An assessment of serum ^{57}Co cyanocobalamin as an index of vitamin B_{12} absorption¹

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SYNOPSIS A technique has been developed for accurately assaying serum and urine ^{57}Co cyanocobalamin during the conventional Schilling test. Serum and urine radioactivity in a large number of patients has been correlated and the precise normal limits have been determined by statistical analysis. The implications of this for investigating conditions other than pernicious anaemia and the malabsorption syndromes are stressed.

Since Schilling's original paper (1953) urine radioactivity measurements have been the most commonly used parameter in the assessment of vitamin B_{12} absorption. Other workers have measured hepatic uptake of isotope (Glass, Boyd, Gellin, and Stephanson, 1954; Townsend, Smith, and Langman, 1968) or whole body radioactivity (Reizenstein, Cronkite, and Cohn, 1961; Meyer, Jansen, Van der Merwe, and Oosthuizen, 1968). Serum radioactivity has been assayed previously (Booth and Mollin, 1956; Doscherholmen and Hagen, 1957; Nelp, McAfee, and Wagner, 1963; McCurdy, 1965; McIntyre and Wagner, 1966; Armstrong and Woodliff, 1966; Coupland, 1966; Woodliff and Armstrong, 1966; Forshaw and Harwood, 1966; Finney and Payne, 1968) but no accurate analysis of the relation between urine and serum levels has been made up to the present. The main reason for this is that serum radioactivity is extremely low following the standard oral dose of $0.5\ \mu\text{Ci}$ ^{57}Co vitamin B_{12} . Thus it takes a long time to measure the serum in order to attain accurate results. For this reason, occasionally, larger doses of isotope have been administered (Coupland, 1966; Armstrong and Woodliff, 1966; Saif, 1967) at the cost of a higher radiation dose to the patient.

The advantages of serum over urine counting are that it excludes errors due to inaccurate urine collection, contamination of urine with extraneous radioactivity, and fallacies associated with impaired renal function (see discussion).

In the course of investigating neurological patients a method was developed for assaying accurately the serum and urine radioactive vitamin B_{12} during a standard Schilling test and the data obtained were subjected to mathematical analysis.

Methods

^{57}Co vitamin B_{12} (Radiochemical Centre, Amersham) was used on account of the longer half-life (270 days) compared with that of ^{58}Co (71 days), the smaller radiation dose to the patient (Armstrong and Woodliff, 1966), and because of the narrow band emission spectrum and therefore higher counting efficiency with a pulse height discrimination counting technique.

In part 1 of the test an accurately known dose of the order of $0.5\ \mu\text{Ci}$ was given orally to patients who had been fasted for 12 hours. At the same time a standard flushing dose of $1,000\ \mu\text{g}$ of non-radioactive vitamin B_{12} was administered intramuscularly in order to saturate the protein-binding sites for the vitamin (Schilling, 1953).

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In part 2, at least three days later, one capsule (approximately 10 mg) of hog intrinsic factor concentrate (Radiochemical Centre, Amersham) was given in addition.

Urine was collected over the following 24 hours, and serum at eight hours after the radioactive dose, preliminary experiments having confirmed the findings of Booth and Mollin (1956), Doscherholmen and Hagen (1957), and Coupland (1966) that following the dose of vitamin B_{12} serum radioactivity at this time is maximal.

Counting Technique

Samples of urine, serum, and standards, each of 8 ml, were counted in a well-type scintillation counter and scaler (I.D.L.) with pulse height discrimination, set for ^{57}Co characteristics, and in such a way as to achieve very great stability (coefficient of variation of high counting standard <1%) for long periods of time at the cost of a moderately high background count. Results for urine values were expressed as percentages of the dose administered. Results for serum counting are given in counts per second, no allowance being made for total blood volume (Finney and Payne, 1968).

TIME FOR COUNTING

Serum was counted for approximately 120 minutes (4×10^4 counts) in order to achieve a coefficient of variation of 16% and a sample error of 8% (see preliminary results). Urine and standards with background, and background alone, were also counted to the same accuracy.

Patients

Patients studied included the following:

- 1 Patients who were vitamin B_{12} deficient (serum B_{12} <150 $\mu\text{g}/\text{ml}$) with early megaloblastic changes in bone marrow, with and without neurological involvement.
- 2 Postgastrectomy patients with neurological complications.
- 3 Patients with undiagnosed peripheral neuropathies not due to pernicious anaemia.
- 4 Patients with hypopituitarism and myxoedema, with and without replacement therapy.
- 5 Folate-deficient (serum folate <3.0 $\text{m}\mu\text{g}/\text{ml}$) epileptic patients on treatment with anticonvulsant drugs.
- 6 Control subjects ('normals') consisted of patients with a wide range of neurological diseases, some of whom had peripheral neuropathies not due to vitamin B_{12} deficiency, and in whom the serum vitamin B_{12} levels were subsequently found to be normal.

Results

PRELIMINARY EXPERIMENTS

Table I compares the degree of accuracy of serum counting with the time taken when the background is high in relation to the serum level (background + sample: background approximately 1:1:1). As time increases, the coefficient of variation decreases so that when the total count is 4×10^4 (approximately two hours), the coefficient of variation is 16% and the sample error better than 8%. Counting for longer than this is not practicable, and shorter counts give a much larger coefficient of variation (greater than 30.9% and a sample error greater than 15%), militating against the production of a smooth tolerance curve as shown in Figure 1. This figure also confirms that the peak of serum counts is at eight hours, when the flushing dose is given at the same time as the oral dose.

These serum results can be compared with similar findings in urine (Table II). Others (Finney and Payne, 1968) have expressed their serum results as percentages of dose per litre. A correction factor of $\times 1.35$ may be used to make our figures comparable. Our preliminary results confirm that all the radioactivity was in serum with none detected in the red cells.

Total No. of Counts	Approximate Time (min)	Mean of 10 Determinations in One Patient (counts/sec)	S.D.	Coefficient of Variation (%)
1×10^4	3	0.62	0.31	50.0
4×10^3	12	0.42	0.15	35.3
1×10^4	30	0.43	0.13	30.9
4×10^4	120	0.51	0.08	16.0

Table I Demonstration of degree of increase in accuracy of serum ^{57}Co counting with total count¹

¹Sample + background/background = approximately 1:1:1

Fig. 1 ^{57}Co vitamin B_{12} tolerance curve after an oral dose of 50 μg when serum count is 4×10^4 .

Total No. of Counts	Approximate Time (min)	Mean of 10 Determinations in One Patient (% excretion in 24 hours)	SD	Coefficient of Variation (%)
1 × 10 ³	0.1	17.3	2.7	15.4
4 × 10 ³	0.5	17.7	2.4	13.6
1 × 10 ³	1.0	17.8	1.0	5.4
4 × 10 ³	5.0	17.7	0.6	3.6
1 × 10 ⁴	10.0	18.0	0.4	2.0
4 × 10 ⁴	50.0	18.0	0.1	0.8

Table II Demonstration of degree of increase in accuracy of urine ⁵⁷Co counting with total count¹

¹Sample + background/background = approximately 2:1

Ninety-six assays were carried out on 59 patients in this series and the data are shown graphically in Figure 2. The percentage urine excretion is represented as the ordinate (y) and the eight-hour serum radioactivity in counts per second is represented as the abscissa (x).

By inspection there is clearly a positive correlation between the two measurements for this heterogeneous group of patients, which included those with myxoedema, pituitary insufficiency, and the folate-deficient epileptic patients. If serum levels are to be used as criteria of vitamin B₁₂ absorption the observed relationship with percentage urine excretion must be accurately assessed. The scatter of points in Fig. 2 suggests a linear model for this relationship; from the diagram it can be seen that nothing is to be gained by considering more complicated models. A

linear regression line with the equation $y = bx + c$ was fitted (Hoel, 1967) to the data by least squares where b = slope of the line (this can be interpreted as the average increase in percentage urine excretion per unit increase in serum level) and c = the intercept on the y-axis.

Four points on the extreme right hand side of the graph (Fig. 2) were omitted from the calculations as they were found to be unrepresentative of the main body of data on the basis of Chauvenet's criterion (Anscombe, 1960) for the detection of outliers. (This involves computing the ratio, deviation of point/SD of data, for all data points and rejecting those whose ratios exceed a certain extreme value.)

The regression calculations resulted in a value of 16.7 for the slope (b), the 95% confidence interval for this parameter being (13.4, 20.0). The test statistic (Student's t test with 90 degrees of freedom) for b was found to be 10.14 which is significant at the 0.1% level (the chance of uncorrelated variables giving such a value of b is less than 1 in 1,000). These figures confirm our supposition of positive correlation.

The intercept on the y axis (c) is 2.3 and is not significantly different from zero, the test statistic (Student's t test) in this case being 1.8 which is smaller than the 5% significance point, 1.99. This result gives weight to the use of a linear model, as a zero value of serum level would, in theory, correspond to a zero value of percentage urine excretion. As the value of b suggests, the

Fig. 2 Correlation of 24-hour urinary excretion and eight-hour serum levels of ⁵⁷Co vitamin B₁₂ in Schilling tests.

correlation between the variables is high, the sample correlation coefficient being 0.72. These calculations are summarized in Table III.

Table IV summarizes the normal values for urine and serum using the above counting technique.

Coefficient of correlation (r)	= 0.72
Regression equation	$y = 16.7x + 2.3$
Test statistic index for slope which is greater than 1.99 and therefore the slope is significantly different from zero.	= 10.14
95% confidence limits for the slope	= 13.4 to 20.0
Test statistic index for intercept which is less than 1.99 and therefore the intercept is not significantly different from zero	= 1.8
95% confidence limits for the intercept of the fitted line	= -0.2 to +4.8

Table III Data from graph showing correlation of 24-hour urinary excretion and eight-hour serum levels in Schilling tests

Patient	24-hour Urinary Excretion (% of dose administered)	Eight-hour Serum Level (counts/sec/8 ml specimen)
1	22.0	0.84
2	14.1	0.72
3	19.3	0.93
4	14.1	0.42
5	16.3	0.74
6	16.8	0.70
7	15.0	0.94
8	17.2	0.94
9	25.8	0.56
10	20.6	0.97
Mean	18.1	0.78
SD	3.8	0.18
Range (with 95% confidence limits)	10.5-25.7	0.42-1.14

Table IV Schilling test results on 10 normal subjects

Patient	24-hour Urinary Excretion Values on Repeat Tests (% of dose administered)	Eight-hour Serum Values on Repeat Tests (counts/sec/8 ml specimen)	Maximum Difference between Urine Values (% of dose administered)	Maximum Difference between Serum Values (counts/sec/8 ml specimen)
1	19.6 23.9	0.69 0.75	4.3	0.06
2	14.3 13.4	0.64 0.98	0.9	0.33
3	16.9 14.2	0.81 0.66	2.7	0.15
4	12.9 19.6	1.06 1.01	6.7	0.06
5	7.1 6.4	0.54 0.47	0.7	0.07
6	3.8 8.0	0.49 0.94	3.3 2.4	0.05 0.20
7	10.4 6.3	0.74 0.16	0.9	0.15
8	7.2 9.2	0.31 0.51	1.2	0.15
9	10.4 6.7	0.66 0.19	2.4	0.32
	9.1 8.8	0.52 0.40		
Mean			2.1	0.21
Range			2.5 0.7-6.7	0.16 0.05-0.33

Table V Biological variation in repeat Schilling tests

Table V summarizes the variation obtained in nine subjects in whom repeat Schilling tests were carried out.

Discussion

Schilling tests have been a routine laboratory test for many years, and from general experience the range of normal results is fairly wide. Serum counting has been advocated as a useful parameter by several workers (vide supra). No precise statistical studies on the normal values in the serum are available, and few for the urine (Woodliff and Armstrong, 1966; Finney and Payne, 1968).

We agree with Finney and Payne (1968) that it is of value to study serum radioactivity. This has several advantages over urine counting alone: (1) as an independent check on the urine findings; (2) to overcome the problem of inadequacies in urine collection, a particularly important consideration in neurological patients; (3) in neurological practice the vastly increased use of isotopes for brain scanning has now caused a major diagnostic problem due to interference with other laboratory tests involving the use of isotopes. The presence of these artefacts is immediately apparent from serum counting, whereas the accidental contamination of an already moderately radioactive urine might pass unnoticed; (4) in order to avoid giving false impressions of vitamin B₁₂ absorption in patients who have renal impairment: in such subjects the urine excretion in the first 24 hours is low, but the eight-hour serum level is normal. Moreover, excretion of vitamin B₁₂ in the urine may continue for four or five days, and the 24-hour serum level of radioactive vitamin B₁₂ is considerably higher than in normal patients (personal observations). Thus unsuspected renal insufficiency may come to light by the finding of an abnormal ratio between serum and urine radioactivity.

The main disadvantage of serum determinations is that the low level of radioactivity present requires a long time for counting.

The Schilling test was performed initially in the control subjects in order to rule out the possibility of an abnormality of vitamin B₁₂ absorption. The subjects were on normal diets, and no abnormalities were detected in the blood films. The mean, standard deviation, and range for these normal controls is shown in Table IV, both for the urine and serum values.

The physical variation, ie, the scatter of results due to inaccuracy of counting technique, has a standard deviation of 0.08 counts per second at a level of 0.51 counts per second in the serum. These figures do not include errors due to pipetting.

The biological variation, ie, the scatter of results obtained in one patient when the test is

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repeated several times should also be known, but clearly for ethical reasons cannot be obtained directly. A number of patients, however, did require repeat tests (Table V) for various reasons. The mean difference between repeats over a range of urine excretion between 3.8% and 23.9% was 2.5%, with a range of differences from 0.7% to 6.7%. For serum levels, the mean difference over a range of levels from 0.16 to 1.06 counts per second was 0.16 counts per second, with a range of differences from 0.05 to 0.33 counts per second (see Tables I and II).

The other patients included in Fig. 2, investigated in this series, are described in a later paper.

Thus the normal range with 95% confidence limits has been established in the Schilling test, both for urine and for serum. The degree of correlation between the two parameters has also been calculated and the established reproducibility in different individuals allows precision in the diagnosis of marginally abnormal states. Hence it is now possible to investigate reliably the vitamin B₁₂ absorption of various groups of patients, for example, in those following partial gastrectomy, in those who are folate deficient, and in those with endocrinopathies associated with neurological disease.

We have not investigated malabsorption states but we have examined postgastrectomy patients. Our experience of the postgastrectomy group is discussed in another paper. It is interesting that Adams and Cartwright (1963) in their paper quoted a wide variation in the urine excretion of radioactive vitamin B₁₂ in patients with primary malabsorption and after partial gastrectomy, but serum values are not quoted. Adams and Seaton (1961) quoted a similar wide variation for a patient with coeliac disease.

The diagnosis of pernicious anaemia is straightforward, but with the extended counting technique it would also now be possible to examine the relatives of these patients for evidence of defects in vitamin B₁₂ absorption and to correlate the results with the studies of others (te Velde, Abels, Anders, Arends, Hoedemaeker, and Nieweg, 1964; Wangel, Callender, Spray, and Wright, 1968; Whittingham, Ungar, MacKay, and Mathews, 1969) on autoimmune antibodies in these subjects.

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