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Vitamin B12

REF

E27-393

Enzyme Immunoassay for the Quantitative Determination of Vitamin B₁₂ in Food

FOR RESEARCH USE ONLY

General Information

The Immunospec Vitamin B₁₂ as a trace element belongs to the biologically important chelate formers. The basic unit consists of a corrin ring with cobalt as a central atom. Cobalt is sixfold coordinated by four nitrogen atoms, one cyanide and a dimethylbenzimidazol group. Vitamin B₁₂ forms a stable complex, which is absorbed in the lower part of the small intestine, with the so-called *intrinsic factor* present in the gastric juice. A lack of vitamin B₁₂ can lead among other things to pernicious anemia. This disease is not generated by an insufficient supply of vitamin B₁₂, but by the absence of *intrinsic factor*. A pernicious anemia can be treated by a high dosage of vitamin B₁₂.

The existing detection procedures are mainly microbiological methods (1), but also HPLC and thin-layer chromatography, all of which are associated with a high amount of time and instrumentation.

With the present test kit it is possible, to determine vitamin B₁₂ quantitatively in vitaminated food (2) in a significantly faster way (2.5 to 4 hours inclusive sample pretreatment) compared with a conventional microbiological assay (24 to 48 hours).

Principle of the Test

The Immunospec Vitamin B₁₂ quantitative test is based on the principle of the enzyme linked immunosorbent assay. An antibody directed against vitamin B₁₂ is bound on the surface of a microtiter plate. Vitamin B₁₂ containing samples or standards and a vitamin B₁₂-peroxidase conjugate are given into the wells of the microtiter plate. Enzyme labeled and free vitamin B₁₂ compete for the antibody binding sites. After one hour incubation at room temperature, the wells are washed with diluted washing solution to remove unbound material. A substrate solution is added and incubated for 20 minutes, resulting in the development of a blue colour. The colour development is

inhibited by the addition of a stop solution, and the colour turns yellow. The yellow colour is measured photometrically at 450 nm. The concentration of vitamin B₁₂ is indirectly proportional to the colour intensity of the test sample.

Precautions

Full compliance of the following good laboratory practices (GLP) will determine the reliability of the results:

1. Prior to beginning the assay procedure, bring all reagents to room temperature (20-25°C).
2. All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
3. Once the assay has been started, all subsequent steps should be completed without interruption and within the recommended time limits.
4. Replace caps in all the reagents immediately after use. Do not interchange vial stoppers.
5. Use a separate disposable tip for each specimen to prevent cross-contamination.
6. All specimens and standards should be run at the same time, so that all conditions of testing are the same.
7. Do not mix components from different batches.
8. Do not use reagents after expiration date.
9. Check both precision and accuracy of the laboratory equipment used during the procedure (micropipets, ELISA reader etc.).

Health and safety instructions

1. Do not smoke or eat or drink or pipet by mouth in the laboratory.
2. Wear disposable gloves whenever handling patient specimens.
3. Avoid contact of substrate and stop solution with skin and mucosa (possible irritation, burn or toxicity hazard). In case of contact, rinse the affected zone with plenty of water.
4. Handling and disposal of chemical products must be done according to good laboratory practices (GLP).

Reagents

The kit contains reagents for 96 determinations. They have to be stored at 2-8°C. Expiry data are found on the labels of the bottles and the outer package.

1. Microtiter plate consisting of 12 strips with 8 breakable wells each, coated with anti-vitamin B₁₂.
2. Vitamin B₁₂ Standards (0; 0.4; 1; 4; 10; 40 ng/mL): 6 vials with 0.5 mL each, dyed red, ready-to-use.
3. Conjugate (Vitamin B₁₂-Peroxidase): 6 mL, dyed red, ready-to-use.
4. Substrate Solution (TMB): 15 mL; ready-to-use.
5. Stop Solution (0.5 M H₂SO₄): 15 mL; ready-to-use.
6. Sample Diluent (PBS): 2 x 60 mL; dyed red, ready-to-use.
7. Washing Solution (PBS + Tween 20): 30 mL as 10x concentrate, dyed blue. Dilute 1+9 with distilled water. If during the cold storage crystals precipitate, the concentrate should be warmed up to 37°C for 15 minutes.
8. Two plastic foils to cover the strips during the incubation.
9. Plastic bag to store unused microtiter strips.
10. Instruction Manual.

Additional Instrumentation and Reagents (not provided)

Instrumentation

- 10 - 1000 µL-micropipets
- Volumetric flask
- Mortar, mixer
- Centrifuge
- ELISA reader (450 nm)

Reagents

- Potassiumhexacyanoferrate(II)-3-hydrate (150 g/L; Carrez I)
- Zincsulfate-7-hydrate (300 g/L; Carrez II)
- Double-distilled water
- 1 M caustic soda solution
- 1 M hydrochloric acid

Sample Preparation

The vitamin is extracted from the sample by double-distilled water. After the dissolution, the pH is adjusted by 1 M caustic soda solution or 1 M hydrochloric acid to 6-7. Afterwards potential turbid matter is precipitated by Carrez I (150 g/L Potassiumhexacyanoferrate(II)-3-hydrate) and Carrez II (300 g/L Zincsulfate-7-hydrate). The extract is filled up to a defined volume and is centrifuged. Samples which are difficult to dissolve in cold water can be brought in solution by gentle warming. After the centrifugation, the samples are further diluted by the supplied sample diluent. To exclude interfering matrix or pH effects, a minimal dilution of 1 in 5 should be followed. We

recommend a dilution to 1-10 ng/mL, in order to obtain an optimal accuracy during the measurement.

Grain products normally contain low concentrations of vitamin B₁₂. In order to avoid high dilutions, the sample can be extracted directly by sample diluent instead of double-distilled water. The amount of sample diluent supplied in the kit is not sufficient in this case. The buffer can however be ordered separately from **Immunospec**.

Multivitamin Tablets and Capsules

The tablets and capsules are dissolved in double-distilled water, and the pH value is adjusted to 6-7. Then 0.5 mL each of Carrez I and Carrez II are added, and the solution is filled up to a defined volume by double-distilled water. The solid matter is separated by centrifugation, and the upper phase is further diluted by sample diluent. To dissolve the capsules, heating to 30-40°C is recommended.

Multivitamin Juices

The juice is adjusted to pH 6-7, 0.5 mL each of Carrez I and Carrez II are added, and the solution is filled up to a defined volume by double-distilled water. The solid matter is separated by centrifugation, and the upper phase is further diluted by sample diluent.

Multivitamin Jam

The jam is homogenised in a mixer, and approximately 8 grams are extracted by double-distilled water, the pH is adjusted to 6-7 and 0.5 mL each of Carrez I and Carrez II are added. Afterwards the solution is filled up to a defined volume by double-distilled water. The solid matter is separated by centrifugation, and the upper phase is further diluted by sample diluent.

Grain Products (Corn Flakes and Muesli)

3-5 grams of sample are homogenised by a mortar or a mixer, extracted by double-distilled water, the pH is adjusted to 6-7, and 0.5 mL each of Carrez I and Carrez II are added. Afterwards the solution is filled up to a defined volume by double-distilled water. The solid matter is separated by centrifugation, and the upper phase is further diluted by sample diluent. Grain products normally contain low concentrations of vitamin B₁₂. In order to avoid high dilutions, the sample can be extracted directly by sample diluent instead of double-distilled water.

Multivitamin Sweets

The sweets are dissolved by gentle heating (if necessary) in double-distilled water, the pH is adjusted to 6-7, and 0.5 mL each of Carrez I and Carrez II are added. Afterwards the solution is filled up to a defined volume by double-distilled water. The solid matter is separated by centrifugation, and the upper phase is further diluted by sample diluent.

Procedure

1. Prepare samples as described above.
2. Pipet 50 µL standards or prepared samples in duplicate into the appropriate wells of the microtiter

plate. Immediately add 50 μL vitamin B₁₂-peroxidase conjugate into each well.

- Cover the microtiter plate with a plastic foil and incubate for 60 minutes at room temperature on a microtiter plate shaker (or 90 minutes without shaker).
- Wash the plate three times as follows: Discard the contents of the wells (dump or aspirate). Pipet 300 μL of diluted washing solution into each well. After the third repetition empty the wells again and remove residual liquid by striking the plate against a paper towel. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbencies.
- Pipet 100 μL of substrate solution into each well.
- Allow the reaction to develop in the dark (e.g. cupboard or drawer; the chromogen is light sensitive) for 20 minutes at room temperature.
- Stop enzyme reaction by adding 100 μL of stop solution (0.5 M H₂SO₄) into each well. The blue colour will turn yellow upon addition.
- After thorough mixing, measure absorbance at 450 nm (reference wavelength 620 nm), using an ELISA reader. The colour is stable for 30 minutes.

Calculation of results

- Calculate the average optical density (OD 450 nm) for each set of reference standards or samples.
- Construct a standard curve by plotting the mean optical density obtained for each reference standard against its concentration in ng/mL on semi-log graph paper with the optical density on the vertical (y) axis and the concentration on the horizontal (x) axis.
- Using the mean optical density value for each sample, determine the corresponding concentration of vitamin B₁₂ in ng/mL from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.
- The diluted samples must be further converted by the appropriate dilution factor. The factor is dependent on the sample preparation procedure employed.

Typical Standard Values

The following table contains an example for a typical standard curve. The binding is calculated as percent of the absorption of the 0 ng/mL standard. These values are only an example and should not be used instead of the standard curve which has to be measured in every new test.

Vitamin B₁₂ (ng/mL) (% binding of 0 ng/mL)

0	100
0.4	86
1.0	70
4.0	24
10	10
40	4

Performance

Sensitivity

The sensitivity of the **Immunospec Vitamin B₁₂ test** is 0.3 ng/mL (based on the standard curve).

Recovery

The recovery of spiked samples was determined to 98%.

Intra-assay Precision

The intra-assay variation of the vitamin B₁₂ test was determined to 3%.

Cross-reactivity relative to vitamin B₁₂ (=100%)

Hydroxycobalamine	29%
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