Effect of drugs on vitamin B₁₂ levels obtained using the *Lactobacillus leichmanii* method

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SYNOPSIS In 750 consecutive assays of serum vitamin B₁₂ levels using *Lactobacillus leichmanii*, 25 (3.3%) showed inhibition. A satisfactory history of drug therapy was obtained in 23 cases. Seventeen of these were receiving ampicillin at the time. Two patients showed inhibition while receiving phenoxymethyl penicillin or phenethicillin. A prospective study of 11 patients detected inhibition in seven at varying intervals during ampicillin therapy. It was shown that the vitamin B₁₂ as measured by the radioisotope and *Euglena gracilis* methods was not affected. Inhibition of *L. leichmanii* was reproduced *in vitro* with concentrations of ampicillin corresponding to those obtained in therapy. Benzylpenicillin, streptomycin, and chloramphenicol had no such effect.

A prospective study of patients receiving chlorpromazine yielded negative results.

Inhibition may occasionally be seen in the absence of any known intake of drugs. Measurement by other methods may be required to distinguish this from vitamin B₁₂ depletion.

Where serum B₁₂ is estimated using *Lactobacillus leichmanii*, the phenomenon of inhibition is encountered not infrequently. Boczarow (1961) reported on the effect of penicillin on the test organism and found that penicillin therapy invalidated the test. However, Watts (1967) claimed that the dilution and heating involved in the assay procedure resulted in inactivation of the antibiotic, except where novobiocin was given. In view of this discrepancy we decided to measure the incidence of the phenomenon of inhibition and its possible relationship to antibiotic and drug therapy.

MATERIAL AND METHOD

Serum vitamin B₁₂ was estimated using *Lactobacillus leichmanii* (ATCC 7830) and DANO-B₁₂ assay medium (Dano Chemicals, Copenhagen). The method (Thompson, Dietrich, and Elvehjem, 1950; Spray, 1955) was basically as described by Dano Chemicals, except that the serum dilution was adjusted so that the vitamin B₁₂ activity was measured using both 0.1 and 0.25 ml of serum (corresponding to dilutions of 1:100 and 1:40 respectively). The tests were done in duplicate. All the assays were carried out by one of us (C.D.) who has been using the method for five years. It was first established that there was insufficient diurnal or daily variation to invalidate

the study and that the normal levels were between 120 and 600 µg/ml.

The *Euglena gracilis* and ⁵⁷Co methods were performed according to the descriptions of Ross (1952) and Lau, Gottlieb, Wasserman, and Herbert (1965).

The incidence of inhibition was determined by assessing the results of the last 750 consecutive vitamin B₁₂ assays, 480 from cases in a psychiatric hospital and 270 from general medical cases. All the accepted cases showed a marked reduction (usually more than twofold) of the vitamin B₁₂ level with the larger volume of serum (1:40 dilution). Drug ingestion was then investigated.

When it was realized that ampicillin was implicated, tests *in vivo* and *in vitro* were carried out with this antibiotic. Eleven patients who required therapy were studied. Blood was taken to establish a basal serum vitamin B₁₂ level before ampicillin was given in a dose of 250 mg intramuscularly every eight hours. Subsequent samples were collected half an hour after the morning injection on the third and fifth treatment days. A final sample was collected between five and seven days after the cessation of therapy. A similar procedure was adopted in 10 patients receiving chlorpromazine hydrochloride 50 mg twice daily.

A further five patients were studied during ampicillin therapy in order that parallel vitamin B₁₂ levels by the radioisotope and/or *E. gracilis* methods could be determined in the inhibitory phase.

Tests *in vitro* with ampicillin and other antibiotics were carried out by adding the antibiotics to the serum before the assay. The quantities added were adjusted to give levels which might be encountered *in vivo*.

RESULTS

INCIDENCE OF INHIBITION Twenty-five instances of probable inhibition were found among the 750 consecutive vitamin B₁₂ assays (incidence 3·3%). Only three instances were in the psychiatric group, while 22 occurred in the much smaller general group. The patients' ages ranged from 44 to 92 years. A reliable drug history could not be obtained in two patients. No drugs had been prescribed in five patients. One patient was receiving phenethicillin. The remaining 17 were all on ampicillin (Table I). Patients 4 (a case of pernicious anaemia) and 15 had been given cytamien; patient 6 was also on chlorpromazine, imipramine, and promazine hydrochloride; and patient 11 was on streptomycin. The corresponding figures for the five patients for whom no drugs had been prescribed are also shown.

TABLE I

VITAMIN B₁₂ LEVELS IN 22 PATIENTS¹

Patient	0·1 ml Serum (1:100 dilution)	0·25 ml Serum (1:40 dilution)
<i>Patients receiving ampicillin</i>		
1	40	0
2	120	75
3	350	30
4	1,300	140
5	50	0
6	580	340
7	55	0
8	1,200	600
9	50	10
10	110	20
11	45	0
12	300	95
13	130	80
14	0	0
15	1,700	860
16	135	90
17	20	0
<i>Patients receiving no drugs</i>		
18	75	30
19	420	250
20	50	20
21	50	20
22	830	530

¹Results are expressed in $\mu\text{g/ml}$.

Many other drugs, including tetracycline, frusemide, digoxin, imipramine hydrochloride, methyl-dopa, and barbiturates, were being administered to the other patients in this survey at the time of sampling. None of these were associated with inhibition.

PROSPECTIVE STUDY OF AMPICILLIN THERAPY The effect of ampicillin was studied prospectively in 11 patients (Table II). Seven showed clear evidence of inhibition on the third and/or fifth days of therapy. Case 23 showed almost complete inhibition on both days; four of the others (cases 24, 28, 29, and 31)

TABLE II

EFFECT OF AMPICILLIN ON VITAMIN B₁₂ LEVELS¹

Case No.	Basal Level		Days after Ampicillin					
			Third Day		Fifth Day		Five to Seven	
	0·1 ml	0·25 ml	0·1 ml	0·25 ml	0·1 ml	0·25 ml	0·1 ml	0·25 ml
23	320	300	0	0	20	0	280	260
24	170	190	0	0	160	0	150	160
25	120	120	110	110	—	—	80	110
26	370	380	380	380	350	0	410	380
27	210	230	190	200	350	350	220	280
28	650	500	240	0	550	470	770	660
29	430	410	0	0	390	360	250	300
30	230	230	280	280	0	0	Died	
31	620	510	370	10	440	350	770	660
32	80	90	60	70	50	10	110	130
33	220	220	210	190	270	290	220	250

¹Results are expressed in $\mu\text{g/ml}$.

showed marked inhibition on the third day, but only one of these (case 24) continued to show clear evidence of inhibition on the fifth day. On the other hand two patients (cases 26 and 30) showed inhibition on the fifth day only. Sometimes the degree of inhibition was complete with both 0·1 and 0·25 ml of serum. In others the presence of an inhibitor was indicated by the higher B₁₂ levels in the more dilute sample. This difference may be marked, for example, in case 26 on the fifth day from 350 $\mu\text{g/ml}$ to zero. On the other hand, the difference may be much smaller and more difficult to interpret, for example, in case 32 from 50 to 10 $\mu\text{g/ml}$, or in case 31, in which both pre- and post-antibiotic levels were above 600 $\mu\text{g/ml}$, from 440 to 350 $\mu\text{g/ml}$ on the fifth day.

A further five patients were studied to check the effect of ampicillin therapy on vitamin B₁₂ levels, as measured by the radioisotope and *E. gracilis* methods. Because of insufficient serum it was not possible to perform all three methods on all samples. The first samples were collected before ampicillin therapy was begun, and the second on the third day of treatment (Table III). Three of the five (M.T., H.H., and M.E.H.) showed typical inhibition with *L. leichmanii*, but when measurements were performed by the other two methods levels of vitamin B₁₂ were within the normal range. The ampicillin levels in two of the sera that showed inhibition (M.T. and H.H.) were 0·4 μg and 3·1 μg per ml respectively.

Patient F.G. is of interest in that the low serum vitamin B₁₂ levels obtained with *L. leichmanii* were not related to ampicillin therapy, as shown by a similar level after treatment. This patient, aged 88 years, was on thyroid therapy for myxoedema, but was not anaemic. Assay with *E. gracilis* gave the same reading as with *L. leichmanii* and a true vitamin B₁₂ deficiency was confirmed by a Schilling radioisotope absorption test which showed only 2·6% excretion in the first 24 hours.

TABLE III

COMPARISON OF ASSAY METHODS FOR SERUM B₁₂ IN PATIENTS GIVEN AMPICILLIN

Patient	Sample	<i>L. leichmanii</i>		⁵⁷ Co	<i>E. gracilis</i>
		0.1 ml Serum	0.25 ml Serum		
M.T.	1	250	250	—	—
	2	110	0	270	—
H.H.	1	290	300	—	—
	2	250	80	594	—
E.H.	1	200	220	—	—
	2	240	240	530	327
M.E.H.	1	360	330	—	—
	2	340	40	—	373
F.G.	1	30	0	—	—
	2	50	30	—	—
	Thereafter	30	30	—	30

Sample 1 refers to that taken before the start of ampicillin, and sample 2 on the third day of therapy. Results are expressed in $\mu\text{g/ml}$.

EFFECT OF ADDING AMPICILLIN IN VITRO The effect of adding ampicillin *in vitro* to normal serum before vitamin B₁₂ assay is shown in Table IV. The B₁₂ value before the addition of ampicillin was 210 $\mu\text{g/ml}$. Ampicillin was added to give levels of 1.0 to 10.0 μg per ml and it was shown that 4.0 μg and above produced inhibition.

The specificity of the inhibition was confirmed, as shown in Table IV, where 2 and 8 μg of ampicillin were added to the pre-antibiotic serum of case 26 (Table II). Slight inhibition was seen in the 0.25 ml sample at 2 μg , and complete inhibition in 0.1 ml and 0.25 ml at 8 g. This patient subsequently showed inhibition on the fifth day of ampicillin therapy.

Ampicillin sensitivity of *L. leichmanii* The organism was tested using the standard assay medium in the presence of ampicillin. The organism was found to be highly sensitive, giving a minimum inhibitory concentration of 0.04 μg per ml.

TABLE IV

EFFECT OF ADDING AMPICILLIN TO CONTROL SERUM IN VITRO BEFORE VITAMIN B₁₂ ASSAY

Ampicillin (μg)	Vitamin B ₁₂	
	0.1 ml Serum	0.25 ml Serum
1.0	190	180
2.0	170	70
4.0	100	0
6.0	0	0
8.0	0	0
10.0	0	0
No ampicillin	210	210

TABLE IVA

EFFECT ON AMPICILLIN IN VITRO IN CASE 26 (TABLE II)

	0.1 ml Serum	0.25 ml Serum
Basal sample	340	310
2 μg ampicillin added	340	170
8 μg ampicillin added	0	0

INHIBITION WITH PHENOXYMETHYL PENICILLIN One patient, investigated after completion of the survey, showed complete inhibition of *L. leichmanii* at all serum dilutions. This patient had been given phenoxy methyl penicillin, 250 mg four times daily, and complete inhibition was observed on the third and sixth days of treatment. The levels obtained by *E. gracilis* and radioisotope methods showed no inhibition. A level of B₁₂ of 230 μg per ml was found two weeks after cessation of treatment.

When more than 5 μg per ml of phenoxy methyl penicillin was added *in vitro* complete inhibition was found at both serum dilutions.

EFFECT OF ADDING BENZYL PENICILLIN IN VITRO The vitamin B₁₂ levels obtained when sodium benzyl penicillin (Crystapen, Glaxo) was added before assay showed no evidence of inhibition (Table V).

TABLE V

EFFECT OF ADDING BENZYL PENICILLIN IN VITRO BEFORE VITAMIN B₁₂ ASSAY

Benzylpenicillin (units)	Vitamin B ₁₂	
	0.1 ml Serum	0.25 ml Serum
0	280	270
0.5	270	270
1.0	290	270
2.0	290	280
3.0	290	270
5.0	290	270
10.0	290	240
15.0	290	270
20.0	270	300

OTHER ANTIBIOTICS Chloramphenicol and streptomycin (both in strengths of up to 50 μg per ml) were without any inhibitory effect when tested *in vitro*.

EFFECT OF AMPICILLIN ON HIGH VITAMIN B₁₂ LEVELS Two additional patients who showed high vitamin B₁₂ levels were studied. The first had received vitamin B₁₂ therapy until three weeks before admission. Ampicillin was prescribed and the vitamin B₁₂ results obtained on the second day of therapy were 1,300 μg per ml with 0.1 ml serum and 160 μg per ml with 0.25 ml serum. The second patient presented with diabetes mellitus and features consistent with liver damage. She had received neither vitamin B₁₂ nor antibiotic therapy, but B₁₂ levels were 1,100 μg per ml with 0.1 ml serum and 640 μg per ml with 0.25 ml serum.

PROSPECTIVE STUDY OF CHLORPROMAZINE THERAPY The B₁₂ levels were obtained before, during, and after chlorpromazine therapy but only the mean value for the dilutions of 0.1 ml and 0.25 ml are

TABLE VI

VITAMIN B₁₂ LEVELS DURING CHLORPROMAZINE THERAPY

Basal B ₁₂ Level	Chlorpromazine Therapy		
	Third Day	Fifth Day	Five to Seven Days Later
250	240	240	230
120	120	110	100
140	140	140	150
210	430	500	310
280	290	240	400
620	650	500	540
340	290	—	360
140	260	290	300
240	—	220	—
270	240	220	230
190	110	190	120

FIG. 1. Vitamin B₁₂ levels during courses of ampicillin and chlorpromazine (C.P.Z.) therapy in one patient

given in Table VI because no significant differences were seen.

One patient who received ampicillin followed by chlorpromazine therapy showed complete inhibition on ampicillin but no inhibition while receiving chlorpromazine (Fig. 1).

DISCUSSION

In this survey it has been shown that when *L. leichmanii* is used for the assay of serum vitamin B₁₂ and the serum tested at dilutions of 1:40 and 1:100, approximately 3.3% of samples showed inhibition of the test organism. A large number of inhibitory samples were from patients receiving ampicillin. Patients in a psychiatric hospital, although being treated with a variety of drugs, showed a much lower incidence of inhibition.

A prospective study has confirmed that the *L. leichmanii* used in vitamin B₁₂ assay is often inhibited in the course of ampicillin therapy. In this investigation the phenomenon was observed in approximately 60% of the patients receiving ampicillin.

The stage at which inhibition was observed was also variable. In some it was present within three days, but in others it was not manifest until the fifth day. Alternatively inhibition may appear within three days, only to disappear by the fifth. The degree of inhibition also varies. It may be complete in both dilutions as tested, or may only become apparent as the concentration of serum is increased. The volumes used in this method, namely 0.1 and 0.25 ml, appear to be appropriate, otherwise partial inhibition at one level could be misleadingly interpreted as a low value. Even when two volumes are used partial inhibition may still be difficult to assess, especially over the lower range of vitamin B₁₂ levels. We have, in fact, noticed that the low levels obtained in proven examples of pernicious anaemia showed higher readings in the more diluted serum sample. This may be related to the observation that traces of serum usually depress the growth of *E. gracilis* (Anderson, 1964). The additional cases reported showed that inhibition can occur even with the high vitamin B₁₂ levels induced by cytamene therapy. Occasional instances occur where no history of drug therapy can be obtained. If the B₁₂ levels are below the normal range, then other steps must be taken to exclude true deficiency. Thus the results of *L. leichmanii* assays are not reliable as a solitary index of vitamin B₁₂ deficiency in large surveys.

The range of blood ampicillin levels that might be expected within 30 minutes of intramuscular injection (as supplied by Beecham Limited) determined the levels used in our tests *in vitro*. These confirmed that ampicillin-induced inhibition occurs at the expected therapeutic blood levels 2 to 6 µg per ml. Sera from patients, free from inhibition before ampicillin therapy, showed a degree of inhibition similar to that seen *in vitro* on the addition of comparable concentrations of ampicillin. It is of interest to speculate on the mechanisms that might account for inconstancy of inhibition *in vivo*. Presumably this is not a simple penicillin versus organism equation. Although among the penicillins ampicillin shows the least degree of protein binding, a variable degree of binding may be one possible explanation.

Boczrow (1961) found that *L. leichmanii* showed a sensitivity to penicillin similar to that of the Oxford staphylococcus. He examined five patients being treated with penicillin, and, although he did not specify the type of penicillin, or the time of sampling, he found complete inhibition with serum dilutions of 1:20 and 1:50. Boczrow further stated that even low concentrations of penicillin invalidated the B₁₂ result in practically every case. We have confirmed the occurrence of inhibition with ampicillin but have noted inexplicable exceptions.

Watts (1967) did not specify the heating procedure adopted, although in the method supplied by the Danochemie Company the diluted serum is steamed for 30 minutes. This is the procedure we adopted. Our results are in conflict with those of Watts, who confirmed the sensitivity *in vitro* of *L. leichmanii* to penicillin (and many other antibiotics), but claimed that dilution, heat, and precipitation of protein removed the antibiotic.

Ross (1952) showed that several antibiotics, including penicillin, had no effect on *E. gracilis* when added *in vitro* in concentrations comparable to those found during treatment. We have confirmed that this also applies *in vivo*. The radioisotope method is also unaffected.

Herbert, Gottlieb, and Altschule (1965) reported chlorpromazine inhibition of *E. gracilis* in vitamin B₁₂ assays. In the correspondence that followed, Forshaw and Harwood (1966) showed that this did not occur when *L. leichmanii* was used as the test organism. Davis and Nicol (1966) thought that there might be differences in the effect of Largactil and Thorazine, the latter being the trade name under

which chlorpromazine is sold in the United States. However, they failed to show inhibition with either, although it is not clear which test organism was used. Our results confirm those of Forshaw and Harwood with the *L. leichmanii* assay and suggest that this is a valuable routine method in a psychiatric hospital (of 2,000 beds) where chlorpromazine and other psychotropic drugs are used extensively.

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