Existence of vitamin B12 analogs in biological samples: A reality

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Due to the fact that the existence of vitamin B12 analogs in biological materials has been much debated, we realized on plasma, bile, and feces series of experiences to demonstrate that analogs assayed by radioisotopic method in these biological media using intrinsic factor and haptocorrin as binders could not be extraction products or dosage artifacts. To this purpose, exposure to light was used to transform all corrinoids (true B12 + analogs) to their hydroxo forms. After that all corrinoids were transformed with KCN by cyanilation to their cyano forms. This permitted us to separate corrinoids on high performance liquid chromatography into only two peaks. These peaks will be subsequently identified by radioisotopic dilution assay as cyanocobalamin α and/or β cyanocobinamides. Because the binding ability of an intrinsic factor to corrinoids is limited to its cobalamin forms, our method enabled us to emphasize the existence in biological materials of corrinoids that only recognize haptocorrin (analogs of vitamin B12) and not intrinsic factors.

Keywords: vitamin B12; analogs; analysis; methodology

Introduction

Two proteins can bind vitamin B12 at the level of the digestive tract: haptocorrin (Hc) and gastric intrinsic factor (IF).[1,2,3] Corrinoids represent a family of tetrapyrrol components. One of these components is vitamin B12 (cobalamin). Only cobalamin forms hydroxo (OH-cbl), cyano (CN-cbl), deoxyadenosyl (Ado-cbl), and methyl (CH3-cbl) bind to IF.[4,5] We call analogs, corrinoids that bind Hc and not IF or have a better affinity for Hc analogs. Total corrinoids represent the sum of cobalamin and analogs.

Among analogs, we must distinguish cobinamides devoid of nucleotide from cobamides that possess, compared with cobalamins, a modified benzimidazol in the α position with regard to the tetrapyrrol plane.[6,7] Cobinamides do not bind IF. Cobamides have a lower affinity for IF compared with Hc.

Corrinoids are only synthesized by microorganisms, i.e., bacteria, yeasts, or algae.[8] Bacteria present in the digestive flora synthesize them.[9,10] High levels of cobalamin analogs can be associated with neurologic abnormalities in cases of cobalamin deficiency.[11] In human[12] and animal tissues,[13] cobalamin analogs are found and can inhibit growth and development.[14] These observations are at the origin of the development of analysis techniques for the evaluation of these analogs. Beside the laborious bioautographic techniques, there are numerous publications using a radioisotopic dilution assay (RIDA) for both cobalamin and vitamin B12 analogs,[12,15] as well as more elaborated techniques using high pressure liquid chromatography (HPLC) for the identification of the different forms of cobalamin and analogs.[16,17,18] We recently published a technique associating HPLC and RIDA applied to biological material,[19] and another study comparing HPLC and high performance capillary electrophoresis.[20]

Techniques developed with RIDA have been questioned several times, as they are founded on the utilization of affinity reactions whose constants are unknown.[21,22] Moreover, some reagents used for preliminary extraction of corrinoids could, under certain conditions, transform cobalamin into other corrinoids forms and modify its affinity towards the IF binding protein. Van Kapel[17] has shown that OH-cobalamin, in the presence of sodium azide, was transformed into a corrinoid whose retention time in HPLC was close to that of ado-cobalamin. Further, Gimsing[22] has shown that when dithiothreitol was added, the affinity of cobalamins for IF was decreased.

The aim of this study was to show that vitamin B12

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analogs that appear after HPLC are not measurement artifacts, but correspond effectively to corrinoids having no affinity for IF. Two types of studies have been developed; one of them consisted of a transformation of all present corrinoids (cobalamins + analogs) in their CN-forms to obtain a better identification of corrinoids present after HPLC, by differentiation of "true vitamin B12" and analogs. The second study analyzed the binding ability of corrinoids for both carriers, IF and Hc, to prove the existence of corrinoids having no affinity with IF.

Methods and materials

Chemicals

Crystalline OH-Cbl, CN-Cbl, MeCbl, and AdoCbl were obtained from Sigma (St. Louis, MO USA), crystalline CN-Cobinamide from Calbiochem (La Jolla, CA USA), and CN (^{57}Co) Cbl (specific activity 220 μCi/μg) from the Radiochemical Center (Amersham, France). Cadmium acetate, glacial acetic acid, triethanolamine, *tert.*-butanol, methanol, acetonitrile, Amberlite XAD2 and RP18 column (5 μm silica, 250 mm length × 5 mm internal diameter) were obtained from Merck (Darmstadt, Germany). Sep Pak C18 cartridges were purchased from Waters Associates (Milford, MA USA). IF and protein R (non IF) were obtained from porcine gastric mucosa (respectively, ref. I-6006 and N-1883 from Sigma).

Biological fluids

All samples were handled in the dark or in dim-red illumination. Plasma was separated from the whole blood collected on EDTA (1 mg/mL), within 1 hour after puncture. Bile and feces were collected with no additive. Bile was collected by T-tube draining of the common bile duct of hospitalized patients after surgery on the biliary tract.

Extraction of corrinoids

Corrinoids from plasma, bile, and feces were extracted with hot ethanol to denature and precipitate corrinoids binding proteins as described.[19] To avoid the nonspecific binding of OH-Cbl to thiol and/or histidine residues of proteins, the samples were pre-incubated for 2 hours at room temperature with an excess of cadmium acetate (0.2 mol/L).[23]

Reverse phase HPLC

Before HPLC, the extracts of plasma were desalted on a Sep Pak C18 cartridge as described previously.[24] Bile and feces were desalted by two successive treatments with Amberlite XAD2 and with Sep Pak C18.[19]

HPLC was performed at room temperature using a two-pump gradient system (Gilson, Villers-le-Bal, France) and a detection at 365 nm using an Holochrome (Gilson) spectrophotometer. Evaporated extract was dissolved in 0.2 mL of bidistilled water and filtered through a 0.45 μm filter (Millipore, Illkirch, France), before injection of the whole volume. Corrinoids were separated on a RP18 column. The elution mobile phases consisted of 0.085 M phosphoric acid titrated to pH 3.0 with triethanolamine (phase A) and acetonitrile (phase B). A 10–50% linear gradient of phase B was performed in 20 min at a flow rate of 0.5 mL/min. The effluent was collected by 0.5 min fractions, each containing 200 μL borate buffer for raising the pH (0.082 mol/L *di*-sodium tetraborate (10 H_2O), pH 9.20, 0.02% bovine serum albumin (BSA) (wt/vol).

Radioisotope dilution assay

The concentration of corrinoids was determined in duplicate in the HPLC fractions using a RIDA described previously[19] and adapted from Kolhouse.[12] The fractions eluted from HPLC were evaporated to dryness and redissolved in 500 μL of bidistilled water. One hundred μL of the sample were needed per test tube. The test tubes were protected from light during the assay. Test tubes containing HPLC fractions and CN-cobalamin dilutions used as a standard curve were extracted at 100° C for 15 min after adding 24 fmoles of CN (^{57}Co)Cbl in 1 mL of 0.082 M *di*-sodium tetraborate (10 H_2O) buffer (pH 9.2) containing 0.02 M NaN3, 0.1 M urea, 0.3 mmol/L KCN, 0.3% thioglycerol, 0.05% DTT (wt/vol) (pH 9.2), and 0.02% BSA (wt/vol).

A hemoglobin-coated charcoal suspension was used to adsorb the free corrinoids and to remove them by centrifugation at 2000*g* for 15 min as described.[25] The results obtained with IF-RIDA were considered to represent the "true Cbl" concentration and those obtained with Hc-RIDA the total corrinoid concentration. The standard curve of the two RIDAs was established using six solutions with respective CN-Cbl concentrations of 55, 110, 220, 440, 886, and 1770 pmol/L.

Effect of dithiothreitol and sodium azide on cobalamins was checked. For this purpose, RIDA was performed with and without these two components. The study was realized on six plasma samples.

Transformation of corrinoids by light exposure and KCN treatment

Standards of OH-, CN-, Ado-, CH3-cobalamin, and CN-cobinamides, as well as samples extracted from plasma, bile, and feces were exposed to a 100-watt tungsten light at a distance of 50 cm for 90 minutes. Standard concentrations were of 15 μmol/L. Dilutions of biological samples were those of the samples before HPLC analysis. Under these conditions, Ado- and CH_3- forms were transformed into the OH-form.

After this first step, different preparations were incubated for 90 minutes with 0.2 M 10^{-2} KCN to transform OH-forms into CN-cobalamin and CN-cobinamide. The treated samples were then analyzed in HPLC, and the binding capacity of present corrinoids toward the carriers IF and Hc was measured in the isolated fractions.

Results

Test of stability of corrinoids

To plasma with a low B12 and analogs level were added individually the different forms of corrinoids: OH-, CN-, ado-, CH3- cobalamin and CN-cobinamide. An extraction with ethanol was then carried out in the same standard conditions. After centrifugation, the supernatant was analyzed in HPLC, and corrinoids measured in fraction after RIDA. For each cobalamin form, only one peak appeared whose retention time was that of the standard form (*Table 1*). When CN-cobinamide was added to the plasma, two peaks were identified, their retention times corresponded to those of α and β- CN-cobinamides.

There was no denaturation of cobalamins, at least as far as their affinity with IF is concerned. This stability has been verified at each step of extraction procedure: ethanol extraction, Sep-pak, chromatography, and HPLC followed by RIDA.

Table 1 Percent corrinoids having an affinity with IF in the different steps of the analysis.

Corrinoid	% true B12 after extraction	% true B12 after Sep-pack	% true B12 after HPLC	RT (min.) after HPLC	RT (min.) standards
OH-Cbl	97 ± 2	99 ± 4	95 ± 3	14.5 ± 0.5	14.7 ± 0.5
CN-Cbl	100 ± 1	99 ± 1	96 ± 2	17.2 ± 0.2	17.2 ± 0.3
Ado-Cbl	98 ± 2	97 ± 5	97 ± 2	18.5 ± 0.3	18.7 ± 0.4
CH_3-Cbl	100 ± 0	97 ± 3	98 ± 2	20.4 ± 0.4	20.6 ± 0.5
CN-Cobinamide	0 ± 2	1 ± 1	2 ± 2	16.0 ± 0.5	15.9 ± 0.5
				18.3 ± 0.5	18.5 ± 0.2

The initial B12 value of the plasma was not taken into account. RT = retention time. Each corrinoid analysis was performed in triplicate. True B12 was defined as corrinoids detected by IF-RIDA.

Effect of dithithreitol (DTT) and sodium azide (NaN_3)

Effect of DTT and NaN_3 on cobalamin extraction and RIDA is indicated in *Table 2*. No significant differences were observed between vitamin B12 or total corrinoids level with or without DTT and NaN_3.

Transformation of corrinoids after light exposure and KCN treatment

Development on corrinoids standards Light exposure of corrinoids standards enabled us to produce, under our experimental conditions, a complete transformation of ado- and CH_3-cobalamin forms into OH-cobalamin. The incubation with 0.2 10^{-2} M KCN provokes a transformation of OH-cobalamin into CN-cobalamin (*Figure 1*). There was no alteration in standards of CN-cobalamin and CN-cobinamide. pH conditions in HPLC led to the presence of two CN-cobinamide peaks, corresponding to the α and β CN forms.[16,24]

Application to biological samples In plasma, few analogs are present. Before treatment, several peaks were present in HPLC. They correspond to a function of their affinity toward IF and Hc, either to cobalamin forms, or to cobinamides, or to a mixture of both of them. After KCN treatment and light exposure, a main peak corresponding to CN-cobalamin, and two minor peaks, whose constituents only bound Hc, were obtained. The retention time of these two peaks were that of α and β CN-cobinamides (*Figure 2*).

In bile, analog levels vary considerably from one patient to another. Two extreme cases were chosen. The first bile presented a 3% analog level (bile #1), the second one, a 95% analog level (bile #2) (*Figure 3*). In the first case, before treatment two main peaks were found. Their retention times were that of ado and CH_3-cobalamin. They disappeared after treatment and were replaced by a new peak whose retention time was that of CN-cobalamin. In the second case, results before and after treatment were similar: there was a peak whose retention time was that of CN-cobinamide, this product was not transformed after treatment.

The identification of each peak was realized from its retention time and the binding capacity of its constituent toward IF and Hc carriers. In the first bile, corrinoids present recognized both two carriers, while in the second bile, corrinoids present, identified by the main peak, only recognized HC (*Figure 3*).

In feces, where the analog level is always very high, the action of light and KCN in the same standard conditions provoked a transformation of present corrinoids, and the appearance of only three peaks, whose retention times were that of CN-cobalamin and α and β CN-cobinamides (*Figure 4*).

Discussion

The checking of the composition of plasma supplemented with the different corrinoid forms (OH-, CN-, Ado-, CH3-cbl, and CN-cobinamide) showed that the different extraction and measurement steps did not produce artificial vitamin B12 analogs. In the same way, analogs were not transformed into vitamin B12. In fact, for each B12 form no corrinoid recognized Hc only.

This procedure showed that the whole handling of vitamin B12 enables true quantitative B12 values to be obtained, without any measurement artifacts. Moreover, when analogs were extracted alone (without B12) there was no binding with IF. All these observations validate the techniques used with regard to the specificity towards analogs.

Table 2 Effect of DTT and NaN_3 on cobalamin extraction and RIDA

	with DTT & NaN_3		without DTT & NaN_3	
	Vitamin B12 (pg/mL)	Total corrinoids (pg/mL)	Vitamin B12 (pg/mL)	Total corrinoids (pg/mL)
M ± S.D.	215 ± 86	233 ± 78	208 ± 85	229 ± 54

Mean values and standard deviations are expressed as M ± S.D. (n = 6).

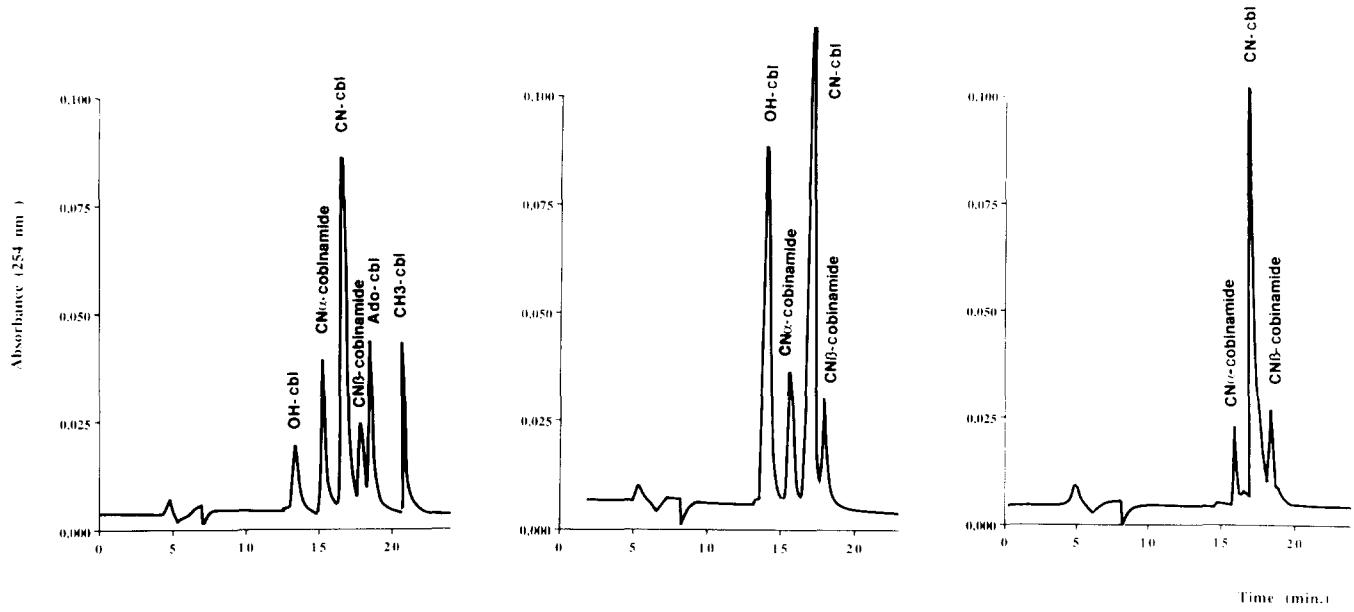

Figure 1 Effect of light exposure and 0.2M KCN treatment on the structure of corrinoids used as standards: OH-cbl, CN-cbl, Ado-cbl, CH3-Cbl, and CN-cobinamide. Under the present pH conditions, CN-cobinamide was composed of the two α and β forms. (Values used for all figures are means of three experiences performed on 3 different days).

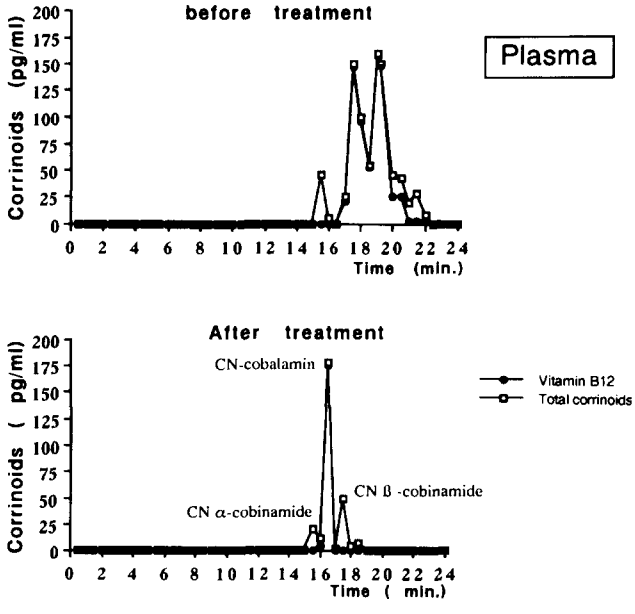

Figure 2 Effect of light exposure and 0.2M KCN treatment on corrinoids extracted from human plasma. Total corrinoids represent the sum of vitamin B12 plus analogs.

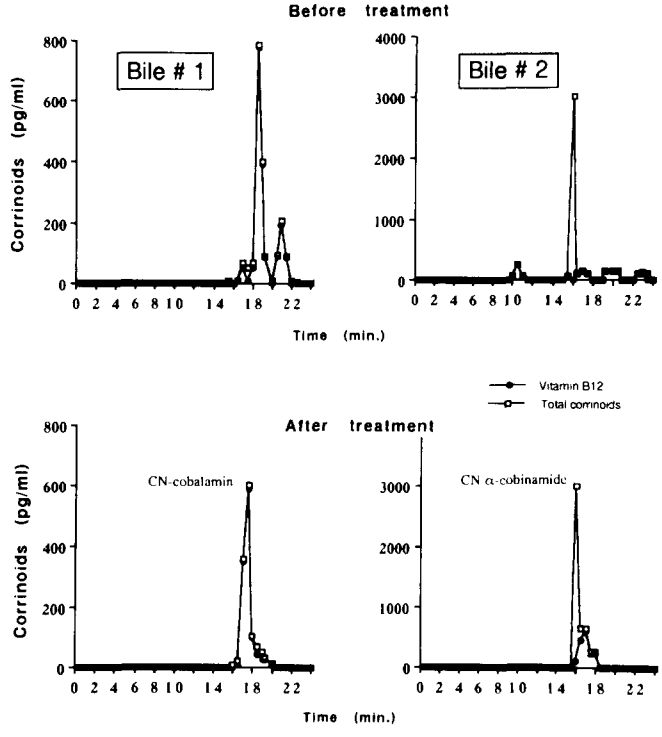

Figure 3 Effect of light exposure and 0.2M KCN treatment on 2 different human biles. Bile #1 was mainly composed of cobalamin. Bile #2 was composed of cobinamide. Total corrinoids represent the sum of vitamin B12 plus analogs.

HPLC fractions were collected each 0.5 minutes in a tube containing 200 μL 0.082 M dinatrium tetraborate-decahydrate buffer, 0.02 M bovine serum albumin, pH 9.2. Thus, a good discrimination between ado-cbl and CN-cobinamide was obtained, and the resolution improved compared with our initial procedure.[19]

The study of the composition of different biological samples (plasma, bile, feces) has shown that variable proportions of analogs can be observed. Plasma proportion is very low in normal subjects (5–15%). Analogs are the main constituent of feces corrinoids (70–90%). In this study, we describe two extreme cases of biles containing 3 and 95% analogs. These observations show

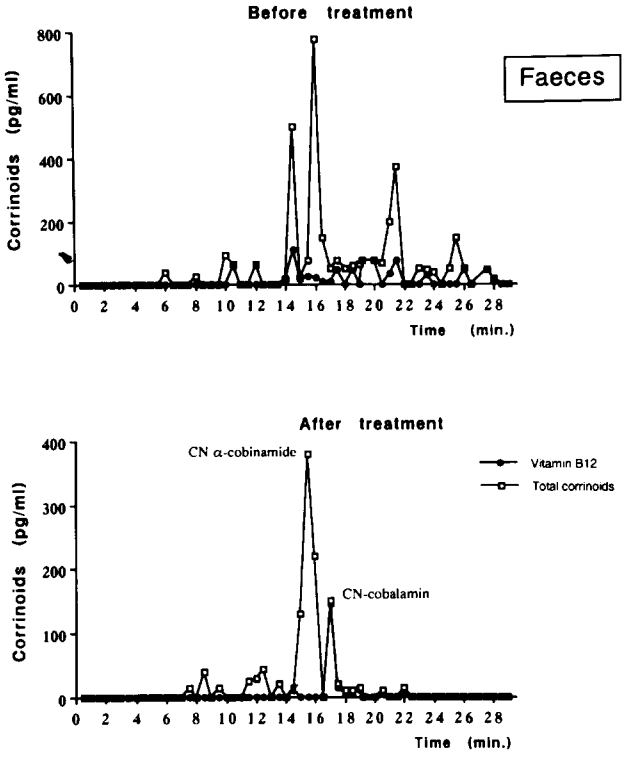

Figure 4 Effect of light exposure and 0.2M KCN treatment of human feces. Total corrinoids represent the sum of vitamin B12 plus analogs.

that the presence of analogs after extractions and RIDA cannot be due to an artifact, as they are not systematically present. These results confirm those obtained with the supplemented plasma and affirm the existence of vitamin B12 analogs in vivo.

Two constituents of the extraction medium included in the RIDA: (DTT),[26] and NaN_3,[16] could, respectively, alter the cobalamin structure and transform OH-cbl into a product having a retention time in HPLC close to that of ado-cbl. Our present results show that, in our usual extraction method (HPLC and RIDA conditions) there was no alteration in the B12 forms studied, at least until the HPLC step. The DTT and NaN_3 components intervene only in the last step, which is that of the RIDA. At this stage, each corrinoid has already been identified by its retention time, and the only perturbation that could alter the assay would be a transformation of true vitamin B12 into cobinamide. We demonstrated that this was not the case. An eventual transformation of OH-cbl into ado-cbl or other cbl form is not a problem, as all forms are voluntary transformed in the CN-cbl and CN-cobinamide forms by adding KCN to homogenize the affinity against carriers, at least for cobalamins. Anyway, to be sure that DTT and NaN_3 have really no effect, plasma was analyzed after RIDA, both in the presence and the absence of these two components. The results obtained were similar in both cases.

As far as bile is concerned, earlier studies have shown that corrinoid concentration was four times that of plasma, this difference being due only to analogs.[27] Herbert demonstrated in the human the existence of an enterohepatic circulation of corrinoids.[28,29] We also showed that bile takes part in the clearance of free analogs or those bound to Hc.[30]

Contrary to bile and feces, in which the only cobinamide peak, which occurred after light exposure and KCN treatment, had a retention time corresponding to α CN-cobinamide, the major cobinamide peak in plasma had a retention time similar to that of the β CN-cobinamide. The peak whose retention time was similar to α CN-cobinamide was very low. These results lead us to conclude that the partition α and β depends on the medium. We have shown that there is an equilibrium of the α to β ratio after HPLC as a function of the KCN concentration.

The existence of analogs has been previously demonstrated by other authors. Kolhouse[6] has shown that in the rabbit most analogs bound with a low affinity to IF, and their ileal capture and absorption was very low compared with cobalamin. Analogs that bind tightly to IF are captured by the ileum, but in most cases they remain at that location. At the blood level, most analogs bound with a great affinity to transcobalamin II, and they are all transferred towards tissues in the same way as cobalamin.

The transformation of cobalamin and cobinamide forms into CN-cbl and α and β CN-cobinamide in biological fluids like plasma, bile, and feces, followed by a RIDA, enable us to simplify the identification profile of corrinoids, and to better differentiate what binds IF (Cbl) from what only binds HC (analogs).

In conclusion, we demonstrated that corrinoids that do not recognize IF exist in biological material at variable concentrations. The fact that some corrinoids do not bind to IF is not due to an alteration in vitamin B12 during the different phases of extraction and chromatography, or during the subsequent RIDA.

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