PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :
G01N 33/82, 33/543, 33/58
G01N 33/547

A1

(11) International Publication Number: **WO 89/12826**

(43) International Publication Date: 28 December 1989 (28.12.89)

(21) International Application Number: PCT/US89/02193

(22) International Filing Date: 19 May 1989 (19.05.89)

(30) Priority data:
207,139 15 June 1988 (15.06.88) US

(71) Applicant: BECKMAN INSTRUMENTS, INC. [US/US]; 2500 Harbor Boulevard, Fullerton, CA 92634 (US).

(72) Inventors: OH, Chan, S. ; 23323 Wagon Trail Road, Diamond Bar, CA 91765 (US). STONE, Linda, J. ; 15554 Del Norte Avenue, Chino, CA 91709 (US).

(74) Agents: MAY, William, H. et al.; Legal Department, Beckman Instruments, Inc., 2300 Harbor Boulevard, Fullerton, CA 92634 (US).

(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP (Utility model), LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: VITAMIN B_{12} ASSAY

(57) Abstract

A competitive binding assay for vitamin B_{12} in a test sample gives a steep dose-response curve in the clinically significant concentration regions, yielding great accuracy. The assay uses immobilized vitamin B_{12} to compete with the vitamin B_{12} in the test sample for a binding partner. The quantity of the binding partner bound to the immobilized vitamin B_{12} can be measured and is inversely proportional to the concentration of free vitamin B_{12} in the test sample. The binding partner is typically biotinylated intrinsic factor prepared so that substantially all the molecules of intrinsic factor are biotinylated and the binding activity of the intrinsic factor for vitamin B_{12} is not diminished. The bound biotinylated intrinsic factor can then be determined by reacting it with avidin coupled to a reporter group. The reporter group is typically an enzyme such as horseradish peroxidase, but can be another detectable label. The immobilized vitamin B_{12} is typically bound to a solid support such as diazotized amine-derivatized beads, and can be bound through a protein linker. Other binding partners such as anti-vitamin B_{12} antibody are also usable. The binding partner can be labeled directly with the reporter group. The method is adaptable to the assay of serum, and methods of inactivating endogenous vitamin B_{12}-binding proteins in serum are described.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Fasso	HU	Hungary	NO	Norway
BG	Bulgaria	IT	Italy	RO	Romania
BJ	Benin	JP	Japan	SD	Sudan
BR	Brazil	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic			SN	Senegal
CG	Congo	KR	Republic of Korea	SU	Soviet Union
CH	Switzerland	LI	Liechtenstein	TD	Chad
CM	Cameroon	LK	Sri Lanka	TG	Togo
DE	Germany, Federal Republic of	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		
ES	Spain	MG	Madagascar		

VITAMIN B_{12} ASSAY

BACKGROUND

This invention relates to a vitamin B_{12} assay usable for determining vitamin B_{12} concentrations in body fluids such as serum.

Cyanocobalamin (vitamin B_{12}) is an essential vitamin in the human diet. It participates as a coenzyme in several reactions of intermediary metabolism, including the conversion of acetyl-coenzyme A to methylmalonyl-coenzyme A by the enzyme methylmalonyl-coenzyme A mutase in lipid metabolism, and the reduction of ribonucleotides to deoxyribonucleotides by ribonucleotide reductase, essential for the synthesis of DNA.

Low levels of vitamin B_{12} in a patient's blood serum are indicative of the disease pernicious anemia. This serious form of megaloblastic anemia also affects the digestive and nervous systems. The nervous system damage can lead to ataxia and psychotic symptoms. Before the identification of vitamin B_{12} as a vitamin in 1926, this disease was incurable and usually fatal. Although pernicious

anemia can be caused by a dietary deficiency of vitamin B_{12} from a strictly vegetarian diet, the most common cause is poor absorption of the vitamin from the intestine. This is caused by decreased synthesis of a mucoprotein called intrinsic factor (IF) by the stomach lining. Pernicious anemia, once diagnosed, is readily treated with doses of vitamin B_{12}.

Vitamin B_{12} has traditionally been assayed by microbiological assays using the growth response of organisms requiring the vitamin, such as the bacterium Lactobacillus lactis and the protozoan Euglena gracilis. Although such microbiological assays are still widely accepted in research and clinical laboratories, they are difficult to perform and time consuming. In recent years, attempts have been made to devise alternative vitamin B_{12} assays based on a competitive binding reaction between the vitamin B_{12} contributed by a test sample and a known quantity of labeled vitamin B_{12} as an assay reagent. The test sample vitamin B_{12} and the labeled vitamin B_{12} compete for a limited amount of binding partner for the vitamin B_{12}. Typical binding partners include binding proteins such as IF and protein R as well as antibodies raised against vitamin B_{12}. Competitive binding assays provide a quicker and equal reliable alternative to the traditional microbiological assays.

The particular binding protein used in a competitive binding assay for vitamin B_{12} can be important. In particular, some binding proteins whose use has previously been suggested are believed to bind cobalamin analogues as well as vitamin B_{12} itself. For example, it has been shown that transcobalamin II and R proteins bind to cobalamin analogues present in human serum as well as to vitamin B_{12}. This lack of specificity leads to erroneous results in vitamin B_{12} assays. It is generally accepted that IF is the preferred binding protein for use in competitive binding assays because it is highly selective for 5'-deoxyadenosylcobalamin, the physiologically active form of vitamin B_{12} in human serum.

Generally, assays based on competitive binding employ a labeled ligand or ligand analogue carrying a chemical moiety or group that can be readily identified or quantified. Such labels typically include radioactive isotopes, fluorescent groups, and enzymes. Radioactively labeled vitamin B_{12} derivatives have been the most commonly used ligand analogues in competitive binding assays for vitamin B_{12}, and several different isotopes have been successfully used.

The earliest isotope to be used successfully in such assays was ^{57}Co. Syntheses of ^{57}Co-labeled vitamin B_{12} is a tedious and complicated procedure, in particular because the single cobalt atom in the cyanocobalamin molecule lies at the center of its porphyrin ring structure and thus is relatively unreactive to chemical substitution, necessitating the incorporation of the isotope in the biosynthesis of vitamin B_{12}. The ^{57}Co-vitamin B_{12} produced by this procedure is thus relatively expensive and of low specific activity. The low specific activity necessitates relatively lengthy counting times of at least one minute in competitive binding assays using this labeled vitamin B_{12}. These lengthy counting times slow processing of samples and are undesirable.

Another approach has been to prepare ^{125}I-vitamin B_{12} derivatives. According to U.S. Patent No. 4,209,614 to Bernstein et al., such derivatives can be prepared by reacting vitamin B_{12} with a glutaric anhydride derivative that binds to the vitamin B_{12} via the sugar ring of the B_{12}. The glutaric anhydride-derived substituent is then iodinated.

Yet another process for preparing ^{125}I-vitamin B_{12} derivatives is described in U.S. Patent 3,981,863 to Niswender et al. Natural vitamin B_{12} contains a number of

amide substituents on its porphyrin rings. Mild hydrolysis cleaves some of these amide substituents to form a mixture of monocarboxylic acids containing mostly the (e)-isomer. The mixture of acids is then reacted with a p-(aminoalkyl) phenol to introduce a phenol group by reaction with one of the free carboxylic acid groups. The mixed substituent vitamin B_{12} derivatives are then iodinated on the phenol group substituent. These ^{125}I-labeled mixed derivatives are useful in the radioimmunoassay of vitamin B_{12}, using antibodies raised against the mixture.

Attempts have also been made to develop vitamin B_{12} derivatives that bind IF strongly. U.S. Patent No. 4,465,775 to Houts discloses that labeled derivatives formed from the (d)-monocarboxylic acid isomer of partially hydrolyzed vitamin B_{12} have a much greater affinity for IF than do the (b)- and (e)-isomer derivatives. Accordingly, these derivatives are preferred for use as labeled analogues in vitamin B_{12} competitive binding assays.

The use of radioactive compounds in these immunoassays presents many disadvantages. Extensive safety precautions must be taken in their storage, use, and disposal, such as use of lead shielding and special waste treatment procedures. Expensive equipment is needed for

radioactive counting. The isotope ^{125}I decays with a half-life of approximately 60 days. This radioactive decay not only reduces the amount of radioactivity available for detection, but also may initiate chemical reactions that damage the remaining reagents, reducing sensitivity further. Thus storage of ^{125}I-labeled reagents is limited to several months. These difficulties are magnified if the other commonly used iodine isotope, ^{131}I, is used to label. This isotope has a half-life of less than 9 days.

As a result of these disadvantages, there have been increasing efforts in recent years to develop alternative labeling schemes for vitamin B_{12} lacking them.

Attempts have been made to utilize enzyme-labeled vitamin B_{12} in competitive binding assays for vitamin B_{12}. Enzyme-labeled vitamin B_{12} derivatives are also prepared by labeling the monocarboxylic acid derivatives of vitamin B_{12}. Leonidas et al., Biotechniques 4, 42-55 (1986) suggest that the use of ligand-selective binding proteins such as IF can be superior to the use of antibody to bind vitamin B_{12} in such a competitive binding assay.

According to the procedure of Leonidas et al., IF is first immobilized on the surface of a solid support

material such as agarose beads. The immobilized IF is then contacted with the test sample containing vitamin B_{12} and allowed to incubate for a designated period of time. A given amount of the enzyme-labeled vitamin B_{12} is subsequently added to the reaction mixture, followed by a further incubation. Following the second incubation period, the solid phase is washed to remove unbound material and the amount of bound enzyme determined by adding the appropriate substrates for the enzymatic reaction. Where appropriate substrates are used, the amount of bound enzyme can be determined colorimetrically, typically by spectrophotometry. A change in absorbance results from the action of the enzyme on the substrate, and the extent of absorbance change is inversely proportional to the concentration of unlabeled vitamin B_{12} present in the test sample.

In a sequential binding assay of this type, the test sample vitamin B_{12} and enzyme-labeled vitamin B_{12} "compete" for the available binding sites on the immobilized IF. Because of the sequential addition, this assay is more properly described as "pseudo-competitive."

This enzyme-labeled sequential binding method of Leonidas et al. has not been found to produce consistently good data in a clinical environment. This dose-response

curve achieved with enzyme-labeled vitamin B_{12} was not as steep as that achieved in sequential binding assays for other analytes using comparable natural binding proteins for those analytes. Leonidas et al. theorize that the lower dose-response curve may be due in part to the impaired ability of immobilized IF to assume the proper three-dimensional conformation for folding around vitamin B_{12} once the IF has been bound to a solid support.

An additional assay using enzyme-labeled IF and vitamin B_{12} coated onto magnetic chromium dioxide particles has been disclosed by Wang et al. in a publication distributed at the American Association of Clinical Chemists Meeting in July 1987. In this assay, free vitamin B_{12} in the test sample competes with the vitamin B_{12} coated onto the particles for the intrinsic factor present. The chromium dioxide particles are then removed by application of magnetic force and the remaining supernatant assayed for enzymatic activity.

It would be greatly advantageous to have a competitive or pseudo-competitive binding assay for vitamin B_{12} that would be capable of producing consistently accurate data in a clinical environment and eliminate the problems seen by Leonidas et al.

SUMMARY

A competitive binding assay method for vitamin B_{12} in a test sample satisfies these needs. The essential feature of this assay method is the use of a binding partner for vitamin B_{12}, generally intrinsic factor, free in solution, and of an immobilized vitamin B_{12} derivative. Even though the same competition reaction occurs as in previous assays, the reversal of the roles of these reagents causes an unexpected improvement of the efficiency of the assay, especially within the clinically significant concentration ranges.

Another significant feature of many embodiments of the present invention is the use of a preparation of biotinylated intrinsic factor in which substantially all of the molecules of intrinsic factor are covalently linked to at least one biotin molecule, and in which the biotin molecules are coupled to the amino groups of the intrinsic factor. In this procedure, the biotin can be replaced by another molecule, such as a hapten, capable of being specifically recognized by a macromolecule carrying a "reporter group." The term "reporter group," as hereinafter used, includes enzymes, enzyme cofactors, enzyme inhibitors, enzyme

modulators, fluorescent labels, chemiluninescent labels, and electrolytically detectable labels.

Most generally, one version of the assay method comprises the steps of:

(1) combining a test sample containing vitamin B_{12} with:

(a) a binding partner for vitamin B_{12} capable of subsequently binding a reporter group; and

(b) sufficient immobilized vitamin B_{12} that substantially all of the binding partner is bound by the immobilized vitamin B_{12} in the absence of vitamin B_{12} in the test sample; and

(2) then determining the quantity of the binding partner bound to the immobilized vitamin B_{12}, the quantity of vitamin B_{12} in the sample being inversely proportional to the quantity of the binding partner bound to the immobilized vitamin B_{12}.

The more free vitamin B_{12} in the test sample, the greater is the fraction of the binding partner that binds to the free vitamin B_{12}, and the smaller the fraction of the binding partner that binds to the immobilized vitamin B_{12}. Only the fraction of binding partner that binds to the immobilized vitamin B_{12} is actually determined.

Typically, the immobilized vitamin B_{12} is cross-linked to a solid support. The solid support can be activated cross-linked polyacrylamide, activated cellulose, or diazotized amine-derivatized polystyrene. The linkage between the vitamin B_{12} and the solid support can be through a protein linker. The protein linker can be bovine serum albumin or IgG. The linkage can be formed by the steps of:

(1) activating the carboxyl group of a vitamin B_{12}-monocarboxylic acid with carbonyldiimidazole;

(2) reacting the activated vitamin B_{12}-monocarboxylic acid with N-hydroxysuccinimide to give the N-hydroxysuccinimidyl ester of the monocarboxylic acid; and

(3) reacting the N-hydroxysuccinimidyl ester of the monocarboxylic acid with the amino groups of the protein, thereby coupling the vitamin B_{12} to the protein by the formation of amide linkages. Typically, the vitamin B_{12}-monocarboxylic acid is the (e)-isomer. The vitamin B_{12}-monocarboxylic acid can also be succinylated on its primary alcohol group to improve its affinity for the binding partner.

The reporter group can be an enzyme, an enzyme cofactor, an enzyme inhibitor, an enzyme modulator, or a fluorescent, chemiluminescent, or electrolytically detectable

label. When the reporter group is an enzyme, the enzyme can be horseradish peroxidase, alkaline phosphatase, or β-galactosidase.

When the binding partner is biotinylated intrinsic factor, the step of determining the quantity of the biotinylated intrinsic factor bound to the immobilized vitamin B_{12} can comprise:

(a) reacting the bound biotinylated intrinsic factor with avidin labeled with the reporter group; and

(b) then determining the quantity of avidin bound to the intrinsic factor by assaying the reporter group bound to the intrinsic factor. This assay is an assay of the bound enzymatic activity when the reporter group is an enzyme. The avidin can be succinylated to reduce its non-specific binding.

When the intrinsic factor is conjugated to a hapten instead of to biotin, anti-hapten antibody then replaces avidin in the above method.

Unconjugated intrinsic factor can alternatively be used, and the avidin or anti-hapten antibody is then replaced by anti-intrinsic factor antibody bound to a receptor group.

The binding partner for vitamin B_{12} capable of subsequently binding a reporter group can be an antibody. The antibody can be biotinylated, and avidin covalently bound to a reporter group can be used. Alternatively, a second antibody specific for the first antibody and covalently bound to a reporter group can be used.

The binding partner for vitamin B_{12} can itself be covalently linked to a reporter group. When it is, the assay method can comprise the steps of:

(1) combining a test sample containing vitamin B_1 with:

(a) a binding partner for vitamin B_{12} covalently linked to the reporter group; and

(b) sufficient immobilized vitamin B_{12} that substantially all of the binding partner is bound by the immobilized vitamin B_{12} in the absence of vitamin B_{12} in the test sample; and

(2) then determining the quantity of the binding partner bound to the immobilized vitamin B_{12} by assaying the reporter group bound to the immobilized vitamin B_{12}. In this embodiment of the method, the binding partner can be intrinsic factor or anti-vitamin B_{12} antibody.

Specifically, one important version of the test comprises the steps of:

(1) combining the test sample with:

(a) a biotinylated intrinsic factor such that substantially all of the molecules of intrinsic factor are covalently linked to at least one biotin molecule, the biotin molecules being coupled to the amino groups of the intrinsic factor; and

(b) sufficient immobilized vitamin B_{12} that substantially all of the biotinylated intrinsic factor is bound by the immobilized vitamin B_{12} in the absence of vitamin B_{12} in the test sample, the immobilized vitamin B_{12} being covalently linked to a solid support of diazotized amine-derivatized beads polystyrene beads by a linker of IgG;

(2) separating the solid support and any intrinsic factor bound to the solid support from unbound intrinsic factor;

(3) incubating the solid support with horseradish peroxidase covalently linked to succinylated avidin so that any intrinsic factor bound to the solid support will bind horseradish peroxidase; and

(4) then assaying the horseradish peroxidase bound to the solid support, the enzymatic activity of horseradish peroxidase present being inversely proportional to the

quantity of free vitamin B_{12} originally present in the test sample.

When the sample is serum, certain preliminary steps are needed to inactivate endogenous serum proteins with intrinsic factor-like activity. A version of the assay specifically useful for serum comprises the steps of:

(1) treating serum with NaOH and dithiothreitol to inactivate any endogenous vitamin B_{12}-binding activity present in the serum, in the presence of KCN to stabilize the vitamin B_{12};

(2) neutralizing the treated serum;

(3) adding biotinylated intrinsic factor to the neutralized treated serum and incubating the resulting solution to permit binding of the vitamin B_{12} to the intrinsic factor;

(4) then adding a solid support to which vitamin B_{12} has been covalently coupled and incubating the support in the solution to bind any remaining unbound biotinylated intrinsic factor in the solution;

(5) washing the solid support to remove any unbound material;

(6) adding succinylated avidin conjugated to horseradish peroxidase to the solid support and incubating again;

(7) washing the excess avidin-peroxidase conjugate from the solid support;

(8) adding the peroxidase substrate o-phenylenediamine to the solid support, thereby producing a colored product, the quantity of colored product produced being inversely proportional to the quantity of free vitamin B_{12} originally present in the serum sample; and

(9) measuring the absorbance of the colored product produced thereby.

Another aspect of the present invention is compositions of matter comprising modified intrinsic factors suitable for the assays of the present invention. Most generally, such a composition of matter comprises modified intrinsic factor covalently coupled to a ligand through the amino groups of the intrinsic factor such that substantially all the molecules of intrinsic factor are covalently linked to at least one ligand. The composition of matter, for example, can comprise biotinylated intrinsic factor or intrinsic factor covalently coupled to a hapten. The ligand molecules can contain a carboxyl group and can then be coupled to the molecules of intrinsic factor through amide linkages. The amide linkages can be produced by the reaction of the carboxyl group of the ligand with N-hydroxysuccinimide to form a N-hydroxysuccinimidyl ester and

the subsequent reaction of the N-hydroxysuccinimidyl ester with the amino groups of intrinsic factor.

Still another important aspect of the invention is a process for the biotinylation of intrinsic factor, as well as a composition of matter comprising biotinylated intrinsic factor produced by the process. The process comprises the steps of:

(1) reacting the intrinsic factor with the N-hydroxysuccinimidyl ester of biotinylamidocaproic acid at a pH of about 7.4 and at a ratio of biotin moieties to intrinsic factor molecules of from about 6.3:1 to about 1375:1; and

(2) then deactivating the remaining N-hydroxysuccinimidyl ester of biotinylamidocaproic acid by reacting it with an excess of ethylenediamine.

In a competitive assay for vitamin B_{12} wherein the vitamin B_{12} is reacted with a binding partner, an improvement can comprise using as the binding partner any of the modified intrinsic factor preparations described above.

DRAWING

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description, appended claims, and accompanying drawing where:

The single figure is a standard curve for the assay of the present invention using immobilized vitamin B_{12} and biotinylated intrinsic factor.

DESCRIPTION

A novel competitive binding assay for vitamin B_{12} is provided in accordance with the present invention. As used hereinafter, the term "competitive binding assay" is intended to include both true competitive binding assays in which the competing species are present simultaneously, and pseudocompetitive binding assays in which the competing species are present sequentially.

The critical element of the assay of the present invention is the use of biotinylated IF prepared in such a manner that substantially all the IF molecules are biotinylated without any significant degradation in the ability of the biotinylated IF to bind vitamin B_{12}. This

biotinylated IF also serves as the way of introducing an enzyme or other reporter group, such as enzyme cofactors, enzyme modulators, enzyme inhibitors, fluorophores, chemiluminophores, or electrolytically detectable species, into the assay. The reporter group is used for subsequent determination of the quantity of IF bound to the solid support. The enzyme or other reporter group is introduced as avidin labeled with the enzyme or other reporter group and binds tightly and specifically to the biotin moiety of the biotinylated IF.

The use of biotinylated IF also allows the vitamin B_{12} to be coupled to a solid support instead of being labeled. This role reversal of the competitive B_{12} reagent and the IF reagent has unexpectedly been found to improve the efficiency of the assay, especially within the clinically significant concentration ranges. This unexpected improvement occurs even though the same competitive reaction takes place, the insolubilized vitamin B_{12} competing with the test sample vitamin B_{12} for the same binding partner, typically biotinylated IF.

In the performance of the assay, an excess of the immobilized vitamin B_{12} is typically added to the test sample containing vitamin B_{12} along with a limited amount of the

biotinylated IF. The insolubilized vitamin B_{12} reagent competes with the test sample vitamin B_{12} for the limited number of available IF binding sites. Enzyme-labeled avidin is then added following incubation of the biotinylated IF with the immobilized vitamin B_{12} and the test sample vitamin B_{12} for a predetermined period of time. The solid phase is then separated from the solution and the enzyme activity associated with the solid phase measured to determine the quantity of labeled IF reagent bound to the solid phase through the immobilized vitamin B_{12}. The quantity of bound enzyme activity is inversely proportional to the vitamin B_{12} content of the sample.

1. Biotinylation of Intrinsic Factor

Two essential requirements exist for biotinylated IF that is to serve as binding partner for vitamin B_{12} in this assay. The first requirement is the preservation of the binding capacity of the IF for vitamin B_{12} after biotinylation. The second requirement is the biotinylation of substantially all of the IF. If one of these requirements is not met, the resulting preparation of biotinylated IF is unsuitable for use as a binding partner for vitamin B_{12} in a competitive binding assay. Many naturally occurring binding proteins have amino functional groups distributed throughout

the binding surface. Some of those amino groups can generally be found at or near the critical binding site. Biotinylating agents are usually in the form of species reactive with amino groups. When biotin reacts with those amino groups at or near the binding site, that particular binding protein becomes denatured and loses its binding activity. In the present invention, unexpectedly, IF did not lose its binding activity even after exhaustive biotinylation, indicating that IF does not have amino groups at or near its binding site for vitamin B_{12}.

Incomplete biotinylation of IF can result in a poor inhibition curve, because those vitamin B_{12} molecules bound to non-biotinylated IF would not be detected. Non-biotinylated IF, whether bound to vitamin B_{12} or not, cannot be bound to an enzyme carrying avidin. On the other hand, the biotinylated IF, if not bound to vitamin B_{12}, can bind to the solid phase vitamin B_{12} moiety, eventually attracting enzyme-labeled avidin. Analysis of prior preparations of biotinylated IF revealed that as many as 35-40% of the IF molecules in the biotinylated preparations lacked any biotin.

In accordance with these requirements, a procedure for synthesizing biotinylated IF was developed that resulted in a superior preparation of biotinylated IF. This

preparation was 100% biotinylated and showed no decrease in its capacity to bind vitamin B_{12}.

This biotinylated IF was prepared by reacting IF with the N-hydroxysuccinimidyl ester of biotinylamidocaproic acid. This ester activates the carboxyl group of the above biotin derivative, and results in the coupling of the biotin moiety to either the ε-amino group of lysine or the amino-terminus of the IF protein. The amino groups of IF are believed not to be located in or adjacent to the active site, and therefore can be derivatized extensively without altering either the three-dimensional structure or the activity of IF. The ratio of biotin moieties to IF molecules in the starting reaction mixture can range from 6.3 to 1375; even at the lowest ratio of biotin moieties to IF molecules, all of the IF molecules were biotinylated. Further details on the coupling reaction are given below in Example 1.

These results lead to the conclusion that any ligand or chemical species that either has a free carboxyl group or can be converted to a carboxylic acid derivative can then be converted to an NHS ester and reacted with IF exhaustively without denaturing the IF. These species include haptens such as 2,4-dinitrophenol; drugs; reporter groups such as enzymes, enzyme cofactors, enzyme inhibitors,

enzyme modulators, fluorescent labels, chemiluminescent labels, and electrolytically active labels detectable by a change of pH or electrical potential in solution.

Furthermore, this method is not limited to either ligands containing carboxyl groups or coupling through NHS esters. The amino groups of IF provide suitable sites for derivatization by many techniques. For example, thiol-containing ligands can be coupled to amino groups by use of such cross-linkers as m-maleimidobenzoyl-N-hydroxysuccinimide ester and 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester. Carboxyl groups can be coupled to the amino groups of IF through carbodiimides or through conversion of the carboxyl group of the ligand to an acid anhydride.

2. Coupling of Vitamin B_{12} to a Solid Support

In this assay, the vitamin B_{12} is coupled to a solid support. A variety of solid supports and coupling reactions are usable. As solid supports, cross-linked acrylamide, agarose, cellulose, and nylon were successfully used.

As was mentioned above, the vitamin B_{12} derivative has weaker binding characteristics towards IF than unmodified vitamin B_{12}. The binding becomes even weaker when the vitamin B_{12} derivative is bound to a bulky protein molecule. IF, which itself is a relatively bulky protein molecule, has difficulty in binding protein-bound vitamin B_{12} moieties. One way to improve the stable binding of IF to the vitamin B_{12} derivative is to use a plurality of vitamin B_{12} molecules immobilized on a solid phase.

There are several methods for immobilizing vitamin B_{12} molecules. Solid surface functional groups can be suitably chemically derivatized and reacted with vitamin B_{12}-monocarboxylic acid. Alternatively, vitamin B_{12}-monocarboxylic acid can be attached to a suitable proteinaceous carrier molecule and this vitamin B_{12}-protein conjugate can be attached to a solid phase by many known methods.

In these immobilization reactions, the vitamin B_{12} is partially hydrolyzed to a monocarboxylic acid, preferably the (e)-isomer of monocarboxylic acid, which is the most tightly binding of the monocarboxylic acid isomers. The monocarboxylic acid is then covalently coupled to a protein,

either bovine serum albumin (BSA) or immunoglobulin G (IgG), typically horse IgG or bovine IgG.

This coupling occurs by activation of the carboxyl group of the vitamin B_{12}-monocarboxylic acid with carbonyldiimidazole (CDI), followed by reaction of the activated vitamin B_{12} derivative with N-hydroxysuccinimide (NHS) to give the N-hydroxysuccinimidyl ester of vitamin B_{12}-monocarboxylic acid. The activated ester then reacts with the amino groups of the protein to couple the vitamin B_{12} derivative to the protein by the formation of an amide linkage.

Preferably the vitamin B_{12} is also succinylated by the reaction of the primary alcohol group of the vitamin B_{12} with succinic anhydride. The succinylation of the vitamin B_{12} improves the binding of vitamin B_{12} to IF when vitamin B_{12} is subsequently coupled to a solid support through protein.

Vitamin B_{12} can be coupled to a number of solid supports, depending upon the requirements of the assay.

a. Pel 102

Pel 102 (TM) is a proprietary cross-linked polyacrylamide available from Amicon, preactivated with NHS and ready to couple species containing amino groups. If a vitamin B_{12} monocarboxylate-BSA conjugate is added to Pel 102, the amino groups on the BSA will become linked to the solid support.

b. CDI-Activated Cellulose

CDI activates the hydroxyl groups of the cellulose for reaction with amino groups of the protein in a vitamin B_{12} monocarboxylate-protein conjugate. The protein can be either BSA or IgG.

An elaboration of this reaction can be used to provide a spacer molecule between the cellulose and the vitamin B_{12} monocarboxylate-protein conjugate to reduce the possibility of steric hindrance. In this elaboration, CDI-activated cellulose can be derivatized with hexanediamine. The free end of the hexanediamine spacer, bearing an amino group, can then be reacted with succinic anhydride. Additional hexanediamine spacers can then be incorporated by

coupling the free carboxyl group of the succinyl moiety to additional hexanediamine.

c. Polystyrene Beads

The optimum solid support in terms of minimizing leakage of immobilized vitamin B_{12} and suitability for use with automated analyzer equipment has proven to be polystyrene beads of 1/4 inch (6.35 mm). The derivatization of polystyrene beads to produce immobilized vitamin B_{12} proceeds by a series of reactions:

1) nitration of the polystyrene beads, using either nitronium tetrafluoroborate, or concentrated nitric acid in concentrated sulfuric acid;

2) reduction of the nitropolystyrene beads to aminopolystyrene beads with tin metal in concentrated hydrochloric acid;

3) conversion of the aminopolystyrene beads to diazoniumpolystyrene beads with sodium nitrite in HCl; and

4) binding of a vitamin B_{12}-IgG conjugate to the diazoniumpolystyrene beads by reaction in 0.1 M sodium borate buffer, pH 9.2. Further details on these procedures are given below under Examples 2 and 3.

Still other coupling procedures are possible and can be useful in particular assay schemes. For example, a monocarboxylate derivative of vitamin B_{12} can be activated with CDI and coupled to ethylenediamine, giving a free amino group. The amino derivative can then be coupled with succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate. The product can then be coupled to any protein containing thiol groups.

4. Preparation and Use of Avidin-Labeled Enzyme

a. Preparation of Conjugated Avidin

Avidin has an exceptionally high affinity for biotin, with the binding constant being approximately 10^{15} M^{-1}. Thus, the coupling of an enzyme to avidin is an excellent method for introducing an enzymatic label into an immunological reaction in which one of the reagents is labeled with biotin.

The enzyme used can be any easily assayable enzyme producing a detectable product whose activity is not affected by the conjugation with avidin or by the binding to biotin-labeled IF. Preferably, the detectable product is visually detectable. Typically, the enzyme is horseradish peroxidase

(HRPO), but β-galactosidase, glucose oxidase, and alkaline phosphatase are all suitable enzymes.

When HRPO is used as the enzyme, several coupling methods can be used to couple the HRPO to the avidin. Two efficient methods are periodate-mediated conjugation and conjugation by the reaction of maleimide with thiols.

(1) Periodate-Mediated Conjugation

The periodate-mediated conjugation procedure used is modified, by slight changes of reaction time and reagent volume, from the procedures described by P. Tijssen and E. Kurstak, "Highly Efficient and Simple Methods for the Preparation of Peroxidase and Active Peroxidase-Antibody Conjugates for Enzyme Immunoassays," Anal. Biochem. 136, 451-457 (1984), and P.K. Nakane and A. Kawaoi, "Peroxidase Labeled Antibody: A New Method of Conjugation," J. Histochem. Cytochem. 22, 1084-1091 (1974).

(2) Conjugation by Reaction of Maleimide with Thiols

The conjugation reaction with maleimide is a modification of the method described by M. Imagawa et al., "Characteristics and Evaluation of Antibody-Horseradish

Peroxidase Conjugates Prepared by Using a Maleimide Compound, Glutaraldehyde and Periodate," J. Applied Biochem. 4, 41-57 (1982). The reaction conditions and purification procedure are slightly modified from those given. In this reaction, the maleimide group is introduced into HRPO by treatment with a 1:200 molar excess of sulfo-succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate for 1 to 2 hours in sodium phosphate buffer, pH 7.0. The avidin is treated with dithio-bis-succinimidyl propionate followed by dithiothreitol to introduce thiol groups. To effect the conjugation reaction, the two proteins are mixed in equimolar amounts for from 20 minutes to 120 minutes. The conjugate is then isolated using the same carboxymethyl-Sephadex procedure used for the periodate conjugate.

b. Use of the Avidin-HRPO Conjugate in the Assay

Avidin-HRPO conjugate is typically introduced into the assay following a 30 minute incubation of the sample with biotinylated IF and a further 30 minute incubation after addition of the immobilized vitamin B_{12}. To prevent non-specific binding, the incubation of avidin-HRPO is optimally performed in "avidin buffer," containing, per liter of solution, 29.8 g KCl, 1.2 g Tris base, 10.0 g BSA, and 0.5 g Thimerosal (a preservative).

c. Use of Succinylated Avidin

Avidin is an extremely basic protein, with an isoelectric point of approximately pH 11. Its high basicity leads to a high level of non-specific binding of the avidin conjugate. To reduce this non-specific binding, incubation with the conjugate must be performed with avidin buffer as stated above. However, avidin buffer is incompatible with performance of the assay in certain automated analyzer systems. To eliminate this, the avidin can be succinylated by reaction with succinic anhydride. This succinylated avidin, after conjugation with HRPO, can be used in the assay without avidin buffer, using normal saline in the wash buffer.

5. Application to Assay of Serum

In the use of such an assay on actual serum samples, it is necessary to inactivate endogenous sample proteins with intrinsic factor-like activity. These proteins bind vitamin B_{12} and distort the assay results. These proteins have been traditionally inactivated by boiling, but a simpler and more easily performed serum pretreatment step is highly desirable for rapid processing of serum samples.

Such a pretreatment step can comprise a 15 minute incubation of the serum sample with NaOH and dithiothreitol (DTT) for 15 minutes at room temperature, followed by neutralization of the NaOH with Tris buffer (Example 4).

Also, the presence of KCN is very important in the assay mixture. The KCN is believed to stabilize the vitamin B_{12} in the form of cyanocobalamin during the performance of the assay.

6. Alternative Embodiments of the Assay

As mentioned previously, it is not necessary for the reporter group on avidin to be an enzyme. Other reporter groups, such as enzyme cofactors, modulators, or inhibitors; chemiluminescent or fluorescent labels; or electrolytically detectable labels, can be used instead. In fact, the reporter group need not be on the avidin or other molecule binding specifically to the IF, but can be located on the IF itself.

Instead of being covalently linked to biotin and an avidin-labeled enzyme being added later, the binding partner for vitamin B_{12} can be covalently conjugated with the

enzyme or other reporter group. In this embodiment, there is no need to use the avidin-biotin system.

It is also possible to use antibody that is specific for the binding partner of vitamin B_{12}, such as anti-intrinsic factor antibody. In this embodiment, the enzyme or other reporter group can be directly attached to the antibody. Alternatively, a first and second antibody can be used, with the first antibody specific for the binding partner, and the second antibody specific for the first antibody. In this alternative, the enzyme or other reporter group would be attached to the second antibody. For example, if the binding partner were IF, the first antibody could be rabbit anti-IF antibody and the second antibody could be goat anti-rabbit IgG covalently conjugated to HRPO.

Although the binding partner for vitamin B_{12} is typically intrinsic factor, other binding partners are also usable in the assay of the present invention, such as Protein R and anti-vitamin B_{12} antibodies.

If the IF is labeled with a hapten such as 2,4-dinitrophenol rather than biotin, highly specific tight-binding antibodies for the hapten can replace avidin in the assay. The antibodies can be labeled with any of the

reporter groups previously mentioned. Alternatively, as mentioned above, the IF itself can be labeled with any of these reporter groups.

EXAMPLES

Example 1 - Biotinylation of Intrinsic Factor

To each of three 0.5 ml aliquots of intrinsic factor (IF) at 0.5 mg/ml in water there was added 0.1 ml of 0.5 M sodium phosphate, pH 7.4, with slow stirring To the first aliquot, 5.7 µl of a 2.5 mg/ml solution of N-hydroxysuccinimidyl ester of biotinylamidocaproic acid (Biotin-X-NHS) was added. To the second aliquot, 5.7 µl of a 25 mg/ml solution of Biotin-X-NHS was added. To the third aliquot, 62.5 µl of a 50 mg/ml solution of Biotin-X-NHS was added. The tubes containing the reaction mixtures were stirred at room temperature for 2 to 3 hours and then stirred overnight at 4°C. The reaction mixtures remained clear. To the first and second aliquot were then added 0.1 ml of 0.1 M ethylenediamine in phosphate buffered saline (PBS). To the third aliquot was added 0.2 ml of 0.1 M ethylenediamine solution. After the addition of the ethylenediamine, the solutions were stirred for at least 2 hours at room temperature. Finally, to each tube was added 0.1 ml of

bovine serum albumin (BSA) at 0.1 g/ml. The contents of each tube were then transferred to dialysis tubing and dialyzed against 3 changes of 50 mM Tris-HCl, pH 7.4, and then against two further changes of phosphate buffered saline, pH 7.4, diluted to 10 mM.

The ratio of biotin moieties to IF molecules was 6.3:1 in the first reaction mixture, 63:1 in the second, and 1375:1 in the third. Analysis of the vitamin B_{12}-binding capacity of the biotinylated IF, using ^{57}Co-labeled vitamin B_{12} and BSA-treated charcoal which binds free vitamin B_{12} but not vitamin B_{12} bound to intrinsic factor, showed that there was no decrease in the vitamin B_{12}-binding capacity of the biotinylated IF preparations. Analysis of the extent of biotinylation was also carried out using ^{57}Co-labeled vitamin B_{12}, BSA-treated charcoal, and avidin immobilized on cellulose. In this analysis, any labeled vitamin B_{12} binding to IF not carrying at least one biotin moiety is bound neither by the charcoal nor by the cellulose-avidin and remains in solution. This analysis showed that substantially all of the IF molecules were biotinylated, even at the lowest biotin:IF ratio of 6.3:1.

Example 2 - Preparation of Immobilized Vitamin B_{12} on Polystyrene Beads Using Nitration of the Polystyrene with Nitronium Tetrafluoroborate

About 7000 polystyrene beads of 1/4 inch (6.35 mm) diameter were placed in a flask and treated with 700 ml of 0.25 M nitronium tetrafluoroborate in dry acetonitrile. The beads in the flask were shaken slowly for 45 minutes at room temperature, and then washed three times with 700 ml of acetonitrile, and then with copious amounts of water.

To reduce the nitropolystyrene beads to aminopolystyrene, the beads were suspended in 750 ml of concentrated HCl. To the beads was added 150 g of tin powder, and the mixture was shaken on a rocker with occasional hand shaking for 1 hour. At that point, 200 g more tin and 1000 ml concentrated HCl were added, and the mixture was shaken overnight at room temperature, after which 500 ml of concentrated HCl was again added. The beads were washed with copious amounts of water. The presence of amino groups on the beads was confirmed by a test with 2,4,6-trinitrobenzenesulfonic acid (TNBS).

For the conversion of the aminopolystyrene beads to diazopolystyrene beads, sodium nitrite in hydrochloric acid was used. A small portion of the amine-containing beads was

treated with 5 volumes of a 1:1 mixture of 4% (w/v) $NaNO_2$ and 2 M HCl, with shaking for 10 minutes at 4°. The beads were washed with 0.01 M HCl.

For immobilization of the vitamin B_{12}-IgG conjugate on the beads, 30 ml of the diazonium beads were treated with the conjugate in 0.1 M sodium borate, pH 9.2. The conjugate itself had been prepared separately as described in Example 3. The beads were shaken overnight at 4°C with the conjugate, and then washed with borate buffer.

Example 3 - Preparation of Immobilized Vitamin B_{12} on Polystyrene Beads Using Nitration of the Polystyrene with Nitric Acid

For the nitration, 440 ml of concentrated H_2SO_4 and 340 ml of concentrated HNO_3 were added in portions in a 2-liter Erlenmeyer flask, with shaking and cooling in ice. To the mixture of acids was added approximately 1200 ml of polystyrene beads. The mixture was shaken by hand for 1 hour in an ice bath, and then filtered and rinsed.

For the reduction to amine-containing beads, 500 ml of the nitrated polystyrene beads was placed in a 1-liter double-necked round-bottomed flask with an overhead stirrer. Then 100 g of tin shot was added followed by 400 ml of

concentrated HCl. The flask containing the beads was stirred for 8 hours at room temperature, and then left standing overnight at room temperature. The beads were then washed with copious amounts of water and stored under H_2O.

The vitamin B_{12}-IgG conjugate was prepared separately before the diazotization of the beads. For the preparation of the vitamin B_{12}-IgG conjugate, 87.5 mg of the (e)-isomer of the monocarboxylic acid derivative of vitamin B_{12} was heated to 70° -80°C in 2 ml of dimethylformamide. Not all of the monocarboxylic acid derivative of vitamin B_{12} dissolved. 87.5 mg of carbonyldiimidazole was then added, and the temperature maintained at 70° - 80°C for 10 minutes. A clear red solution formed. The solution was then cooled to room temperature, and 87.5 mg of N-hydroxysuccinimide (NHS) added, with stirring for 4 hours at room temperature. IgG was then dissolved into 0.1 M phosphate buffered saline (PBS) at 10 mg/ml. To each of six tubes was added 2.5 ml of the IgG solution, 1.3 ml of PBS, then the quantity of activated vitamin B_{12} given in Table 1. The ratio of activated B_{12} to IgG present in the reaction mixture ranged from 1:1 (Tube 1) to 1:500 (Tube 5). The reactions between the activated vitamin B_{12} and the IgG were then allowed to proceed overnight at 4°C. The products were dialyzed against 10 mM PBS, then passed through a Sephadex (TM) G-25 column, and

then dialyzed again except for the two products with the greatest ratio of vitamin B_{12} to IgG (Tubes 1 and 2).

For the coupling reaction, the IgG conjugate was diluted with 15 ml of 0.1 M sodium borate, pH 9.2. For the diazotization and coupling, to an aliquot of beads was added 10 ml of 4% $NaNO_2$ and 10 ml 2M HCl; the tubes containing the beads were shaken and allowed to stand for 5 minutes at 4°C. The solution was then filtered from the beads, and the beads washed with 0.01 M HCl. The vitamin B_{12}-IgG conjugate was then added, and enough PBS added to cover the beads. The conjugate and beads were allowed to react for 3 days with shaking.

TABLE 1
QUANTITIES OF ACTIVATED VITAMIN B_{12} AND IgG USED IN FORMATION OF CONJUGATES (EXAMPLE 3)

TUBE NO.	VOLUME OF IgG (10 mg/ml), ml	VOLUME OF ACTIVATED B_{12} µl	RATIO OF B_{12} TO IgG
1	2.5	1238.00	1:1
2	2.5	619.00	1:2
3	2.5	123.80	1:10
4	2.5	12.38	1:100
5	2.5	6.19	1:500

Example 4 - Assay of Vitamin B_{12} in Serum

Into 12 x 75 mm polystyrene tubes was added 200 µl of the serum sample or standard, 100 µl of 0.1% KCN, 0.2% DTT in 0.85% NaCl, and 50 µl of 1 N NaOH. The samples were incubated 15 minutes at room temperature. Then 500 µl of 1 M Tris, pH 8, and 100 µl of a preparation of biotinylated IF with a ratio of 50:1 of biotin moieties per IF molecule at a dilution of 1/2000 were added. The mixture was incubated at 37°C for 30 minutes. A polystyrene bead containing a vitamin B_{12}-IgG conjugate, prepared by the method using HCl/HNO_3 nitration (Example 3) and prewashed three times with 0.1 M Tris, pH 8 was added, and the reactions were incubated for an additional 30 minutes at 37°C on a rotator at 190 rpm. The beads were washed in avidin buffer, and 300 ml of a 1/2000 dilution of avidin-HRPO (obtained commercially from Boehringer-Mannheim) was added. After an additional incubation for 30 minutes at 37°C on the rotator, the beads were then washed three times with avidin buffer, and 300 µl of o-phenylenediamine at 2 mg/ml in citric acid buffer was added as a substrate for the HRPO. To determine enzymatic activity, the reactions were incubated for 15 minutes at room temperature. The reactions were stopped with 1 ml of 0.9 M H_2SO_4, and the absorbance at 490 nm (A_{490}) was read for each sample. A substrate blank and a non-specific binding control without biotinylated IF were also run. The value for the substrate blank was subtracted from the mean

value of the A_{490} from the assays for each concentration of vitamin B_{12} to obtain a corrected mean absorbance proportional to the quantity of biotinylated IF bound to the immobilized vitamin B_{12} (B). The B value for each concentration of vitamin B_{12} was then divided by the B value at zero concentration of vitamin B_{12} (B_0) to give B/B_0, the fraction of biotinylated IF binding compared to the fraction binding at zero concentration. Fig. 1 shows the results of a plot of B/B_0 versus vitamin B_{12} concentration. The results on the standards are shown in Table 2 and Fig. 1. Table 3 shows the results obtained on a number of sera with this assay compared with the results on the same sera obtained using two commercially available radioimmunoassay kits from Amersham and Corning. The correlation between the assay of the present invention, designated EIA in Table 3, and either the Amersham and Corning assays is at least as good or better than that obtained between the Amersham and Corning assays themselves. A SmithKline Bioscience radioimmunoassay was also performed on the same samples, and the correlation between the assay of the present invention and the SmithKline Bioscience assay was better than that between the Corning assay and the SmithKline Bioscience assay. The square of the correlation coefficient, R^2, was 0.561 between the assay of the present invention and the Corning assay, and was 0.614 between the assay of the present invention and the SmithKline Bioscience assay. It was 0.459 between the Corning and SmithKline Bioscience assay. It was approximately zero between

the assay of the present invention and the Amersham assay, and between the Amersham and Corning assays.

TABLE 2

RESULTS OF EIA ASSAY ON STANDARDS (EXAMPLE 4)

VITAMIN B_{12} CONCENTRATION (pg/ml)	ABSORBANCE (A_{490}) ON SAMPLES	MEAN A_{490} MINUS SUBSTRATE BLANK	B/B_0 (%)
SUBSTRATE BLANK	0.0110	0	-----
NON-SPECIFIC BINDING (NO BIOTINYLATED IF)	0.0700, 0.0430, 0.0610	0.0470	-----
0	1.0205, 0.8930, 1.1480	1.0095*	100.0
100	1.130, 0.9980, 0.9430	1.1030	100.3
250	0.7170, 0.8430, 0.7560	0.7610	74.2
500	0.7130, 0.4410, 0.5860	0.5690	54.2
1000	0.1780, 0.1760, 0.1540	0.1583	11.6
2000	0.0450, 0.0610, 0.0570	0.0433	<0

* This value was taken as B_0.

TABLE 3
ASSAY OF SERUM SAMPLES FOR VITAMIN B_{12} CONCENTRATION
(EXAMPLE 4)

SAMPLE NO.	VITAMIN B_{12} CONCENTRATION, pg/ml, WITH:		
	EIA	AMERSHAM RIA	CORNING RIA
1	526.7	1308.0	656.5
2	342.2	625.7	1278.0
4	343.3	455.0	311.8
10	348.3	238.0	448.1
11	500.4	628.7	715.3
13	424.2	490.7	343.6
15	427.3	695.0	623.1
16	476.4	743.3	546.2
18	511.1	762.7	921.9
19	366.3	348.7	581.4
21	918.0	628.4	1539.0
23	1147.0	843.3	1934.4
24	306.8	811.4	417.9
28	1033.9	969.5	1639.5
30	579.8	619.9	1814.8

The column labeled "EIA" refers to assays performed by the enzyme immunoassay method of the present invention

The method of assaying vitamin B_{12} concentration of the present invention achieves the goals that have been sought, and possesses a number of significant advantages over the assays previously used. The assay is accurate and precise, especially in the clinically significant concentration ranges. It gives a better dose-response curve than the assay of Leonidas et al. It can use the theoretically most desirable binding partner for vitamin B_{12}, intrinsic factor. In using biotinylated intrinsic factor, the assay avoids the problems of either underbiotinylation of the intrinsic factor or inactivation of the vitamin B_{12}-binding activity of the intrinsic factor, problems that have previously hampered the use of the biotin-avidin system in assays for vitamin B_{12} using intrinsic factor. The assay is readily performed on serum and accommodates simple methods for inactivating the intrinsic vitamin B_{12}-binding proteins of serum. It does not require the use of radioactivity, giving it the advantages of greater stability of reagents and greater shelf life. Furthermore, the avoidance of radioactivity means that the assay can be performed by workers with less training, at less risk to themselves, co-workers, or the environment.

Although the present invention has been described in considerable detail with regard to certain preferred versions thereof, other versions are possible. Therefore, the spirit and

scope of the appended claims should not be limited to the descriptions of the preferred versions contained herein.

What is claimed is:

1. A competitive binding assay method for detecting vitamin B_{12} in a test sample, comprising the steps of:

(a) combining the test sample with:

(i) a binding partner for vitamin B_{12} capable of subsequently binding a reporter group; and

(ii) sufficient immobilized vitamin B_{12} that substantially all of the binding partner is bound by the immobilized vitamin B_{12} in the absence of vitamin B_{12} in the test sample; and

(b) then determining the quantity of the binding partner bound to the immobilized vitamin B_{12}, the quantity of vitamin B_{12} in the test sample being inversely proportional to the quantity of the binding partner bound to the immobilized vitamin B_{12}.

2. The method of claim 1 wherein the reporter group is selected from the group consisting of enzymes, enzyme cofactors, enzyme inhibitors, enzyme modulators, fluorescent labels, chemiluminescent labels, and electrolytically detectable labels.

3. A competitive binding assay method for detecting vitamin B_{12} in a test sample, comprising the steps of:

(a) combining the test sample with:

(i) intrinsic factor capable of subsequently binding a reporter group; and

(ii) sufficient immobilized vitamin B_{12} that substantially all of the intrinsic factor is bound by the immobilized vitamin B_{12} in the absence of vitamin B_{12} in the test sample; and

(b) then determining the quantity of the intrinsic factor bound to the immobilized vitamin B_{12}, the quantity of vitamin B_{12} in the test sample being inversely proportional to the quantity of the intrinsic factor bound to the immobilized vitamin B_{12}.

4. The method of claim 3 wherein the intrinsic factor is biotinylated such that substantially all of the molecules of intrinsic factor are covalently linked to at least one biotin molecule, the biotin molecules being coupled to the amino groups of the intrinsic factor.

5. The method of claim 4 wherein the step of determining the quantity of the biotinylated intrinsic factor bound to the immobilized vitamin B_{12} comprises:

(i) reacting the bound biotinylated intrinsic factor with avidin labeled with the reporter group; and

(ii) then determining the quantity of avidin bound to the intrinsic factor by assaying the reporter group bound to the intrinsic factor.

6. The method of claim 5 wherein the reporter group is an enzyme and wherein the step of determining the quantity of avidin bound to the intrinsic factor comprises assaying the enzymatic activity bound to the intrinsic factor.

7. The method of claim 6 wherein the enzyme labeling the avidin is selected from the group consisting of horseradish peroxidase, alkaline phosphatase, and β-galactosidase.

8. The method of claim 5 wherein the avidin is succinylated to reduce non-specific binding of the avidin.

9. The method of claim 3 wherein the intrinsic factor is covalently conjugated with a hapten such that substantially all of the molecules of the intrinsic factor are covalently linked to at least one hapten molecule, the hapten molecules being coupled to the amino groups of the intrinsic factor.

10. The method of claim 3 wherein the step of determining the quantity of the conjugated intrinsic factor bound to the immobilized vitamin B_{12} comprises:

(i) reacting the bound conjugated intrinsic factor with anti-hapten antibody labeled with the reporter group; and

(ii) then determining the quantity of anti-hapten antibody bound to the intrinsic factor by assaying the reporter group bound to the intrinsic factor.

11. The method of claim 10 wherein the reporter group is an enzyme and wherein the step of determining the quantity of anti-hapten antibody bound to the intrinsic factor comprises assaying the enzymatic activity bound to the intrinsic factor.

12. The method of claim 11 wherein the enzyme labeling the avidin is selected from the group consisting of horseradish peroxidase, alkaline phosphatase, and β-galactosidase.

13. The method of claim 3 wherein the step of determining the quantity of the intrinsic factor bound to the immobilized vitamin B_{12} comprises:

(i) reacting the bound intrinsic factor with an anti-intrinsic factor antibody bound to a reporter group; and

(ii) then determining the quantity of anti-intrinsic factor antibody bound to the intrinsic factor by assaying the reporter group bound to the intrinsic factor.

14. The method of claim 1 wherein the immobilized vitamin B_{12} is covalently linked to a solid support.

15. The method of claim 14 wherein the solid support is selected from the group consisting of activated cross-linked polyacrylamide, activated cellulose, and diazotized amine-derivatized polystyrene.

16. The method of claim 14 wherein the linkage between the vitamin B_{12} and the solid support is through a protein linker.

17. The method of claim 16 wherein the protein linker is selected from the group consisting of bovine serum albumin and IgG.

18. A competitive binding assay method for detecting vitamin B_{12} in a test sample, comprising the steps of:

(a) combining the test sample with:

(i) a binding partner for vitamin B_{12} capable of subsequently binding a reporter group; and

(ii) sufficient immobilized vitamin B_{12} that substantially all of the binding partner is bound by the immobilized vitamin B_{12} in the absence of vitamin B_{12} in the test sample; the immobilized vitamin B_{12} being covalently linked through a protein linker to a solid support, the linkage between

the protein linker and the vitamin B_{12} being formed by the steps of:

[a] activating the carboxyl group of a vitamin B_{12}-monocarboxylic acid with carbonyldiimidazole;

[b] reacting the activated vitamin B_{12}-monocarboxylic acid with N-hydroxysuccinimide to give the N-hydroxysuccinimidyl ester of the monocarboxylic acid; and

[c] reacting the N-hydroxysuccinimidyl ester of the monocarboxylic acid with the amino groups of the protein, thereby coupling the vitamin B_{12} to the protein by the formation of amide linkages; and

(b) then determining the quantity of the binding partner bound to the immobilized vitamin B_{12}, the quantity of vitamin B_{12} in the test sample being inversely proportional to the quantity of the binding partner bound to the immobilized vitamin B_{12}.

19. The method of claim 18 wherein the vitamin B_{12}-monocarboxylic acid is the (e)-isomer.

20. The method of claim 18 wherein the vitamin B_{12}-monocarboxylic acid is also succinylated on its primary alcohol group to improve its affinity for the binding partner.

21. The method of claim 1 wherein the binding partner for vitamin B_{12} is an antibody.

22. The method of claim 21 wherein the anti-vitamin B_{12} antibody is biotinylated.

23. The method of claim 22 wherein the step of determining the quantity of the anti-vitamin B_{12} antibody bound to the immobilized vitamin B_{12} comprises:

(i) reacting the anti-vitamin B_{12} antibody bound to the immobilized vitamin B_{12} with avidin labeled with a reporter group; and

(ii) then determining the quantity of avidin bound to the anti-vitamin B_{12} antibody by assaying the reporter group bound to the anti-vitamin B_{12} antibody.

24. The method of claim 23 wherein the reporter group is an enzyme and wherein the step of determining the quantity of avidin bound to the anti-vitamin B_{12} antibody comprises assaying the enzymatic activity bound to the anti-vitamin B_{12} antibody.

25. The method of claim 24 wherein the enzyme labeling the avidin is selected from the group consisting of horseradish peroxidase, alkaline phosphatase, and β-galactosidase.

26. The method of claim 23 wherein the avidin is succinylated to reduce non-specific binding of the avidin.

27. The method of claim 21 wherein the step of determining the quantity of the anti-vitamin B_{12} antibody bound to the immobilized vitamin B_{12} comprises:

(i) reacting the anti-vitamin B_{12} antibody bound to the immobilized vitamin B_{12} with a second antibody specific for the anti-vitamin B_{12} antibody and covalently linked to a reporter group; and

(ii) then determining the quantity of second antibody bound to the anti-vitamin B_{12} antibody by assaying the reporter group bound to the anti-vitamin B_{12} antibody.

28. The method of claim 27 wherein the reporter group is an enzyme and wherein the step of determining the quantity of second antibody bound to the anti-vitamin B_{12} antibody comprises assaying the enzymatic activity bound to the anti-vitamin B_{12} antibody.

29. The method of claim 28 wherein the enzyme covalently linked to the second antibody is selected from the group consisting of horseradish peroxidase, alkaline phosphatase, and β-galactosidase.

30. A competitive binding assay method for detecting vitamin B_{12} in a test sample, comprising the steps of:

(a) combining the test sample with:

(i) a binding partner for vitamin B_{12} covalently linked to a reporter group; and

(ii) sufficient immobilized vitamin B_{12} that substantially all of the binding partner is bound by the immobilized vitamin B_{12} in the absence of vitamin B_{12} in the test sample; and

(b) then determining the quantity of the binding partner bound to the immobilized vitamin B_{12} by assaying the reporter group bound to the immobilized vitamin B_{12}.

31. The method of claim 30 wherein the binding partner is intrinsic factor.

32. The method of claim 30 wherein the binding partner is an antibody.

33. The method of claim 30 wherein the reporter group is an enzyme and wherein the step of determining the quantity of binding partner bound to the immobilized vitamin B_{12} comprises assaying the enzymatic activity bound to the immobilized vitamin B_{12}.

34. The method of claim 33 wherein the enzyme is selected from the group consisting of horseradish peroxidase, alkaline phosphatase, and β-galactosidase.

35. A specific binding enzyme assay method for vitamin B_{12} in a test sample, comprising the steps of:

(a) reacting the test sample with:

(i) a biotinylated intrinsic factor such that substantially all of the molecules of intrinsic factor are covalently linked to at least one biotin molecule, the biotin molecules being coupled to the amino groups of the intrinsic factor; and

(ii) sufficient immobilized vitamin B_{12} that substantially all of the biotinylated intrinsic factor is bound by the immobilized vitamin B_{12} in the absence of vitamin B_{12} in the test sample, the immobilized vitamin B_{12} being covalently linked to a solid support of diazotized amine-derivatized beads polystyrene beads by a linker of IgG;

(b) separating the solid support and any intrinsic factor bound to the solid support from unbound intrinsic factor;

(c) incubating the solid support with horseradish peroxidase covalently linked to succinylated avidin so that any intrinsic factor bound to the solid support will bind horseradish peroxidase; and

(d) then assaying the horseradish peroxidase bound to the solid support, the enzymatic activity of horseradish peroxidase present being inversely proportional to the quantity of free vitamin B_{12} originally present in the test sample.

36. A specific binding enzyme assay method for vitamin B_{12} in serum comprising the steps of:

(a) treating serum with NaOH and dithiothreitol to inactivate any endogenous vitamin B_{12}-binding activity present in the serum, in the presence of KCN to stabilize the vitamin B_{12};

(b) neutralizing the treated serum;

(c) adding biotinylated intrinsic factor to the neutralized treated serum and incubating the resulting solution to permit binding of the vitamin B_{12} to the intrinsic factor;

(d) then adding a solid support to which vitamin B_{12} has been covalently coupled and incubating the support in the solution to bind any remaining unbound biotinylated intrinsic factor in the solution;

(e) washing the solid support to remove any unbound material;

(f) adding succinylated avidin conjugated to horseradish peroxidase to the solid support and incubating again;

(g) washing the excess avidin-peroxidase conjugate from the solid support;

(h) adding the peroxidase substrate o-phenylenediamine to the solid support, thereby producing a colored product, the quantity of colored product produced being inversely proportional to the quantity of free vitamin B_{12} originally present in the serum sample; and

(i) measuring the absorbance at 490 nanometers of the colored product produced thereby.

37. A composition of matter comprising modified intrinsic factor covalently coupled to a ligand through the amino groups of the intrinsic factor such that substantially all the molecules of intrinsic factor are covalently linked to at least one ligand.

38. The composition of matter of claim 37 wherein the ligand molecules contain a carboxyl group and wherein the ligand molecules are coupled to the molecules of intrinsic factor through amide linkages between the carboxyl groups of the ligand molecules and the amino groups of intrinsic factor.

39. The composition of matter of claim 38 wherein the amide linkages are produced by the reaction of the carboxyl group of the ligand with N-hydroxysuccinimide to form an N-hydroxysuccinimidyl ester and the subsequent reaction of the N-

hydroxysuccinimidyl ester with the amino groups of intrinsic factor.

40. A composition of matter comprising biotinylated intrinsic factor such that substantially all of the molecules of intrinsic factor are covalently linked to at least one biotin molecule, the biotin molecules being coupled to the amino groups of the intrinsic factor.

41. A composition of matter comprising intrinsic factor covalently coupled to a hapten through the amino groups of the intrinsic factor such that substantially all of the molecules of the intrinsic factor are covalently linked to at least one hapten molecule.

42. A process for the biotinylation of intrinsic factor comprising the steps of:

(a) reacting the intrinsic factor with the N-hydroxysuccinimidyl ester of biotinylamidocaproic acid at a pH of about 7.4 and at a ratio of biotin moieties to intrinsic factor molecules of from about 6.3:1 to about 1375:1; and

(b) then deactivating the remaining N-hydroxysuccinimidyl ester of biotinylamidocaproic acid by reacting it with an excess of ethylenediamine.

43. A composition of matter comprising biotinylated intrinsic factor produced by the process of claim 42.

44. In a competitive assay for vitamin B_{12} wherein the vitamin B_{12} is reacted with a binding partner, the improvement comprising using as the binding partner the composition of matter of claim 37.

45. In a competitive assay for vitamin B_{12} wherein the vitamin B_{12} is reacted with a binding partner, the improvement comprising using as the binding partner the composition of matter of claim 38.

46. In a competitive assay for vitamin B_{12} wherein the vitamin B_{12} is reacted with a binding partner, the improvement comprising using as the binding partner the composition of matter of claim 39.

47. In a competitive assay for vitamin B_{12} wherein the vitamin B_{12} is reacted with a binding partner, the improvement comprising using as the binding partner the composition of matter of claim 40.

48. In a competitive assay for vitamin B_{12} wherein the vitamin B_{12} is reacted with a binding partner, the improvement

comprising using as the binding partner the composition of matter of claim 41.

49. In a competitive assay for vitamin B_{12} wherein the vitamin B_{12} is reacted with a binding partner, the improvement comprising using as the binding partner the composition of matter of claim 43.

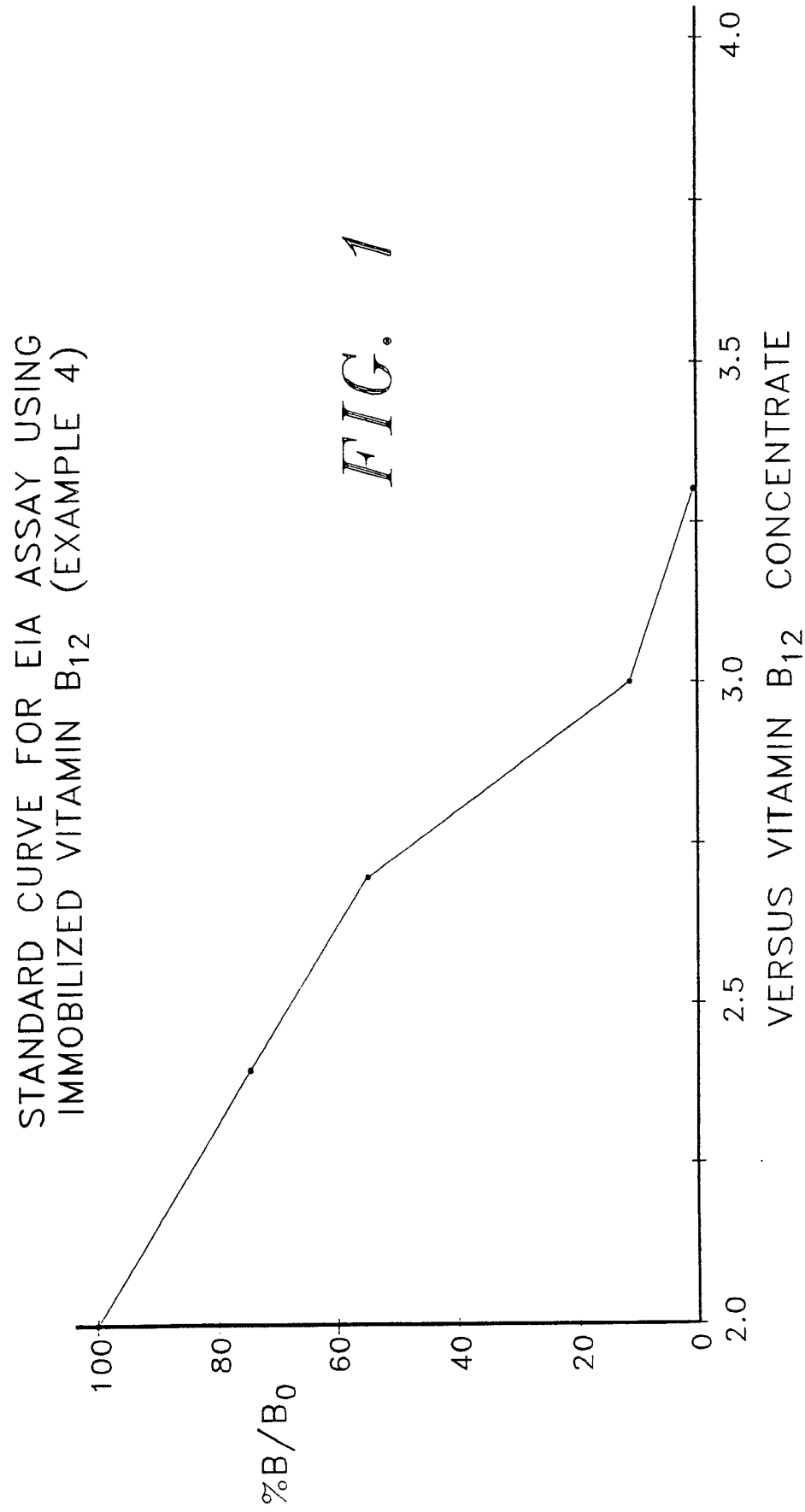
STANDARD CURVE FOR EIA ASSAY USING
IMMOBILIZED VITAMIN B12 (EXAMPLE 4)
%B/B0
100
80
60
40
20
0
2.0
2.5
3.0
3.5
4.0
VERSUS VITAMIN B12 CONCENTRATE

FIG. 1

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/02193

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: G 01 N 33/82, 33/543, 33/58, 33/547

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	FR, A, 2412074 (TECHNICON INSTRUMENTS CO.) 13 July 1979, see the whole document	1,3,30,31
X	US, A, 4333918 (TECHNICON INSTRUMENTS CO.) 8 June 1982, see the whole document	1,30
X	EP, A, 0069450 (TECHNICON INSTRUMENTS CO.) 12 January 1983, see the whole document	1-3,21,30-34
A	GB, A, 2084320 (AMERSHAM INTERNATIONAL LTD) 7 April 1982, see the whole document	36
A	FR, A, 1540895 (PHARMACIA AB) 27 September 1968	

---

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th September 1989	06 OCT 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8902193 SA 29420

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 29/09/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-A- 2412074	13-07-79	AU-A- 4239678	21-06-79
		CH-A- 638053	31-08-83
		DE-A,C 2853500	21-06-79
		JP-A- 54116293	10-09-79
		NL-A- 7812106	18-06-79
		AU-B- 519485	03-12-81
		BE-A- 872681	12-06-79
		CA-A- 1104911	14-07-81
		GB-A,B 2011070	04-07-79
		SE-B- 433669	04-06-84
		SE-A- 7812615	15-06-79
US-A- 4333918	08-06-82	None	
EP-A- 0069450	12-01-83	CA-A- 1180273	01-01-85
		JP-A- 58000997	06-01-83
		US-A- 4465775	14-08-84
GB-A- 2084320	07-04-82	DE-A,C 3137668	27-05-82
		US-A- 4426455	17-01-84
FR-A- 1540895		BE-A- 705412	01-03-68
		CH-A- 476493	15-08-69
		DE-A- 1673015	04-05-72
		GB-A- 1151608	14-05-69
		NL-A- 6714282	22-04-68
		US-A- 3505019	07-04-70
		US-E- RE29480	22-11-77

EPO FORM P0479