	6.4941	6.4753	2.624		1.439	**1.36**

* Negligible background contamination in the shovel and container washings with concentrations of these elements typical of the municipal water supply.

Pre-treatment manure diluted 5-fold with 1-part manure added to 4-parts water. # Horse A - total manure 33.0 kg added to 150 L water therefore dilution rate = (150 + 33) / 33 = 5.545. Multiply pre-treatment result by 5 / 5.545 to align dilution rates then subtract from post treatment result to correct for baseline concentration of measured element. # Horse B total manure 29.5 kg added to 150 L water therefore dilution rate = (150 + 29.5) / 29.5 = 6.085. Multiply pre-treatment result by 5 / 6.085 to align dilution rates then subtract from post treatment result to correct for baseline concentration of measured element.

Table A4-9.9 Iron concentrations in manure collected over 84 hours from Horses A and B.

Liquid portion of manure						Manure after acid digest							
	Fe	Fe	Av. Fe	Fe excretion corrected for dilution (mg)	Baseline corrected Fe excretion (mg)		Weight	Fe	Reagent blank corrected	Fe	Background adjusted Fe	Fe excretion corrected for dilution	Baseline corrected Fe excretion
	($\mu g\ L^{-1}$)	---------- (mg) ---------					(g)	($\mu g\ L^{-1}$)		---------- (mg) ---------			
Shovel Washings *	20	0.020				Shovel Washings *		20					
Container Washings *	2.0	0.002				Container Washings *		2.0					
						Reagent blank		10.0188	10.0000				
A Pre Rx	1090	1.090	0.983			A Pre Rx	0.40159	54.9022	54.8834	28.700	25.879		
A Post (Aliquot) 1	6112	6.112		5.129		A Post (Aliquot) 1	0.40842	442.0127	441.9939	227.263		201.384	
A Post (Aliquot) 2	6630	6.630		5.647		A Post (Aliquot) 2	0.54334	673.2183	673.1995	260.190		234.312	
A Post (Aliquot) 3	7001	7.001		6.018	**5.60**	A Post (Aliquot) 3	0.22144	295.4911	295.4723	280.208		254.329	**230**
B Pre Rx	2136	2.136	1.755			B Pre Rx	0.42260	172.3647	172.3459	85.643	70.372		
B Post (Aliquot) 1	5704	5.704		3.949		B Post (Aliquot) 1	0.45471	527.082	527.0632	243.415		173.043	
B Post (Aliquot) 2	5936	5.936		4.181		B Post (Aliquot) 2	0.40673	787.9543	787.9355	406.821		336.449	
B Post (Aliquot) 3	6215	6.215		4.460	**4.20**	B Post (Aliquot) 3	0.51815	782.8346	782.8158	317.266		246.894	**252**

* Negligible background contamination in the shovel and container washings with concentrations of these elements typical of the municipal water supply.

Pre-treatment manure diluted 5-fold with 1-part manure added to 4-parts water. # Horse A - total manure 33.0 kg added to 150 L water therefore dilution rate = (150 + 33) / 33 = 5.545. Multiply pre-treatment result by 5 / 5.545 to align dilution rates then subtract from post treatment result to correct for baseline concentration of measured element. # Horse B total manure 29.5 kg added to 150 L water therefore dilution rate = (150 + 29.5) / 29.5 = 6.085. Multiply pre-treatment result by 5 / 6.085 to align dilution rates then subtract from post treatment result to correct for baseline concentration of measured element.

5. Results of analysis for pilot studies described in Sections 3.6.5 - 3.6.7, 3.6.9 and 3.6.15.

Table A5-9.10 Results of analysis for pilot studies described in Sections 3.6.5 - 3.6.7, 3.6.9 and 3.6.15. Supplement administered immediately after sample collection at the times shown where HP denotes IV injection of Hemoplex® and NV denotes IV injection of 1 mg mL^{-1} solution of Nature Vet Vitamin B12. Renal efficiency calculated as urine Co divided by plasma Co.

Hemoplex® (Section 3.6.5)	Correlation (Section 3.6.6)	Accumulation (Section 3.6.7)	Cyanocobalamin (Section 3.6.9)	Creatinine vs SG (Section 3.6.15)	Volume of supplement injected	Mare	Sample number	Collection date and time	Cumulative time (h)	Urine specific gravity	Urine cobalt (µg L^{-1})	Urine creatinine (g L^{-1})	Urine cobalt (µg g^{-1} creatinine)	Plasma cobalt (µg L^{-1})	Blood cobalt (µg L^{-1})	Renal efficiency
✓	✓	✓	x	x	40 HP	A	19/2/8/A	19/02 8:00	0	–	6.6	1.52	4.4	1.6	1.2	4.1
✓	✓	✓	x	x	40 HP	A	21/2/2/A	21/02 14:00	54	–	5.7	1.57	3.6	1.5	1.1	3.8
✓	✓	✓	x	x	40 HP	A	24/2/11/A	24/02 11:00	123	–	46.8	1.91	24.5	21.6	13.6	2.2
✓	✓	✓	x	x	40 HP	A	25/2/8/A	25/02 8:00	144	–	61.3	1.65	37.1	27.5	15.8	2.2
✓	✓	x	x	x	–	A	25/2/12/A	25/02 12:00	148	–	199.1	0.76	262.7	44.1	28.0	4.5
✓	✓	x	x	x	–	A	25/2/16/A	25/02 16:00	152	–	122.6	1.23	99.4	40.3	23.7	3.0
✓	✓	x	x	x	–	A	25/2/20/A	25/02 20:00	156	–	46.3	0.97	47.6	36.1	20.9	1.3
✓	✓	x	x	x	–	A	26/2/12/A	26/02 12:00	172	–	57.9	1.63	35.5	36.7	17.2	1.6
✓	✓	x	x	x	–	A	26/2/16/A	26/02 16:00	176	–	45.6	1.54	29.6	34.3	20.0	1.3
✓	✓	x	x	x	–	A	26/2/20/A	26/02 20:00	180	–	44.3	1.59	27.8	31.6	18.2	1.4
✓	✓	✓	x	x	40 HP	A	4/3/8/A	4/03 8:00	312	–	42.5	1.55	27.4	35.4	21.5	1.2

✓	✓	x	x	x	–	A	4/3/12/A	4/03 12:00	316	–	501.4	1.20	418.2	51.8	28.6	9.7
✓	✓	x	x	x	–	A	4/3/14/A	4/03 14:00	318	–	264.9	1.65	160.4	49.3	30.0	5.4
✓	✓	x	x	x	–	A	4/3/16/A	4/03 16:00	320	–	129.5	1.64	79.0	46.6	26.2	2.8
✓	✓	x	x	x	–	A	4/3/20/A	4/03 20:00	324	–	62.8	1.54	40.8	45.2	25.8	1.4
✓	x	✓	x	x	40 HP	A	–	5/03 8:00	336	–	–	–	–	–	–	–
x	✓	✓	x	x	20 HP	A	5/3/17/A	5/03 17:00	345	–	186.4	1.01	185.1	60.7	33.5	3.1
x	✓	x	x	x	–	A	6/3/12/A	6/03 12:00	364	–	71.7	1.41	50.7	54.3	29.0	1.3
x	✓	x	x	x	–	A	6/3/14/A	6/03 14:00	366	–	77.4	1.67	46.2	53.5	31.4	1.4
x	✓	x	x	x	–	A	6/3/16/A	6/03 16:00	368	–	126.0	2.77	45.5	52.5	31.1	2.4
x	✓	x	x	x	–	A	6/3/18/A	6/03 18:00	370	–	52.9	1.35	39.3	49.7	29.4	1.1
x	✓	✓	x	x	40 HP	A	9/3/9/A	9/03 9:00	433	–	56.8	1.14	49.7	50.5	28.9	1.1
x	x	✓	x	x	40 HP	A	–	10/03 6:00	454	–	–	–	–	–	–	–
x	✓	x	x	x	–	A	11/3/8/A	11/03 8:00	480	–	62.3	0.96	64.8	57.0	25.9	1.1
x	✓	x	x	x	–	A	11/3/12/A	11/03 12:00	484	–	45.3	1.07	42.2	51.6	28.8	0.9
x	✓	x	x	x	–	A	11/3/1330/A	11/03 13:30	485	–	79.1	1.78	44.5	52.9	26.6	1.5
x	✓	x	x	x	–	A	11/3/15/A	11/03 15:00	487	–	65.7	1.57	41.8	50.5	24.7	1.3
x	✓	x	x	x	–	A	11/3/17/A	11/03 17:00	489	–	65.2	1.76	36.9	48.2	26.0	1.4
x	✓	x	x	x	–	A	11/3/19/A	11/03 19:00	491	–	75.2	1.53	49.2	48.7	25.4	1.5
x	✓	✓	x	x	40 HP	A	12/3/8/A	12/03 8:00	504	–	30.6	1.18	26.0	41.8	22.9	0.7
x	✓	x	x	x	–	A	12/3/12/A	12/03 12:00	508	–	208.3	0.76	274.8	61.9	34.5	3.4
x	✓	x	x	x	–	A	12/3/14/A	12/03 14:00	510	–	176.3	1.23	143.0	57.9	30.5	3.0
x	✓	x	x	x	–	A	12/3/16/A	12/03 16:00	512	–	124.8	1.06	117.4	56.0	31.4	2.2
x	✓	x	x	x	–	A	12/3/18/A	12/03 18:00	514	–	65.6	0.98	66.7	57.1	28.0	1.1
x	✓	x	x	✓	–	A	18/3/9/A	18/03 9:00	649	1.020	15.2	0.36	42.0	50.3	29.0	0.3
x	✓	x	x	✓	–	A	18/3/15/A	18/03 15:00	655	1.026	46.7	1.20	38.9	49.4	29.6	0.9
x	✓	✓	x	✓	40 HP	A	18/3/18/A	18/03 18:00	658	1.024	23.3	0.60	38.9	47.5	26.0	0.5
x	✓	x	x	✓	–	A	19/3/12/A	19/03 12:00	676	1.028	25.2	0.93	27.2	41.4	20.6	0.6
x	✓	x	x	✓	–	A	19/3/1330/A	19/03 13:30	677	1.018	17.3	0.60	28.9	44.9	25.0	0.4
x	✓	x	x	✓	–	A	19/3/16/A	19/03 16:00	680	1.030	39.5	1.32	29.8	40.3	25.1	1.0
x	✓	✓	x	✓	40 HP	A	19/3/18/A	19/03 18:00	682	1.028	21.1	0.76	27.8	39.4	22.5	0.5

x	✓	x	x	✓	–	A	20/3/8/A	20/03 8:00	696	1.012	20.9	0.45	46.2	47.0	27.8	0.4
x	✓	x	x	✓	–	A	20/3/11/A	20/03 11:00	699	1.030	51.8	1.12	46.3	45.5	25.8	1.1
x	✓	x	x	✓	–	A	20/3/13/A	20/03 13:00	701	1.035	70.2	1.59	44.0	46.7	28.2	1.5
x	✓	x	x	✓	–	A	20/3/15/A	20/03 15:00	703	1.034	44.9	1.30	34.5	45.3	27.2	1.0
x	✓	x	x	✓	–	A	20/3/17/A	20/03 17:00	705	1.034	43.8	1.32	33.1	43.9	26.1	1.0
x	x	✓	x	x	40 HP	A	–	22/03 21:00	757	–	–	–	–	–	–	–
x	✓	x	x	✓	–	A	23/3/11/A	23/03 11:00	771	1.030	32.1	0.87	36.9	51.1	28.7	0.6
x	✓	x	x	✓	–	A	23/3/12/A	23/03 12:00	772	1.040	92.7	2.52	36.7	52.9	27.3	1.8
x	✓	x	x	✓	–	A	23/3/1330/A	23/03 13:30	773	1.044	65.5	1.75	37.4	–	–	–
x	✓	x	x	✓	–	A	23/3/15/A	23/03 15:00	775	1.036	36.4	1.05	34.6	44.3	25.5	0.8
x	✓	✓	x	✓	–	A	23/3/18/A	23/03 18:00	778	1.040	45.2	1.43	31.7	41.0	24.3	1.1
x	✓	✓	x	✓	–	A	7/04/8/A	7/04 8:00	1128	1.026	12.2	0.76	16.1	22.5	12.7	0.5
✓	✓	✓	x	x	40 HP	B	19/2/8/B	19/02 8:00	0	–	1.8	0.97	1.9	0.8	0.6	2.3
✓	✓	✓	x	x	40 HP	B	21/2/2/B	21/02 14:00	54	–	0.8	0.53	1.5	0.8	0.7	1.0
✓	✓	✓	x	x	40 HP	B	24/2/11/B	24/02 11:00	123	–	13.2	0.43	30.7	28.1	18.2	0.5
✓	✓	✓	x	x	40 HP	B	25/2/8/B	25/02 8:00	144	–	80.8	1.80	44.9	31.1	20.1	2.6
✓	✓	x	x	x	–	B	25/2/12/B	25/02 12:00	148	–	411.1	0.62	660.8	53.5	31.9	7.7
✓	✓	x	x	x	–	B	25/2/16/B	25/02 16:00	152	–	243.5	1.22	199.3	47.4	29.0	5.1
✓	✓	x	x	x	–	B	25/2/20/B	25/02 20:00	156	–	123.7	1.31	94.3	43.3	24.2	2.9
✓	✓	x	x	x	–	B	26/2/12/B	26/02 12:00	172	–	74.8	1.47	50.9	41.9	22.9	1.8
✓	✓	x	x	x	–	B	26/2/16/B	26/02 16:00	176	–	71.2	1.67	42.5	40.3	23.6	1.8
✓	✓	x	x	x	–	B	26/2/20/B	26/02 20:00	180	–	62.7	1.80	34.9	39.7	23.0	1.6
✓	✓	✓	x	x	40 HP	B	4/3/8/B	4/03 8:00	312	–	44.0	1.35	32.7	36.6	21.3	1.2
✓	✓	x	x	x	–	B	4/3/12/B	4/03 12:00	316	–	164.5	0.29	559.3	58.4	30.1	2.8
✓	✓	x	x	x	–	B	4/3/14/B	4/03 14:00	318	–	255.1	0.80	317.6	52.7	30.5	4.8
✓	✓	x	x	x	–	B	4/3/16/B	4/03 16:00	320	–	306.0	2.24	136.6	48.4	28.4	6.3
✓	✓	x	x	x	–	B	4/3/20/B	4/03 20:00	324	–	142.5	1.78	80.2	48.5	26.5	2.9
x	x	✓	x	x	40 HP	B	–	5/03 8:00	336	–	–	–	–	–	–	–
x	✓	✓	x	x	20 HP	B	5/3/17/B	5/03 17:00	345	–	61.6	0.29	209.4	66.4	39.8	0.9
x	✓	x	x	x	–	B	6/3/12/B	6/03 12:00	364	–	93.3	1.40	66.5	59.5	31.8	1.6

x	✓	x	x	x	–	B	6/3/14/B	6/03 14:00	366	–	51.4	1.00	51.6	56.9	32.8	0.9
x	✓	x	x	x	–	B	6/3/16/B	6/03 16:00	368	–	80.0	1.67	47.8	54.7	30.2	1.5
x	✓	x	x	x	–	B	6/3/18/B	6/03 18:00	370	–	75.6	1.69	44.9	55.3	30.6	1.4
x	✓	✓	x	x	40 HP	B	9/3/9/B	9/03 9:00	433	–	110.5	1.53	72.4	54.3	33.4	2.0
x	x	✓	x	x	40 HP	B	–	10/03 6:00	454	–	–	–	–	–	–	–
x	✓	x	x	x	–	B	11/3/8/B	11/03 8:00	480	–	94.7	1.48	63.9	52.5	27.8	1.8
x	✓	x	x	x	–	B	11/3/12/B	11/03 12:00	484	–	71.2	1.27	56.2	51.7	27.0	1.4
x	✓	x	x	x	–	B	11/3/1330/B	11/03 13:30	485	–	92.2	1.82	50.6	50.1	25.2	1.8
x	✓	x	x	x	–	B	11/3/15/B	11/03 15:00	487	–	69.8	1.36	51.4	46.5	24.8	1.5
x	✓	x	x	x	–	B	11/3/17/B	11/03 17:00	489	–	87.3	1.70	51.4	43.6	23.3	2.0
x	✓	x	x	x	–	B	11/3/19/B	11/03 19:00	491	–	66.0	2.01	32.8	46.1	23.1	1.4
x	✓	✓	x	x	40 HP	B	12/3/8/B	12/03 8:00	504	–	19.4	0.66	29.6	40.9	24.2	0.5
x	✓	x	x	x	–	B	12/3/12/B	12/03 12:00	508	–	333.7	0.97	343.0	63.0	36.7	5.3
x	✓	x	x	x	–	B	12/3/14/B	12/03 14:00	510	–	159.6	0.98	162.2	57.9	32.9	2.8
x	✓	x	x	x	–	B	12/3/16/B	12/03 16:00	512	–	159.7	1.23	129.5	54.5	30.1	2.9
x	✓	x	x	x	–	B	12/3/18/B	12/03 18:00	514	–	127.1	1.33	95.2	51.0	28.7	2.5
x	✓	x	x	✓	–	B	18/3/9/B	18/03 9:00	649	1.018	24.1	0.49	49.5	50.6	31.4	0.5
x	✓	x	x	✓	–	B	18/3/15/B	18/03 15:00	655	1.018	34.8	0.81	42.7	52.0	30.5	0.7
x	✓	✓	x	✓	40 HP	B	18/3/18/B	18/03 18:00	658	1.030	81.3	1.83	44.4	49.7	26.1	1.6
x	✓	x	x	✓	–	B	19/3/12/B	19/03 12:00	676	1.024	22.1	0.70	31.5	42.0	24.4	0.5
x	✓	x	x	✓	–	B	19/3/1330/B	19/03 13:30	677	1.020	25.0	0.86	29.1	44.0	24.5	0.6
x	✓	x	x	✓	–	B	19/3/16/B	19/03 16:00	680	1.031	53.3	1.65	32.3	42.8	23.7	1.2
x	✓	✓	x	✓	40 HP	B	19/3/18/B	19/03 18:00	682	1.030	31.8	1.15	27.6	40.7	23.1	0.8
x	✓	x	x	✓	–	B	20/3/8/B	20/03 8:00	696	1.011	32.1	0.57	56.8	54.2	31.9	0.6
x	✓	x	x	✓	–	B	20/3/11/B	20/03 11:00	699	1.022	77.1	1.41	54.5	52.4	28.6	1.5
x	✓	x	x	✓	–	B	20/3/13/B	20/03 13:00	701	1.030	90.9	1.92	47.3	53.3	31.5	1.7
x	✓	x	x	✓	–	B	20/3/15/B	20/03 15:00	703	1.034	91.8	2.12	43.4	50.6	29.8	1.8
x	✓	x	x	✓	–	B	20/3/17/B	20/03 17:00	705	1.032	72.1	1.75	41.1	51.1	28.7	1.4
x	x	✓	x	x	40 HP		–	22/03 21:00	757	–	–	–	–	–	–	–
x	✓	x	x	✓	–	B	23/3/11/B	23/03 11:00	771	1.034	76.9	1.93	39.8	51.1	31.4	1.5

x	✓	x	x	✓	–	B	23/3/12/B	23/03 12:00	772	1.030	71.7	1.75	40.9	52.8	26.7	1.4
x	✓	x	x	x	–	B	23/3/1330/B	23/03 13:30	773	–	NS	NS	NS	48.0	24.9	NS
x	✓	x	x	✓	–	B	23/3/15/B	23/03 15:00	775	1.036	81.6	2.22	36.8	48.4	26.3	1.7
x	✓	✓	x	✓	–	B	23/3/18/B	23/03 18:00	778	1.032	52.3	1.46	35.8	47.9	26.0	1.1
x	✓	✓	x	✓	–	B	7/04/8/B	7/04 8:00	1128	1.008	3.9	0.23	17.2	27.6	16.9	0.1
✓	✓	x	x	x	–	C	19/2/8/C	19/02 8:00	0	–	6.4	2.01	3.2	0.7	0.6	9.1
✓	✓	x	x	x	–	C	21/2/2/C	21/02 14:00	54	–	1.9	0.71	2.7	0.8	0.7	2.4
✓	✓	x	x	x	–	C	24/2/11/C	24/02 11:00	123	–	2.6	0.95	2.7	0.9	0.8	2.9
✓	✓	x	x	x	40 HP	C	25/2/8/C	25/02 8:00	144	–	5.2	1.91	2.7	0.9	0.8	5.8
✓	✓	x	x	x	–	C	25/2/12/C	25/02 12:00	148	–	540.5	1.69	320.7	17.8	11.4	30.4
✓	✓	x	x	x	–	C	25/2/4/C	25/02 16:00	152	–	246.1	2.29	107.7	14.6	9.1	16.9
✓	✓	x	x	x	–	C	25/2/20/C	25/02 20:00	156	–	52.1	1.71	30.5	11.7	7.0	4.5
✓	✓	x	x	x	–	C	26/2/12/C	26/02 12:00	172	–	43.6	2.38	18.4	11.1	6.1	3.9
✓	✓	x	x	x	–	C	26/2/4/C	26/02 16:00	176	–	40.9	2.78	14.7	10.1	5.9	4.0
✓	✓	x	x	x	–	C	26/2/8/C	26/02 20:00	180	–	42.5	2.98	14.3	9.2	5.4	4.6
✓	✓	x	x	x	40 HP	C	4/3/8/C	4/03 8:00	312	–	4.8	0.95	5.1	3.1	2.2	1.5
✓	✓	x	x	x	–	C	4/3/12/C	4/03 12:00	316	–	564.2	1.28	441.4	21.3	12.6	26.5
✓	✓	x	x	x	–	C	4/3/2/C	4/03 14:00	318	–	379.2	1.74	217.7	18.6	11.0	20.4
✓	✓	x	x	x	–	C	4/3/4/C	4/03 16:00	320	–	234.0	2.42	96.7	16.7	9.6	14.0
✓	✓	x	x	x	–	C	4/3/20/C	4/03 20:00	324	–	49.7	1.48	33.5	14.1	8.0	3.5
x	✓	x	x	x	–	C	9/3/9/C	9/03 9:00	433	–	8.3	1.55	5.4	6.3	3.7	1.3
x	✓	x	x	x	–	C	12/3/10/C	12/03 10:00	506	–	8.0	1.29	6.2	4.9	2.9	1.6
x	✓	x	x	✓	–	C	18/3/9/C	18/03 9:00	649	1.028	3.1	0.70	4.4	3.3	2.1	0.9
x	✓	x	x	✓	–	C	20/3/8/C	20/03 8:00	696	1.025	50.6	1.40	36.1	14.8	8.7	3.4
x	✓	x	x	✓	–	C	20/3/11/C	20/03 11:00	699	1.031	58.9	1.95	30.3	14.1	8.2	4.2
x	✓	x	x	✓	–	C	20/3/13/C	20/03 13:00	701	1.027	24.1	1.03	23.4	13.2	7.9	1.8
x	✓	x	x	✓	–	C	20/3/15/C	20/03 15:00	703	1.026	19.1	1.02	18.8	13.2	7.6	1.4
x	✓	x	x	✓	–	C	20/3/17/C	20/03 17:00	705	1.028	21.1	1.22	17.3	11.8	6.9	1.8
x	✓	x	x	x	–	C	2/4/9/C	2/04 9:00	1009	–	18.7	2.64	7.1	4.5	2.9	4.2
x	✓	x	x	x	–	C	2/4/11/C	2/04 11:00	1011	–	16.5	1.86	8.9	4.6	2.8	3.6

x	✓	x	x	✓	–	C	2/4/13/C	2/04 13:00	1013	1.042	13.5	2.07	6.5	4.6	2.8	2.9
x	✓	x	x	✓	–	C	2/4/15/C	2/04 15:00	1015	1.042	13.3	2.01	6.6	4.3	2.6	3.1
x	✓	x	x	✓	–	C	2/4/18/C	2/04 18:00	1018	1.026	8.2	1.46	5.6	4.3	2.5	1.9
x	✓	x	x	✓	–	C	7/04/8/C	7/04 8:00	1128	1.036	9.5	1.71	5.6	3.6	2.3	2.6
x	✓	x	x	x	–	D	19/2/8/D	19/02 8:00	0	–	5.2	1.09	4.8	1.2	1.0	4.3
x	✓	x	x	x	–	D	21/2/14/D	21/02 14:00	54	–	1.6	0.40	4.0	1.3	1.0	1.2
x	✓	x	x	x	–	D	24/2/11/D	24/02 11:00	123	–	3.4	0.67	5.1	1.3	1.0	2.6
x	✓	x	x	x	–	D	25/2/8/D	25/02 8:00	144	–	7.5	1.39	5.4	1.2	0.9	6.3
x	✓	x	x	x	–	D	4/3/8/D	4/03 8:00	312	–	3.7	0.92	4.0	1.1	0.9	3.4
x	✓	x	x	x	–	D	9/3/9/D	9/03 9:00	433	–	4.8	1.13	4.2	1.1	1.0	4.4
x	✓	x	x	x	–	D	12/3/10/D	12/03 10:00	506	–	4.5	0.71	6.3	1.2	0.9	3.8
x	✓	x	x	✓	–	D	18/3/9/D	18/03 9:00	649	1.021	2.2	0.45	4.9	NS	NS	NS
x	✓	x	x	✓	–	D	20/3/8/D	20/03 8:00	696	1.016	15.7	0.54	28.9	9.7	5.8	1.6
x	✓	x	x	✓	–	D	20/3/11/D	20/03 11:00	699	1.030	29.7	0.93	32.0	9.2	5.4	3.2
x	✓	x	x	✓	–	D	20/3/13/D	20/03 13:00	701	1.033	17.4	0.69	25.2	7.9	5.2	2.2
x	✓	x	x	✓	–	D	20/3/15/D	20/03 15:00	703	1.028	13.7	0.68	20.2	8.3	5.1	1.7
x	✓	x	x	✓	–	D	20/3/17/D	20/03 17:00	705	1.026	10.9	0.63	17.2	7.4	4.8	1.5
x	✓	x	x	x	–	D	2/4/9/D	2/04 9:00	1009	–	6.6	0.98	6.7	3.8	2.5	1.7
x	✓	x	x	✓	–	D	2/4/11/D	2/04 11:00	1011	1.030	5.0	0.80	6.2	3.7	2.3	1.4
x	✓	x	x	✓	–	D	2/4/13/D	2/04 13:00	1013	1.034	7.6	1.30	5.8	3.9	2.4	1.9
x	✓	x	x	✓	–	D	2/4/15/D	2/04 15:00	1015	1.034	7.6	1.36	5.6	3.5	2.3	2.2
x	✓	x	x	✓	–	D	2/4/18/D	2/04 18:00	1018	1.027	3.7	0.84	4.4	3.4	2.3	1.1
x	✓	x	x	✓	–	D	7/04/8/D	7/04 8:00	1128	1.026	4.9	0.70	7.0	3.0	1.9	1.6
x	✓	x	x	x	–	E	19/2/8/E	19/02 8:00	0	–	3.7	0.86	4.3	1.4	1.3	2.6
x	✓	x	x	x	–	E	21/2/14/E	21/02 14:00	54	–	1.2	0.20	5.9	1.5	1.3	0.8
x	✓	x	x	x	–	E	24/2/11/E	24/02 11:00	123	–	0.9	0.17	5.3	1.6	1.3	0.6
x	✓	x	x	x	–	E	25/2/8/E	25/02 8:00	144	–	2.0	0.36	5.5	1.5	1.2	1.3
x	✓	x	x	x	–	E	4/3/8/E	4/03 8:00	312	–	1.7	0.40	4.3	1.4	1.0	1.2
x	✓	x	x	x	–	E	9/3/9/E	9/03 9:00	433	–	4.2	0.81	5.2	1.5	1.2	2.8
x	✓	x	x	x	–	E	12/3/10/E	12/03 10:00	506	–	1.7	0.29	5.8	1.5	0.9	1.1

x	✓	x	x	✓	–	E	18/3/9/E	18/03 9:00	649	1.004	0.4	0.11	3.5	1.4	1.1	0.3
x	✓	x	x	✓	–	E	20/3/8/E	20/03 8:00	696	1.008	4.6	0.14	33.9	15.2	9.3	0.3
x	✓	x	x	✓	–	E	20/3/11/E	20/03 11:00	699	1.025	28.3	0.94	30.1	14.8	8.3	1.9
x	✓	x	x	✓	–	E	20/3/13/E	20/03 13:00	701	1.030	29.4	1.12	26.3	13.8	8.0	2.1
x	✓	x	x	✓	–	E	20/3/15/E	20/03 15:00	703	1.028	23.1	1.06	21.7	13.4	8.4	1.7
x	✓	x	x	✓	–	E	20/3/17/E	20/03 17:00	705	1.028	22.1	1.17	19.0	12.7	7.8	1.7
x	✓	x	✓	✓	10 HP	E	23/3/6/E	23/03 6:00	0	1.010	2.5	0.21	11.6	8.3	5.1	0.3
x	✓	x	✓	✓	–	E	23/3/8/E	23/03 8:00	2	1.014	142.1	0.43	330.6	16.7	9.7	8.5
x	✓	x	✓	✓	–	E	23/3/10/E	23/03 10:00	4	1.028	64.4	0.66	98.2	13.7	7.8	4.7
x	✓	x	✓	✓	–	E	23/3/12/E	23/03 12:00	6	1.024	30.2	0.58	52.3	12.2	6.8	2.5
x	✓	x	✓	✓	–	E	23/3/15/E	23/03 15:00	9	1.024	17.9	0.62	28.8	10.6	6.4	1.7
x	✓	x	✓	✓	–	E	23/3/18/E	23/03 18:00	12	1.026	12.0	0.57	21.2	10.2	5.9	1.2
x	✓	x	x	✓	–	E	7/04/8/E	7/04 8:00	362	1.026	4.8	0.61	7.9	3.4	2.1	1.4
x	✓	x	x	x	–	F	19/2/8/F	19/02 8:00	0	–	6.8	1.07	6.3	1.7	1.3	4.0
x	✓	x	x	x	–	F	21/2/2/F	21/02 14:00	54	–	7.5	1.26	6.0	1.7	1.4	4.4
x	✓	x	x	x	–	F	24/2/11/F	24/02 11:00	123	–	10.3	1.41	7.3	1.9	1.5	5.4
x	✓	x	x	x	–	F	25/2/8/F	25/02 8:00	144	–	9.8	1.22	8.0	1.8	1.5	5.4
x	✓	x	x	x	–	F	4/3/8/F	4/03 8:00	312	–	8.5	1.30	6.5	1.6	1.2	5.3
x	✓	x	x	x	–	F	9/3/9/F	9/03 9:00	433	–	5.8	0.97	6.0	1.7	1.4	3.4
x	✓	x	x	x	–	F	12/3/10/F	12/03 10:00	506	–	5.5	0.78	7.0	1.9	1.1	2.9
x	✓	x	x	✓	–	F	18/3/9/F	18/03 9:00	649	1.031	6.2	0.69	9.0	1.8	1.2	3.4
x	✓	x	x	✓	–	F	20/3/8/F	20/03 8:00	696	1.032	33.5	0.90	37.0	9.8	6.1	3.4
x	✓	x	x	✓	–	F	20/3/11/F	20/03 11:00	699	1.040	49.9	1.50	33.2	8.7	5.7	5.7
x	✓	x	x	✓	–	F	20/3/13/F	20/03 13:00	701	1.028	30.6	1.00	30.7	8.6	5.5	3.6
x	✓	x	x	✓	–	F	20/3/15/F	20/03 15:00	703	1.032	21.5	0.88	24.4	8.3	5.4	2.6
x	✓	x	x	✓	–	F	20/3/17/F	20/03 17:00	705	1.028	16.7	0.78	21.4	8.1	5.5	2.1
x	✓	x	✓	✓	23 NV	F	23/3/6/F	23/03 6:00	0	1.028	7.7	0.62	12.4	5.4	3.4	1.4
x	✓	x	✓	✓	–	F	23/3/8/F	23/03 8:00	2	1.042	956.4	1.79	535.1	12.4	7.2	77.1
x	✓	x	✓	✓	–	F	23/3/10/F	23/03 10:00	4	1.032	52.1	0.71	73.1	8.3	5.2	6.3
x	✓	x	✓	✓	–	F	23/3/12/F	23/03 12:00	6	1.034	43.7	0.92	47.7	7.9	4.7	5.5

x	✓	x	✓	✓	–	F	23/3/15/F	23/03 15:00	9	1.042	36.4	1.39	26.2	6.8	4.0	5.4
x	✓	x	✓	✓	–	F	23/3/18/F	23/03 18:00	12	1.030	10.1	0.57	17.9	6.2	4.2	1.6
x	✓	x	x	✓	–	F	7/04/8/F	7/04 8:00	362	1.030	6.5	0.60	10.8	2.8	1.8	2.3
x	✓	x	x	✓	–	G	18/3/9/G	18/03 9:00	0	1.006	0.5	0.17	2.9	0.8	0.5	0.6
x	✓	x	x	✓	–	G	20/3/8/G	20/03 8:00	47	1.014	8.5	0.60	14.2	6.0	3.9	1.4
x	✓	x	x	✓	–	G	20/3/11/G	20/03 11:00	50	1.022	10.8	0.84	12.9	5.8	3.9	1.9
x	✓	x	x	✓	–	G	20/3/13/G	20/03 13:00	52	1.024	12.0	1.07	11.2	5.5	3.7	2.2
x	✓	x	x	✓	–	G	20/3/15/G	20/03 15:00	54	1.024	10.0	1.09	9.2	5.0	3.5	2.0
x	x	x	x	x	–	G	20/3/17/G	20/03 17:00	56	–	NS	NS	NS	5.0	3.3	NS
x	✓	x	x	✓	–	G	25/3/8/G	25/03 8:00	167	1.021	14.7	0.70	21.0	12.1	5.8	1.2
x	✓	x	x	✓	–	G	25/3/10/G	25/03 10:00	169	1.025	18.1	0.96	18.8	12.5	6.8	1.4
x	✓	x	x	✓	–	G	25/3/12/G	25/03 12:00	171	1.023	13.7	0.79	17.3	11.2	6.5	1.2
x	✓	x	x	✓	–	G	7/04/8/G	7/04 8:00	479	1.022	5.3	0.64	8.2	3.1	2.1	1.7
x	✓	x	x	✓	–	H	18/3/9/H	18/03 9:00	0	1.014	2.1	0.45	4.6	1.2	0.9	1.8
x	✓	x	x	✓	–	H	20/3/8/H	20/03 8:00	47	1.010	3.7	0.21	17.2	8.6	5.5	0.4
x	✓	x	x	✓	–	H	20/3/11/H	20/03 11:00	50	1.028	18.8	1.20	15.7	8.9	5.4	2.1
x	✓	x	x	✓	–	H	20/3/13/H	20/03 13:00	52	1.030	16.6	1.06	15.6	8.4	5.1	2.0
x	✓	x	x	✓	–	H	20/3/15/H	20/03 15:00	54	1.024	8.3	0.66	12.7	8.4	5.1	1.0
x	✓	x	x	✓	–	H	20/3/17/H	20/03 17:00	56	1.026	10.2	0.92	11.1	7.9	5.0	1.3
x	✓	x	x	✓	–	H	25/3/8/H	25/03 8:00	167	1.009	3.3	0.17	19.4	13.0	7.2	0.3
x	✓	x	x	✓	–	H	25/3/10/H	25/03 10:00	169	1.020	16.7	0.89	18.7	12.4	7.0	1.3
x	✓	x	x	✓	–	H	25/3/12/H	25/03 12:00	171	1.035	28.4	1.65	17.2	11.4	7.2	2.5
x	✓	x	x	✓	–	H	7/04/8/H	7/04 8:00	479	1.022	4.9	0.62	7.9	3.8	2.5	1.3
x	✓	x	x	✓	–	I	18/3/9/I	18/03 9:00	0	1.016	1.4	0.63	2.2	0.7	0.5	2.0
x	✓	x	x	✓	–	I	20/3/8/I	20/03 8:00	47	1.016	6.8	0.37	18.2	3.7	2.4	1.8
x	✓	x	x	✓	–	I	20/3/11/I	20/03 11:00	50	1.028	17.5	1.11	15.8	3.8	2.4	4.6
x	✓	x	x	✓	–	I	20/3/13/I	20/03 13:00	52	1.024	9.7	0.67	14.5	3.5	2.4	2.8
x	✓	x	x	✓	–	I	20/3/15/I	20/03 15:00	54	1.024	7.8	0.69	11.3	3.8	2.3	2.1
x	✓	x	x	✓	–	I	20/3/17/I	20/03 17:00	56	1.026	6.6	0.72	9.1	3.6	2.2	1.8
x	✓	x	x	✓	–	J	18/3/9/J	18/03 9:00	0	1.026	2.6	0.77	3.4	0.9	0.8	2.9

x	x	x	x	✓	−	J	20/3/8/J	20/03 8:00	47	1.014	7.9	0.35	22.5	NS	NS	NS
x	✓	x	x	✓	−	J	20/3/11/J	20/03 11:00	50	1.029	19.3	1.15	16.7	4.6	3.4	4.2
x	✓	x	x	✓	−	J	20/3/13/J	20/03 13:00	52	1.029	17.0	1.11	15.3	4.5	3.1	3.8
x	✓	x	x	✓	−	J	20/3/15/J	20/03 15:00	54	1.030	12.1	1.09	11.1	4.3	2.9	2.8
x	✓	x	x	✓	−	J	20/3/17/J	20/03 17:00	56	1.030	13.1	1.32	9.9	4.4	3.0	3.0
x	✓	x	x	✓	−	J	25/3/8/J	25/03 8:00	167	1.021	12.6	0.70	18.0	11.4	6.8	1.1
x	✓	x	x	✓	−	J	25/3/10/J	25/03 10:00	169	1.035	22.9	1.35	17.0	10.7	7.3	2.1
x	✓	x	x	✓	−	J	25/3/12/J	25/03 12:00	171	1.033	16.6	1.20	13.8	10.5	7.2	1.6
x	✓	x	x	✓	−	J	7/04/8/J	7/04 8:00	479	1.028	6.0	0.74	8.2	3.8	2.6	1.6
x	✓	x	x	✓	−	K	18/3/9/K	18/03 9:00	0	1.006	0.7	0.20	3.4	1.1	0.7	0.6
x	✓	x	x	✓	−	K	20/3/8/K	20/03 8:00	47	1.006	2.1	0.14	15.5	10.7	5.8	0.2
x	✓	x	x	✓	−	K	20/3/11/K	20/03 11:00	50	1.022	14.1	1.03	13.7	10.8	5.9	1.3
x	✓	x	x	✓	−	K	20/3/13/K	20/03 13:00	52	1.025	12.9	0.97	13.3	9.6	5.6	1.3
x	✓	x	x	✓	−	K	20/3/15/K	20/03 15:00	54	1.028	14.9	1.29	11.6	9.5	5.1	1.6
x	✓	x	x	✓	−	K	20/3/17/K	20/03 17:00	56	1.022	9.4	0.93	10.1	8.6	5.0	1.1
x	✓	x	x	✓	−	K	25/3/8/K	25/03 8:00	167	1.008	2.6	0.16	16.4	14.5	7.0	0.2
x	✓	x	x	✓	−	K	25/3/10/K	25/03 10:00	169	1.025	17.8	1.10	16.2	13.9	7.8	1.3
x	✓	x	x	✓	−	K	25/3/12/K	25/03 12:00	171	1.031	20.2	1.39	14.5	13.0	7.7	1.6
x	✓	x	x	✓	−	K	7/04/8/K	7/04 8:00	479	1.012	1.7	0.34	5.0	3.9	2.2	0.4
x	✓	x	x	✓	−	L	18/3/9/L	18/03 9:00	0	1.030	3.7	1.13	3.3	0.7	0.4	5.3
x	✓	x	x	✓	−	L	20/3/8/L	20/03 8:00	47	1.018	15.5	0.85	18.3	4.7	2.8	3.3
x	✓	x	x	✓	−	L	20/3/11/L	20/03 11:00	50	1.020	18.2	1.10	16.6	4.4	2.7	4.1
x	✓	x	x	✓	−	L	20/3/13/L	20/03 13:00	52	1.022	10.0	0.70	14.3	4.2	2.6	2.4
x	✓	x	x	✓	−	L	20/3/15/L	20/03 15:00	54	1.030	6.8	0.59	11.6	4.1	2.4	1.7
x	✓	x	x	✓	−	L	20/3/17/L	20/03 17:00	56	1.020	6.5	0.70	9.3	4.3	2.5	1.5
x	✓	x	x	✓	−	L	25/3/8/L	25/03 8:00	167	1.020	27.0	0.95	28.4	14.1	7.8	1.9
x	✓	x	x	✓	−	L	25/3/10/L	25/03 10:00	169	1.034	62.6	2.69	23.3	13.6	7.6	4.6
x	✓	x	x	✓	−	L	25/3/12/L	25/03 12:00	171	1.030	30.3	1.37	22.1	13.6	7.5	2.2
x	✓	x	x	✓	−	L	7/04/8/L	7/04 8:00	479	1.024	6.2	0.98	6.3	3.8	2.2	1.6
x	✓	x	x	✓	−	M	1/4/1630/M	1/04 16:30	0	1.032	11.2	1.18	9.5	6.3	4.1	1.8

x	✓	x	x	✓	–	M	2/4/13/M	2/04 13:00	20	1.030	40.4	1.04	38.8	7.6	4.2	5.3
x	✓	x	x	✓	–	M	2/4/15/M	2/04 15:00	22	1.030	31.2	1.44	21.7	6.9	4.5	4.5
x	✓	x	x	✓	–	M	2/4/17/M	2/04 17:00	24	1.038	26.3	1.91	13.8	6.7	4.0	3.9
x	✓	x	x	✓	–	N	1/4/1630/N	1/04 16:30	0	1.040	13.2	1.70	7.8	5.0	3.3	2.6
x	✓	x	x	✓	–	N	2/4/13/N	2/04 13:00	20	1.038	33.1	1.78	18.6	7.0	3.4	4.7
x	✓	x	x	✓	–	N	2/4/15/N	2/04 15:00	22	1.058	55.6	3.47	16.0	5.8	3.7	9.6
x	✓	x	x	✓	–	N	2/4/17/N	2/04 17:00	24	1.034	22.3	1.69	13.2	5.5	3.4	4.1
x	x	x	x	x	10 HP	R	–	19/02 8:00	0	–	–	–	–	–	–	–
x	x	x	x	x	10 HP	R	–	21/02 14:00	54	–	–	–	–	–	–	–
✓	✓	x	x	x	40 HP	R	25/2/8/R	25/02 8:00	144	–	15.8	1.22	12.9	NS	NS	NS
✓	✓	x	x	x	–	R	25/2/12/R	25/02 12:00	148	–	254.8	0.88	288.8	27.4	18.3	9.3
✓	✓	x	x	x	–	R	25/2/4/R	25/02 16:00	152	–	130.3	1.48	87.9	22.4	15.4	5.8
✓	✓	x	x	x	–	R	25/2/20/R	25/02 20:00	156	–	49.7	1.18	42.2	19.8	12.6	2.5
✓	✓	x	x	x	–	R	26/2/12/R	26/02 12:00	172	–	43.5	1.45	30.0	20.2	11.2	2.2
✓	✓	x	x	x	–	R	26/2/4/R	26/02 16:00	176	–	55.2	2.00	27.6	18.6	11.2	3.0
✓	✓	x	x	x	–	R	26/2/8/R	26/02 20:00	180	–	41.1	1.70	24.2	16.7	11.1	2.5
✓	✓	x	x	x	40 HP	R	4/3/8/R	4/03 8:00	312	–	6.9	0.90	7.6	9.8	6.3	0.7
✓	✓	x	x	x	–	R	4/3/12/R	4/03 12:00	316	–	195.2	0.58	338.4	25.6	16.4	7.6
✓	✓	x	x	x	–	R	4/3/2/R	4/03 14:00	318	–	116.9	0.75	156.6	22.4	14.6	5.2
✓	✓	x	x	x	–	R	4/3/4/R	4/03 16:00	320	–	109.9	1.17	94.3	21.8	13.4	5.0
✓	✓	x	x	x	–	R	4/3/20/R	4/03 20:00	324	–	28.4	0.69	41.2	19.5	11.2	1.5
x	✓	x	x	x	–	R	5/3/8/R	5/03 8:00	336	–	41.7	1.74	23.9	20.3	11.0	2.1
x	✓	x	x	x	40 HP	R	12/3/10/R	12/03 10:00	506	–	4.9	0.71	6.9	8.9	5.2	0.6
x	✓	x	x	x	–	R	12/3/14/R	12/03 14:00	510	–	355.4	0.98	361.1	17.5	11.3	20.3
x	✓	x	x	x	–	R	12/3/16/R	12/03 16:00	512	–	297.4	1.11	268.3	17.8	10.0	16.7
x	✓	x	x	x	–	R	12/3/18/R	12/03 18:00	514	–	151.8	0.94	161.7	16.1	10.1	9.4
x	✓	x	x	✓	40 HP	R	18/3/18/R	18/03 18:00	658	1.030	6.7	0.95	7.1	9.5	6.8	0.7
x	✓	x	x	✓	–	R	19/3/12/R	19/03 12:00	676	1.026	25.0	0.83	30.3	17.9	10.1	1.4
x	✓	x	x	✓	–	R	19/3/14/R	19/03 14:00	678	1.028	25.7	0.93	27.7	16.9	9.8	1.5
x	✓	x	x	✓	–	R	19/3/16/R	19/03 16:00	680	1.030	30.0	1.27	23.7	15.8	9.6	1.9

x	✓	x	x	✓	−	R	19/3/18/R	19/03 18:00	682	1.031	23.3	1.03	22.6	28.2	17.2	0.8
x	x	x	x	✓	−	R	20/3/8/R	20/03 8:00	696	1.032	80.3	1.39	57.7	NS	NS	NS
x	✓	x	x	✓	−	R	20/3/11/R	20/03 11:00	699	1.032	49.9	1.05	47.4	26.3	14.9	1.9
x	✓	x	x	✓	−	R	20/3/13/R	20/03 13:00	701	1.030	39.1	0.89	43.8	26.1	15.8	1.5
x	✓	x	x	✓	−	R	20/3/15/R	20/03 15:00	703	1.034	43.6	1.23	35.4	23.7	14.4	1.8
x	✓	x	x	✓	−	R	20/3/17/R	20/03 17:00	705	1.032	32.8	1.01	32.6	23.8	14.5	1.4
x	✓	x	✓	✓	23 NV	R	23/3/6/R	23/03 6:00	0	1.032	22.6	0.93	24.4	24.0	12.9	0.9
x	✓	x	✓	✓	−	R	23/3/8/R	23/03 8:00	2	1.036	457.4	1.09	421.2	27.9	16.5	16.4
x	✓	x	✓	✓	−	R	23/3/10/R	23/03 10:00	4	1.032	88.7	0.76	117.0	24.5	14.9	3.6
x	✓	x	✓	✓	−	R	23/3/12/R	23/03 12:00	6	1.030	63.4	0.83	76.8	23.4	13.5	2.7
x	✓	x	✓	✓	−	R	23/3/15/R	23/03 15:00	9	1.032	50.2	1.19	42.3	23.0	13.3	2.2
x	✓	x	✓	✓	−	R	23/3/18/R	23/03 18:00	12	1.032	25.8	0.83	31.2	22.7	13.3	1.1
x	✓	x	x	✓	−	R	7/04/8/R	7/04 8:00	362	1.028	4.7	0.72	6.5	7.8	5.7	0.6

NS = No sample as uncooperative horse resulted in specimen being unable to be collected.

6. Results of analysis for cobalt displacement study described in Section 3.6.11.

Table A5-9.11 Results of analysis for Co displacement study described in Section 3.6.11.

Mare	Time	Sample number	Urine cobalt	Creatinine	Creatinine adjusted cobalt	Plasma cobalt	Blood cobalt
	(hours)		(µg L^{-1})	(g L^{-1})	(µg g-1 creatinine)	(µg L^{-1})	(µg L^{-1})
A	0	29/7/8/A	2.8	1.6	1.8	1.0	0.7
A	2	29/7/10/A	13.4	3.1	4.4	1.4	1.1
A	5	29/7/13/A	4.8	2.3	2.1	1.2	1.0
A	9	29/7/17/A	3.7	2.6	1.4	1.2	0.7
A	24	30/7/10/A	2.1	2.0	1.1	1.2	0.8
B	0	29/7/8/B	3.3	1.8	1.9	1.1	0.6
B	2	29/7/10/B	5.5	3.8	1.4	1.1	0.7
B	5	29/7/13/B	1.4	1.2	1.1	1.0	0.5
B	9	29/7/17/B	2.0	2.0	1.0	0.7	0.5
B	24	30/7/10/B	3.2	3.7	0.9	0.7	0.6
C	0	29/7/8/C	7.1	0.7	10.3	13.0	11.7
C	2	29/7/10/C	35.8	2.5	14.1	12.5	10.7
C	5	29/7/13/C	11.3	1.3	8.5	11.5	11.5
C	9	29/7/17/C	13.3	2.0	6.7	11.1	11.7
C	24	30/7/10/C	1.5	0.2	7.0	12.7	11.1
D	0	29/7/8/D	5.4	0.5	10.6	16.1	18.5
D	2	29/7/10/D	23.9	2.2	10.9	17.2	18.9
D	5	29/7/13/D	19.9	2.1	9.4	16.0	19.2
D	9	29/7/17/D	21.8	2.6	8.3	16.5	19.0
D	24	30/7/10/D	11.1	1.4	8.0	14.5	18.6
E	0	29/7/8/E	10.8	2.5	4.3	10.3	9.3
E	2	29/7/10/E	20.4	2.6	7.9	8.7	8.9

E	5	29/7/13/E	15.3	2.5	6.2	8.6	10.1
E	9	29/7/17/E	12.5	2.7	4.6	8.2	8.8
E	24	30/7/10/E	14.3	3.3	4.3	8.5	7.9
F	0	29/7/8/F	7.5	1.4	5.2	8.0	9.0
F	2	29/7/10/F	10.6	2.0	5.3	8.2	8.6
F	5	29/7/13/F	5.2	1.0	5.2	8.6	9.0
F	9	29/7/17/F	8.2	2.0	4.1	6.6	7.5
F	24	30/7/10/F	1.8	0.4	4.8	6.5	8.1
G	0	28/7/8/G	17.0	2.0	8.4	17.4	13.2
G	2	28/7/10/G	45.7	3.0	15.4	15.4	14.4
G	5	28/7/13/G	34.8	3.0	11.6	16.8	14.6
G	9	28/7/17/G	28.7	3.1	9.4	16.7	14.2
G	24	29/7/10/G	31.1	3.8	8.3	15.6	14.3
H	0	28/7/8/H	10.8	1.3	8.5	12.5	13.1
H	2	28/7/10/H	21.4	2.3	9.1	12.5	13.4
H	5	28/7/13/H	30.0	3.3	9.1	12.3	13.9
H	9	28/7/17/H	19.5	2.2	8.8	12.2	13.8
H	24	29/7/10/H	NS	NS	NS	NS	NS

NS = No sample as uncooperative horse resulted in specimen being unable to be collected.

7. Results of analysis for administration trials described in Chapters 5 and 6.

Table A7-9.12 Results of analysis for administration trials described in Chapters 5 and 6. Creatinine adjusted Co calculated by dividing average urine Co by urine creatinine concentration. Red cell Co calculated from plasma Co, whole blood Co, and haematocrit using the formula described in Section 5.4.5.

Collection date	Mare	Sample ID	Creatinine	Urine SG	Urine cobalt	Urine cobalt	Urine cobalt	Av. Co	Creatinine adjusted Co	Plasma cobalt	Plasma cobalt	Plasma cobalt	Mean plasma cobalt	Blood cobalt	Blood cobalt	Blood cobalt	Mean blood cobalt	Red Cell Cobalt	Haematocrit
			g L^{-1}		µg L^{-1} Run 1	µg L^{-1} Run 2	µg L^{-1} Run 3	µg L^{-1}	µg g^{-1}	µg L^{-1} Run 1	µg L^{-1} Run 2	µg L^{-1} Run 3	µg L^{-1}	µg L^{-1} Run 1	µg L^{-1} Run 2	µg L^{-1} Run 3	µg L^{-1}	µg L^{-1}	L L^{-1}
9/05/2017	A	9/5/10/A	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.36
9/05/2017	B	9/5/10/B	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.39
9/05/2017	C	9/5/10/C	0.945	1.025	3.5	3.7	3.1	3.4	3.6	1.7	1.8	2.0	1.8	1.2	1.2	1.2	1.2	0.4	0.43
9/05/2017	D	9/5/10/D	1.123	1.033	6.1	6.2	5.3	5.9	5.2	2.5	2.4	2.7	2.6	1.7	1.7	1.8	1.7	0.4	0.37
9/05/2017	E	9/5/10/E	1.457	1.031	2.3	2.5	2.0	2.3	1.6	1.2	1.2	1.3	1.2	0.7	0.8	0.8	0.7	0.0	0.40
9/05/2017	F	9/5/10/F	0.846	1.024	3.6	3.7	3.3	3.5	4.2	2.2	2.2	2.4	2.3	1.6	1.6	1.7	1.7	0.6	0.37
9/05/2017	G	9/5/10/G	1.310	1.033	2.2	2.3	2.0	2.2	1.7	0.8	0.9	0.9	0.9	0.4	0.4	0.5	0.4	0.0	0.49
9/05/2017	H	9/5/10/H	0.534	1.020	1.7	1.8	1.6	1.7	3.2	1.0	1.0	1.1	1.0	0.7	0.7	0.7	0.7	0.2	0.39
16/05/2017	A	16/5/14/A	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.45
16/05/2017	B	16/5/14/B	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.45
16/05/2017	C	16/5/14/C	0.560	1.012	17.8	18.1	18.4	18.1	32.3	41.7	41.6	44.5	42.6	24.3	24.4	25.0	24.6	-0.3	0.42
16/05/2017	D	16/5/14/D	1.773	1.036	60.4	61.6	57.3	59.8	33.7	44.8	45.2	47.3	45.8	27.8	28.3	28.7	28.2	2.0	0.40
16/05/2017	E	16/5/14/E	1.355	1.034	17.4	17.5	16.6	17.2	12.7	16.9	17.2	18.1	17.4	11.1	11.0	11.3	11.1	3.2	0.44
16/05/2017	F	16/5/14/F	0.468	1.012	6.9	6.9	7.4	7.1	15.1	21.8	22.1	22.9	22.3	13.5	13.7	14.1	13.8	-0.7	0.37
16/05/2017	G	16/5/14/G	0.891	1.023	32.2	32.6	33.3	32.7	36.7	38.9	39.3	41.0	39.7	21.2	21.5	21.9	21.5	-3.6	0.42

16/05/2017	H	16/5/14/H	0.561	1.019	20.9	21.1	22.3	21.4	38.2	32.9	32.9	34.9	33.6	20.6	21.0	21.1	20.9	1.9	0.40
23/05/2017	A	23/5/9/A	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.40
23/05/2017	B	23/5/9/B	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.39
23/05/2017	C	23/5/9/C	0.541	1.010	22.0	22.3	24.6	23.0	42.5	61.9	62.9	65.4	63.4	34.1	34.1	35.2	34.5	0.5	0.46
23/05/2017	D	23/5/9/D	2.136	1.037	104.1	104.6	104.8	104.5	48.9	62.4	63.3	65.0	63.6	39.5	39.8	40.6	40.0	8.6	0.43
23/05/2017	E	23/5/9/E	1.893	1.035	40.1	40.3	40.8	40.4	21.4	29.7	30.0	30.9	30.2	17.2	17.4	17.8	17.5	-0.8	0.41
23/05/2017	F	23/5/9/F	1.244	1.025	26.7	26.9	28.7	27.4	22.0	30.6	31.1	31.4	31.0	20.4	20.5	21.2	20.7	3.1	0.37
23/05/2017	G	23/5/9/G	1.857	1.036	104.2	104.4	106.2	104.9	56.5	66.8	67.8	70.9	68.5	36.9	37.5	38.6	37.7	2.9	0.47
23/05/2017	H	23/5/9/H	1.085	1.024	64.0	65.2	69.7	66.3	61.1	52.9	53.5	55.7	54.1	34.1	34.3	35.6	34.7	-1.3	0.35
30/05/2017	A	30/5/8/A	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.45
30/05/2017	B	30/5/8/B	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.45
30/05/2017	C	30/5/8/C	1.471	1.032	67.8	64.3	63.9	65.3	44.4	73.2	79.8	75.7	76.2	42.8	42.1	39.1	41.3	-1.3	0.45
30/05/2017	D	30/5/8/D	1.799	1.034	105.1	98.4	100.0	101.2	56.2	87.0	82.1	85.6	84.9	51.2	47.0	50.2	49.5	6.2	0.45
30/05/2017	E	30/5/8/E	2.443	1.040	57.1	55.2	55.2	55.8	22.9	43.6	43.0	42.0	42.9	23.6	20.3	20.8	21.6	0.3	0.50
30/05/2017	F	30/5/8/F	1.980	1.036	43.7	35.7	36.4	38.6	19.5	37.7	39.9	38.4	38.7	24.1	24.8	23.2	24.0	4.6	0.43
30/05/2017	G	30/5/8/G	2.364	1.042	136.4	128.2	129.2	131.3	55.5	86.9	93.5	90.8	90.4	44.7	40.5	39.5	41.6	-7.3	0.50
30/05/2017	H	30/5/8/H	2.002	1.034	126.7	127.2	124.9	126.3	63.1	71.9	72.7	70.6	71.7	46.8	42.6	41.2	43.5	6.2	0.43
6/06/2017	A	6/6/8/A	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.44
6/06/2017	B	6/6/8/B	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.42
6/06/2017	C	6/6/8/C	1.527	1.028	69.7	66.0	67.4	67.7	44.3	83.7	91.2	89.4	88.1	49.7	51.1	49.6	50.1	5.6	0.46
6/06/2017	D	6/6/8/D	1.595	1.030	85.7	82.6	81.6	83.3	52.2	91.1	98.4	92.6	94.0	59.5	61.2	59.2	60.0	18.3	0.45
6/06/2017	E	6/6/8/E	3.179	1.038	76.2	73.2	77.3	75.6	23.8	54.1	54.4	53.0	53.8	29.5	29.4	27.9	28.9	-5.5	0.42
6/06/2017	F	6/6/8/F	1.595	1.029	41.2	39.4	40.0	40.2	25.2	44.7	47.1	44.8	45.5	29.5	27.6	28.1	28.4	2.7	0.40
6/06/2017	G	6/6/8/G	2.330	1.040	153.4	142.6	139.3	145.1	62.3	106.1	109.0	108.0	107.7	57.4	57.4	54.2	56.3	-11.8	0.43
6/06/2017	H	6/6/8/H	2.036	1.038	156.0	144.6	146.2	148.9	73.1	89.3	91.8	89.2	90.1	56.9	51.7	51.6	53.4	-1.7	0.40
13/06/2017	A	13/6/8/A	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.45
13/06/2017	B	13/6/8/B	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.42
13/06/2017	C	13/6/8/C	1.403	1.025	75.8	74.2	73.6	74.5	53.1	100.2	106.6	109.5	105.4	53.5	51.1	59.2	54.6	-2.7	0.47

13/06/2017	D	13/6/8/D	2.115	1.038	115.6	110.3	108.0	111.3	52.6	113.5	116.1	112.7	114.1	71.0	72.0	66.7	69.9	0.8	0.39
13/06/2017	E	13/6/8/E	3.405	1.045	120.5	108.3	110.8	113.2	33.2	63.4	66.0	65.1	64.8	36.6	36.9	32.9	35.5	-0.4	0.45
13/06/2017	F	13/6/8/F	1.923	1.030	60.0	55.9	57.0	57.6	30.0	52.3	57.5	55.7	55.2	28.6	28.0	28.7	28.4	-1.7	0.47
13/06/2017	G	13/6/8/G	3.903	1.055	351.3	313.5	324.3	329.7	84.5	123.3	128.3	126.8	126.1	58.3	55.8	54.3	56.1	-11.1	0.51
13/06/2017	H	13/6/8/H	3.009	1.048	256.1	249.8	245.4	250.4	83.2	105.9	105.5	110.2	107.2	65.0	60.4	60.4	61.9	-3.2	0.41
20/06/2017	A	20/6/8/A	1.538	NT	1.1	1.2	0.9	1.1	0.7	1.8	1.3	1.1	1.4	0.7	0.6	0.7	0.7	-0.4	0.40
20/06/2017	B	20/6/8/B	2.885	NT	1.9	1.9	1.8	1.9	0.6	0.9	1.1	1.1	1.0	0.6	0.5	0.6	0.6	-0.2	0.38
20/06/2017	C	20/6/8/C	0.882	1.016	38.1	37.4	35.6	37.0	42.0	62.9	72.3	69.9	68.4	44.2	43.2	41.7	43.0	5.0	0.40
20/06/2017	D	20/6/8/D	2.014	1.040	106.4	100.8	109.8	105.7	52.5	80.8	76.7	82.7	80.1	56.3	54.4	55.6	55.4	18.5	0.40
20/06/2017	E	20/6/8/E	3.756	1.044	110.9	116.1	114.8	113.9	30.3	58.2	61.5	62.8	60.8	42.4	40.6	39.8	40.9	9.8	0.39
20/06/2017	F	20/6/8/F	1.685	1.030	42.1	39.5	41.4	41.0	24.3	46.7	50.9	46.8	48.1	34.0	31.5	31.7	32.4	7.8	0.39
20/06/2017	G	20/6/8/G	3.371	1.050	228.3	228.2	234.1	230.2	68.3	112.7	120.4	121.6	118.2	71.3	67.7	70.4	69.8	5.6	0.43
20/06/2017	H	20/6/8/H	2.070	1.036	150.3	146.6	150.7	149.2	72.1	95.0	99.3	103.1	99.1	65.6	63.7	62.0	63.8	10.7	0.40
27/06/2017	A	27/6/8/A	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.41
27/06/2017	B	27/6/8/B	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.43
27/06/2017	C	27/6/8/C	0.419	1.010	23.5	23.6	23.5	23.5	56.2	82.6	85.2	83.6	83.8	49.5	49.7	46.9	48.7	7.5	0.46
27/06/2017	D	27/6/8/D	2.466	1.040	173.3	174.3	176.7	174.8	70.9	95.5	97.9	98.3	97.2	67.2	66.3	64.9	66.1	23.2	0.42
27/06/2017	E	27/6/8/E	3.405	1.045	109.5	106.3	106.3	107.4	31.5	65.3	64.0	64.4	64.6	39.5	36.9	38.2	38.2	7.2	0.46
27/06/2017	F	27/6/8/F	1.120	1.017	34.2	32.4	34.3	33.6	30.0	52.6	52.4	54.1	53.0	33.1	30.9	30.1	31.4	5.9	0.46
27/06/2017	G	27/6/8/G	2.907	1.043	179.8	179.6	176.1	178.5	61.4	109.7	120.9	119.3	116.6	64.9	67.0	63.9	65.3	11.8	0.49
27/06/2017	H	27/6/8/H	2.070	1.038	139.5	139.8	137.0	138.8	67.0	88.0	93.4	93.5	91.6	57.7	58.6	56.5	57.6	14.3	0.44
4/07/2017	A	4/7/8/A	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.43
4/07/2017	B	4/7/8/B	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.39
4/07/2017	C	4/7/8/C	2.330	1.039	57.9	53.7	57.5	56.4	24.2	41.1	42.4	43.5	42.3	26.6	25.9	27.5	26.7	6.7	0.44
4/07/2017	D	4/7/8/D	2.930	1.050	84.9	77.9	77.2	80.0	27.3	50.9	53.7	50.5	51.7	40.5	36.4	38.3	38.4	18.5	0.40
4/07/2017	E	4/7/8/E	6.346	1.065	111.3	104.8	106.8	107.6	17.0	29.7	31.7	32.7	31.4	21.8	21.1	22.4	21.8	7.4	0.40
4/07/2017	F	4/7/8/F	2.930	1.040	40.3	36.7	36.2	37.7	12.9	25.6	25.2	26.2	25.7	19.4	19.0	19.6	19.3	9.8	0.40
4/07/2017	G	4/7/8/G	1.606	1.029	45.1	44.8	45.6	45.2	28.1	55.8	58.1	59.1	57.7	34.2	33.6	34.8	34.2	7.7	0.47

4/07/2017	H	4/7/8/H	2.262	1.044	65.6	64.0	62.4	64.0	28.3	41.9	43.7	44.2	43.3	31.2	30.2	32.1	31.2	17.0	0.46
12/07/2017	A	12/7/8/A	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.42
12/07/2017	B	12/7/8/B	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.46
12/07/2017	C	12/7/8/C	1.697	1.028	21.0	21.1	20.3	20.8	12.3	22.5	24.5	23.7	23.6	17.6	17.2	17.7	17.5	9.8	0.44
12/07/2017	D	12/7/8/D	2.828	1.050	40.9	39.2	42.6	40.9	14.5	29.6	30.2	28.9	29.6	27.3	25.3	27.4	26.7	22.7	0.42
12/07/2017	E	12/7/8/E	2.941	1.040	25.0	24.4	26.2	25.2	8.6	16.7	17.7	18.2	17.5	13.5	13.7	14.5	13.9	8.5	0.40
12/07/2017	F	12/7/8/F	2.364	1.032	18.1	17.4	17.6	17.7	7.5	14.8	15.3	14.6	14.9	12.7	11.2	12.8	12.2	7.9	0.38
12/07/2017	G	12/7/8/G	3.099	1.051	46.9	45.2	48.0	46.7	15.1	32.1	31.4	33.2	32.2	21.1	19.9	20.1	20.4	7.5	0.48
12/07/2017	H	12/7/8/H	2.738	1.045	42.4	41.0	43.1	42.2	15.4	22.1	23.4	23.0	22.8	19.7	16.1	19.8	18.5	12.1	0.40
18/07/2017	A	18/7/8/A	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.48
18/07/2017	B	18/7/8/B	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.39
18/07/2017	C	18/7/8/C	1.007	1.018	8.7	8.8	8.4	8.6	8.6	17.9	19.3	18.6	18.6	14.6	14.1	13.9	14.2	8.6	0.44
18/07/2017	D	18/7/8/D	2.738	1.047	26.7	27.8	27.3	27.3	10.0	22.6	23.5	23.2	23.1	22.9	21.0	21.8	21.9	20.3	0.43
18/07/2017	E	18/7/8/E	3.145	1.040	18.0	17.7	18.0	17.9	5.7	13.6	13.1	13.7	13.5	10.8	11.2	11.1	11.0	7.7	0.42
18/07/2017	F	18/7/8/F	1.369	1.020	8.2	8.6	9.0	8.6	6.3	11.1	11.7	11.6	11.5	9.9	10.2	10.3	10.1	8.0	0.38
18/07/2017	G	18/7/8/G	3.303	1.050	38.2	38.3	37.4	38.0	11.5	25.1	26.8	25.0	25.6	16.8	16.3	17.1	16.7	9.2	0.54
18/07/2017	H	18/7/8/H	NS	NS	NS	NS	NS	NS	NS	17.7	18.8	18.8	18.4	16.3	18.0	15.9	16.7	14.2	0.40
28/07/2017	G	28/7/8/G	2.014	1.040	17.0	17.4	17.2	17.2	8.5	17.4	17.4	16.9	17.2	13.2	14.5	14.8	14.2	11.0	0.49
28/07/2017	H	28/7/8/H	1.267	1.030	10.8	11.1	11.0	11.0	8.7	12.5	12.3	12.7	12.5	13.1	13.6	13.6	13.4	14.8	0.40
29/07/2017	A	29/7/8/A	1.595	1.030	2.8	3.0	3.0	2.9	1.8	1.0	1.0	1.4	1.1	0.7	0.8	0.7	0.7	0.2	0.42
29/07/2017	B	29/7/8/B	1.765	1.033	3.3	3.8	4.0	3.7	2.1	1.1	0.9	1.1	1.0	0.6	0.9	0.6	0.7	0.3	0.44
29/07/2017	C	29/7/8/C	0.690	1.011	7.1	7.3	7.1	7.2	10.4	13.0	12.9	13.0	13.0	11.7	11.3	11.6	11.5	9.4	0.40
29/07/2017	D	29/7/8/D	0.509	1.013	5.4	5.8	5.9	5.7	11.2	16.1	15.7	16.7	16.2	18.5	17.7	17.8	18.0	20.9	0.39
29/07/2017	E	29/7/8/E	2.489	1.034	10.8	11.4	11.4	11.2	4.5	10.3	9.2	9.9	9.8	9.3	9.2	9.5	9.3	8.7	0.44
29/07/2017	F	29/7/8/F	1.437	1.026	7.5	8.3	7.9	7.9	5.5	8.0	7.7	8.0	7.9	9.0	8.3	8.3	8.5	9.7	0.35
23/08/2017	A	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.42
23/08/2017	B	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.47
23/08/2017	C	23/08/14/C	1.912	1.030	9.7	9.2	9.7	9.5	5.0	6.5	6.4	6.5	6.5	7.6	7.5	7.2	7.4	8.7	0.43

23/08/2017	D	23/08/14/D	2.217	1.043	14.5	14.5	14.7	14.6	6.6	8.0	8.4	7.9	8.1	14.3	13.5	13.9	13.9	21.3	0.44
23/08/2017	E	23/08/14/E	3.371	1.047	10.1	9.9	10.0	10.0	3.0	5.2	5.2	5.3	5.2	6.1	6.2	6.2	6.2	7.4	0.42
23/08/2017	F	23/08/14/F	0.939	1.017	2.9	2.9	3.6	3.1	3.3	4.9	4.8	4.7	4.8	6.4	6.3	6.3	6.3	8.7	0.39
23/08/2017	G	23/08/14/G	1.131	1.023	5.6	5.2	5.5	5.4	4.8	7.4	8.4	8.0	7.9	9.2	8.9	9.2	9.1	10.5	0.46
23/08/2017	H	23/08/14/H	0.509	1.010	2.5	2.3	2.5	2.4	4.8	5.6	6.2	6.2	6.0	8.8	8.8	9.0	8.9	13.2	0.40
13/09/2017	A	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.44
13/09/2017	B	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.43
13/09/2017	C	13/09/10/C	1.561	1.020	6.5	6.5	6.6	6.5	4.2	4.7	4.0	4.7	4.5	5.8	6.4	6.9	6.4	9.1	0.41
13/09/2017	D	13/09/10/D	2.749	1.045	13.3	13.1	13.1	13.2	4.8	5.4	4.9	5.7	5.4	11.2	12.1	12.1	11.8	20.4	0.43
13/09/2017	E	13/09/10/E	4.683	1.044	13.7	13.3	13.9	13.6	2.9	3.5	3.1	3.7	3.4	5.0	5.1	5.1	5.1	7.6	0.40
13/09/2017	F	13/09/10/F	2.613	1.042	7.2	7.3	7.3	7.3	2.8	3.3	2.9	3.8	3.3	5.1	5.0	5.2	5.1	8.1	0.38
13/09/2017	G	13/09/10/G	2.183	1.033	7.7	7.6	7.2	7.5	3.4	5.7	5.5	5.8	5.7	7.6	8.1	7.3	7.7	9.7	0.49
13/09/2017	H	13/09/10/H	2.342	1.041	8.9	9.0	9.5	9.1	3.9	4.3	4.3	4.6	4.4	7.5	7.7	7.7	7.6	12.7	0.39

NT = not tested as these collection time points were superfluous to the study design.

NS = no sample as specimen was physically unable to be collected.

8. Press release – Expert joint letter calling for a moratorium in racing cobalt charges

PRESS RELEASE – 29.8.19
EXPERT JOINT LETTER CALLS FOR
MORATORIUM IN RACING COBALT CHARGES

A group of veterinarians and scientists have urged Australian racing regulators to implement an immediate moratorium on prosecutions for alleged misuse of cobalt. In a recent communication to Australian Racing Board, Australian Harness Racing Board and Australian Greyhound Board, the group set out their concerns.

The current test method employed to detect cobalt salts in urine is inappropriate and prone to "false positives" due to Vitamin B12 and urine concentration effects and could therefore result in convictions of innocent parties.

It is clear that some trainers have incurred "positives" from cobalt exposure in feed and environment outside of their knowledge or control.

The experts question the use of **population studies** on race day samples from horses with unknown exposure in feed supplements and the environment, to set a "threshold".

They believe there is **confusion** and **misinformation** regarding both the potency and potential toxicity of cobalt salts.

A further request to regulators is that they provide financial and administrative support to a multi-disciplinary Committee of Inquiry whose charge is to find a consensus approach to future regulation of cobalt use in racing animals.

The signatories emphasise that they endorse the efforts of regulators to identify and punish those who seek to gain an advantage by unfair means – which includes use of performance-enhancing substances.

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***CO-SIGNATORIES**
Dr Andrew Clarke, Mr David Dawson
Dr Derek Major, Mr Neale Scott,
Mr Ross Tinniswood, Mr Ross Wenzel

9. Published manuscripts

Major, D. & Wenzel, R. 2016, 'Commentary on Paper: “Controlling the misuse of cobalt in horses”', *Drug testing and analysis*, vol. 8, no. 8, pp. 880-81.

Bishop, D.P., Blanes, L., Wilson, A.B., Wilbanks, T., Killeen, K., Grimm, R., Wenzel, R., Major, D., Macka, M., Clarke, D., Schmid, R., Cole, N. & Doble, P.A. 2017, 'Microfluidic high performance liquid chromatography-chip hyphenation to inductively coupled plasma–mass spectrometry', *Journal of Chromatography A*, vol. 1497, pp. 64-9.

Wenzel, R., Major, D., Hesp, K. & Doble, P. 2018, 'Determination of Vitamin B12 in Equine Urine by Liquid Chromatography–Inductively Coupled–Plasma Mass Spectrometry', *Journal of Trace Elements in Medicine and Biology*, vol. 50, pp. 634-39.

Wenzel, R., Major, D., Hesp, K., Hall, E. & Doble, P. 2019, 'Cobalt accumulation in horses following repeated administration of cobalt chloride', *Australian Veterinary Journal*, vol. 97, no. 11, pp. 465-72.