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R. Mohammed, M. Lamand

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## THE DIAGNOSIS OF VITAMIN B12 DEFICIENCY IN SHEEP : COMPARISON OF SERUM VITAMIN B12 LEVELS MEASURED BY A MICROBIOLOGICAL AND A RADIOISOTOPE DILUTION TECHNIQUE

R. MOHAMMED and M. LAMAND

Laboratoire des Maladies Nutritionnelles, INRA, CRZV de Theix, 63122 Ceyrat, France

### Résumé

DIAGNOSTIC DE LA CARENCE EN VITAMINE B12 CHEZ LE MOUTON : COMPARAISON DES TAUX DE VITAMINE B12 SÉRIQUE MESURÉS PAR DES TECHNIQUES MICROBIOLOGIQUES ET DE DILUTION RADIOISOTOPIQUE. — La vitamine B12 sérique de six moutons recevant une ration subcarence en cobalt (0,06 mg de cobalt par kg de matière sèche) a été dosée par une méthode de radiodilution isotopique. Les résultats obtenus ont été comparés avec les valeurs trouvées avec un dosage microbiologique (avec *Lactobacillus leichmannii* — ATCC 7830). Ce dernier dosage est spécifique de la vitamine B12 active et n'enregistre pas les isomères inactifs de la vitamine. Pendant les 16 semaines de l'expérience, les concentrations de vitamine B12 sérique ont été quatre à six fois plus élevées avec la méthode de radiodilution qu'avec la méthode microbiologique. Pendant toute l'expérience et contrairement aux résultats obtenus par la méthode microbiologique, les valeurs de vitamine B12 sérique obtenues par radiodilution ne franchissent jamais le seuil de carence (établi par voie microbiologique) de 200 pg/ml. Par ailleurs, adopter un seuil de carence plus élevé et propre à la méthode de radiodilution, serait peu crédible en raison de la corrélation faible (non significative) entre les résultats obtenus par les deux méthodes. On peut considérer qu'avec les réactifs utilisés (le kit commercial de dosage de la vitamine B12 de Amersham Radiochemical Centre), la méthode de radiodilution ne permet pas le diagnostic de la carence subclinique en cobalt chez le mouton.

The use of the RID technique in the rapid diagnosis of vitamin B12 deficiency in human subjects gave rise to the idea of adapting this method to veterinary medicine. The use of microbiological methods in the confirmation of vitamin B12 deficiency is limited by the length of time needed to carry out the analysis, the presence of antibiotics in the serum as well as the need for a specially equipped laboratory.

Other biochemical parameters have been used as indicators of vitamin B12 deficiency both in man and animals. In cobalt-vitamin B12 deficient sheep, the urinary excretion of methylmalonic acid has been used as an index for vitamin B12 de-

fiency (Gawthorne, 1968). The current analytical techniques used in the quantification of this acid are just as time consuming and costly as the microbiological techniques.

The analysis of urinary methylmalonic acid by gas chromatography (Frenkel and Kitchens, 1975) involves a number of complicated steps, the major limitation being the loss of this acid from the urine sample by volatilisation before analysis. A practical point of interest is the difficulties involved in obtaining urine samples from animals at pasture, as opposed to the ease with which blood samples may be obtained for the analysis of vitamin B12.

The need to quantify serum vitamin B12 rapidly

in the ruminant animal is of increasing importance since the quantity of "true" vitamin B12 synthesised in the rumen of animals fed a high concentrate ration diminishes considerably. The physiological needs of the host for this vitamin are consequently not met (Elliot *et al.*, 1972; Elliot, 1973; Elliot and Bauman, 1980).

The need for increasing amounts of vitamin B12 in highly productive dairy cows, particularly during early lactation, when more glucose is needed for milk production (Elliot *et al.*, 1979), is likely to deplete hepatic reserves of this vitamin, to the extent that a subclinical vitamin B12 deficiency may be induced.

In the light of these facts, the present study was undertaken to establish whether the RID technique may be used in the rapid diagnosis of vitamin B12 deficiency in ruminants.

## Materials and Methods

### Induction of vitamin B12 deficiency

Six healthy, adult, rumen-cannulated sheep (Texel), weighing between 65 to 70 kg bodyweight were each fed 1.4 kg DM of Timothy hay per day over a 16 weeks period. The hay contained 0.06 mg cobalt per kg DM. At the start of the experiment each sheep received intramuscular injections of copper (60 mg) and zinc (300 mg as oxide) (Prolontex<sup>®</sup>, Roussel Uclaf). Thiamine (625 mg) and pyridoxine (62.5 mg) were administered intramuscularly to each animal at thirty days intervals.

Animals were housed in individual wooden pens and had water and a salt block (NaCl) at their disposal. The salt block contained less than 0.004 mg cobalt per kg DM. The first four weeks were considered as an adaptation period at the end of which sample collection was initiated.

### Samples

Blood samples (20 ml) were obtained by jugular vein puncture once every four weeks just before feeding (8 a.m.). Samples were allowed to stand in the dark at room temperature for two hours, centrifuged at 1500 × *g* for 15 minutes and the serum obtained was divided into 3 ml aliquot portions before storage at -20 °C until analysis.

Rumen samples were procured via the cannula and were analysed for cobalt by the method of Hocquellet (1974) in an effort to monitor cobalt concentrations in the rumen. Approximately 60 g of rumen contents were sampled on the same day that blood samples were taken. Rumen samples were homogenised and stored at -20 °C until analysis.

### Microbiological analysis

A 2.5 ml aliquot of all serum samples was first analysed for vitamin B12 by the *Lactobacillus leichmannii* (ATCC 7830) method proposed by Hansen and Hauschildt (1974), in order to establish the "true" vitamin B12 status of the animals.

Once established, serum samples were selected from animals displaying a chronic state of deficiency (threshold value: 200 pg B12/ml) as well as those from animals showing acute deficiency (< 200 pg B12/ml). Samples for the chronic state of deficiency were pooled as well as those for the acute state of deficiency.

Table 1. — Serum vitamin B12 concentrations (pg/ml) determined by the *Lactobacillus leichmannii* method.

Week	Sheep						Mean ± SEM	CV (%)
	A	B	C	D	E	F		
4	853	387	720	400	1 140	467	661.2 ± 122.6	45
8	567	445	696	233	277	344	427.0 ± 72.9	42
12	151	193	195	142	145	208	172.3 ± 12.0	17
16	115	100	117	92	91	233	124.7 ± 22.1	43

CV: Coefficient of variation.

Table 2. — Serum vitamin B12 concentrations (pg/ml) determined by the RID method.

Week	Sheep						Mean ± SEM	CV (%)
	A	B	C	D	E	F		
4	2 050	1 600	1 920	960	900	410	1 306.7 ± 264.8	50
8	830	810	1 210	540	800	890	846.7 ± 87.7	25
12	700	725	1 000	600	515	650	698.3 ± 67.6	24
16	850	525	1 375	575	600	675	766.7 ± 103.1	42

CV: Coefficient of variation.

Analyses were performed on the sample pool. Pooled serum samples from an animal receiving an adequate supply of cobalt (0.1 mg/kg DM) during the 16 weeks period served as a control.

*RID analysis*

Serum samples collected throughout the experimental period were analysed for vitamin B12 by a commercial kit supplied by Amersham Radiochemical Centre (Buckinghamshire, England) based on the method of Lau *et al.* (1965). Each sample pool (control, chronic and acute) was divided into five equal portions and each of the latter was analysed by two methods.

**Results**

Cobalt concentrations in the rumen varied between 0.05 and 0.07 mg/kg DM during the experimental period.

*Lactobacillus leichmannii method*

The mean vitamin B12 concentrations obtained by this method are shown in table 1. In general, values ranged between  $661.2 \pm 122.6$  and  $124.7 \pm 22.1$  pg/ml during the 16 weeks, experimental

Table 3. — Serum vitamin B12 concentrations (pg/ml) as determined by the *Lactobacillus leichmannii* and the RID methods for different deficiency states.

Method	Number of repetitions					Mean $\pm$ SDM	CV (%)
	1	2	3	4	5		
<i>Lactobacillus leichmannii</i>							
Control	523	520	525	524	523	$523.0 \pm 1.87$	0.36
Chronic	206	208	208	208	206	$207.2 \pm 1.09$	0.53
Acute	149	149	146	150	149	$148.6 \pm 1.52$	1.02
Radio Isotope Dilution							
Control	3 375	3 375	3 380	3 375	3 370	$3 375.0 \pm 3.53$	0.10
Chronic	1 000	990	1 000	1 000	998	$997.6 \pm 4.33$	0.43
Acute	600	600	600	598	600	$599.6 \pm 0.89$	0.15

CV: Coefficient of variation.

Table 4. — Ratio of serum vitamin B12 concentrations (pg/ml) in different deficiency states (control, chronic and acute) between the *Lactobacillus leichmannii* and the RID methods of analysis. (means from table 3).

Deficiency state	Method		Ratio (1/2)
	RID (1)	<i>Lactobacillus leichmannii</i> (2)	
Control	3 375.0	523.0	6.4
Chronic	997.6	207.2	4.8
Acute	599.6	148.6	4.0

Table 5. — Ratio of serum vitamin B12 concentrations (pg/ml) during the 16 weeks experimental period between the *Lactobacillus leichmannii* and the RID methods of analysis. (means from tables 1 and 2).

Week	Method		Ratio (1/2)
	RID (1)	<i>Lactobacillus leichmannii</i> (2)	
4	1 306.7	661.2	2.0
8	846.7	427.0	2.0
12	698.3	172.3	4.0
16	766.7	124.7	6.1

period. Values were significantly lower ( $P < 0.05$ ) between weeks 4 and 12. There was, however, no significant difference between weeks 12 and 16.

The coefficient of variation (CV) decreased between weeks 4 and 12 (47 % to 17 % respectively). At week 16 the CV increased to approximately the same value observed at week 8 of the experiment.

#### RID method

Mean serum vitamin B12 concentrations obtained by this method are shown in table 2. Values ranged between  $1\,036.7 \pm 264.8$  and  $698.3 \pm 67.6$  pg/ml for weeks 4 to 12 respectively. The difference was at the limit of significance ( $P = 0.05$ ) for the latter period. The increase in vitamin B12 concentration was not however significantly different between weeks 12 and 16.

The coefficient of variation followed a similar pattern to that observed for the *Lactobacillus leichmannii* method. A decrease between weeks 4 and 12 (50 % to 24 % respectively) with an increase at week 16 (42 %) was observed.

#### State of deficiency

Mean vitamin B12 concentrations obtained by the two methods for the control, chronic and acute states of deficiency after a series of 5 repetitions are shown in table 3. For the control, values varied between 520 and 525 pg/ml with a CV of 0.36 % for the *Lactobacillus leichmannii* method. Values obtained for the same samples analysed by the RID method ranged between 3 370 and 3 380 pg/ml with a CV of 0.10 %. The ratio of the means between the two methods (table 4) indicates that the latter method gave values 6.4 times greater than the microbiological method.

Analysis of samples for the chronic state of deficiency gave values between 206 and 208 pg/ml (CV: 0.53 %) by the *Lactobacillus leichmannii* method whilst values ranged between 990 and 1 000 pg/ml (CV: 0.43 %) for the RID method. Vitamin B12 concentrations were thus 4.8 times greater for the latter method than those observed with the microbiological method.

For the acute state of deficiency, values ranged between 146 and 150 pg/ml (CV: 1.02 %) as analysed by the microbiological method. RID values ranged between 598 and 600 pg/ml (CV: 0.15 %). Mean RID concentrations were 4.0 times greater than values obtained by the *Lactobacillus leichmannii* method (see table 4).

A comparison of the ratios between the mean vitamin B12 concentrations obtained during the 16 weeks experimental period (table 5), as analysed by the two methods, indicates that as the state of deficiency changes from chronic to acute (between weeks 8 and 12), the ratio of the values

increases from 2.0 to 4.0. Between weeks 12 and 16, the ratio increases further from 4.0 to 6.1 respectively.

Linear regression analysis between the two methods gave a coefficient ( $r$ ) of 0.535 ( $P < 0.05$ ). Only 28 % of the variance ( $r^2$ ) was explained by the equation

$$Y = aX + b.$$

#### Discussion

The *Lactobacillus leichmannii* method is specific for the physiologically active forms (hydroxo-, methyl and adenosyl cobalamines) of vitamin B12 (Matthews, 1962). In adult sheep, serum vitamin B12 concentrations greater than 400 pg/ml are considered to be normal (Dawbarn *et al.*, 1957). Concentrations lower than 200 pg/ml are considered deficient, in spite of the absence of clinical symptoms, as determined by the *Lactobacillus leichmannii* method (Andrews and Stephenson, 1966; Underwood, 1977).

Mean vitamin B12 concentrations during the 16 weeks experimental period indicate therefore that a state of deficiency was attained at week 12 ( $172.3 \pm 12.0$  pg/ml). In sheep fed a hay diet supplying 0.04 mg cobalt/kg DM, serum vitamin B12 activity as measured by the *Lactobacillus leichmannii* method, attained deficiency threshold values after a period of 7 to 8 weeks (Smith and Marston, 1970; Tressol and Lamand, 1979). The delay (7 to 8 *versus* 12 weeks) in which deficiency threshold was attained may be explained by the difference in the quantity of cobalt ingested (0.04 *versus* 0.06 mg cobalt/kg DM).

Serum vitamin B12 concentrations obtained by the radioassay method during the course of the experiment were superior in all cases to those obtained by the microbiological method (tables 1 and 2). This may be attributed to non-specific binding by the hog intrinsic factor (IF) used during the radioassay. Millar and Penrose (1980) observed that radioassay kits containing purified IF to eliminate problems with non-specific, binding proteins, gave lower concentrations of vitamin B12 in the serum of cobalt-deficient sheep when compared to kits containing non-purified IF.

The increase in the ratio of the means between the RID and the *Lactobacillus leichmannii* methods (table 5) is probably due to the presence of increasing quantities of "non-active" vitamin B12 analogues. Concentrations of the latter were inversely correlated to "true" vitamin B12 activity in the sera of sheep when cobalt intake decreased from 0.10 to 0.06 mg/kg DM. It is interesting to note that when vitamin B12 activity is superior to 400 pg/ml (661.2 and 427.0 pg/ml for weeks 4 and 8 respectively) the ratios are of the same order of magnitude (table 5).

The contribution made by "true" vitamin B12 to the values obtained by the RID method is of a decreasing order of magnitude as the degree of deficiency is intensified, 68.3%, 50.4%, 24.7% and 16.3% for weeks 4, 8, 12 and 16 respectively. These results are in accordance with those of Gawthorne (1970).

Using a radioassay method, Sutherland (1978) has recommended a serum vitamin B12 level of 1 000 pg/ml as the critical value for sheep. Reference to table 2 indicates that at week 4, three of the sheep (D, E and F), and all but one of the sheep (C) were vitamin B12 deficient from week 8 onwards.

Radioassay of the serum sample pool for the chronic state of deficiency (table 3) more or less corresponds to the critical value recommended by Sutherland (1978) since the values obtained by the microbiological method agree with the threshold value (200 pg/ml) recommended by Andrews and Stephenson (1966) and Underwood (1977).

The question arises of whether the critical value recommended by Sutherland (1978) for the radioassay technique would be credible in the presence of greater than normal amounts of inactive

analogues whose production is reputedly favoured by high concentrate diets (Elliot, 1980; Sutton and Elliot, 1972).

Results for the analysis of vitamin B12 in the present study by the microbiological and RID methods show poor correlation ( $r = 0.53$ ;  $P > 0.05$ ) between the two methods. Our results are in disagreement with those obtained by Millar and Penrose (1980) for which a strong correlation was shown ( $r = 0.92$ ;  $P < 0.001$ ) in cobalt deficient sheep, and by Mac Pherson (1982) in cobalt-deficient calves.

The two analytical methods used for measuring vitamin B12 according to degree of deficiency had low coefficients of variation, less than 1.1% (table 3). In spite of this, the RID technique appears to be less reliable than the *Lactobacillus leichmannii* method for the analysis and diagnosis of "true" vitamin B12 deficiency in sheep. It can therefore be concluded that the Radiochemical Centre Radioassay kit is not suitable for the rapid diagnosis of vitamin B12 deficiency in subclinically cobalt-deficient sheep.

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## Summary

Serum vitamin B12 from 6 sheep fed a diet containing 0.06 mg cobalt per kg dry matter (DM) was analysed by a radioisotope dilution (RID) technique. The results were compared with values obtained by a microbiological method (*Lactobacillus leichmannii* - ATCC 7830) specific for "true" vitamin B12. Serum vitamin B12 concentrations were four to six times greater during the 16 weeks experimental period with the RID method as compared to the microbiological technique. During the course of the experiment, values obtained by the RID method remained superior to the threshold value (200 pg B12/ml) of the microbiological method. This investigation indicates that the RID method is not suitable for the rapid diagnosis of vitamin B12 deficiency in subclinically cobalt-deficient sheep.

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