Sensitivity of Serum Methylmalonic Acid and Total Homocysteine Determinations for Diagnosing Cobalamin and Folate Deficiencies

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PURPOSE: **Patients with cobalamin (vitamin B12) deficiency usually lack many of the classic features of severe megaloblastic anemia; because of the low diagnostic specificity of decreased serum cobalamin levels, demonstrating the deficiency unequivocally is often difficult. We examined the sensitivity of measuring serum concentrations of methylmalonic acid and total homocysteine for diagnosing patients with clear-cut cobalamin deficiency and compared the results with those of patients with clear-cut folate deficiency.**

PATIENTS AND METHODS: **Serum metabolites were measured for all patients seen from 1982 to 1989 at two university hospitals who met the criteria for cobalamin and folate deficiency states and for such patients seen from 1968 to 1981 from whom stored sera were available. In all, 406 patients had 434 episodes of cobalamin deficiency and 119 patients had 123 episodes of folate deficiency. Criteria for deficiency states included serum vitamin levels, hematologic and neurologic findings, and responses to therapy. Responses were documented in 97% of cobalamin-deficient patients and 76% of folate-deficient patients. Metabolite levels were measured by modified techniques using capillary-gas chromatography and mass spectrometry.**

RESULTS: **Most of the cobalamin-deficient patients had underlying pernicious anemia; two thirds were blacks or Latinos. Hematocrits were normal in 28% and mean cell volumes in 17%. Of the 434 episodes of cobalamin deficiency, 98.4% of serum methylmalonic acid levels and 95.9% of serum homocysteine levels were elevated (greater than 3 standard deviations above the mean in normal subjects). Only one patient had normal levels of both metabolites. Serum homocysteine levels were increased in 91% of the 123 episodes of folate deficiency. Methylmalonic acid was elevated in 12.2% of the folate-deficient patients; in all but one, the elevation was attributable to renal insufficiency or hypovolemia.**

CONCLUSIONS: **For the cobalamin-deficient patients, measuring serum metabolite concentrations proved to be a highly sensitive test of deficiency. We conclude that normal levels of both methylmalonic acid and total homocysteine rule out clinically significant cobalamin deficiency with virtual certainty.**

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Cobalamin (vitamin B^{12}) deficiency is common. Early diagnosis and treatment can prevent severe anemia or potentially irreversible damage to the nervous system from developing, but the majority of patients lack the textbook features of advanced deficiency of the vitamin [1–4]. Serum cobalamin concentration is widely used as a screening test for deficiency but has major limitations. Its sensitivity is less than perfect [5,6], and many workers [4,7–12] have found that a substantial proportion—perhaps 25% to 50% [4,12]—of patients with low serum cobalamin levels do not appear to be deficient in the vitamin or to have underlying disorders predisposing them to deficiency.

The metabolites methylmalonic acid and homocysteine accumulate when the two mammalian cobalamin-dependent enzymatic reactions are impaired [13,14]. We found that serum levels of both metabolites are markedly elevated (more than three standard deviations [SD] above the mean in healthy control subjects) in most deficient patients, suggesting that these tests are a useful and convenient way to establish a diagnosis of cobalamin deficiency [4,13–15]. The assays were modified in early 1988 to further increase their specificity and precision [16,17].

We report here using the modified techniques to assay more than 400 patients with clinical evidence of cobalamin deficiency. To our knowledge, this series, accumulated over 21 years, is the largest group of patients with well-documented cobalamin deficiency reported in the literature. For comparison, we assayed a series of 119 patients with folate deficiency. In previous studies involving relatively small numbers of folate-deficient patients, total homocysteine concentrations were elevated in most cases [14,18,19].

TABLE I
Criteria for Cobalamin Deficiency in 434 Episodes in 401 Patients

Serum Cobalamin < 200 pg/mL and			
Diagnostic Marrow +/or Blood Smear	Response to Cbl Rx*	No. of Episodes	%
+	+	309	71.2
+	NA	13	3.0
NA	+	107	24.6
0	+	5	1.2
Total		434	100

NA = not available; Cbl = cobalamin; Rx = therapy.
*See Table II for definition of responses.

TABLE II
Responses to Cobalamin Treatment in 434 Episodes of Cobalamin Deficiency in 409 Patients

	No. of Episodes	%
Decrease in mean cell volume ≥ 5 fL	369	85.0
Increase in hematocrit ≥ 0.05	286	65.9
Resolution of thrombocytopenia	110	25.3
Resolution of leukopenia	50	11.5
Improvement in neuropsychiatric abnormalities*	112	25.8
More than one of above responses to treatment	325	74.9
Other responses†	9	2.1
No documentation of response	13	3.0

*Clear-cut improvement in neuropsychiatric abnormalities within the first three months of treatment. This was the only response to treatment in a single episode.

†Only one of the following responses was documented: reticulocytosis (five patients); correction of neutrophil hypersegmentation (three patients); and resolution of atrophic glossitis (one patient).

TABLE III
Criteria for Folate Deficiency in 123 Episodes in 119 Patients

	No. of Episodes	%
Diagnostic marrow or blood smear, or both	123	100
Serum folate < 4 ng/mL*	123	100
Serum cobalamin > 300 pg/mL†	123	100
Underlying disorder associated with folate deficiency	123	100
Documented response to folic acid	94	76.4

*Serum folate < 2.1 ng/mL in 99 episodes and 2.1 to 3.9 ng/mL in 24.
†Serum cobalamin 301 to 350 pg/mL in 7 episodes, 351 to 400 pg/mL in 10, 401 to 500 pg/mL in 20, 501 to 1,000 pg/mL in 60, and greater than 1,000 pg/mL in 26. None of the patients had been treated with vitamin B^{12} before study.

PATIENTS AND METHODS

Patients

All cases of cobalamin and folate deficiency seen at Harlem Hospital Center and Columbia-Presbyterian Medical Center between July 1968 and June 1989 were reviewed. The majority of the patients were evaluated in consultation with one of the coauthors as part of prospective studies of megaloblastic anemias, and the hematologic and neurologic findings have been the subject of previous reports [4,13–15,20–26].

Methylmalonic acid and total homocysteine levels were measured in all patients seen between 1968 and 1981 who met the study's criteria for cobalamin and folate deficiency and from whom serum stored at –20°C was available. From 1982 to 1989, serum metabolite levels were measured in each of 267 consecutively seen patients who met the cirteria for cobalamin deficiency (219 patients) or folate deficiency (48 patients).

Methods

When available, smears of peripheral blood and bone marrow from cobalamin- and folate-deficient patients were coded and interspersed with those of patients without evidence of deficiency for blinded review by one of the authors. Hypersegmentation in blood smears was defined as more than five cells with five lobes per 100 neutrophils or any cells with six or more lobes. Serum cobalamin levels were determined by radioassay using purified intrinsic factor (Quantaphase, Bio-Rad Laboratories, Richmond, CA) or by microbiologic assay with *Lactobacillus leichmannii* [27]. Serum folate concentrations were measured by milk-binder radioassays or by microbiologic assay with *Lactobacillus casei* [28].

Serum concentrations of methylmalonic acid [29] and total homocysteine [30] were measured by modified [16,17] techniques using capillary-gas chromatography and mass spectrometry. The normal ranges (calculated as the mean ± 3 SD after log transformation to correct for skewing toward higher values) are 53 to 376 nmol/L for methylmalonic acid and 4.1 to 21.3 µmol/L for total homocysteine [16]. For 160 of the patients with cobalamin deficiency and 19 with folate deficiency, serum metabolite levels were measured by methods used before our modifications were made, and the results were reported earlier [4,13–15].

Standard statistical methods, including Student's *t*–test and the chi-squared test with Yates' correction for continuity [31], were used to analyze the data.

RESULTS

Criteria of Deficiency

Serum methylmalonic acid and total homocysteine levels were measured in 406 patients who had 434 episodes of cobalamin deficiency; 25 patients had one or more recurrent episodes of deficiency because they discontinued maintenance treatment with the vitamin.

The diagnostic criteria for including cobalamin-deficient patients in this series are summarized in **Table I.** All patients had low serum cobalamin levels. Morphologic abnormalities (megaloblastic bone marrow or hypersegmented neutrophils, or both, with or without macroovalocytes, on blood smear) were found in 322 episodes; clear-cut responses to cobalamin therapy (as defined in **Table II**) were documented for 309 of the 322 episodes. Although marrow and blood films were not available for 107 episodes before therapy began, clear-cut responses were doc-

TABLE IV
Etiology of Cobalamin and Folate Deficiencies in 529 Patients

Etiology	Patients
Cobalamin deficiency	
Pernicious anemia	
Proven	234
Probable*	76
Tropical sprue	
Proven	11
Probable†	28
Gastrectomy	13
Ileal disease or resection, or both	13
Gastric and ileal resections	1
Jejunal diverticula	6
Vegetarianism	2
Food cobalamin malabsorption	3
Not established	19
Total	406
Folic acid deficiency	
Alcoholism	103
Malnutrition	11
Malabsorption syndrome	1
? Oral contraceptives	1
Pregnancy	1
Sickle cell anemia	1
Tropical sprue	1
Total	119

*One or more of the following: cobalamin malabsorption in part 1 of the Schilling test, achlorhydria, and autoimmune thyroid disease.

†Malabsorption syndrome in a person who had lived in the tropics (intestinal biopsy not performed).

TABLE V
Clinical Characteristics of 434 Episodes of Cobalamin Deficiency in 406 Patients and 123 Episodes of Folate Deficiency in 119 Patients

	Cobalamin Deficiency		Folate Deficiency	
Characteristics*	No./Total†	%	No./Total†	%
Sex				
Male	151/406	37.2	70/119	58.8
Female	255/406	62.8	49/119	41.2
Ethnicity				
Black	168/406	41.3	105/119	88.2
White	135/406	33.3	9/119	7.6
Latino	101/406	24.9	5/119	4.2
Other	2/406	0.5	0/119	0.0
Alcoholism	59/434	13.6	107/123	87.0
Anemia	313/434	72.1	123/123	100.0
Macrocytosis				
MCV > 100 fL	334/402	83.1	68/91	74.7
MCV > 110 fL	224/402	55.7	50/91	54.9
Leukopenia‡	73/433	16.9	22/123	17.9
Thrombocytopenia‡	136/384	35.4	78/118	66.1
Pancytopenia	48/384	12.5	21/118	17.8
LDH > 1,000 U/L	96/330	29.1	24/83	28.9
Atrophic glossitis	111/434	25.6	27/123	22.0
Neuropsychiatric syndrome§	156/434	35.9	36/123	29.3

MCV = mean corpuscular volume; LDH = lactate dehydrogenase.

*Mean age 49 ± 17 years in folate-deficient group and 66 ± 18 years in cobalamin-deficient patients.

†Total number of cases for which information was available; denominator = number of patients, for sex and ethnic background, and number of episodes for other items.

‡Leukopenia = leukocyte count less than 4,000/μL for white and Latino patients and less than 3,000/μL for blacks; thrombocytopenia = platelet count less than 150,000/μL.

§Consistent with those syndromes seen in cobalamin deficiency [26]. Peripheral neuropathy, ataxia, and cerebral dysfunction were often seen in folate-deficient alcoholics.

umented; all but one of these episodes showed at least one of the hematologic responses listed in Table II. In five episodes showing a therapeutic response, pretreatment blood smears were normal or nondiagnostic (marrow aspirates were not obtained). Of the 434 episodes, 421 (97%) responses to treatment with cyanocobalamin were documented (Table II).

Serum metabolites were measured in 123 episodes of folate deficiency diagnosed in 119 patients (four patients had two episodes). Our definition of folate deficiency was based on the clinical, morphologic, and biochemical criteria summarized in **Table III**. Hematologic responses to treatment with folic acid were documented for 94 episodes; adequate hematologic follow-up data were not obtained for the others.

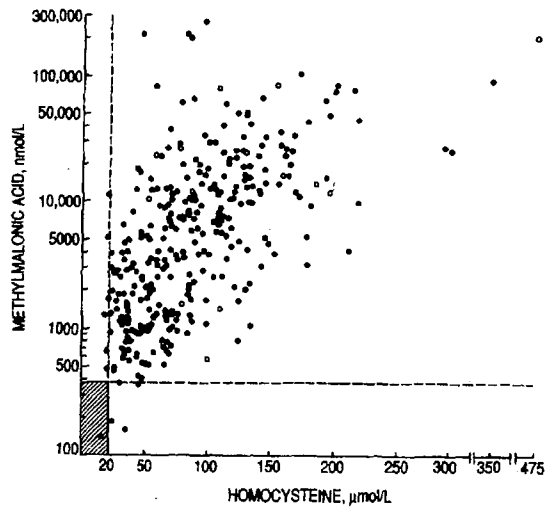
Patients were considered folate or cobalamin deficient based solely on the stated criteria and not on the results of the metabolite assays. The majority of the patients (86%) were studied before 1986, when the metabolite assays became available, and the percent of patients with each deficiency with elevated metabloite levels was similar for patients who were evaluated before and after 1986.

CAUSES OF DEFICIENCY STATES

Diagnostic studies were adequate to determine the etiology of cobalamin deficiency for most patients (**Table IV**). Pernicious anemia, demonstrated by serial Schilling tests or serum antibody to intrinsic factor, was established for 57.6% and considered likely for another 18.7%. The serum folate level was less than 2.1 ng/mL in 17 patients and between 2.1 ng/mL and 3.9 ng/mL in 57 patients in the cobalamin-deficiency group. All 74 patients had one or more findings of serum antibodies to intrinsic factor, malabsorption of vitamin B^{12} in Schilling tests, or neurologic dysfunction responsive to cobalamin treatment. Of these 74 patients, 19 had proven or probable tropical sprue, and it is likely that some had folate deficiency along with cobalamin deficiency. The 19 cobalamin-deficient patients for whom diagnostic evaluation was inadequate to determine the etiology of the deficiency (Table IV) had serum folate levels greater than 4 ng/mL in all cases. Alcoholism caused 87% of the episodes of folate deficiency (Table IV).

Characteristics of Episodes

The clinical features of the cobalamin- and folate-deficient patients are summarized in **Table V**. The



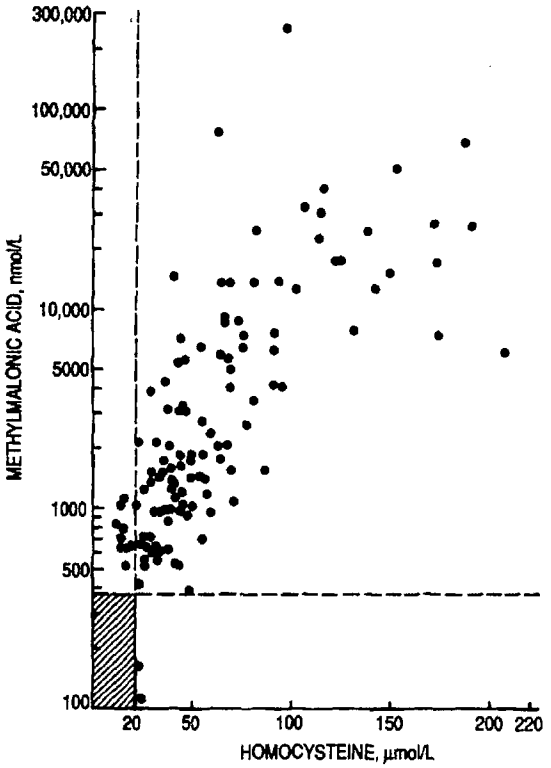


Figure 1 Top. Serum concentrations of methylmalonic acid and total homocysteine in 313 episodes of megaloblastic anemia due to cobalamin deficiency. **Dashed lines** indicate three standard deviations above the mean for normal controls for each metabolite; **hatched area** includes values for both metabolites less than three standard deviations above the mean for normal controls; **open circles** indicate 16 cobalamin deficiency episodes associated with anemia with elevated serum creatinine.

Figure 1 Bottom. Serum concentrations of methylmalonic acid and total homocysteine in 121 episodes of cobalamin deficiency in which the hematocrit was normal (greater than or equal to 40% in men, greater than or equal to 35% in women). **Dashed lines** indicate three standard deviations above the mean for normal controls for each metabolite; **hatched area** includes values for both metabolites less than three standard deviations above the mean for normal controls.

TABLE VI
Serum Concentrations of Methylmalonic Acid and Total Homocysteine in Episodes of Cobalamin Deficiency With and Without Anemia

	Anemia Present*		Anemia Absent		All Episodes	
	No.	%	No.	%	No.	%
Total number of episodes	313		121		434	
Episodes with						
Elevated§ MMA	308	98.4	119	98.3	427	98.4
Elevated§ HCYS	306	97.8†	110	90.9†	416	95.9
Both MMA, HCYS elevated	302	96.5‡	108	89.3‡	410	94.5
MMA alone elevated	6	1.9	11	9.2	17	3.9
HCYS alone elevated	4	1.3	2	1.7	6	1.4
MMA, HCYS both normal	1	0.3	0	0	1	0.2

MMA = methylmalonic acid; HCYS = total homocysteine.
*Hematocrit less than 40% in males; less than 35% in females. The mean corpuscular volume was normal (80 to 100 fL) in 9.9% of the episodes when anemia was present and in 33.3% of those when anemia was absent.
†$p < 0.005$.
‡$p < 0.01$.
§More than 3 SD above the mean in normal control subjects.

TABLE VII
Serum Metabolite Concentrations in Anemia Caused by Cobalamin or Folate Deficiency

	Cobalamin Deficiency		Folate Deficiency	
	No.	%	No.	%
Total no. of episodes*	297		98	
Elevated MMA	292	98.3	4†	4.1
Elevated HCYS	290	97.6	88	89.8
Both MMA, HCYS elevated	286	96.2	4	4.1
Elevated MMA, normal HCYS	6	2.0	0	0
Elevated HCYS, normal MMA	4	1.3	84	85.7
MMA, HCYS both normal	1	0.3	10	10.2

MMA = methylmalonic acid; HCYS = total homocysteine.
*Sixteen patients with cobalamin deficiency and 25 with folate deficiency with elevated serum creatinine levels were excluded from the analysis.
†Severe volume depletion present in three episodes.

substantial numbers of black and Latino patients with cobalamin deficiency reflect the distribution of ethnic groups in the catchment areas of the two hospitals and are consistent with previous reports of a high prevalence of pernicious anemia in these populations [32–34]. The higher proportion of black patients and anemic patients in the folate-deficient group came from including several series of alcoholics with anemia studied at Harlem Hospital Center [23,25].

The mean corpuscular volume (MCV) was normal in 17% of cobalamin-deficient patients and in 25% of folate-deficient patients. Only a minority of patients with either deficiency state had leukopenia, pancytopenia, atrophic glossitis, or marked elevations of serum lactate dehydrogenase. More than a third of the cobalamin-deficient group has neuropsychiatric symptoms and signs (Table V) compatible with that vitamin deficiency [26]. Peripheral neuropathy, ataxia, and cerebral dysfunction were frequently associated with alcoholism in the folate-deficient patients.

Serum Metabolites in Cobalamin Deficiency

Serum methylmalonic acid levels were markedly increased (more than 3 SD above the mean in normal controls) in 427 (98.4%) of the 434 episodes of cobalamin deficiency (**Figure 1, Table VI**); serum total homocysteine concentrations were markedly increased (more than 3 SD above the mean in normal controls) in 416 (95.9%). In 410 episodes (94.5%), both metabolite levels were markedly elevated. In only one cobalamin-deficient patient (0.2%) were both metabolites normal.

The hematocrit was normal (Figure 1 Bottom; Table VI) in 121 of the 434 episodes of cobalamin deficiency. Nonetheless, serum methylmalonic acid values were elevated in 98.3% of the episodes without anemia. In contrast, homocysteine levels were elevated in 90.9% of the nonanemic patients compared with 97.8% of anemic patients (Table VI; chi squared = 8.66, $p < 0.005$). Thus serum methylmalonic acid levels were more sensitive for identifying nonanemic cobalamin-deficient patients than serum homocysteine levels ($p < 0.005$). Both metabolites were elevated in 89.3% of the episodes in which the hematocrit was normal. Mean homocysteine levels were significantly higher in anemic than nonanemic patients (89.4 ± 55.0 μmol/L versus 60.2 ± 41.3 μmol/L, $p < 0.001$). Mean methylmalonic acid concentrations were also higher in anemic patients, but not significantly so (14,663 ± 30,698 nmol/L versus 8,599 ± 25,554 nmol/L, $p > 0.05$).

The clinical, hematologic, and neurologic features of the 18 episodes with normal serum homocysteine levels did not differ from those of the 416 with elevated levels, except that anemia was generally less severe when they were normal (mean hematocrit, 37.2% versus 28.3%, $p < 0.001$). The seven patients with normal methylmalonic acid values did not differ significantly in any respect from the 427 with elevated values.

Serum creatinine was elevated (open circles, Figure 1 Top) in 16 cobalamin deficiency episodes, all of which were anemic. The mean serum homocysteine was higher in these 16 episodes than in the 297 associated with anemia but with normal serum creatinine (139.5 ± 103.0 μmol/L versus 86.7 ± 50.0 μmol/L, $p < 0.001$). Similarly, mean methylmalonic acid values were higher when megaloblastic anemia was associated with renal failure (36,527 ± 48,036 nmol/L versus 13,450 ± 29,098 nmol/L, $p < 0.005$).

Even though many of the serum specimens had been stored at –20°C for 10 to 20 years, there was no indication that methylmalonic acid or homocysteine levels varied inversely with increased storage time. For example, metabolite levels in serum obtained from cobalamin-deficient patients between 1968 and 1973 did not differ significantly from those in serum obtained during the most recent 5 years of the study, 1984 to 1989 (data not shown).

In a series of 86 consecutively studied patients with cobalamin deficiency [4] tested by earlier assay methods, 12 (14%) did not have elevated methylmalonic acid and 13 (15.1%) did not have increased homocysteine. Sera were available from most of these patients for assay by the modified techniques used in this study. Seven of 10 patients with methylmalonic acid levels less than 3 SD above the normal mean using the older method had elevated concentrations using the current assay system. Of 11 previously normal homocysteine values, four were increased when reassayed by the modified method.

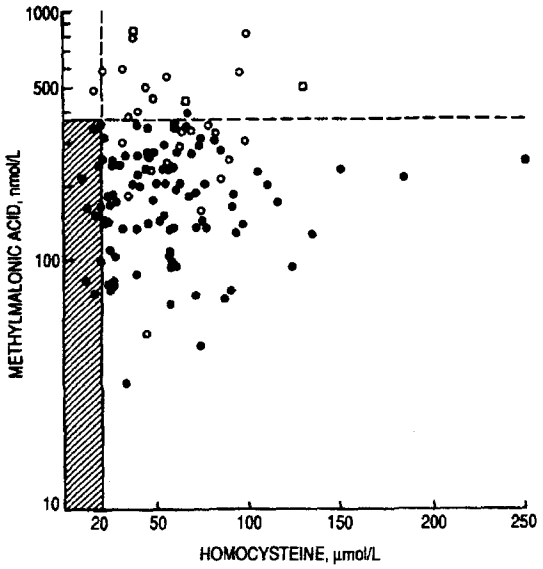


Figure 2. Serum concentrations of methylmalonic acid and total homocysteine in 123 episodes of megaloblastic anemia due to folate deficiency. **Open circles** indicate patients with elevated serum creatinine concentrations; boxes indicate three patients with severe hypovolemia and normal creatinine levels. **Dashed lines** indicate three standard deviations above the mean for normal controls. (Note that the scale for methylmalonic acid values differs from that in Figure 1.)

Serum Metabolites in Folate Deficiency

Serum homocysteine levels were markedly elevated in 112 (91.0%) of the 123 episodes of folate deficiency (**Figure 2**). The 11 patients with normal levels did not differ from the others in severity of anemia, megaloblastic morphologic changes, methylmalonic acid elevation, time in hospital before study, reticulocyte counts, or serum levels of folate, cobalamin, creatinine, bilirubin, or albumin (data not shown).

Serum methylmalonic acid was increased in 15 (12.2%) of the 123 episodes of folate deficiency (Figure 2); however, 14 of these patients had renal dysfunction (11 with serum creatinine elevation and 3 with clinical evidence of severe volume depletion).

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