Value of Estimating Methylmalonic Acid Excretion in Anaemia

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Summary: A rapid technique suitable for routine pathology laboratories has been used to estimate methylmalonic acid excretion in a 24-hour urine collection following a 10g. valine load. Levels above 40 mg./24 hours were found only in patients with vitamin B₁₂ deficiency. Patients with pernicious anaemia treated more than 24 hours before urine collection and patients with other types of anaemia had methylmalonic acid levels below 25 mg./24 hours.

This method of demonstrating vitamin B₁₂ deficiency can be applied rapidly in debilitated patients so that specific treatment can be instituted within 36 hours of admission.

Introduction

It has been established that vitamin B₁₂ is required in the metabolism of propionic acid to succinic acid, acting as a coenzyme for the conversion of methylmalonic acid to succinic acid (White, 1962). Methylmalonic acid has been shown to be excreted in small amounts in normal urine, but to be present in excess in vitamin B₁₂ deficiency (Cox and White, 1962; Barness, Young, Mellman, Kahn, and Williams, 1963). There is an overlap between the normal and abnormal ranges, but this can be abolished by giving a valine load before collecting urine (Gompertz, Hywel Jones, and Knowles, 1967).

Methods of estimating methylmalonic acid in urine have been described using gas chromatography, which is accurate but unsuitable for use in most clinical laboratories (Cox and White, 1962) and thin-layer chromatography, which is only semi-quantitative (Bashir, Hinterberger, and Jones, 1966). The development of a colorimetric method suitable for use in a routine laboratory (Green, 1968) stimulated the present study to find whether methylmalonic acid excretion would be of value in clinical situations in which a severe megaloblastic anaemia existed.

The definitive diagnosis of megaloblastic anaemia may be lengthy, yet in some clinical situations rapid treatment with the deficient haematinic is essential. Megaloblastic anaemia is usually gradual in development and often occurs in the elderly, who are slow to complain. The problem is whether to give vitamin B₁₂ or folic acid therapy. Assay of blood levels of serum vitamin B₁₂ and folate and other diagnostic procedures are relatively slow. Intrinsic factor and intrinsic factor antibody determinations can be carried out rapidly in some centres but are not abnormal in all cases of vitamin B₁₂ deficiency. The possible risk of provoking neurological symptoms encourages most physicians to treat the patient with vitamin B₁₂ before investigations are complete. When the anaemia has been due to folate deficiency we have seen the delay in response so caused to have serious consequences.

Patients and Methods

Thirty-eight patients with haemoglobin levels between 3.2 and 12.8 g./100 ml. have been studied. Thirty-seven were

admitted to hospital with symptoms of anaemia and one with neurological symptoms of subacute combined degeneration of the cord (Hb 12.8 g./100 ml.). A marrow sample was aspirated from the sternum or iliac crest in each case. Thirty-one had megaloblastic erythropoiesis, seven were normoblastic.

Serum vitamin B₁₂ levels were assayed with *Lactobacillus leichmannii* (normal level 120-400 µg./ml.).

Serum folic acid activity and red cell folate were assayed with *L. casei* (normal serum level 5-25 µmg./ml.; normal red cell level 165-640 µmg./ml.).

Valine Loading.—All but five patients were given 10 g. of valine dissolved in water and flavoured with fruit juice.

Collection of Urine.—Twenty-four-hour urine specimens were collected into bottles containing 10 ml. of concentrated hydrochloric acid as a preservative. The total volumes were recorded and aliquots stored at -20° C. until the estimation was carried out.

Estimation of Methylmalonic Acid.—The method is described in detail elsewhere (Green, 1968). Acidified urine was saturated with ammonium sulphate and extracted with ethanol and ether, and the extract was purified with a strongly basic ion exchange resin. The methylmalonic acid was eluted and a colour developed with diazotized *p*-nitroaniline.

Results

Patients with Vitamin B₁₂ Responsive Anaemia.—Twenty-three patients aged 40 to 83 were studied; all had megaloblastic erythropoiesis and serum vitamin B₁₂ levels below 100 µg./ml. The Schilling test was abnormal in all those patients in whom this was performed (see Table). All patients responded to vitamin B₁₂ therapy as shown by a rise in reticulocyte count and a significant increase in haemoglobin concentration. Each of the 15 patients whose urine was collected before treatment was started had excretion rates of methylmalonic acid above 50 mg./24 hours (range 50-320 mg./24 hours). Two patients treated with vitamin B₁₂ immediately before receiving the valine and starting the 24-hour urine collection had levels of 63 and 45 mg./24 hours respectively. Urine from six patients who had been given vitamin B₁₂ more than a day before the 24-hour urine was collected had levels below 20 mg./24 hours (see Chart).

Valine Loading.—Eighteen of the vitamin B₁₂ deficient patients received valine before collection of the 24-hour urine; five were not given valine. In 12 patients who had received no therapy the excretion of methylmalonic acid in the urine 24 hours after the valine was more than 60 mg./24 hours (range 60-320 mg./24 hours). Three untreated patients who were not given valine had levels of 50 and 100 (two cases) mg./24 hours. Valine loading in two patients immediately after vitamin B₁₂ treatment gave methylmalonic acid levels of 45 and 63 mg./24 hours. Valine was given to four patients who had been treated with vitamin B₁₂ for more than 24 hours; in these the excretion of methylmalonic acid was 15 and 5 (three cases) mg./24 hours. In all the untreated cases of vitamin B₁₂ deficiency (15) the 24-hour excretion of methylmalonic acid was above 50 mg./24 hours. In the 24 hours following therapy raised levels were still obtained, but after a greater interval the excretion fell below 20 mg./24 hours.

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Table of Results

Case No.	Age	Hb (g./100 ml.)	Marrow	Serum B ₁₂ (μg./ml.)	Serum folate (μg./ml.)	Diagnosis	Methylmalonic Acid Excretion (mg./24 hours)
Vitamin B₁₂ Responsive Anaemias							
1	83	7.4	Megaloblastic	10	7.0	PA	105
2	73	5.9	"	42		PA	95
3	51	6.7	"	20	7.0	PA	45†
4	40	5.8	"	1	3.7	PA	100*
5	66	12.8	"	10	26.5	SACD	320
6	71	4.9	"	10	8	PA	108*
7	81	5.1	"	<10	20.5	PA	100
8	68	10.2	"	14	6.5	PA	70
9	64	5.9	"	10	20.5	PA	84
10	84	7.5	"	20	8	PA	61
11	67	3.2	"		6.25	PA	63†
12	66	9.0	"	10	18.5	PA	65
13	49	9.3	"	40	23.5	PA	72
14	67	6.7	"	<10	12.3	PA	75
15	65	4.3	"	40	7.5	PA	15‡
16	83	6.1	"	10	20.8	PA	5*‡
17	73	4.9	"	60	2.5	PA	5*‡
18	80	5.9	"	100	2.5	PA	5‡
19	72	3.4	"	20	5.0	Total gastrectomy	5‡
20	71	8.7	"	30	47.0	PA	7‡
21	83	9.5	"	60	8.7	PA	50*
22	83	11.1	"	40	14.0	PA	221
23	53	5.5	"	20	23.25	PA	99
Other Anaemias							
24	52	7.9	"	48	3.5	Folate deficiency	5
25	62	7.7	"	140	40 RC	"	<5
26	45	3.3	Normoblastic	180	12.5	Iron deficiency	<5
27	55	11.3	"	120	3.5	Post-gast. anaemia	<5
28	79	4.9	"	400	5.0	Iron deficiency	<5
29	74	7.6	"	—	—	Chronic lymph. leuk. aemia	<5
30	67	8.2	"	110	4.0	Cirrhosis of liver	<5
31	65	5.1	Megaloblastic	350	131 RC	Folate deficiency	<5
32	75	4.8	"	470	3.0	"	<5
33	69	4.9	"	60	142 RC	"	<5
34	74	10.5	Normoblastic	50	5	Iron deficiency	6
35	51	4.2	Megaloblastic	70	51 RC	Folate deficiency	21
36	52	4.0	"	40	2.75	"	24
37	96	6.4	"	328	2.5	"	<5
38	78	8.8	Normoblastic	400	9.5	Iron deficiency	<5

* No valine loading.

† Collected in the 24 hours following vitamin B₁₂ injection.‡ Valine given and urine collection started at least 24 hours after administration of vitamin B₁₂.

RC = Red cell folate level. PA = Pernicious anaemia. SACD = Subacute combined degeneration of the cord.

Patients with Other Anaemias.—This group consists of 15 patients with a variety of disorders, which have been divided into two groups. Group 1: those with a megaloblastic anaemia and evidence of folate deficiency (eight cases). In these the serum vitamin B₁₂ levels varied between 48 and 470 μg./ml. They were all given valine, and urine was collected before therapy, and in each case the 24-hour excretion of methyl-

malonic acid was below 25 mg./24 hours (see Table and Chart). Five patients who had a low serum vitamin B₁₂ level but did not respond to vitamin B₁₂ therapy all had methylmalonic acid levels below 25 mg./24 hours. This suggests that the methylmalonic acid excretion is not related to the serum vitamin B₁₂ level, but is increased only where a true vitamin B₁₂ deficiency exists. Group 2 consisted of seven anaemic patients who had normoblastic erythropoiesis. The level of methylmalonic acid excretion following the valine loading was never higher than 5 mg./24 hours.

Discussion

In this series an increased excretion of methylmalonic acid was found only in vitamin B₁₂ deficient conditions; this agrees with the findings of Cox and White (1962) and Barness *et al.* (1963). It is of interest that the low serum vitamin B₁₂ levels associated with folate deficiency do not lead to an increased excretion of methylmalonic acid. One patient has presented on two occasions in six years with a severe megaloblastic anaemia responding to folic acid but not to vitamin B₁₂ therapy. On each occasion there was a low serum vitamin B₁₂ level but she did not have a raised level of methylmalonic acid in the urine before treatment.

The highest excretion of methylmalonic acid found in this series was in a patient with subacute combined degeneration of the cord and no significant anaemia. Gompertz *et al.* (1967) found the highest levels of methylmalonic acid excretion in a patient with cerebral involvement.

The estimation of methylmalonic acid in urine provides a rapid means of confirming the diagnosis of vitamin B₁₂ deficiency. The estimation takes one and a half hours and 95% recovery can be achieved. The technique used is relatively simple to perform in a routine laboratory. By administering a valine load borderline levels were avoided, and the procedure increases the value of this estimation as a routine diagnostic test. The use of valine does not prevent the rapid fall of methylmalonic acid in the urine after treatment with vitamin B₁₂, so that this test is of no value if the urine is collected more than 24 hours after therapy has been instituted. During the 24-hour period following therapy, raised levels were found in two cases, but were in the lower part of our abnormal range. The test should therefore be used before therapy begins.

We have not yet confirmed whether 24-hour specimens are essential as stated by Kahn *et al.* (1965), who found that the excretion is not constant throughout the day. It may be possible to collect six-hour samples following valine administration. The reliability of this is being evaluated.

The method is valuable in the acute situation. It is often difficult clinically to say which deficiency is present, and usually vitamin B₁₂ therapy is begun without a definite diagnosis being established. If the patient is folate-deficient there will be a delay in effective treatment. The amount of methylmalonic acid excreted can be measured within 36 hours and specific treatment begun with less delay.

Though the estimation of methylmalonic aciduria with this method is most useful in situations where rapid answers are required, it may also replace vitamin B₁₂ assay in smaller laboratories with limited time and facilities.

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REFERENCES

- Barness, L. A., Young, D., Mellman, W. J., Kahn, S. B., and Williams, W. J. (1963). *New Engl. J. Med.*, 268, 144.
- Bashir, H. V., Hinterberger, H., and Jones, B. P. (1966). *Brit. J. Haemat.*, 12, 704.
- Cox, E. V., and White, A. M. (1962). *Lancet*, 2, 853.
- Gompertz, D., Jones, J. H., and Knowles, J. P. (1967). *Lancet*, 1, 424.
- Green, A. (1968). *J. clin. Path.*, 21, 221.
- Kahn, S. B., *et al.* (1965). *J. Lab. clin. Med.*, 66, 75.
- White, A. M. (1962). *Biochem. J.*, 84, 41P.