Experience with a commercial kit for the radioisotopic assay of vitamin B₁₂ in serum: the Phadebas B₁₂ Test

J. L. RAVEN¹ AND M. B. ROBSON

From the Department of Haematology, St George's Hospital, London, and the Department of Haematology, St Vincent's Hospital, Darlinghurst, Australia

SYNOPSIS The first commercial kit for the radioisotopic assay of vitamin B₁₂ in serum—the Phadebas B₁₂ Test produced higher values than the radioisotopic method of Raven, Robson, Walker, and Barkham (1969) and the *Lactobacillus leichmannii* microbiological assay. Its normal range was 300-1100 pg/ml and its reproducibility was similar to that of the other radioisotopic method. It should be possible to lower the results obtained by the Phadebas method by modifying its standard curve and to reduce the time taken for the assay by shortening its incubation period.

Since 1961, many different radioisotopic methods have been described for the assay of vitamin B₁₂ in serum. One of these, the method of Wide and Killander (1971) is now available in kit form as the Phadebas B₁₂ Test (Pharmacia AB, Uppsala, Sweden). This is the first and, very likely, the first of many commercial kits and is being marketed in Great Britain, Europe, the USA, and Australia. It is said by the Editor of the *Year Book of Nuclear Medicine* (see Meyer and Gizis, 1972) to be 'receiving rapid acceptance throughout the United States'.

This paper describes experience with the Phadebas B₁₂ Test and compares its results with those obtained by a standard microbiological method and another radioisotopic method.

Materials and Methods

The Phadebas B₁₂ Test was supplied by Pharmacia AB, Uppsala, Sweden. Each kit contained sufficient lyophilized Sephadexintrinsic factor, lyophilized vitamin B₁₂ standard, lyophilized ⁵⁷Co-vitamin B₁₂, and Tween solution for the preparation of a standard curve and the assay of 18 test sera in duplicate. The basis of the assay is similar to that of all other radioisotopic methods for the assay of vitamin B₁₂ in serum: (1) vitamin B₁₂ is extracted from its binding serum proteins (by heating in the presence of glutamic acid); (2) ⁵⁷CoB₁₂ is added to the serum extract; (3) a representative sample of the mixture of radio-

active and non-radioactive vitamin B₁₂ is bound by the vitamin B₁₂ binder (intrinsic factor); (4) the free and bound forms of vitamin B₁₂ are separated (Sephadex); (5) the radioactivity in test and standard solutions is compared and the serum vitamin B₁₂ value calculated (from a standard curve). In the Phadebas assay, steps 3 and 4 are combined because the intrinsic factor and Sephadex are coupled, the intrinsic factor being bound covalently to Sephadex so that its vitamin B₁₂-binding capacity is retained intact.

The second radioisotopic method was that of Lau, Gottlieb, Wasserman, and Herbert (1965) modified by Raven, Robson, Walker, and Barkham (1968, 1969) and Raven and Barkham (1973). Individual serum backgrounds were used for each serum sample. The normal range with this method is 200-1000 pg/ml.

The *Lactobacillus leichmannii* method was that of Rosenthal and Sarett (1952), as modified by Spray (1955) and Matthews (1962). The normal range with this method is 150-850 pg/ml although some sera from patients with untreated pernicious anaemia may have vitamin B₁₂ values of between 150 and 200 pg/ml (Ardeman, Chanarin, Krafchik, and Singer, 1966; Raven, Robson, Morgan, and Hoffbrand, 1972).

Five hundred and twenty-six sera from a variety of normal subjects and hospital patients were assayed. Thirty-three of the sera were from patients with untreated pernicious anaemia, the diagnosis having been proven by peripheral blood and bone marrow examination, serum vitamin B₁₂ and folate assays,

¹Please send reprint requests to J. L. Raven, Department of Haematology, St George's Hospital, London, SW17 0QT

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Fig 1 Comparison of the vitamin B₁₂ values obtained for 526 sera by the Phadebas method and the radio-isotopic method of Raven et al (1969).

In this and subsequent figures, the line drawn through the ordinate represents the line of identity for the two results on the same serum.

Fig 2 Comparison of the vitamin B₁₂ values obtained for 245 sera by the Phadebas and *Lactobacillus leichmannii* methods.

Fig 3 Comparison of the serum vitamin B₁₂ values obtained by the Phadebas method and the radioisotopic method of Raven et al (1969) for 217 sera from normal subjects and 33 sera from patients with untreated pernicious anaemia. The horizontal lines represent mean values.

Fig 4 Comparison of the duplicate values obtained for 100 sera by the Phadebas method.

gastric antibody studies, Schilling's test of vitamin B₁₂ absorption, and haematological response to vitamin B₁₂ therapy. The vitamin B₁₂ levels of all 526 sera were assayed by the Phadebas method and by the radioisotopic method of Raven *et al* (1969) and 245 were assayed by the *L. leichmannii* method.

Results

GENERAL COMPARISON OF PHADEBAS, OTHER RADIOISOTOPIC, AND *L. LEICHMANNII* RESULTS

The comparisons between the Phadebas assay and the method of Raven *et al* (1969) and the *L. leichmannii* assay are shown in figures 1 and 2. The Phadebas assay tended to give higher results than the other two methods.

NORMAL RANGE FOR THE PHADEBAS ASSAY

The vitamin B₁₂ values obtained by the Phadebas method and the method of Raven *et al* (1969) for sera from 217 normal subjects and 33 patients with untreated pernicious anaemia are shown in figure 3. The results obtained by the Phadebas assay for the normal sera are higher (range 280-1590 pg/ml, mean 570 pg/ml) than those obtained by the method of Raven *et al* (1969) (range 220-1130 pg/ml, mean 500 pg/ml). In addition, the Phadebas values for the sera from patients with untreated pernicious anaemia

are higher (range 50-330 pg/ml, mean 175 pg/ml) than those obtained by the other radioisotopic method (range 5-195 pg/ml, mean 90 pg/ml). Despite the slight overlapping of results obtained for normal and pernicious anaemia sera by the Phadebas method (one normal serum giving a value of 280 pg/ml and one pernicious anaemia serum a value of 330 pg/ml), the lower limit of normal for the Phadebas assay would seem to be 300 pg/ml. Of the 526 sera assayed by the Phadebas method, 62 gave values of less than 300 pg/ml. One of the 62 was obtained from a normal subject (280 pg/ml), 32 from patients with untreated pernicious anaemia, and 29 from patients with folate deficiency, the causes of which included malabsorption, malnutrition, malignancy, and drugs.

REPRODUCIBILITY OF THE PHADEBAS ASSAY

The duplicate results for 100 sera assayed by the Phadebas method show satisfactory correlation (fig 4). The reproducibility of the Phadebas method both within a single assay and in different assays was similar to that of the method of Raven *et al* (1969).

THE NEED FOR A THREE-HOUR INCUBATION TIME FOR THE PHADEBAS ASSAY

Fifty sera were assayed by the Phadebas method using the recommended three-hour incubation time

Fig 5 The effect of incubation time on the vitamin B₁₂ results obtained for 50 sera by the Phadebas method.

Fig 6 Comparison of the vitamin B₁₂ values obtained for 106 sera by the radioisotopic method of Raven *et al* (1969), using its N/4 HCl extraction process, and by the same method modified to include the Phadebas glutamic acid extraction process.

and the same sera were re-assayed using a one-hour incubation time. There was little difference between the results obtained (fig 5).

STUDY OF THE REASONS FOR DIFFERENCE BETWEEN THE RESULTS OBTAINED BY THE PHADEBAS METHOD AND THE METHOD OF RAVEN *ET AL* (1969)

Phadebas extraction process

One hundred and six sera were assayed by the radioisotopic method of Raven *et al* (1969) using the N/4 HCl extraction process employed by that method and the assays were repeated by the same method, modified to incorporate the glutamic acid extraction process used by the Phadebas assay. There was little difference between the results obtained with the two extraction processes (fig 6).

Phadebas standard curve

Standard curves for the Phadebas assay were prepared in two different ways. For the first curve, lyophilized vitamin B₁₂ was diluted in buffer of pH 4.1 and doubling dilutions were prepared in the same buffer in accordance with the instruction sheet. For the second curve, lyophilized vitamin B₁₂ was diluted in serum with a vitamin B₁₂ content of less than 10 pg/ml (as determined by radioisotopic and micro-

biological assay) and doubling dilutions were prepared in the same serum. Then 0.5 ml of each of the vitamin B₁₂-serum standards was added to 2 ml of buffer of pH 3.1 and the solutions were heated in the

Fig 7 Two different standard curves obtained in a Phadebas assay. The upper curve was prepared in accordance with the instruction sheet. In the lower curve, the standard vitamin B₁₂ solutions were prepared in vitamin B₁₂-deficient serum and heated. In the Phadebas kit used for this figure, the intrinsic factor-Sephadex was diluted in 45 ml and the ⁵⁷CoB₁₂ in 7 ml.

Fig 8 As for fig 7 except that a different batch of the Phadebas kit was used, in which the intrinsic factor-Sephadex was diluted in 30 ml and the $^{57}\text{CoB}_{12}$ in 5.5 ml.

same way as for test sera. The two standard curves thus obtained for each of two different batches of the Phadebas kit (one in which the intrinsic factor-Sephadex was diluted in 45 ml and the $^{57}\text{CoB}_{12}$ in 7 ml, and the other in which dilutions were 30 ml and 5.5 ml respectively) are shown in figures 7 and 8. The difference between the two curves is such that when a serum vitamin B₁₂ value is calculated from a standard curve prepared according to the manufacturer's instructions, that value will be approximately 100 pg/ml higher than when the calculation is made from a standard curve containing vitamin B₁₂-free serum.

Discussion

Except for early difficulty with reproducibility of results the Phadebas assay proved easy to introduce into laboratory use. The reproducibility problems were due to loss of $^{57}\text{CoB}_{12}$ during the stage of triple washing of the fine Sephadex-intrinsic factor deposit and these problems disappeared when a Pharmacia suction nozzle was used during this stage of the assay.

The Phadebas assay fulfilled the main function of any serum vitamin B₁₂ assay in that it was able to identify sera from patients with untreated vitamin B₁₂ deficiency. In this respect, the results of this were more favourable than those of Wide and Killander (1971), who found that there was considerable overlap between the results obtained in normal subjects and those with vitamin B₁₂ deficiency. Undoubtedly the explanation for the difference between these two

trials lies in the method of identification of vitamin B₁₂-deficient sera. This trial employed only sera from patients with untreated pernicious anaemia, the diagnosis having been made by full haematological investigation, whereas the vitamin B₁₂-deficient sera employed by Wide and Killander (1971) were identified as such only by the *Euglena gracilis* assay. The *E. gracilis* assay may give falsely low values for sera containing antibiotics and other inhibitors.

The results of this study suggest that the lower limit of the normal range for the Phadebas assay is 300 pg/ml, which is consistent with the findings of Wide and Killander (1971) and Kubasik and Murray (1972). In general the Phadebas assay gave higher serum vitamin B₁₂ values than the radioisotopic method of Raven *et al* (1969) and the *L. leichmannii* assay. Differences between Phadebas and *E. gracilis* results have been noted by Wide and Killander (1971). The possibility that the Phadebas assay produces higher serum vitamin B₁₂ values than other assays because it has a more efficient extraction process can be discounted (fig 6) and there remains the possibility that the Phadebas results are falsely high. Despite the findings of Wide and Killander (1971), this present study suggests that serum vitamin B₁₂ values obtained by the Phadebas assay are approximately 100 pg/ml lower when the vitamin B₁₂ standards are diluted in serum and heated in the same way as for test sera. It has been a common finding by those interested in radioisotopic assays using intrinsic factor that accurate serum vitamin B₁₂ values are only obtained when standards and test sera have a similar protein composition and this subject has been reviewed by Hillman, Oakes, and Finholt (1969), Raven (1970), and Mortensen (1972). With other assays using intrinsic factor, the presence of protein in standard solutions raises the serum vitamin B₁₂ values obtained, whereas with the Phadebas assay lower values are obtained. Presumably intrinsic factor-Sephadex behaves differently from intrinsic factor alone.

The only other comment that one might make about the Phadebas kit is about the time required for the assay. During this study, the best time for the assay of 18 sera was six hours (excluding counting time), the three-hour incubation time in the middle of the assay being largely wasted time. This means that the technician's peak working activity is at the start and especially towards the end of the working day. It is known from other studies that the reaction between intrinsic factor and vitamin B₁₂ is largely completed by the end of 30 minutes (Herbert, Gottlieb, and Lau, 1966) and the present study suggests that the incubation period for the Phadebas assay could well be reduced from three hours to

one hour (fig 5). The Phadebas assay would then be more convenient for general laboratory use.

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