METHODS OF VITAMIN ANALYSIS

Specimen requirements;

- Fasting plasma or serum
- Lithium Heparin is the anticoagulant of choice for vitamins such as; thiamine, riboflavin, retinol, tocopherols & cholecalciferol
- EDTA could be used for vitamins B6 and folates

Methods and their selection;

Selection of a method depends upon the vitamin being analyzed. Some vitamins such as vit-A, K, B12 have special features which allow use of some specific methods in a cost effective (cheap) manner.

Some methods measure vitamins directly and give a quantitative measurement. These are recommended for use particularly for clinically useful vitamins. However these are expensive and not every laboratory can provide these assays. These methods include:

- HPLC *(reference method)*
- Immunoassays (ELISA, RIA, FIA)
- Colorimetric and Spectrophotometric assays
- Fluorometric assay & Chemiluminescence assay
- Amperometric assay

Since vitamins function as co-factors and substrates in many body reactions, some methods utilize this property and indirectly measure vitamin by measuring activity of enzyme under their influence. These methods are comparatively cheaper and could be semi-quantitative or qualitative. However some of these methods are used today for research only and these include:

- Metabolite loading test
- Erythrocyte enzyme activation assay
- Eryhtrocyte fragility test
- Prothrombin time
- Microbiological method
- Bioassays (*in*-vivo & *in*-vitro)

High Performance Liquid Chromatography (HPLC)

This is a reference method to analyze any type of vitamin.

Also used to determine iso-forms of the vitamins

ADVANTAGE	DISADVANTAGE
- Direct - Quantitative - Precise & accurate - Sensitive and specific - Automated - High through put	- Requires sample pretreatment such as extraction & filtration - Requires careful selection of mobile phase & sample pumping rate - Initially requires optimization. In order to obtain high resolution a low baseline and low back ground noise has to be maintained - Requires internal standards, calibrators and HPLC grade reagents - Expensive to buy and maintenance costs are high

Immunoassays

There are several types of immunoassays such as:

- Enzyme Linked Immuno- Sorbant Assay that utilizes antibodies linked to enzyme.

- Radio-immunoassays instead utilize a radio-isotope and due to radioactive disposal concerns these are now out dated.
- Fluorescent immunoassays utilize antibodies labeled with fluorophore but lack sensitivity as compared to previous two.

Vitamin-B12 and Vitamin-D can be analyzed by immunoassays. These assays however depend upon availability of monoclonal antibodies that determine their specificity and sensitivity.
For few samples immunoassays are considered expensive but cost can be reduced if sufficient samples are 'batch' analyzed on a 96-well format.
Immunoassays are the second most reliable method for vitamin measurement after HPLC.

Colorimetric and Spectrophotometric assays

A chemical reaction between a chromogen and vitamin causes color change. The color development shows presence of vitamin….a qualitative analysis.
To quantify this method, a spectrophotometer is used to measure the color intensity 'end point', where color intensity is proportional to vitamin concentration.
Mainly used for vit-C (ascorbic acid) determination.

ADVANTAGE	DISADVANTAGE
- A direct quantitative assay - Requires small sample volume - Automated - Fast turn-around time - Could be applied to single or several samples at a time	- Low sensitivity and specificity hence several other substances may interfere with the chromogen or enzyme used. - Can not be used for all types of vitamins - Proper controls and calibrators should be used

Fluorometric assay & Chemiluminescence assay

Certain vitamins have ability to produce fluorescence when reacted with a fluorophore. This fluorescence is directly proportional to vitamin concentration.

In Chemiluminescence assays, luminol is used to react with vitamin of interest. The mixture is injected at a controlled rate through capillary where 'kinetic reaction' is measured and a quantitative measurement is given.
Advantages and disadvantages are similar to colorimetric and Spectrophotometric assays.

Amperometric assays

Some vitamins can undergo electro-chemical oxidation.
This reaction at an electrode causes change in electrical potential that is directly proportional to vitamin concentration in the sample.
The major disadvantage of this method is the lack of specificity and is rarely used, sometimes in parallel with HPLC for confirmation of certain results.

Indirect methods for vitamin determination

Metabolite loading test

These assays have been extremely popular in the past.

It's is an indirect assay that determines vitamin activity used as a cofactor for certain metabolite.

Steps involved:

- The patient is given orally a large, measured dose of certain metabolite that is used by the vitamin of interest for its conversion.
- Blood or urine sample is obtained from the patient after ~ 6hrs of ingestion.
- The same metabolite is measured in this sample:
  Increased metabolite above the normal range shows vitamin deficiency
  Significantly reduced metabolite shows normal vitamin activity
- E.g. Tryptophan is a loading metabolite for vit-B6 and Histidine is for folate

ADVANTAGE	DISADVANTAGE
- Inexpensive method - Minimum side effects	- Indirect method - *In*-vivo therefore not reliable due to interference by other factors - Not applicable for all vitamins - Invasive

Erythrocyte enzyme activation test

- Hemolysates of whole blood or RBC are prepared
- Enzyme activity under direct influence of the vitamin of interest is measured. This enzyme activity is known as Activity Coefficient (AC).
- A saturating amount of this particular vitamin is added.
- Enzyme activity is re-measured and compared to enzyme activity before addition.
- An increase in AC indicates vitamin deficiency. In case of normal vitamin or even vitamin saturation, the AC will not change.

- E.g. Transketolase activity is measured for evaluating thiamine levels, Glutathione reductase is measured for evaluating riboflavin status and Aspartate transaminase (AST) activity is measured for vit-B6 analysis.

Erythrocyte fragility test

Used specifically for vitamin-E level assessment. This vitamin is responsible for RBC membrane stability.

Steps involved:

- Patient sample is divided into two aliquots.
- RBCs in one aliquot are washed with 2% H_2O_2 (membrane toxic free radical), while RBCs of 2nd aliquot are washed with dist. H_2O.
- After 3hrs incubation, hemoglobin is measured in both aliquots.
- If hemolysis of RBCs in 1st aliquot (washed with H_2O_2) is 20% more than hemolysis in 2nd aliquot (RBCs washed with dist.H_2O), this will indicate vitamin-E deficiency.

Prothrombin Time

This is specific, yet indirect assay for the assessment of vit-K activity.

Since vit-K is responsible for activation of clotting factors (II, VII, IX, X, protein C, S & Z) increased PT > 2 min indicates vit-K deficiency.

This is considered a screening test, however to confirm HPLC or Spectrophotometric analysis is recommended.

Microbiological Methods *(research use only)*

Some micro-organisms e.g. Lactobacillus Casel and Lactobacillus Plantoides depend upon certain vitamins for their growth.

These micro-organisms are grown in a broth or culture agar containing sample with unknown concentration of vitamin of interest.

A negative control that has no vitamin at all is also used.

Incubate for several days

The growth density is measured by turbidimetry

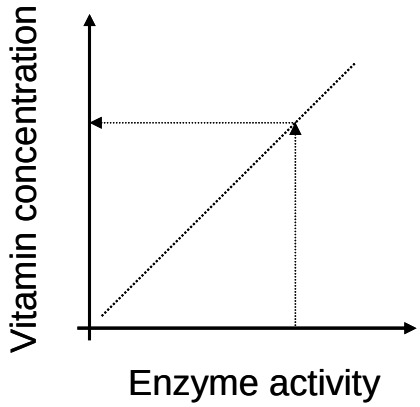
Also lactic acid formation can be quantified which is directly proportional to vitamin concentration in the sample

Bioassays for vitamins

These are research tools only and are rarely used clinically for vitamin analysis. They measure the enzyme under the influence of vitamin and the phenotypic effects of their deficiency. There are two types of bio-assays:

In-vivo Bioassays

- Requires live animals preferably small in size such as rats or chicks
- The animal's physical properties such as weight, behavior, respiration rate, pulse, limb and eye movements and other physical observations are recorded.
- The enzyme activity under the direct influence of the vitamin to be analyzed is determined in blood sample spectrophotometrically (peak value).
- The animal is then starved of the diet containing that particular vitamin for a specific period.
- When the animal starts developing the disease associated with that vitamin deficiency, another blood sample is drawn and enzyme activity is re-measured. This is the baseline value. Other physical symptoms are recorded during the disease state.
- Now a calculated and gradually increasing dose of vitamin is fed to the animal at certain time points.
- The enzyme activity is regained and physical symptoms start improving.
- Blood sample is drawn at specific time intervals and enzyme activity is measured until the peak (optimum) activity is regained and animal's vital symptoms have fully recovered.
- This assay allows a *vitamin concentration* vs *enzyme activity* standard curve.
- The same standard curve can now be used for analyzing the same vitamin in various animal models since enzyme activity can be measured and using the curve, the expected vitamin concentration can be obtained.



e.g. a rat when starved of vit-D for some time, will develop rickets. Vit-D 25-hydroxylase enzyme activity is affected, which utilizes vit-D as substrate. This triggers a cascade of events that lead to bone deformities in this rat. However when certain calculated dose of vit-D is fed the symptoms recover. Enzyme activity is measured at each dose time point and a standard curve is formulated.

ADVANTAGE	DISADVANTAGE
- Close to reality - Can lead to break through in research	- Indirect method - Requires careful monitoring - Difficult to maintain consistent data due to interference by other factors - Not applicable for all vitamins - Expensive due to animal management - Time consuming - Has no clinical application

In-vitro Bioassays

- These assays involve targeting specific cell lines or tissues in a culture media.
- Affect of vitamins is studied on the growth, proliferation and differentiation of the cells.
- Other factors or chemicals such as: proteins, immunoglobulin, calcium, phosphorous produced by these cells & tissues upon exposure to vitamins are also measured.

  E.g. anti-oxidative effects of vit-C and E an be established by these assays

ADVANTAGE	DISADVANTAGE
- Easier than in-vivo - Better monitoring & control of conditions - Less time consuming & inexpensive - Can be used to analyze effects of several vitamins	- Indirect - Not close to reality - Tissue and cell cultures are at risk of contamination