Interlaboratory comparison of serum vitamin B₁₂ assay

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SUMMARY The results have been compared of microbiological and radioisotope dilution (RID) assay of serum vitamin B₁₂ by participants in national interlaboratory trials in Britain. There was wide variation between the individual participants, especially marked in the *L. leichmannii* microbiological assay and in the RID methods, whereas excellent correlation, reproducibility, and recovery were obtained in reference laboratories by microbiological assay with both *E. gracilis* and *L. leichmannii*. In general, RID gave higher results than microbiological assay. The need for suitable reference sera is emphasised.

Measurement of vitamin B₁₂ in serum or plasma is a widely performed test for the investigation and management of patients suspected of megaloblastic anaemia, for excluding vitamin B₁₂ deficiency as a cause of high MCV in patients whose blood counts are within the normal range, for screening for possible vitamin B₁₂ neuropathy, and for the investigation of liver and myeloproliferative disease.

For many years the serum vitamin B₁₂ was measured by microbiological assay using *Euglena gracilis* or *Lactobacillus leichmannii*, and these methods were used in both routine and reference laboratories. Although it has always been recognised that the techniques are complex and require the use of standard sera, they are precise and reproducible (Mollin *et al.*, 1976) and are consistently able to discriminate vitamin B₁₂ deficiency from normal; they are particularly valuable in the diagnosis of vitamin B₁₂ neuropathy.

The introduction of radioisotope dilution (RID) assays, especially in the form of commercial kits, provided an alternative test with the apparent advantages of being convenient, easy to perform, providing rapid results, and, in contrast to the *L. leichmannii* assay, not being affected by antibiotic or other drugs in serum. This led to widespread adoption of the isotope techniques. However, the results with the RID assays in general tend to be higher than those with the microbiological assays (Mollin *et al.*, 1976), and recent work (Hall, 1977; Mahood, 1977; Cooper and Whitehead, 1978;

Kolhouse *et al.*, 1978) has cast doubts on their reliability in distinguishing untreated pernicious anaemia from normal. There is thus need to evaluate the methods in current use.

Interlaboratory trials provide a means for evaluating the behaviour of a test or a procedure as well as the performance of individual laboratories. In this paper we report the results of interlaboratory trials of serum and plasma vitamin B₁₂ assay carried out in the United Kingdom by participants in the National Haematology Quality Control Scheme.

Material and methods

(a) NORMAL SERUM

This was obtained from normal donors and stored in plastic transfer packs at -20°C until needed. Before freezing, each pack was tested for Australia antigen and microbial contamination; if either test was positive the pack was discarded. Before the trial the required number of packs were thawed at room temperature, filtered aseptically, pooled, and sterilised by gamma radiation in a fully automated system by a ⁶⁰Co source under accurately monitored conditions, the absorbed dose being 2.5 mrad (Gamma Radiation Sources Ltd, Reading, Berks). After sterilisation the pool was dispensed in 4 ml aliquot portions by means of a mixing-bottling unit (Chappell and Ward, 1978).

(b) ABNORMAL SERUM

Small volumes of serum were collected from patients with untreated pernicious anaemia and other known vitamin B₁₂ deficiencies over a period of time and

stored at -20°C . When required for a trial the material was thawed, pooled, irradiated, and dispensed as in (a) above.

(c) NORMAL PLASMA

This was obtained from blood collected into ACD NIH 'A' or heparin anticoagulant, and then processed as in (a) above.

(d) PLASMA/SERUM WITH ADDED
CYANOCOBALAMIN

0.50 ml cyanocobalamin (1000 $\mu\text{g}/\text{ml}$) injection BP (Cytamen: Glaxo Ltd, Greenford, Middlesex) was transferred by 1.0×0.01 ml sterile syringe to 500 ml 0.9% sodium chloride (sterile non-pyrogenic infusion solution) (Boots Co Ltd, Nottingham) and mixed to provide solution A (1.0 $\mu\text{g}/\text{ml}$). Solution B was prepared by diluting 1.0 ml of solution A with 9.0 ml sterile 0.9% saline (0.1 $\mu\text{g}/\text{ml}$). Then 0.1 ml of solution B was added to each 100 ml of normal serum or plasma (a and c above) for an increment in final concentration of 100 ng/l. All additions were carried out aseptically and the final mixing and dispensing followed the method as for (a).

All samples were dispensed into gamma-irradiated plastic or glass vials with plastic stoppers. They were despatched by first-class post to ensure delivery to the majority of participants within 24 hours; after receipt they were kept at -20°C until testing. They were identified only by code, and the participants did not know the composition of the samples at testing.

PARTICIPANT CENSUS

The National Quality Control Scheme has 450 members who receive material for blood counts and

various other tests at regular intervals. All were invited to take part in the vitamin B_{12} trials. Five trials have taken place. A preliminary trial was held in 1973 with 58 participants, but an increasing number have now responded so that in 1978 there were 175 participants. These include both 'small' and 'large' laboratories. The tests consisted of *E. gracilis* (15), *L. leichmannii* (41), RID with charcoal (83), and RID by other techniques (43). A small number of laboratories returned results by more than one method. Three participants with special interest in the investigation of megaloblastic anaemias were invited to be reference laboratories. Two of these used the *E. gracilis* assay as described by Anderson (1964), the third the *L. leichmannii* assay (Spray, 1955).

Results

The results of the five trials are shown in Tables 1-3. The first trial which was a pilot study was on a single sample of plasma with a normal vitamin B_{12} content. Participants gave a mean of 423.1 ng/l with a large SD (155.6 ng/l) and CV 36.8% (Table 1). The subsequent trials showed similar wide variation between different laboratories using the same technique, whether microbiological or RID, and, in general, RID assays gave higher results. Results obtained from the referee laboratories in the last two trials are shown in Table 4. Their results showed close correlation.

In trial 4 participants received an unidentified second sample of the same serum (Table 2, serum a). Correlation of results on the two samples in a Youden plot is shown in Fig. 1 (microbiological

Table 1 Results in trials 1, 2, and 3

Trial	Material	Method	Total No.	No. analysed*	Mean (ng/l)	SD (ng/l)	CV%
1	Normal plasma	Not specified	104	98	423	156	36.8
2	B_{12} deficient serum	<i>E. gracilis</i>	14	13	106	26	24.4
		<i>L. leichmannii</i>	68	54	113	39	34.9
		RID charcoal	27	23	133	37	27.8
	Normal plasma	<i>E. gracilis</i>	14	13	183	50	27.1
		<i>L. leichmannii</i>	67	57	229	58	25.3
		RID charcoal	26	23	288	108	35.7
3	Normal plasma A	<i>E. gracilis</i>	13	13	123	28	22.6
		<i>L. leichmannii</i>	32	31	138	83	60.5
		RID charcoal	20	18	153	58	37.7
		RID others	12	11	180	58	31.8
	Normal plasma B	<i>E. gracilis</i>	13	13	262	53	20.1
		<i>L. leichmannii</i>	32	39	287	82	28.6
		RID charcoal	20	18	288	82	28.6
		RID others	13	12	336	122	36.4
	Normal plasma C	<i>E. gracilis</i>	13	13	392	56	14.5
		<i>L. leichmannii</i>	32	30	416	142	34.1
		RID charcoal	19	18	438	121	27.8
		RID others	13	12	515	143	27.8

*Excluding outliers, the results of which were > 2 SD in a preliminary calculation of all results.

Table 2 Results in trial 4

Serum	Method	Total No.	No. analysed*	Mean (ng/l)	SD (ng/l)	CV%
(a) Borderline B ₁₂ deficient	<i>E. gracilis</i>	15	15	92	18	20.3
	<i>L. leichmannii</i>	38	36	121	51	42.4
	RID charcoal	64	61	157	59	37.3
	RID others	27	23	180	54	30.1
(b) B ₁₂ deficient	<i>E. gracilis</i>	12	11	19	7	38.3
	<i>L. leichmannii</i>	15	14	33	28	84.5
	RID charcoal	23	21	50	36	73.2
	RID others	13	13	60	18	29.4
(c) (a) + 200 ng/l B ₁₂	<i>E. gracilis</i>	15	14	332	147	44.4
	<i>L. leichmannii</i>	37	36	303	102	33.7
	RID charcoal	65	61	351	67	19.6

*Excluding outliers the results of which were > 2 SD in a preliminary calculation of all results.

Table 3 Results in trial 5

Serum	Method	Total No.	No. analysed*	Mean (ng/l)	SD (ng/l)	CV%
(a) B ₁₂ deficient	<i>E. gracilis</i>	15	14	71	18	25.0
	<i>L. leichmannii</i>	41	39	75	30	39.9
	RID charcoal	83	80	97	40	41.4
	RID others	36	34	139	37	26.9
(b) (a) + 100 ng/l B ₁₂	<i>E. gracilis</i>	15	14	160	27	16.6
	<i>L. leichmannii</i>	41	39	159	50	31.4
	RID charcoal	83	79	195	63	32.4
	RID others	36	34	232	46	19.8
(c) (a) + 200 ng/l B ₁₂	<i>E. gracilis</i>	15	14	257	43	16.9
	<i>L. leichmannii</i>	39	38	260	77	29.6
	RID charcoal	82	80	285	84	29.3
	RID others	35	33	343	50	14.8
(d) Normal	<i>E. gracilis</i>	15	15	277	80	28.8
	<i>L. leichmannii</i>	40	38	306	81	26.7
	RID charcoal	81	78	384	87	22.8
	RID others	43	40	476	63	13.2

*Excluding outliers the results of which were > 2 SD in a preliminary calculation of all results.

Table 4 Values obtained by reference laboratories in trials 4 and 5

Laboratory method trial	Serum	A	B	C
		<i>E. gracilis</i> (ng/l)	<i>E. gracilis</i> (ng/l)	<i>L. leichmannii</i> (ng/l)
4	(a) Borderline B ₁₂ deficient	91	110	85
	(b) B ₁₂ deficient	16	25	50
	(c) (a) + 200 ng/l B ₁₂	300	315	357
5	(a) B ₁₂ deficient	64	100	72
	(b) (a) + 100 ng/l B ₁₂	169	200	168
	(c) (a) + 200 ng/l B ₁₂	286	280	257
	(d) Normal	340	310	275

assay) and Fig. 2 (RID). This plasma was borderline B₁₂ deficient. With RID random error was especially marked, while in the Euglena assay all results were subnormal (< 160 ng/l). Figure 3 shows the ratio analysis of results obtained in trial 5 by microbiological assay on two samples, one of which (specimen b) was a subsample of the other (a) with the addition of aqueous B₁₂. An analysis of results on the same two samples by RID assay is shown in Figure 4.

These figures illustrate that there was an unacceptably wide interlaboratory variation in results, and in many cases there was poor recovery. On the other hand, in the reference laboratories, recovery was 97.7-103% (Table 4).

Discussion

This survey has shown that there are major problems

Fig. 1 Ratio analysis of data obtained by microbiological assay of split specimen of serum (a) in trial 4. The reference laboratories identified the serum as borderline B_{12} deficient (mean 95.3 ng/l): $\circ = E. gracilis$; $\bullet = L. leichmannii$.

Fig. 2 Ratio analysis of data obtained by RID on same split specimen as in Fig. 1: $\circ = \text{RID charcoal}$; $\bullet = \text{RID other methods}$.

in the assay of vitamin B_{12} in routine practice. Microbiological methods are accurate and reproducible in reference laboratories and the recovery of B_{12} added to serum was entirely satisfactory. In the routine laboratories the results of microbiological assays showed marked variation, particularly with *L. leichmannii*, and there was even more marked variation with RID assays.

There are a number of reasons for the discrepant results. There is no absolute standard for vitamin B_{12} in its biological form, but from the results of repeated and extensive investigations over the years it has been established that microbiological assay gives measurements which are specific for vitamin B_{12} and which consistently parallel the clinical condition. Unreliable results from microbiological assay

Fig. 3 Ratio analysis of data obtained by microbiological assay in trial 5, comparing serum (a) with its subsample (b), which contained added aqueous vitamin B_{12} (100 ng/l): $\circ = E. gracilis$; $\bullet = L. leichmannii$.

are due not to biological discrepancies but to technical faults in these relatively complex methods which require that every step in the procedure be standardised and strictly adhered to. It should be noted that in the reference laboratories both microbiological methods were equally satisfactory. The major factor in technical error in microbiological assay of vitamin B_{12} is the lack of reference preparations. Thus there is a need for stable and reliable material of known vitamin B_{12} content which can be used to verify the accuracy of the method used in routine practice. Preliminary studies have indicated that freeze-dried human serum is suitable; it is necessary to have several preparations obtained from both normal subjects and patients in order to cover the clinically relevant range of B_{12} levels. The vitamin B_{12} of the sera will need to be measured by one or more reference laboratories using a clearly defined method of known reliability with a high degree of accuracy and precision which can be established as the reference method. The data from the present study, together with many previous published studies, suggest that microbiological assay with *Euglena* can be recommended as the reference method.

The problem of the RID assay kits is more

Fig. 4 Ratio analysis of data obtained by RID (charcoal method) on same specimens as in Fig. 3.

complicated. Some RID methods have been reported to give higher results than RID charcoal methods (Raven and Robson, 1974). These higher levels were reported with assays using either R-binder (Green *et al.*, 1974) or intrinsic factor (Wide and Killander, 1971; Raven *et al.*, 1969; Raven *et al.*, 1972; Cooper and Whitehead, 1978). Pure intrinsic factor (IF) only binds clinically active cobalamin whereas R-binder protein also binds inactive analogues of B₁₂ (Bunge and Schilling, 1958; Kolhouse and Allen, 1977). In 1978 evidence was presented by Kolhouse and his colleagues that RID assay kits, which were reported to use only IF as binder, were in fact mixtures of IF and R-binder protein. Furthermore, the sera of patients and normal subjects appear to contain not only cobalamin but inactive analogues of B₁₂. These observations explain the consistently higher levels obtained in normals and abnormals with the chick serum (R-protein) assay of Green *et al.* (1974), and the variable, confusing, and sometimes dangerous results obtained by RID kits claiming to use only IF as their binder (Kolhouse *et al.*, 1978; Cooper and Whitehead, 1978). With the knowledge now available it should be possible to produce satisfactory RID assay kits capable of measuring only clinically active cobalamin in serum.

The parameters necessary for ensuring the precision and specificity of such kits are now under discussion in the United States by the National Committee for Clinical Laboratory Standards (NCCLS).

The chick serum assay (R-protein assay) has many technical advantages over RID assays using IF and discriminates patients with pernicious anaemia from controls with considerable consistency (Green *et al.*, 1974). This suggests that in most instances the level of the suspected B₁₂ analogues and the serum B₁₂ level in pernicious anaemia parallel each other (Sourial and Mollin, 1979). However, as those authors point out, this assay may fail to discriminate between normals and B₁₂ deficiency in patients in whom B₁₂ deficiency is complicated by folate deficiency and in some patients in whom deficiency is due to conditions other than pernicious anaemia. Whether modifications of the R-binder assay will permit it to discriminate invariably between B₁₂ deficiency and control subjects remains to be determined.

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