Development of an experimental approach to measure vitamin B_{12} production and utilisation in sheep

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ABSTRACT

Cobalt (Co) is required by ruminants to enable synthesis of vitamin B_{12} by rumen micro-organisms. Lack of quantitative information on the synthesis, absorption or utilisation of the vitamin has made determining nutrient requirement difficult. Two trials with sheep previously depleted of vitamin B_{12} and offered low Co diet (0.08 mg/kg DM) are described which attempt to overcome these problems. In the first, ewes were dosed with Co intra-ruminally at rates between 0 – 1 mg Co/day. Responses in plasma vitamin B_{12} were proportional ($P < 0.001$) to the amount of Co infused. In the second trial, vitamin B_{12} was infused into the bloodstream via the jugular vein at rates between 0 – 2500 nmol/day. Plasma vitamin B_{12} plateau values were directly proportional ($P < 0.001$) to infusion rate. The rate of vitamin B_{12} infusion needed to sustain the critical plasma concentration of 370 pmol B_{12} /l was 5.02 nmol B_{12} /day, which together with estimated absorption from the basal diet gives a total requirement for absorption of 8.12 nmol B_{12} /day. This value is in agreement with data in the literature. The model approach, therefore, seems quantitatively robust. This approach, together with estimates of vitamin B_{12} flow from the rumen, will enable study of dietary factors that predispose to poor vitamin B_{12} production in the rumen and its absorption from the alimentary tract, and therefore to low animal vitamin B_{12} status.

Key words: Co/vitamin B_{12}; infusion; sheep; rumen; modelling.

INTRODUCTION

Vitamin B_{12} is required by ruminants in the conversion of methylmalonyl CoA to succinyl CoA, a metabolic pathway that enables a major contribution to their glucose requirements to be provided from propionate produced during rumen fermentation.

The non-specific nature of the signs of Co deficiency in ruminants has made diagnosis difficult. Other conditions such as parasitism and malnutrition have been confused with vitamin B_{12} deficiency. Tissue analysis for vitamin B_{12} provides only an index of general status of the animal and is not reliable in predicting a growth response to supplementation. This is shown in the wide bands of plasma vitamin B_{12} concentration (185-370 pmol/L) used to indicate marginal status (Grace, 1994).

The synthesis of vitamin B_{12} by rumen micro-organisms means that the supply of the vitamin is dependent on an adequate intake of cobalt (Co). While variation in plant species and plant maturity, that influence microbial activity in the rumen, have been suspected as causes of variation in vitamin B_{12} supply, these have not been examined quantitatively because of the unavailability of suitable techniques. The efficiency of conversion of dietary Co into vitamin B_{12} in the rumen is thought to be inversely related to Co intake (Smith & Marston, 1970) and possibly to be lower on high concentrate diets (Sutton & Elliot, 1972). Efficiency of absorption of vitamin B_{12} has been suggested to be < 5%, or even < 3% in adult sheep (Marston, 1970). Estimates of vitamin B_{12} requirement for maintenance (Marston, 1970) are based on the rate of injection of vitamin B_{12} required to maintain plasma and liver concentration discounted by an estimate of rate of ruminal synthesis from the diet.

This study describes the development of an animal model based on the technique developed by Suttle (1974) for copper (Cu) metabolism studies, to determine vitamin B_{12} production and utilisation. The model uses change in blood vitamin B_{12} concentration during repletion of depleted animals to determine the production and utilisation of vitamin B_{12}. The ultimate aim is to identify risk factors and enhance the ability of veterinarians and farmers to interpret tissue analysis and better assess the need for supplementation.

MATERIALS AND METHODS

Ten ewes with marginal vitamin B_{12} status were obtained from a cobalt deficient farm at Montalto, Canterbury, New Zealand and housed in wood-lined pens with plastic troughs and provided with deionised water. They were maintained on hay with low cobalt content (0.08 mg Co/kg DM) to further deplete vitamin B_{12} stores. All animals were fitted with rumen and abomasal cannulae using procedures described by Hecker (1974). The animal procedures were approved by the Lincoln University Animal Ethics Committee.

Experiment 1 – Cobalt doses via the reticulo-rumen

Treatments were set up as a 14-day paired Latin square design randomly allocated. Treatments were 0 (control), 0.05, 0.15, 0.25 or 1.0 mg doses of Co given as a cobalt sulphate solution ($CoSO_4.7H_20$) containing 0.05 mg Co/ml. Blood samples were taken on day 0

prior to treatment and on days 1, 2, 4, 7, 10 and 14, at 0900 h before feeding.

Experiment 2 – Vitamin B_{12} infusions via the jugular vein

Following a recovery period of four weeks on the low Co diet and the return of plasma vitamin B_{12} concentrations to pre-treatment levels, the same animals were confined in wooden metabolism crates to enable a 48-hour intra-jugular infusion of vitamin B_{12}. On the morning infusions commenced a catheter (Vialon™ Insyte™ 16 gauge 1.7 x 45 mm, Becton Dickinson Ltd, Auckland, New Zealand) was positioned in the jugular vein, cloth tape 'flags' were adhered to the top of each catheter, stitched to the sheep's skin and infusion lines were attached. Vitamin B_{12} infusions, (as Prolaject, 1 mg/ml hydroxycobalmin, Bomac Laboratories Ltd, Auckland, New Zealand.) were set to provide five rates of infusion - 0 (control), 2.5, 25, 250 and 2500 nmol vitamin B_{12}/day, carried in sterile isotonic saline at the rate of 864 ml/day. Blood samples were obtained prior to commencement of infusion and after 4, 8, 48, 52, 56 and 60 h and at 0900 h on days 3, 4, 7, 10 and 14 after the start of the infusion.

Blood sampling

Blood collection was done via jugular venipuncture into 10ml vacutainers containing sodium heparin (143 i.u.). Samples were covered from light and centrifuged at 3000 rpm at 4°C for 10 minutes. Plasma was pipetted off using disposable pipettes and stored in clean plastic vials at –20°C until analysed. Samples were analysed by Gribbles Alpha Scientific, Hamilton, New Zealand for vitamin B_{12} by chemiluminescence. From our laboratory comparisons (J. Furlong., personal communication), the chemiluminescence assay was highly correlated with the conventional radioisotope dilution assay (RIDA) ($R^2 = 0.90$, $P < 0.0001$, $n = 50$). Vitamin B_{12} analogues do not interfere with the chemiluminescence assay because it is free of R-proteins (Diagnostic Products Corporation, Los Angeles, United States of America) responsible for binding analogues and hence a value 6% lower than the RIDA method.

Statistical analysis

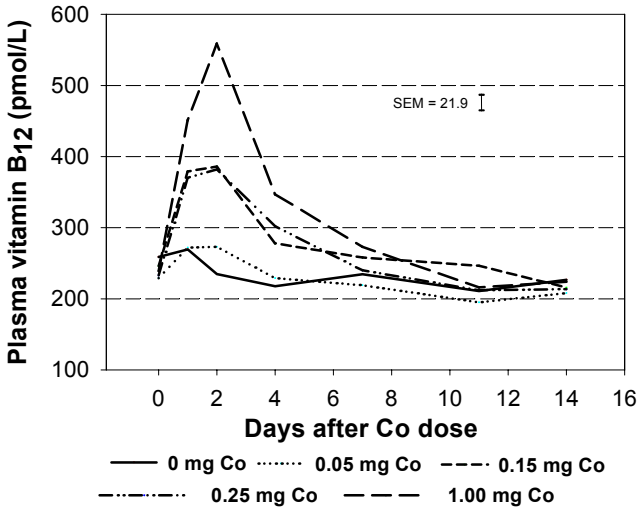
To relate the response curves to a model, double exponential curves were fitted to the data, one each for the appearance and disappearance curves of plasma vitamin B_{12} using the Gauss-Newton method (Draper & Smith, 1988). Data from experiment 1 did not fit to an appearance curve so were analysed by fitting a single decay curve from day 2 (time when plasma vitamin B12 started to decline) and by removing treatment 0 due to the difficulty of fitting a curve to a straight line. Data from experiment 2 were transformed to logarithms ($\log_{10}$) prior to analysis to normalise their distribution. Peak appearance values were obtained from the upper asymptote of the fitted model.

RESULTS

Experiment 1

The plasma vitamin B_{12} response to Co injection is shown in Figure 1. The response was proportional to the amount of Co received, with maximum plasma vitamin B_{12} levels generally being reached by day 2. Subsequently, concentrations decreased following a general decay curve and had returned to pre-treatment levels by day 12.

FIGURE 1: Effect of increasing Co doses via the reticulo-rumen on plasma vitamin B_{12} concentrations in sheep.



Experiment 2

Changes in plasma vitamin B_{12} ($\log_{10}$) in response to intra-jugular infusion of vitamin B_{12} are shown in Figure 2. Concentrations rose rapidly and had reached plateau values at the lower infusion rates and almost reached plateau values at higher infusion rates by 48 h when infusions were stopped. Rates of appearance were proportional to infusion rate. Disappearance from plasma, once the infusions were stopped, followed a common exponential decay rate, irrespective of treatment.

DISCUSSION

One of the important objectives of the work in developing an useful model was to provide the range of cobalt doses or vitamin B_{12} infusions over which plasma concentration would be sensitive to change in vitamin B_{12} entry into the bloodstream. With the exception of the highest rate of Co injection, peak plasma vitamin B_{12} response was achieved within 48 h and there was evidence that plateau (steady state) conditions were developing. When rate of appearance of the vitamin in plasma was plotted against rate of Co dose into the rumen (Figure 3), response to Co diminished with increasing Co dose. This could be due to two factors. Firstly, at low rumen Co concentrations a greater

proportion of the cobalamins synthesised are likely to be true vitamin B_{12} (Gawthorne, 1970), which our assay would have measured and secondly, we have observed (unpublished data) that as rate of flow of vitamin B_{12} from the rumen increases, efficiency of absorption decreases. The rates of change in plasma concentration in response to Co dose were, however, quantitatively similar to observations in previous work (Suttle *et al.,* 1989) in which lambs were dosed orally with much larger range of amounts of Co (1 – 32 mg Co).

FIGURE 2: Effect of increasing infusion rates of vitamin B_{12} via the jugular vein on plasma vitamin B_{12} concentrations in sheep.

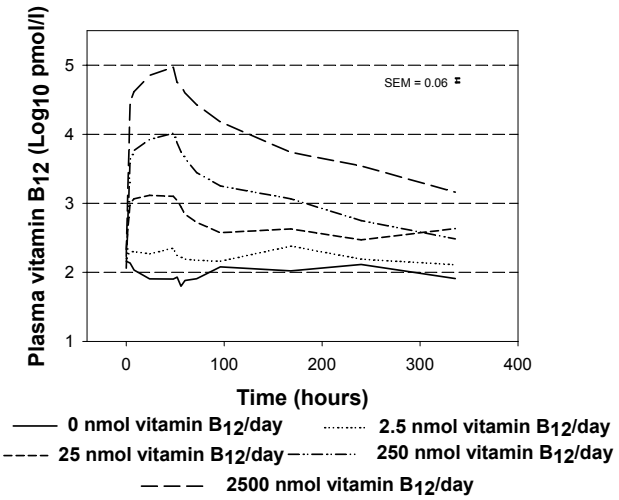
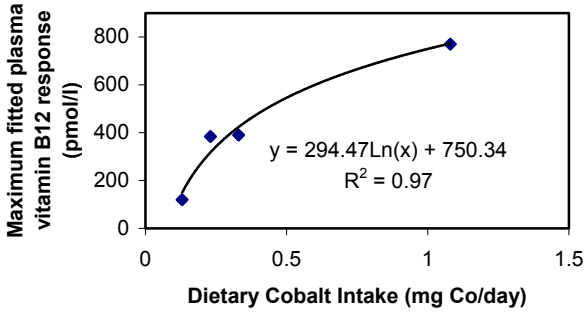


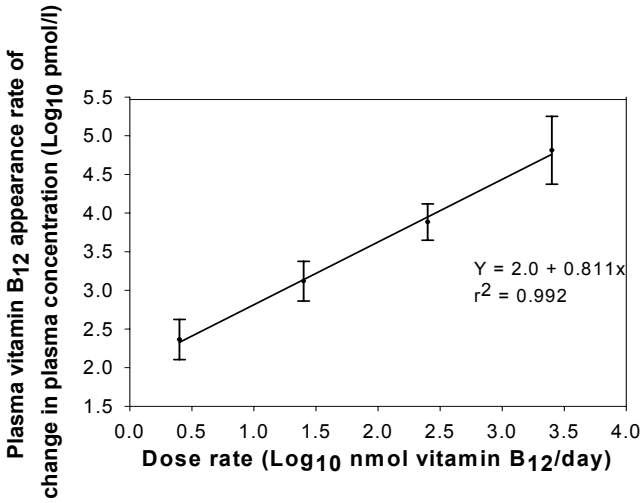
FIGURE 3: Relationship between Co intake and maximum fitted plasma vitamin B_{12} response in cobalt deficient sheep.



A model was fitted to the plasma vitamin B_{12} data from day 2 after giving the Co dose into the rumen, the time after which concentration started to decline. The calculated upper asymptote for each dose was proportional to the amount of Co given. The range of plasma concentrations achieved at the asymptote (120 – 770 pmol/l) encompassed the marginal range of values observed in practice (Grace, 1994). Moreover, the uniformity of the responsiveness and clear separation of concentrations observed for this range of Co dose suggests good sensitivity in detecting a difference in synthesis of the vitamin in the actual diagnostic marginal range.

Peak values of the vitamin following intra-jugular infusions were obtained using the upper asymptote and were related to rates of infusion to estimate the amount of vitamin B_{12} required to maintain a particular concentration of the vitamin in the plasma. As seen in Figure 4, peak plasma response (log_{10}) was linearly related to rate of vitamin infusion (log_{10}).

FIGURE 4: Fitted relationship between rate (log_{10}) of intrajugular infusion of vitamin B_{12} and asymptote (log_{10}) of appearance in the plasma in cobalt deficient sheep.



The amount of vitamin B_{12}, which must have been absorbed (nmol/day) after injection of Co into the rumen can now be calculated by transforming peak responses from intra-ruminal cobalt doses and solving for the relationship in Figure 4. These data are given in Table 1.

TABLE 1: Calculated entry rate of vitamin B_{12} into the bloodstream after cobalt doses via the reticulo-rumen of sheep in Experiment 1.

Reticulo-rumen dose (mg Co)	Peak plasma response (pmol vitamin B_{12}/L)	Intravenous infusion rate required to give the same plasma response (nmol vitamin B_{12}/day)
0.05	120	1.25
0.15	384	5.19
0.25	390	5.35
1.0	770	12.5

Intra-ruminal injections of 0.05 mg Co resulted in a peak plasma response of 120 pmol B_{12} /l. The amount of vitamin B_{12} required to be infused directly into the bloodstream to achieve an equivalent response would be 1.25 nmol B_{12} /day. The daily rate of absorption of the vitamin needed to maintain the plasma concentrations used in diagnosis of marginal deficiency that is in the range of 185 to 370 pmol/l can also be

estimated to be in the range of 2.14 to 5.02 nmol vitamin B_{12} /day infused respectively.

Our quantitative knowledge of vitamin B_{12} requirement is derived from the indirect estimates of Marston (1970). He concluded that daily maintenance requirement was 8.12 nmol vitamin B_{12} /day. This was derived from an estimate of 3.7 nmol vitamin B_{12} /day produced from ruminal synthesis, estimated from previous studies by Smith and Marston (1970) on similar diets, and 4.42 nmol vitamin B_{12} /day being the average rate of injection of the vitamin needed to maintain zero change of vitamin B_{12} concentration in the liver. There has been no other evidence on requirement and those values have been generally accepted.

If Marston's (1970) estimate of basal synthesis from the diet of 3.70 nmol vitamin B_{12} /day is used to provide the basal rate of production of the vitamin from the diet in our sheep, since the diets were similar in Co and other components, our estimate of total requirement of vitamin B_{12} is 8.72 nmol B_{12} /day. Our model approach therefore seems to give quantitatively comparable data to previous observations.

This model can now be used to test the efficacy of other supplements. Gruner (2001) supplemented ewes with two or three Co bullets and obtained long-term plasma vitamin B_{12} responses of 3.33 and 3.56 pmol (log_{10}) vitamin B_{12}/l plasma, respectively. Entering these values into Table 1 suggests equivalent infusion rates to give the same response would be 43 and 84 nmol vitamin B_{12} /day.

Clearly, more precise estimates of rates of production in the rumen and apparent absorption efficiency could be made with information on vitamin B_{12} flow from the rumen. These techniques are currently being developed and will allow a better understanding of the dietary and other factors which influence production and utilisation of the vitamin and a better understanding of the need for supplementation.

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