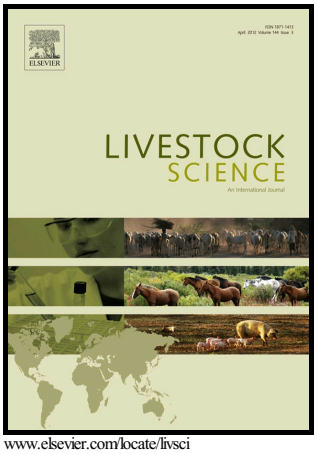

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The efficacy of supplying supplemental cobalt, selenium and vitamin B_{12} via the oral drench route in sheep.

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Abstract

Cobalt and selenium are essential trace elements required for ruminants. There are many different methods of supplementation available to sheep including boluses, in feed, free access minerals, pasture dressing and oral drenches. Recent changes in European legislation have resulted in a reduction in the maximum permitted level (MPL) of cobalt to be included in ruminant diets from 2 mg/kg DM to 1 mg/kg DM with a suggested supplementary rate of 0.3 mg Co/kg DM.

This study aimed to determine the efficacy of cobalt plus/minus vitamin B_{12} and selenium supplementation from oral drenching over a 13 day time period.

Seven groups of grass silage fed Suffolk cross mule lambs (n=56) were randomly allocated to one of 7 treatments, a 5 ml oral drench containing 700, 2300, 12000 mg Co/l, with or without 2300 mg vitamin B_{12}/l in a factoral design, with an additional control group which received no drench. All drenches also contained selenium at 625 mg/l. Lambs were weighed on days 0 and 13. The lambs were sampled by jugular venepuncture on days 0, 1, 2, 3, 4, 5, 6, 7, 9, 11 and 13, with samples analysed for plasma selenium and cobalt by ICP-MS, erythrocyte glutathione peroxidase by colourimetric assay and vitamin B_{12} by immunoassay.

The results showed that cobalt via a drench was able to raise plasma cobalt in a dose dependent manner, with each dose level significantly higher than the previous level at all post drenching time points ($P<0.05$). However, the cobalt containing drenches did not significantly raise vitamin B_{12} concentrations in the plasma. Vitamin B_{12} containing drenches were able to elevate vitamin B_{12} in the plasma for a period of 7 days ($P<0.05$). The selenium content of the drench was able to significantly raise the plasma selenium from day 1 throughout the rest of the trial (day 13) ($P<0.001$). A significant increase in erythrocyte glutathione peroxidase activity of the sheep did eventually occur at day 13 ($P<0.05$).

In summary, the addition of vitamin B_{12} via the drench route resulted in a short term elevation (7 days) of plasma vitamin B_{12} concentrations whereas cobalt sulphate alone was unable to significantly raise vitamin B_{12} concentrations within this trial with marginally adequate cobalt status, despite elevating plasma cobalt concentrations. The drench route was also able to elevate plasma selenium concentrations from a marginal selenium status.

Keywords: Sheep, Drench, Trace Element, Cobalt, Supplementation

Introduction

Cobalt and selenium are essential trace elements required for ruminants. However, their levels in pasture appear to be inadequate in many areas of the UK and worldwide. "In New Zealand, Knowles and Grace (2014) showed that only 54% of pasture from across the country in the trial had adequate cobalt content to fulfil the animals' requirements; the situation was marginally better for selenium, where 76% of pastures contained the adequate concentration. The low concentrations of selenium and cobalt in pasture underlines the necessity of their supplementation especially in grazing ruminants. There are many different methods of supplementation available to sheep including boluses, in feed, free access minerals, pasture dressing and oral drenches (Kendall *et al.,* 2000 and 2001; Kendall and Bone, 2014). The easiest way to supplement animals is via feed. However, when animals are not receiving concentrate feed or total mixed ration (TMR), the easiest to administer, direct to animal treatment is likely to be an oral drench (delivered via a dosing gun). However, the efficacy of single dose oral drench copper supplementation has been shown to be poor (Kendall *et al.,* 2000) and there is a lack published data showing the release profile of cobalt and vitamin B_{12} response to a single oral dose of different amounts of cobalt with or without vitamin B_{12}. Previous work by this group has shown that the administration by oral drenching is quicker than both injecting and bolusing. Administration times for the drench were 30 seconds per sheep with the injection (using a multi-injector) taking 34 seconds per sheep and two different types of bolus taking 75 and 98 seconds per sheep respectively when carried out with ten treatment pens of 10 sheep per administration

method (unpublished data). The bolus reload time made bolus administration slower than the other methods.

Recent changes in European legislation have resulted in a reduction in the maximum permitted level (MPL) of cobalt to be included in ruminant diets from 2 to 1 mg/kg at 88% DM with a suggested supplementary rate of 0.3 mg Co/ kg at 88% DM (Commission Implementing Regulation (EU) No 601/2013 of 24 June 2013 / Amended by Commission Implementing Regulation (EU) No 131/2014 of 11 February 2014). Cobalt within vitamin B_{12} is not included within this calculation. These alterations in the legislation highlight the necessity of determining the effective supplementation time so that the inclusion rates for drench supply of cobalt can fit these revised levels. A typical commercial cobalt, selenium and vitamin B_{12} drench will contain 60 mg Co per dose for a 35 kg lamb which would have an expected dry matter intake of 0.92 kg DM/day (high metabolisability diet (q_m 0.65), McDonald *et al.*, 2011). To be in accordance with the European legislation, this dose of cobalt should be effective for 192 days, a dose of 11.5 mg Co should be effective for 37 days, with a dose of 3.5 mg Co needing to be effective in supplementing cobalt for 12 days. The aim of this study was to determine the efficacy of cobalt and/or vitamin B_{12} supplementation from oral drenching over a 13 day time period. A secondary aim was also to determine the efficacy of the selenium supplementation.

Material and methods

Suffolk cross mule lambs (n=56) were split into 8 allocation groups of seven based on sex and live weight at day 0 then within each allocation group one lamb was randomly allocated to each treatment group. The lambs were housed in two single sex pens of 28 (4 single sex allocation groups per pen). Treatments were randomly

allocated to each treatment group. The treatments, administered on day 0 were a single dose of 5 ml oral drench of 3 different concentrations of cobalt drench (700, 2300, 12000 mg Co/l), with or without vitamin B_{12} (2300 mg vitamin B_{12}/l) in a factoral design, with an additional control group which received no drench. The cobalt was in the form of cobalt sulphate heptahydrate (E3). All drenches contained selenium at 625 mg/l as sodium selenite. Lambs were re-weighed on day 13 at the completion of the trial.

The lambs were housed on allocation and were *ad libitum* fed with grass silage harvested earlier in the year from the field grazed prior to the trial. A sample of the silage was collected and analysed for mineral concentration and contained 0.28 mg/kg DM of cobalt and 0.03 mg/kg DM of selenium.

Lambs were sampled by jugular venepuncture under ASPA licence on day 0, prior to drench administration and then on days 1, 2, 3, 4, 5, 6, 7, 9, 11, 13 at approximately the same time each day. Blood samples were collected via 20g x 1" vacuette needles (Griener Bio One Ltd, Stonehouse, UK) into a single 10 ml lithium heparin (LH) vacuum tube (BD vacutainer, Plymouth, UK). Tubes were gently inverted 6 times before being transported back to the laboratory where after 10 mins of rotational agitation (Mixer 820, Boule diagnostics, Spånga Sweden) 25 µl aliquots of whole blood were taken in auto-analyser cups (Sarstedt, Leicester, UK), capped and stored at -20°C. The blood was subsequently centrifuged (Allegra x-22, Beckman Coulter Ltd, High Wycombe, UK) at 2000 g for 15 mins and haematocrits calculated from estimations of total volume and red blood cell volume made using a ruler and identical volume calibrated tube. Subsequently plasma was removed by pasteur pipette into two 75 x 12 mm polypropylene tubes (Sarstedt, Leicester, UK) which were capped and then stored at -20°C prior to analysis. Analyses were initially

carried out on four of the eight groups. Further analyses were only carried out when additional statistical power was required as indicated by the preliminary results.

Plasma mineral determination (cobalt and selenium)

One aliquot of lithium heparin plasma was thawed and mixed by rotational agitation (Mixer 820, Boule diagnostics, Spånga Sweden) then 0.5ml was pippetted into a 14 ml polypropylene tube (Starstedt, Leicester, UK). Elemental selenium and cobalt concentrations were determined by ICP-MS (Thermo-Fisher XSeries^{II}). Samples and standards were diluted identically (0.5 mL into 10 mL) in a diluent containing 0.1% of a non-ionic surfactant ('Triton X-100' and 'antifoam-B', Sigma-Aldrich Company Ltd., Dorset, UK), 2% methanol and 1% HNO_3 (Trace Analysis Grade, Fisher, UK) including the internal standards Ir (5 µg L^{-1}), Rh (10 µg L^{-1}), Ge (50 µg L^{-1}) and Sc (50 µg L^{-1}). The ICP-MS was run in 'collision cell with kinetic energy discrimination' (CCT-KED) mode using 7% H_2 in He as the hexapole collision cell gas to reduce polyatomic interferences. Aspiration was through a single sample line via a glass concentric nebulizer (Thermo-Fisher Scientific; 1 mL min^{-1}). Following standard dilutions, calibrations were effectively in the range 0–50 µg L^{-1} (Claritas-PPT grade CLMS-2 from Certiprep/Fisher, UK).

Erythrocyte glutathione Peroxidase activity

One aliquot of 25 µl of heparinised whole blood was thawed and diluted with 1ml of RANsel diluent and gently mixed. The diluted whole blood was then assayed using the RANsel kit (Randox laboratories, Belfast, UK) according to manufacturer's instructions on a RX IMOLA clinical chemistry analyser (Randox laboratories, Belfast,

UK). Results were expressed relative to previously estimated haematocrits as U/ml PCV.

Plasma vitamin B_{12} concentration

One aliquot of frozen plasma was packaged and sent to the Animal Health and Plant Agency (AHPA) Biochemistry Laboratory (Shrewsbury, UK) where vitamin B_{12} concentrations were determined. The method used was the vitamin B_{12} assay on the Advia Centaur CP vitamin B_{12} immunoassay system (Siemens, Camberley, Surrey UK) according to manufacturer's recommendations.

Statistical analysis

Cobalt and vitamin B_{12} supplementation was assessed on log transformed data using the 3 x 2 factoral design with full interactions (control group was not included in this analysis) and individual treatment groups (control group included). Plasma selenium and erythrocyte glutathione peroxidase were compared between control and the other experimental groups (all drench groups had same selenium supplementation). Live weights were compared using both the factoral and individual treatment groups without data transformation.

Statistical analysis was carried out using the general linear model procedure within MINITAB 16, with post-hoc individual group comparisons done using Bonferroni corrections. Residuals were checked for approximation to normal distribution and data not following ANOVA assumptions was log transformed (plasma cobalt and vitamin B_{12}) to ensure normality of residuals. A significance of $P<0.05$ was considered significant.

Results

There was no significant effect of oral drench on live weight across the trial with lambs maintaining weight from day 0 at 35.2 kg (± 1.01) until the trial end at day 13 at 34.3 kg (± 2.09).

Cobalt/Vitamin B_{12}

The cobalt containing drenches rapidly increased the plasma cobalt concentrations (Figure 1) in a dose dependent manner irrespective of the inclusion of vitamin B_{12}. The rise in plasma cobalt concentrations were significant ($P<0.001$) from day 1 till the final day of the trial (day 13), with no significant effect of vitamin B_{12} supplementation and no vitamin B_{12}/cobalt interaction. Post-hoc analysis further indicated that high dose resulted in significantly ($P<0.05$) higher values of plasma cobalt concentration than the medium dose which also induced significantly ($P<0.05$) higher cobalt levels than the low dose for the whole experimental period. The plasma cobalt concentrations reached the maximum concentration at the first day post administration (23 hours) and decreased at a steady rate indicating half-lives for the cobalt in plasma as outlined in Table 1. The cobalt level was negatively correlated with the half-life of plasma cobalt with the 700 mg/l supplemented group having significantly greater values than either the medium or high level supplemented cobalt groups ($P<0.05$).

The plasma vitamin B_{12} concentrations (Figure 2) are significantly elevated by the addition of vitamin B_{12} to the drench for days 1 and 2 ($P<0.001$), day 3 ($P<0.01$) and from day 4 to 5 ($P<0.05$) with no significant increase thereafter, although days 6 and 7 both displayed a trend ($P<0.1$). Although the level of cobalt supplementation had no significant effect on vitamin B_{12} concentrations and there were no interactions

between vitamin B_{12} and cobalt supplementation, the low and high but not medium cobalt containing drenches without vitamin B_{12} did have numerically higher plasma vitamin B_{12} concentrations than the control group.

Selenium/Glutathione Peroxidase

The plasma selenium concentrations (Figure 3) were significantly increased ($P<0.001$) for the whole experimental period compared to the undrenched control group.

The erythrocyte glutathione peroxidase activities (Figure 4) appeared to increase in the oral selenium containing drenched groups throughout the 13 day trial, whereas the control lambs exhibited declining erythrocyte glutathione peroxidase activities from a slightly higher starting point. The only day that there was a significant difference between the drenched and the undrenched lambs was for day 13 when erythrocyte glutathione peroxidase activity was significantly higher ($P<0.05$) in the drenched sheep.

Discussion

The results showed that the cobalt containing drenches increased cobalt but not vitamin B12 concentrations in the plasma. On the other hand, vitamin B12 containing drenches were able to elevate vitamin B12 levels in the plasma but only for 7 days. Furthermore, the selenium content of the drench appeared to increase the plasma selenium levels throughout the experimental period and also enhance the erythrocyte glutathione peroxidase activity after 13 days.

By comparing the calculated half-life data (Table 1) and the initial peak plasma cobalt concentration (day 1), the estimated time for the plasma cobalt to return to control

levels are 152, 173 and 184 days for the high, mid and low cobalt levels with additional B12 supplementation and 160, 145 and 164 days for the high, mid and low cobalt levels group without B12 supplementation, respectively.. These time periods are surprisingly independent of dose, however, they do assume a continued constant half-life beyond the day 13 value used for the calculation of half-life. However, when comparing them to the calculated durations required for the different doses from the introduction the low and mid concentrations are longer than the required duration whereas the high dose is shorter than the required 192 day duration. The durations were calculated for a 35 kg lamb which would have an expected dry matter intake of 0.92 kg DM/day (high metabolisability diet (q_m 0.65), McDonald *et al.*, 2011) relative to the recommended maximum supplementary rate for cobalt of 0.3 mg/kg at 88% DM (Commission Implementing Regulation (EU) No 601/2013 of 24 June 2013 / Amended by Commission Implementing Regulation (EU) No 131/2014 of 11 February 2014). This is therefore cobalt dose divided by recommended supplemental intake at 88% DM (in this case 0.3 mg Co/ kg @ 88%DM / (0.92 x 100/88 = 1.045 kg @88% DM)), which gives the number of days over which this supplement requires to show efficacy to fit within EU recommendations. The recommended supplementary level takes into account the likely cobalt intake from the background diet and therefore in order to avoid passing the maximum permissible level (MPL) then supplementation should be restricted. A full mineral audit (Kendall and Bone, 2017) which determines actual cobalt intake would identify the difference between cobalt intake and recommended daily allowance (rda) to give a required supplementary rate. Supplementation regimens should be designed so that the supplement and background will fulfil the rda and should not be targeted to meet the MPL as variations within background diet and supplement release could mean that maximum

permissible levels are exceeded and in some cases, especially in elements with lower toxicity thresholds and lower differences between MPL and rda such as copper and selenium increases in loading and potentially toxicities can occur (Kendall *et al.*, 2015).

Keener *et al.* (1950) showed some recovery from Co deficiency in intravenously treated lambs after daily administration of cobalt sulphate (2,4 and 6 mg/day) giving a response in cobalt deficient sheep, however, higher doses are required and the first signs of recovery are shown much later.

Kercher and Smith (1956) compared oral drenching with intravenous and subcutaneous injections of cobalt (all as 1 mg/day cobalt chloride) administered to cobalt deficient lambs and found that only oral administration raised the vitamin B12 concentration in blood. However, although only oral cobalt supplementation increased rumen content (and omasum-abomasum, duodenum-jejunum and ileum), the injection of cobalt (intravenous and subcutaneous) increased vitamin B12 in the caecum and large intestine to levels comparable to that found after oral cobalt supplementation, suggesting that caecal fermentation may contribute in the production of limited quantities of B12 and thus explaining the findings of Keener et al. (1950) and Kercher and Smith (1956)..

Keener *et al.* (1951) showed some radioactivity in the rumen contents after intravenous administration of labelled cobalt carbonate, however, these levels (180-250 dpm/g) were very low when compared to small (15000-20000 dpm/g) and large intestine contents (12500-25000 dpm/g). Little direct work on cobalt excretion on sheep has been carried out but in humans vitamin B_{12} excretion is predominantly biliary and only a small proportion is excreted in urine (Underwood and Suttle, 1999). Smith and Marston (1970) also showed urinary and faecal excretion, but failed to

confirm the biliary route as this was not directly investigated and was therefore within faecal excretion.

Unfortunately in this study, the cobalt status was above marginal at the beginning and the status increased throughout the trial in the as indicated by the rising plasma cobalt in the control lambs. This finding indicated that the pre-trial management changes (housing and change from grazing to a silage diet) either supplied more cobalt or restricted growth factors which could reduce the vitamin B_{12} requirements for lambs. If the lambs were deficient in cobalt, or fed a cobalt deficient diet as in previous studies (Keener *et al.*, 1950 and 1951; Kercher and Smith, 1956), then it would be more likely to observe a B_{12} response from the elevated plasma cobalt and the post ruminal production of vitamin B_{12} from the potentially recycled biliary cobalt, as the caecal fermentation may be able to supply a part of the sheep's vitamin B_{12} requirement. However, it must be remembered that vitamin B_{12} is also required by the rumen flora for normal metabolism, including the conversion of succinate to propionate (Underwood and Suttle, 1999).

The selenium obtained through the drench resulted in an elevation of the plasma selenium values throughout this short trial and the erythrocyte glutathione peroxidase activity increased across the study in comparison to the declining erythrocyte glutathione peroxidase activity in the control animals, a fact that supports the utilisation of the selenium from the blood for formation of the glutathione peroxidase. Unlike the cobalt status the selenium status of the sheep was marginal both in terms of plasma selenium (deficiency at <0.22 µM) and erythrocyte glutathione peroxidase (marginal 20-40 U/ml PCV), and the target levels for these parameters are 0.8 µM for plasma selenium and 80 U/ml PCV for erythrocyte glutathione peroxidase. The increase in erythrocyte glutathione peroxidase activity would be expected to continue

beyond the supplementation period due to the elevated plasma selenium and marginal background selenium status.

To conclude, cobalt is not effectively supplemented as cobalt sulphate to marginally cobalt adequate sheep by oral single dose drenching as the cobalt does not remain within the rumen long enough to support increased synthesis of vitamin B_{12}. Supplementation with vitamin B_{12} in the single dose drench was effective for approximately 7 days at the dose given (2300 mg) whilst selenium (625 mg) was effective for more than the 13 days of the trial. Overall, this work implies that single dose oral drench is not the most suitable form of cobalt supplementation.

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Fig 1 Mean (±s.e.m.) plasma cobalt concentrations (nmol/l) on a Log (10) scale after oral drenching on day 0 with low (open circle, 700 mg/l), medium (closed circle, 2300 mg/l) and high cobalt doses (square, 12000 mg/l). Supplementation or not with B12 (2300 mg/l) is represented as solid or dotted line, respectively. Control group is marked with triangles and there are also reference lines representing marginal (**–··–··–**) and deficient (**–·–·–**) thresholds at 3 and 5 nmol/l respectively. The 2300 mg/l dose is significantly higher than the 1200 mg/l dose which is significantly higher than the 700 mg/l dose ($P<0.05$) from day 1 to 13 inclusive.

Fig 2 Mean (±s.e.m.) plasma vitamin B_{12} concentrations (pmol/l) after oral drenching on day 0 with low (open circle, 700 mg/l), medium (closed circle, 2300 mg/l) and high cobalt doses (square, 12000 mg/l). Supplementation or not with B12 (2300 mg/l) is represented as solid or dotted line, respectively. Control group is marked with triangles and there are also reference lines representing marginal (**–··–··–**) and deficient (**–·–·–**) thresholds at 188 and 400 pmol/l respectively. The plus vitamin B_{12} groups (dotted lines) are significantly greater than the minus groups from day 1 until day 4 ($P<0.001$) and from day 5 to 7 ($P<0.05$) with no significant increase thereafter.

Fig 3 Mean (±s.e.m.) plasma selenium concentrations (µmol/l) after oral drenching on day 0 for all the experimental groups (different levels of cobalt with or without B12 supplementation) (grey diamond, 625 mg Se/l) and for the control group (black triangles, 0 mg Se/l). Reference lines representing marginal (**–··–··–**) and deficient (**–·–·–**) thresholds at 0.8 and 0.22 µmol/l, respectively. From day 1 to day 13 inclusive the groups were significantly different ($P<0.001$).

Fig 4 Mean (±s.e.m.) erythrocyte glutathione peroxidase activities (U/ml PCV) after oral drenching on day 0 for all the experimental groups (different levels of cobalt with

or without B12 supplementation) (grey diamond, 625 mg Se/l) and for the control group (black triangles, 0 mg Se/l). Linear trend lines are indicated with dashed lines (---). Reference lines representing marginal (**–··–··–**) and deficient (**–·–·–**) thresholds at 40 and 20 U/ml PCV, respectively. There was no significance difference between groups except for day 13 ($P<0.05$).

Table 1 *The half-life of plasma cobalt determined for a single 5 ml drench administration of a cobalt dose (700, 2300, 12000 mg/l), with and without additional vitamin B_{12} administration (2300 mg/l).*

	Half-life of Plasma cobalt (days)					
	With Vit. B_{12}[1]		No Vit. B_{12}		with/without Vit. B_{12}	
Cobalt dose	mean	sem	mean	sem	mean	sem
700 mg/l (3.5 mg Co)	6.60 [a]	0.376	5.90 [ab]	0.376	6.25 [A]	0.269
2300 mg/l (11.5 mg Co)	5.57 [ab]	0.376	4.60 [ab]	0.376	5.09 [B]	0.269
12000 mg/l (60 mg Co)	4.38 [b]	0.376	4.59 [ab]	0.376	4.49 [B]	0.269
all Co doses	5.52	0.217	5.03	0.217		

[1] 2300 mg/l (11.5 mg B_{12}).

[a,b] Values with different superscripts differ significantly at $P<0.05$.

[A,B] Values within a column with different superscripts differ significantly at $P<0.05$.

Highlights

- Drench with single oral dose of cobalt is not effective in marginally adequate cobalt supplemented sheep
- Cobalt does not remain within the rumen to allow synthesis of vitamin B12