Studies on a Long-acting Vitamin B_{12} Preparation

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ABSORPTION of orally administered vitamin B_{12} is very limited and dependent upon a number of variables. For most therapeutic purposes, therefore, parenteral administration of vitamin B_{12} has proved more satisfactory.

However, the very rapid absorption and urinary excretion of vitamin B_{12} by parenteral administration[1] is considered to be a limiting factor in its therapeutic usefulness. The desirability and need for a parenteral vitamin B_{12} with delayed absorption properties has been recognized by ourselves and others.[2]

Preliminary studies toward the objective of a delayed-absorption preparation of vitamin B_{12} compared the effects of several known injectable media on absorption-excretion behavior of vitamin B_{12}. However, the degree of absorption control desired was not achieved by any of the media studied. Attention was then focused on an earlier observation that vitamin B_{12} could be complexed and insolubilized by an interaction of cyanocobalamin, zinc, and tannic acid. The repository or long-acting properties of this complex, cyanocobalamin zinc tannate‡ (Depinar§), are evaluated in the present study.

The absorption-excretion studies with this slow-absorbing vitamin B_{12} complex revealed information with possible wide implications in relation to previous concepts of vitamin B_{12} therapy.

MATERIALS

The vitamin B_{12} used in preparing the various compositions studied was crystalline cyanocobalamin, U.S.P. Comparative studies were conducted with (a) aqueous solution of vitamin B_{12}; (b) vitamin B_{12} in a concentrated (32 per cent), partially hydrolyzed gelatin vehicle; (c) vitamin B_{12} suspended in sesame oil thickened with 2 per cent aluminum monostearate; and (d) vitamin B_{12} complexed to a very insoluble form with zinc and tannic acid. The latter is referred to generically as cyanocobalamin zinc tannate, and in the text of this paper will be abbreviated as CZT. The complex was prepared in lyophilized form in multiple-dose vials, which upon reconstitution (with 5 ml sterile saline or water) provides an aqueous suspension of the insoluble complex containing the equivalent of 500 μg vitamin B_{12}, 1.2 mg zinc, and 2.6 mg tannic acid per ml. The finished suspension also contains methyl and propyl Paraben as preservatives and cysteine as antioxidant.

METHODS

Absorption-excretion studies were conducted in rats by ourselves and in humans by Best[3] and Chow.[4]

Rat Studies

Groups of eight rats (Sprague-Dawley strain) weighing 100–150 g were injected subcutaneously with 1.0 ml (500 μg vitamin B_{12}) of the appropriate preparation per rat. Pooled urine collections were made at suitable intervals for about two weeks or more. Urinalysis for vitamin B_{12} content provided an estimate of daily urinary excretion of vitamin B_{12}. We used the large (human-size) dose in rats in order to provide a more likely index as to the probable behavior of a similar dose at the human injection site. Such a large dose could

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‡ Patent pending.

§ Depinar is a registered trademark of the Armour Pharmaceutical Company, Kankakee, Illinois.

be given because of the complete lack of toxic reactions. This large dose (for rats) also provided sufficient daily urinary excretion of vitamin B_{12}, even from the CZT, to permit chemical determination of the vitamin B_{12} in urine, and thereby provided an index of the absorption-excretion rate behavior of the human-size dose. Pooled urine samples from groups of eight rats each were analyzed for vitamin B_{12} content by the method of Van-Melle.[5]

Human Studies

Similar absorption-excretion studies were carried out in normal subjects by Best[3] and Chow.[4] Urine samples were analyzed for vitamin B_{12} content by microbiologic methods. Vitamin B_{12} serum level determinations were conducted by Chow[4] in which the serum samples were analyzed for vitamin B_{12} content by the microbiologic technique outlined by Gaffney *et al.*[6]

RESULTS

Several experimental lots of CZT have been studied by means of the rat excretion test, and similar experiments were performed with vitamin B_{12} in an isotonic saline solution. Table I shows a summary of the rat excretion data obtained with vitamin B_{12} in an aqueous saline solution and with three separate lots of Depinar. The data on the individual lots demonstrate good reproducibility of absorption characteristics, while the average data from the three lots provide the best estimate of the average absorption rate in rats. Analysis of pooled urine samples resulted in data which allowed only very limited statistical evaluation. Figure 1 illustrates the comparative excretion

TABLE I

Vitamin B_{12} Urinary Excretion (μg) Studies in Normal Rats Receiving Subcutaneously 500 μg Vitamin B_{12} in Saline or Cyanocobalamin Zinc Tannate (CZT)

Time after injection	Vitamin B_{12} in saline	CZT			Average ± Std. Err.
		Lot A	Lot B	Lot C	
0–6 hr	313[a]	49	52	58	53 ± 5.0
6–24 hr	23	20	18	22	20 ± 2.2
24–48 hr	5	57	21	38	39 ± 19.1
3rd day	1.3	—	—	—	—
4th day	0	44	38	47	43 ± 5.0
6th day	0	14	19	20	18 ± 3.5
8th day	0	5	9	6	7 ± 2.4
10th day	—	3	5	4	4 ± 0.8
12th day	—	1.6	4.3	1.6	2.5 ± 1.7
14th day	—	1.6	3.0	1.4	2.0 ± 1.0
20th day	—	—	1.4	—	1.4

[a] Average μg per rat; from pooled urine of 8 rats.

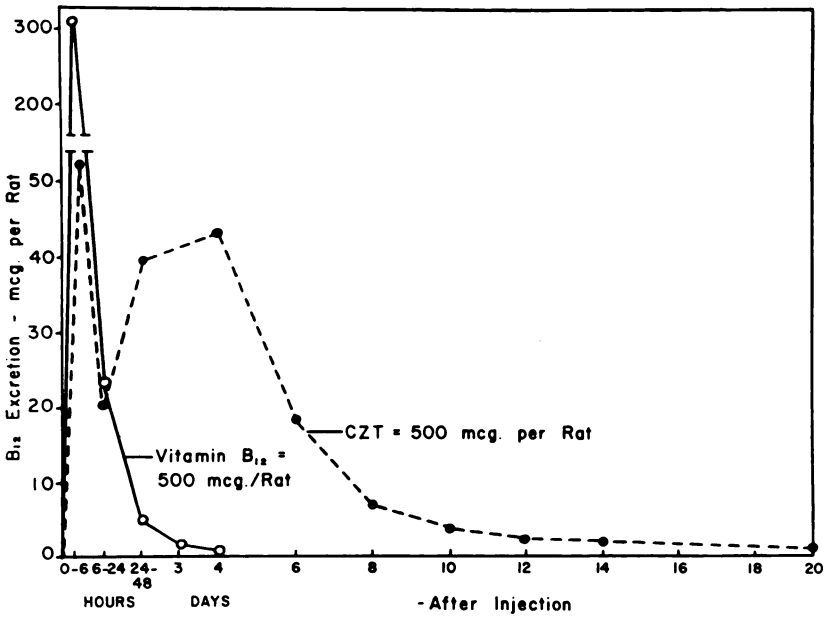

Fig. 1. Urinary B_{12} excretion after subcutaneous injection of vitamin B_{12} and CZT in rats.

TABLE II

Vitamin B_{12} Urinary Excretion Studies in Humans Receiving Intramuscularly Vitamin B_{12} (in Saline) or CZT

Hours after injection	Vit. B_{12} excreted (μg)[a] after injection			
	1000 μg vit. B_{12} in saline i.m.	500 μg vit. B_{12} in saline i.m.	CZT equiv. to 500 μg B_{12}	CZT equiv. to 250 μg B_{12}
	No. subjects			
	5	10	5	5
0–24	753 ± 43	340 ± 25	0	0
24–48	84 ± 12	0	2.4 ± 1.6	0.5 ± 0.3
48–72	1 ± 0.2	0	2.2 ± 0.6	1.5 ± 0.3
72–144	0.1 ± 0.03	0	0.7 ± 0.1	0.8 ± 0.3
144–168	0.1	0	0.2 ± 0.1	0.3 ± 0.2
Total μg excreted	838.1 ± 44	340 ± 25	5.5 ± 1.4	3.1 ± 0.5
% Excreted in urine	83.8 ± 4.4%	68 ± 5%	1.1 ± 0.3%	1.2 ± 0.2%

[a] Average ± Standard error.

rates obtained with vitamin B_{12} in saline and CZT.

Table II summarizes the urinary excretion data in humans utilizing vitamin B_{12} in saline in doses of 1000 and 500 μg, and CZT in doses equivalent to 500 and 250 μg vitamin B_{12}, respectively. The urinary excretion of the vitamin was very high after injection of the aqueous form, as contrasted with the very low excretion of the vitamin following injection of the repository form.

Figure 2 graphically illustrates the comparative urinary excretion in humans following the injection of vitamin B_{12} in aqueous saline solution, in concentrated gelatin, in sesame oil-aluminum monostearate, and in the insoluble complex CZT. The last preparation is the only one of those tested which, for practical purposes, showed almost no urinary excretion, indicating almost complete retention of the 500 μg of the vitamin by each of the human subjects. Statistical analysis of the data in Figure 2 was not attempted because of the small number of subjects involved in the tests. The results from vitamin B_{12} in saline, in sesame oil-aluminum monostearate, and in gelatin agree quite well, however, with those of Aaron *et al.*[7] Injection of vitamin B_{12} suspended in sesame

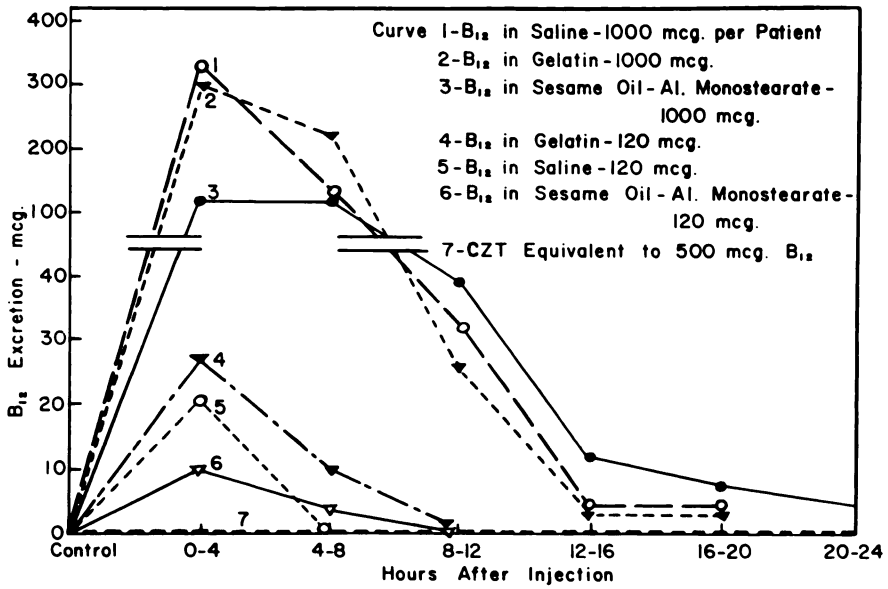

Fig. 2. Urinary vitamin B_{12} excretion after intramuscular injection in humans. Results from one or two subjects for each curve, 1–6; five subjects for curve 7.

oil-aluminum monostearate produces somewhat prolonged excretion of the vitamin, but total loss by urinary excretion is much greater than with injection of CZT. A gelatin medium apparently has very little effect on the absorption excretion behavior of vitamin B_{12}.

TABLE III

Vitamin B_{12} Serum Levels of Subjects Receiving Vitamin B_{12} or CZT by Intramuscular Injection

Days after injection	Vit. B_{12} serum levels ($\mu\mu g$/ml)[a] after injection: 1000 μg vit. B_{12} in saline i.m.	500 μg vit. B_{12} in saline i.m.	CZT equiv. to 500 μg B_{12}	CZT equiv. to 250 μg B_{12}
	No. subjects: 5	10	5	5
0	304 ± 36	340 ± 31	332 ± 61	249 ± 39
1/4	—	8026 ± 1691	—	—
1	> 9000	1728 ± 184	612 ± 138	516 ± 70
3	—	1164 ± 138	—	—
4	851 ± 67	—	868 ± 71	847 ± 106
6	—	555 ± 70	—	—
8	680 ± 78	—	1200 ± 97	1153 ± 155
9	—	446 ± 50	—	—
12	455 ± 30	—	793 ± 90	874 ± 100
16	—	—	744 ± 37	659 ± 93
21	312 ± 46	—	1060 ± 211	—
28	300 ± 37	—	692 ± 90	—

[a] Average ± Standard error.

Table III summarizes the data from vitamin B_{12} serum level studies conducted concurrently on the same subjects which provided the urinary excretion data in Table II. The temporary high serum levels obtained by vitamin B_{12} in aqueous saline were coincident with the period of high urinary excretion (see Table II). In contrast, CZT provided a marked early and sustained elevation in serum vitamin B_{12} level without producing appreciable loss by urinary excretion. The serum level of vitamin B_{12} was still significantly elevated 28 days after the 500 μg dose of CZT, while a 1000 μg dose of aqueous vitamin B_{12} resulted in the decline of the serum level to control values within about 12 days following injection. (With a 500 μg dose of aqueous vitamin B_{12}, serum levels were elevated for approximately 9 days.) The 250 μg dose of CZT provides and sustains a significantly elevated serum vitamin B_{12} level for a longer period than does the 1000 μg aqueous vitamin B_{12} dose. These comparisons are shown graphically in Figure 3.

Tissue uptake studies were conducted in rats. Radioactive vitamin B_{12} uptake was evaluated in various tissues, and the results obtained with radioactive vitamin B_{12} and radioactive CZT are shown in Figure 4.[4] Tissue uptake of radioactive vitamin B_{12} was greater from injection of CZT than from injection of an identical dose of vitamin B_{12} in saline.

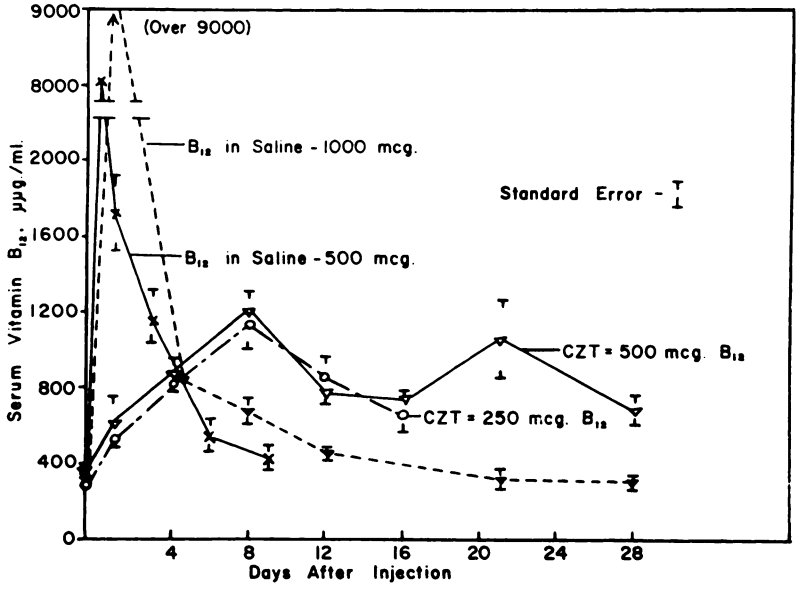

Fig. 3. Vitamin B_{12} serum levels in humans after intramuscular injection of vitamin B_{12} or CZT.

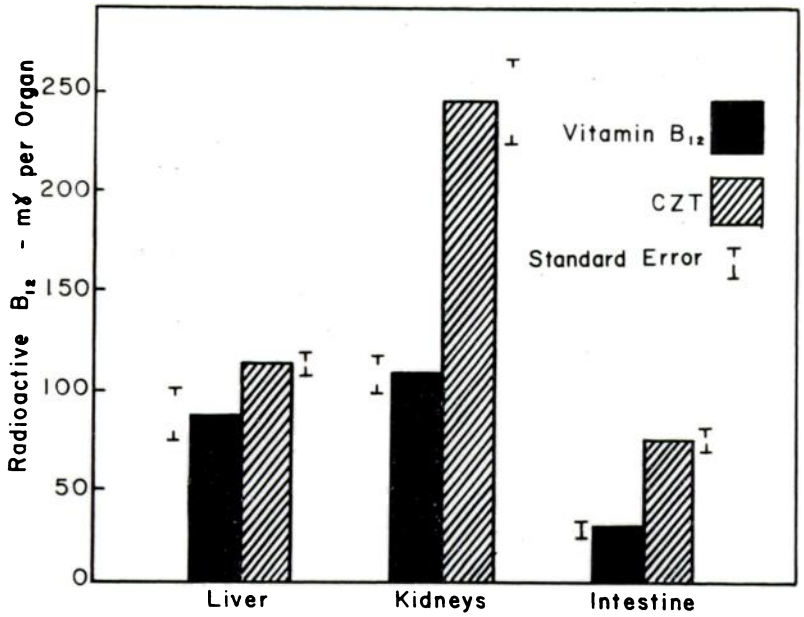

Fig. 4. Comparative tissue uptake after injection of radioactive B_{12} in saline solution and as the aqueous suspension of CZT. Normal rats sacrificed 8 days after injection of equal dosages of 0.84 μg radioactive vitamin B_{12} per rat; five rats in each group.

DISCUSSION

It has been shown that a new repository form of vitamin B_{12}, CZT, allows the administration of large doses (500 μg) of vitamin B_{12} without appreciable loss by urinary excretion. In human subjects the slowly absorbed vitamin B_{12} produces a marked elevation in the serum level of vitamin B_{12}, which is sustained for 28 days or longer following a single 500 μg injection. A certain portion of the vitamin B_{12} (about 10–15 per cent) in the CZT suspension is rapidly absorbed, as shown by the rat excretion during the first six hours after injection (see Fig. 1).

Studies designed to reflect duration of absorption of CZT suggest that serum level elevation in humans outlasts the period of absorption, which finding, in turn, suggests that the sustained serum level elevation of the vitamin is a reflection of the increased "body pool" of vitamin B_{12} accomplished by tissue uptake of nearly all of the vitamin injected.

The serum values for vitamin B_{12} are expressed in micromicrograms per milliliter, and a serum level value of 600 μμg/ml can only account for about 6 μg circulating vitamin B_{12}, as compared with about 500 μg injected. Most of the vitamin B_{12} is probably taken up by the various tissues. This view is supported by the tissue uptake studies in rats wherein the slowly absorbed CZT resulted in greater concentration of the injected vitamin B_{12} in liver, kidneys, and intestinal muscle than did equivalent doses of aqueous vitamin B_{12} (see Fig. 4).

Microbiologic assays of autopsy material by other workers[8, 9] has provided an estimate of average total vitamin B_{12} in the human body to be about 3900 μg; range 790 to 11,100 μg.

The total "body pool" of vitamin B_{12} is predominantly in the tissues, with only a minute fraction circulating in the blood stream. It is probable that the vitamin B_{12} in the tissues is largely functional rather than being present as storage of excess vitamin. It may be questioned as to whether even "average" or "normal" body pool vitamin B_{12} is necessarily optimal. Our studies show that normal subjects can retain large amounts of additional vitamin B_{12} and can even maintain serum levels several times so-called normal values without significant urinary excretion.

The capacity for additional vitamin B_{12}

retention by "normal" humans as demonstrated by our studies with CZT suggests that previous concepts of vitamin B_{12} therapy should perhaps be re-examined. It would now seem more logical that therapeutic application of vitamin B_{12} should consist of four to eight injections of CZT (500 μg vitamin B_{12} per injection) at one- to four-week intervals as convenient, to provide the opportunity for repletion of the body pool of at least 2000–4000 μg vitamin B_{12}. Such dosage of CZT could serve therapeutic, diagnostic, and insured vitamin B_{12} availability objectives.

Diagnostic and Therapeutic Applications

For diagnostic purposes, even serum level vitamin B_{12} determinations might be misleading because of the small fraction (about 0.1 per cent) of total body vitamin B_{12} in the circulating blood (about 3–4 μg vitamin B_{12} in the total blood volume on the average, against a body pool of about 4000 μg in the tissues). A slight shift in this equilibrium could theoretically cause large differences in serum level vitamin B_{12}, whether the body pool is high or low.

Due to limited absorption of vitamin B_{12} on oral administration and because of the rapid absorption and excretion of large doses of ordinary parenteral aqueous vitamin B_{12}, these modes of administration could not be expected to accomplish the above objectives for therapeutic purposes. The administration of CZT, however, appears to simulate a continuous intramuscular infusion of vitamin B_{12}, supplying the body at about the optimal rate to allow for almost total uptake by the body tissues without significant loss by urinary excretion. CZT provides long, sustained therapeutic dosage of vitamin B_{12} at the cellular level, as illustrated by the serum level and tissue uptake studies.

The above-discussed concepts of CZT therapy are especially significant as they relate to the possibility of increased beneficial effects in the fields of mental health,[10] geriatrics,[11] pregnancy,[12,13] neuropathies associated with pernicious anemia and diabetes, liver disease, alcoholism, and others. Clinical studies in these fields are planned. Initial clinical studies[14] in pernicious anemia have confirmed that vitamin B_{12} supplied in the form of CZT retains its biologic activity equivalent to vitamin B_{12} itself. Microbiologic specificity likewise is unimpaired. Preliminary studies involving use of CZT in multiple sclerosis have been reported by O'Connor *et al.*[15]

SUMMARY

Vitamin B_{12} was converted to an insoluble zinc tannate complex. Subcutaneous injection in rats caused continuous absorption and urinary excretion of vitamin B_{12} for two weeks from a single dose equivalent to 500 μg vitamin B_{12}. A similar dose injected intramuscularly in humans showed urinary excretion of less than 2 per cent of the injected dose. Serum level vitamin B_{12} was promptly elevated by several times that of control values, and these levels (above control value) were sustained for 28 days or more from the single dose.

Certain implications of these findings are discussed in relation to previous concepts of vitamin B_{12} therapy. The increased capacity of CZT injection to provide "body pool" vitamin B_{12} repletion and long-sustained elevated vitamin B_{12} serum levels makes it possible to study vitamin B_{12} therapy of a type not attainable with previous preparations of the vitamin, oral or parenteral. Clinical indications and areas for studying the therapeutic potential of CZT are cited.

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You must always be students, learning and unlearning till your life's end, and if, gentlemen, you are not prepared to follow your profession in this spirit, I implore you to leave its ranks and betake yourself of some third-class trade.—Joseph Lister