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MEASUREMENT OF VITAMIN B_{12} CONCENTRATION: A REVIEW ON AVAILABLE METHODS

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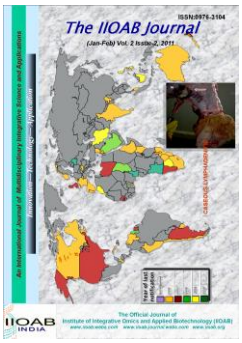
ABSTRACT



Vitamin B12 is a water-soluble vitamin. It is one of the eight vitamins of vitamin B complex, needed for blood and cell maturation. It helps maintain healthy nerve cells and red blood cells, and it is needed in DNA replication. Its deficiency may cause megaloblastic anemia (amidst others health issues). For these and many similar reasons, it sometimes becomes necessary to measure its concentration. This article has carefully reviewed the different methods used for measuring vitamin B12 concentration, and the unique principles involved. The principles, basically, depend on the molecular structure of Vitamin B12 and its reactions with other substances. The methods include microbiological assay and spectrophotometric methods – these are old methods: they were the first available methods, but they are still in use for reference purposes. Another method is electroluminescent (ECL) which involves highly reactive materials. However, inductive-coupled plasma-mass spectrometry (ICP-MS) is a very important method, which is used routinely, even in many research. On the other hand, atomic absorption spectroscopy depends on measuring the amount of energy involved in the reaction; while radioimmunoassay (RIA) is a highly sensitive immunoassay technique. In addition, there are different techniques for separating and preparing samples to be used in the various measurement methods. High-performance liquid chromatography (HPLC) is used for non-validate analyst, while capillary-electrophoresis (CE) that have high resolving power than traditional electrophoresis, which when they are coupled with certain detectors they afford us another principle for measuring this vitamin. Choosing the best method for measuring vitamin B12 concentration depends on many factors – including the type of sample, purpose of the test, necessity of pre-processing, time limitations, cost, sensitivity, specificity.

Keywords: *Electroluminescence; Inductive-Coupled Plasma-Mass Spectrometry; microbiological assay; radioimmunoassay; capillary-electrophoresis; vitamin B12 concentration*

[I] INTRODUCTION

Vitamin B12 is a water-soluble vitamin. It is one of the "B complex vitamins," which play roles in red blood cell formation, nerve cell maintenance, and methyl donation in DNA synthesis. Deficiency of vitamin B12 affects immunologic and hematologic parameter in the body [1].

Human's source of vitamin B12 is of animal origin. It was in 1948 that vitamin B12 was first isolated from liver juice, and it was used in treating pernicious anemia [2]. Vitamin B12 consists of corrin ring (synthesized by bacteria) and cobalt ion; and this cobalt-corrin ring complex gives vitamin B12 its red colouration. Different forms of vitamin B12 are similar in the cobalt central ion, the four parts of the corrin ring and a dimethylbenzimidazole group, but differ in the sixth site which may contain cyano group (CN), hydroxyl group (OH), methyl group (CH3) and/or 5'-deoxyadenosyl group (C-CO) [3,4].

There are several methods to assay and calculate vitamin B12. Some of these methods are used in medical field, and some others in pharmacological studies/investigations. This review

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focuses on some of the weaknesses and strengths of these methods, and aims to identify the best method for measuring the concentration of vitamin B12.

[II] HISTORICAL TECHNIQUES

2.1. Microbiological and spectrophotometric methods

Microbiological method is one of the oldest methods for measuring the concentration of vitamin B12. Information regarding this method has been extensively documented [5].
Ross (1950) was the first scientist to describe microbiological method using *Euglena gracilis var-bacillaris* as test organism. Thereafter there was introduction of Z strain of *Euglena gracilis* so as to shorten the growth period required for the test to as low a five days [6]. Further experiments on measurement of vitamin B12 focused on either changing microorganism test or developing test techniques, such as adding heating step or some substances to the test procedures for converting vitamin B12 to the active free form [6]. Also, several microorganisms were proposed for the microbiological assay. These methods include *Euglena gracilis* tube method, filter paper disc method (FPD), *Escherichia coli* tube method, plate method, *Lactobacillus leishmanii* tube method, bioautographic method, and *Ochromonas malhamensis* tube method [7]. However, *Euglena gracilis* and *Lactobacillus leishmanii* are the most commonly used methods [5,7,8].

Davis et al [6] described a fully automated method for the microbiological measurement of vitamin B12 using chloramphenicol-resistance strain of *Lactobacillus leishmannii* as test organism. Chloramphenicol eliminates the need for sterilization. Using this method, results could be available within 24 hours. This automated method solved the challenge of how to dissociate vitamin B12 from its protein carrier. This was possible by treating the sample with a solution containing glutamic/malic acid [6].

Automated microbiological method was designed to use Mecolab M which is a multi-instrument that provides facilities for sample dilution, reagent addition and mixing, as well as measurement and digital estimation of bacterial growth. It consists of sample preparation unit, autocolorimeter, A/D converter and calculator [6].

Years after, scientists have tried to develop a microbiological method by using microtiter plates. They used chloramphenicol resistance strain of *lactobacillus casei* on serum and red blood cell folates. Then they compared the results with traditional microbiological method. They obtained better results with better intra-assay precision for both serum and red cells (CV% of <5). However, the previous method was more compact, less time consuming, has a lower cost, need smaller amount of sample, and easy to perform in medical laboratories [9].

Microbiological methods are facing difficulties in the assay of vitamin B12, mainly because they are tedious, and time consuming; they have poor precision, and relatively low specificity [10]. Other disadvantage of this method is that whenever the patient serum contains antibiotics, the growth of some assay organisms will be inhibited and false low/negative results would be obtained [10]. Also, *L. leichmannii* assay may give falsely low results in the presence of some antibiotics and antimetabolites. In addition, the *E. gracilis* assays produce falsely low results with sulfonamide and chlorpromazine [11]. The reference normal range of vitamin B12 concentration for which using *Euglena gracilis* method would be good is 200-900 µg/cc [12].

In 1972, scientists started working on photochemistry of vitamin B12 by Pratt [13]. The conversion of cyanocobalamin to hydroxycobalamin takes place readily in the pH range between "3.5 – 6.5" under the action of light. The quantum of the photoaquation reaction of cyanocobalamin is 10^-4 [14]. The photo degradation of cyanocobalamin plays an important role in the stability of vitamin B12 solutions. If the primary photochemical change leading to the formation of hydroxyl cobalamin could be minimized, the photo stability of cyanocobalamin could be enhanced. Spectrophotomery for vitamin B12 measuring was very diverse according to the use of many light spectra like gamma-ray counter spectrophotometer [15]. Ultraviolet (UV) -vis spectrophotometer different types [16, 17] or some reagents were added as 6,7-dimethoxy-1-methyl-2(1H)-quinoxaline-3-prpionyl carboxylic acid hydrazine (DMEQ) to produce a highly fluorescence vitamin B12 derivative [14], and 4,4'-diazobenzenediazoaminoazobenzene (BBDAB) [18]. In UV and visible spectrophotometry, aqueous solutions of cyanocobalamin exhibit maximum UV and visible region at 278nm, 361nm, and 550 nm [19]. However, several factors such as changes in solvent, temperature, and pH can affect the spectrum [20].

Several many colorimetric methods had been reported for the determination of cyanocobalamin. These methods are based on the determination in the content of cobalt which forms complexes with many compounds at different wavelengths. A colorimetric catalytic kinetic method has been developed for the determination of trace amounts of cobalt in vitamin B12 preparation. In acetate buffer (pH.4), cobalt (II) catalyses the reduction of colorless ferric-dipyridyl complex to pink ferrous-dipridyl complex in the dark. The linear determination range is 0-10 mg/10ml cobalt (III) [15].

Finally, application to injections containing vitamin B12 gave results closer to the results obtained by capillary electrophoresis [20]. Spectrophotometric method has low cost and acceptable specificity in comparison with radio ligand assay [20]. However, it is not suitable for complex samples, and the sensitivity is relatively low in such cases – so it is not used routinely [10].

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[III] PRESENT ACTUAL TECHNIQUES

3.1. Electroluminescence (ECL)

Electroluminescence (ECL) is a process in which reaction of highly reactive molecules are generated from stable state electrochemically by an electron flow cell forming highly reacted species on a surface of a platenium electrode producing light [21]. This method uses ruthenium (II)-tris (bipyridyl) [Ru $(bpy)_3$ $]^{2+}$ complex and triproplamine (TPA) and react them with each other to emit light. The applied voltage creates an electrical field that causes the reaction of all materials. Tripropylamine (TPA) oxidized at the surface of the electrode, releases an electron and forms an intermediate which may further react by releasing a proton. In turn the ruthenium complex releases an electron at the surface of the electrode forming an oxidized form of $Ru(bpy)_3^{3+}$ cation, which is the second reaction component for the chemiluminescent reaction. Then this cation will reduce and form $Ru(bpy)_3^{2+}$ and an exited state via energy transfer which is unstable and decays with emission of photon at 620 nm to its original state [21, 22].

The florescence emitted by $Ru(bpy)_3^{2+}$ is detected by standard photomultiplier, and the results are expressed as ECL intensity, which is the measurement of the whole luminescence emitted from the sample [23]. This method employs various test principles (such as competitive principle, sandwich and bridging) for the measurement [22]. The most important one in measuring vitamin B12 concentration is the competitive principle. The competitive principle is applied to low molecular weight molecules. It uses antibodies (intrinsic factor) for vitamin B12 labeled with ruthenium complex. These antibodies are incubated with the sample, then biotinylated vitamin B12 and streptavidin which is coated with paramagnetic miroparticles are added to the mixture. The free binding sites of the labeled antibody become occupied with the formation of an antigen-hapten complex. Then the entire complex is bonded to biotin and streptavidin. After incubation the reaction mixture is transported into the measuring cell where the immune complexes are magnetically entrapped on the working electrode and the excess unbound reagent and sample are washed away. Then the reaction is stimulated electrically to produce light which is indirectly proportional to the amount of vitamin B12 that is measured **[Figure–1]** [22].

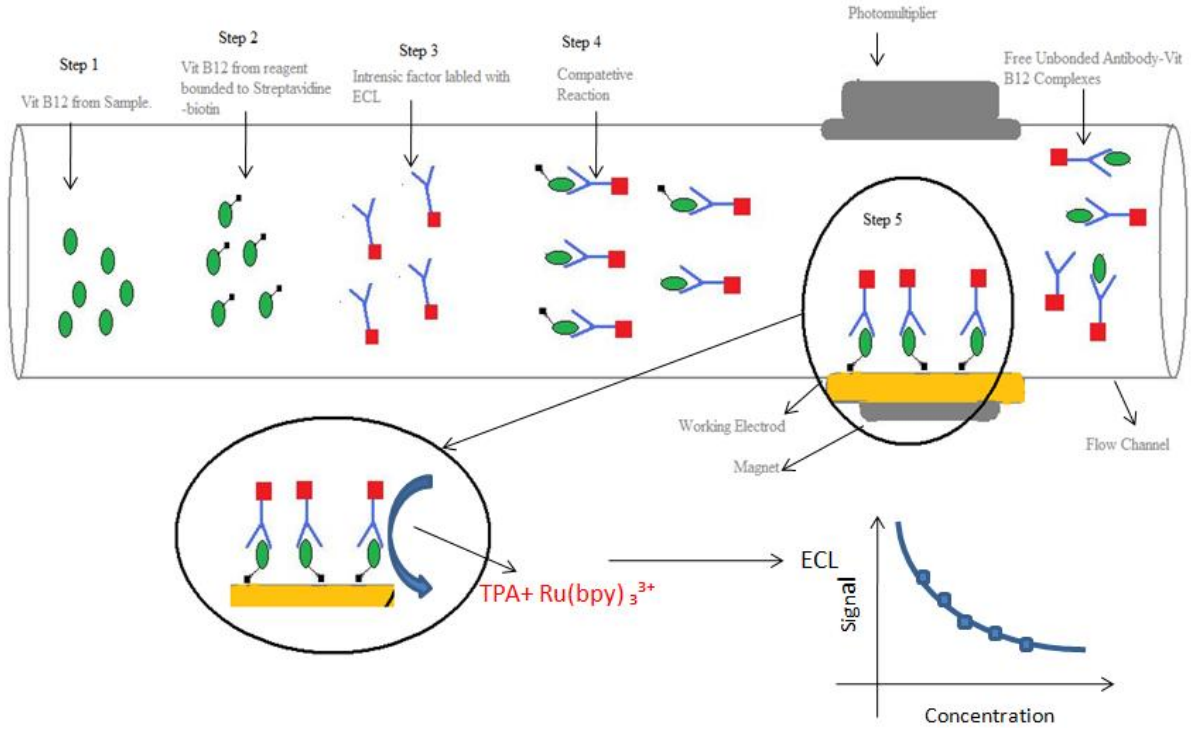

Fig: 1. Electroluminescence method, competitive principle for measuring vitamin B12. **Step 1:** Vitamin B12 from serum sample enters to the flow channel. **Step 2:** Vitamin B12 particles from the reagent are bonded to streptavidin-biotin to help in their attachment to the magnet part. **Step 3:** Intrinsic factor (which acts as antibodies) are bounded to ECL particles to enhance the reaction. **Step 4:** Vitamin B12 from the sample with the particles from the reagent bind to the intrinsic factor. **Step 5:** Only intrinsic factor that is bonded to the vitamin B12 labeled with Streptavidin-biotin particles is attached to the working electrode (by magnetic action) were the ECL reaction will take place and the signal will be measured. The free intrinsic factor with the ones that binds to the vitamin B12 from the sample will be washed away.

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Test sample needed is serum, and the sample duration time is 27 minutes, this test is very sensitive. It can even detect 22 pmol/L (30 pg/ml). It is also very precise (CV% is >10%), and very specific and cross reactivity rarely occurs. This test has high reproducibility, and can be processed easily. Machines used in this technique have extremely long life span with no maintenance costs. An example of such machines is Elecsys 2010 and Cobas e 411. This technique is often used in pharmacological, industrial, clinical and chemical research [24].

3.2. Inductive-coupled plasma (ICP) - mass spectrometry (MS) (ICP-MS)

One of the best methods for the determination of vitamin B12 concentration is mass spectrometry (MS). This is because of its speed, sensitivity, easy (fully automated) and its vast possible application. It is one of the most important instruments for both routine and research applications. In contrast to what its name implies, MS actually measures mass to charge ratio and not just the mass. However, when the charge of all particles (ions) is the same, the mass spectrum plot is simplified to have only mass on the X- axis and the relative abundance on the Y-axis [25].

There are deferent types of MS but they all have three main components in common: an ionization source; mass analyzer; and detector. Ionization process occur in deferent ways in the different types of MS and that is what actually explains the differences between the deferent MS types. Ionization is an important step, and ensures the conversion of the the analyte of interest into gaseous phase ions. The first described ionization source is the electron ionization where the sample must be of low molecular weight, vaporizable and thermally stable. The analytes has to be vaporized and then ionized, and these limited the availability of such method for many biological samples and analytes so there was great need for developmental ionization sources [25, 26]. This lead to the development of electrospray ionization, atmospheric pressure chemical ionization and matrix assisted laser desorption ionization [25].

The first type of ionization source is Electrospray ionization (ESI). It depends on generation of electrons at atmospheric pressure by exposing the sample to different voltage depending on the boiling temperature of the liquid phase sample and the diameter of the inner capillary tube. Most machines with EIS also have additional or optional ionization technique which is Atmospheric Pressure Chemical Ionization or Inductively Coupled Plasma where ionization can also occurs at atmospheric pressure. But these differ from ESI in that sample travels through the different heating zones: when plasma torches it, it becomes dried, vaporized, atomized, and ionized. During this time, the sample is transformed from a liquid aerosol to solid particles, then into a gas, so that they are excited and they gain enough energy to release electrons from their orbits and generate ions. Like ESI and ICP, matrix assisted laser desorption ionization occurs in vacuum where laser irradiation pulsed is the source of ion generation [25, 26, 27].

As Since the ionization sources can differ, there are also several types of mass analyzers. One of the simplest types is “Time of flight mass analyzer” where the velocity of the ions (which depends on the mass to charge ratio) leads to the separation of the ions in different speeds. When fixed potential force them toward the detector, the speed (time) of an ion in reaching the detectors is proportional to its mass to charge ratio – lower ratio is associated with higher velocity. The other type of the mass analyzer is the sector analyzer (magnetic or electric sectors are available). Here the ions are focused toward the detector after they have left the sector, through a split by applying a fixed accelerating potential.

The widely used mass analyzer is the quadruple, especially with gas and liquid chromatography, since it is much smaller, easier, and cheaper than other analyzers. By placing a direct current (DC) field on one pair of rods and a radio frequency (RF) field on the opposite pair, ions of a selected mass are allowed to pass through the rods to the detector, while the others are ejected from the quadrupole. The other ions of different mass to-charge ratios will pass through the spaces between the rods and be ejected **[Figure–2]** [25, 27, 28].

To perform MS one needs to start with pure analyte to be able to use different MS types. So it is important to combined MS with other separation techniques such as capillary electrophoresis, HPLC, gas chromatography, and liquid chromatography, where Cobalamin in human urine and multivitamin tablet solutions can be converted into free cobalt ions in acid medium. The linearity of MS is over the cobalamin concentration range of 1.0 × 10−10 g /mL− to 8.0 × 10−5 g /mL and the limit of detection is 0.05 ng/ mL for both ICP-MS and HPLC-MS. MS is often used in Pharmacology, industries, and in basic research, but not used in clinical field due to its high cost [29].

3.3. Atomic absorption spectroscopy

Atomic absorption spectroscopy is an analytical chemistry technique used for determining concentration of particular metal element in a sample, and it is widely used in pharmaceutics. This technique can be used to analyze the concentration of over 70 different metals in a solution [30]. The discovery of the Fraunhofer lines in the sun's spectrum in 1802 marked the beginning of the main phenomenon behind this technique. However, it was not until 1953 that Sir Alan W (Australian physicist) demonstrated the possibility of using atomic absorption for quantitative analysis [31]. Simply put, atomic absorption spectroscopy has to do with the measurement of the absorption of light by vaporized ground state atoms and then estimating the desired concentration from the absorption. Basically, the incident beam (of light) is attenuated by the absorption by atomic vapor according to Beer’s law [32].

A detector measures the wavelengths of light transmitted by the sample (called the “after wavelengths”), and compares them to the wavelengths, which was passed through the sample (the

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"before wavelengths"). Moreover, a signal processing unit then processes the changes in wavelength, and gives the output for discrete wavelengths as peaks of energy absorption. Since, an atom is unique in its absorption pattern of energy at various wavelengths due to the unique configuration of electrons in its outer shell, the qualitative analysis of a pure sample can be achieved [32]. This, in fact, makes it reasonable for this method to measure the quantity of energy (in the form of photons of light) absorbed by the sample. Using this technique, various metals in organic samples can be analyzed. The basic structure of the machine consists of 4 basic structural elements; a light source (hollow cathode lamp), an atomizer section for atomizing the sample (burner for flame, graphite furnace for electro thermal atomization), a monochromatic for selecting the analysis wavelength of the target element, and a detector for converting the light into an electrical signal **[Figure–3]** [33].

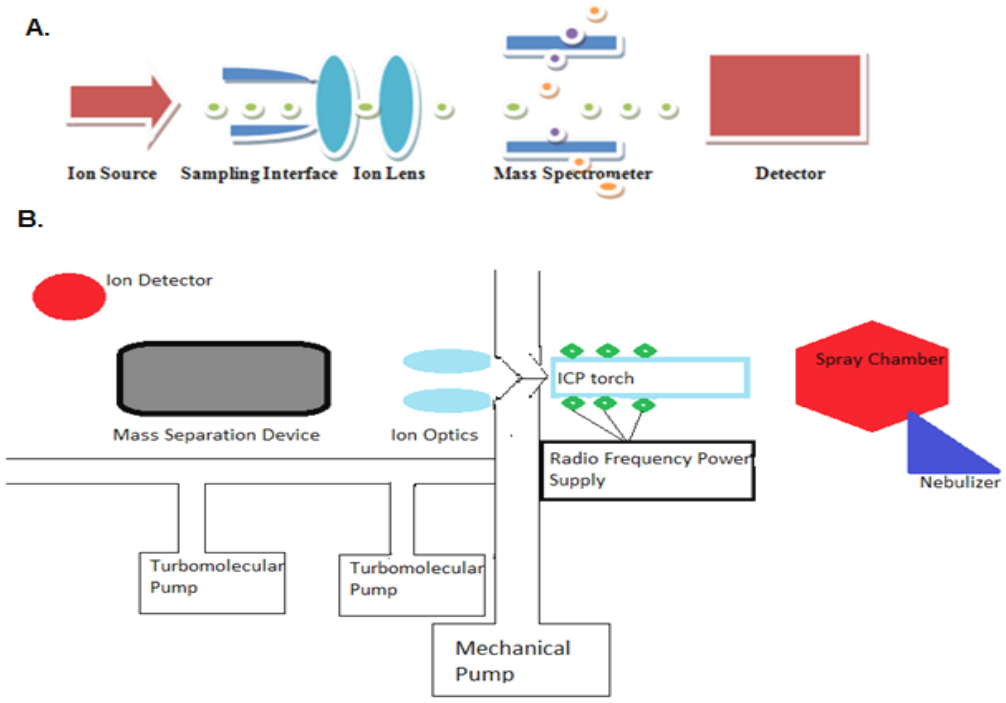

Fig: 2. Inductive-Coupled Plasma-Mass Spectrometry (ICP-MS). A. Mass Spectrometry: Shows the basic components of a typical mass spectrometry. All mass spectrometry shares three main components; an ionization source, mass analyzer, and detector. **B.** Inductive-Coupled Plasma: It serves as the ionization source in some particular types of MS called ICP-MS. The sample travels through the different heating zones and are finally ionized.

Here, the atomizers used are pyrocoated tubes and tubes with centre fixed platforms. In addition, a cobalt hallow cathode lamp is used and a wavelength of 242.5nm could be used for assaying. Argon serves as a protective gas and serum or urine could be introduced into the graphite furnace (GF) directly with equivalent volume of modifiers. H_2O_2 is used to prevent carbon residue formation in graphite tube. The electro thermal atomic absorption correctly and optimally measures Cobalt (and thus, vitamin B12) in serum and urine. It has a detection bound of 0.02 µg/L Co in serum samples with a relative standard deviation of 10-18% [34].

The main advantages of this method is that it has a high sample throughput, it is easy to use, and it has high precision. But the main disadvantages involve its less sensitivity, its requirement of large sample, and the problems with refraction [34]. Another method that is used is the Flame atomic absorption spectrometry. The lowest concentration for quantitative recovery is 4 ng/cm^3 of vitamin B_{12}. The method is used for vitamin B_{12} determination in pharmaceutical samples. It is used in pharmacology, industry, clinical and chemical basic research. [35].

3.4. Radioimmunoassay (RIA)

Radioimmunoassay (RIA) is a highly sensitive laboratory technique used to measure minute amount of substrate (such as, hormones, antigen and drugs) in the body. RIA is a primer immunoassay techniques developed for detecting extremely small concentrations [36]. Berson and Yallow developed the first radio-isotopic technique to study blood volume and iodine metabolism and had used it for the determination of insulin levels in human plasma. Later the technique was adapted for studying how hormones (especially insulin) are being used in the body [35]. This method is so sensitive that it can measure one trillionth of grams of substance per milliliter of blood and only

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small samples are required. These (among other reasons) made RIA to quickly become a standard laboratory tool [37].

RIA is based on the reaction of antigen and antibody in which very small amounts of the radio-labeled antigen competes with endogenous antigen for limited binding sites of the specific antibody against the same antigen. The radio-labeled antigen have been an analogous in biological activity and/or immuno-reactivity to the native antigen. For vitamin B12 we use Modified intrinsic factor (IF) fractions which have R-proteins that bind many porphyrin-ring-containing compounds (i.e., cobinamides) by radio assay with [^{57}Co] vitamin B12 [38].

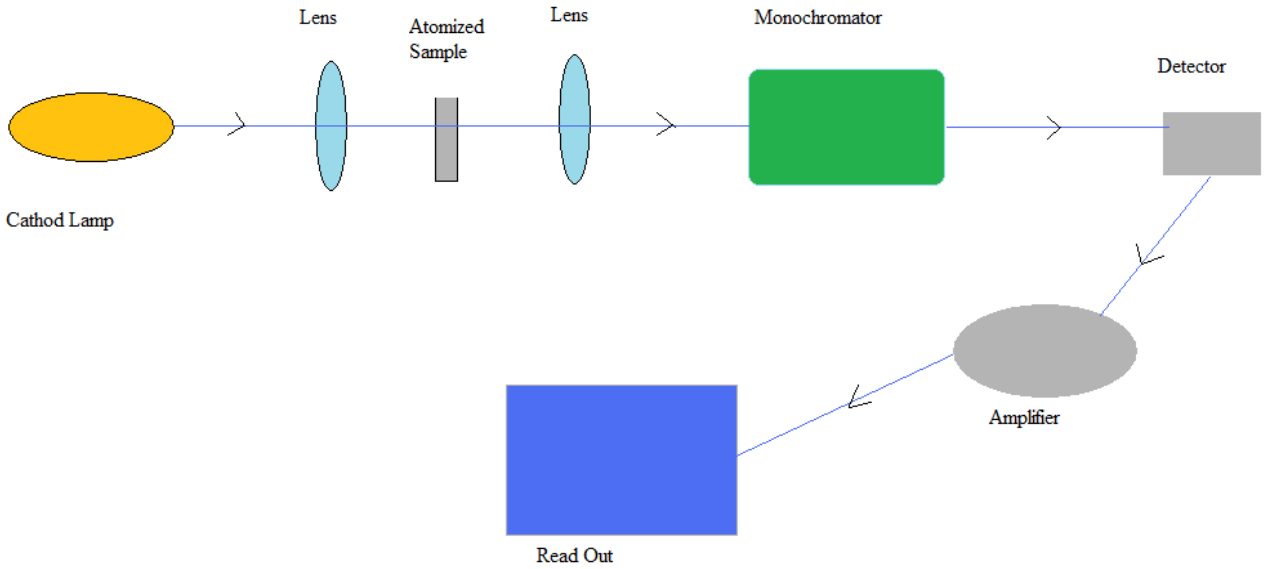

Fig: 3. Schematic diagram of an atomic absorption spectrometer. The basic structure of the machine consists of 4 basic structural elements; a light source (hollow cathode lamp), an atomizer section for atomizing the sample, a monochromatic for selecting the analysis wavelength of the target element, and a detector for converting the light into an electrical signal, amplifier and readout.

Most commonly used radio-isotope in RIA is 125-I. Other emitting isotope such as C14 and H3 have also been used. Some other important aspects of RIA are the use of specific antibody against particular antigen, and the use of pure antigen as the standard or calibrator [37] is attached to tyrosine. These radio labeled IFs are then mixed with a known amount of cyancoblamine, and they become chemically bound to each other. A serum from a patient which contains an unknown quantity of IF is added, so the unlabeled (or "cold") IF from the serum competes with the radio labeled (or "hot") IF for cyanocoblamine binding sites [39].

If the concentration of the "cold" IF increased, more of it binds to cyanocoblamine and this will lead to displacement of the radio-labeled variant, so the ratio of "cyanocoblamine bound to radio labeled antigen" to "free radio labeled IF" is reduced. After that, the bound IF is separated from the unbound ones and the radioactivity of the free IF that remains in the supernatant are measured [39]. The separation of radio-labeled IF bound to cyanocoblamine from unbound radio-labeled IF occurs after optimal incubation conditions (buffer, pH, time and temperatures) [37].

Polyethylene glycol joined with double antibody method is regularly used to separate bound and free radio-labeled IF. Some other techniques in use are the double antigen, charcoal, cellulose, chromatography and solid phase technique [37].
Calibrations or standard curves are formed from sets of known concentrations of the unlabeled standards and from such curves the quantity of IF in the unknown samples can be determined. Improving the sensitivity of the assay is possible by decreasing the amount of radio-labeled analyst and/or antibody, or by disequilibrium incubation format in which radio-labeled IF is added after initial incubation of IF and cyanocoblamine. This technique (just like the others) is supposed to meet the criteria of sensitivity, specificity, precision, recovery and linearity and dilution [39]. For this technique, the precision has been said to be 7.9% for 200 ng/L as the concentration of vitamin B12, 6.6 % for 400 ng/L, and 6.7 % for 800 ng/L. The sensitivity of the assay has also been documented as 37 ± 9 ng/L [39]. RIA is well used in pharmacology, industry, clinical, and chemical research [38].

The principle of radioisotope dilution is based on using unknown quantity of non-radioactive vitamin B12 released from serum to dilute the specific activity of a known quantity of [^{57}Co] vitamin B12. A solution of intrinsic factor concentrate (IFC) with a vitamin B12 binding capacity less than the quantity of added [^{57}Co] vitamin B12 is used to bind a portion of the mixture of radioactive and no radioactive vitamin B12 i.e., to "biopsy" the pool of vitamin B12. The vitamin B12 not bound to IFC is removed by the addition of coated charcoal [40].

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3.5. High-performance liquid chromatography (HPLC)

It is a liquid chromatography used for non-volatile analyst in which the elute do not flow under the force of the gravity but it is derived under a hydrostatic pressure of 5000 to 10000 pounds/square inch through a stainless steel column [41, 42]. The HPLC system uses a mobile-phase pump, a reagent pump, an auto-sampler, a detector and a data system for data processing and system control [43].

The system is a chromatography, in which the eluent is filtered and pumped through the column, then the sample is loaded and injected onto the column and the effluent is monitored using a detector, and the peaks are recorded. The pump of the system must be able to generate high pressure, performing a pulse-free output and deliver flow rates ranging from 0.1 to 10 ml/min [42].

In this method, samples are treated very carefully and the working pH, heating, agitation, centrifugation and filtration are correctly adjusted in accordance with the source of the sample; and the resulting solution is injected into the instrument that does the measurement. he HPLC must be connected to a suitable detector e.g. Micro-mass electrospray mass spectrometer. Its results are often precise, and it is very sensitive with detection limits of 50 nmol/L [43]. An example of this Instrument is Kontron HPLC-system 400. This method is frequently used in pharmacology, industry and basic research [43].

3.6. Capillary electrophoresis

Capillary electrophoresis (CE) was first documented in 1981. It is used to separate peptides. CE have high resolving power than traditional electrophoresis and do not require extremely great skills as high-performance liquid chromatography (HPLC) [44].

CE is quantitative rather than semi quantitative or qualitative, and very small samples (< 10 nL to 1 nL) can be used [43, 44]. The schematic structure for CE is composed of sample vial, two buffer vials (source & destination), capillary, electrodes, high-voltage power supply, detector, and data output. Electroosmotic flow forms the main principle in CE [44]. Generally sample for CE does not require preparations, but in low concentrations biological sample such as serum or plasma, there could be a need for pretreatment to prevent ionic strength and protein-rich matrix from effecting the migration [44,45].
CE can be used for cobalamin separation and for differentiation between different forms of the vitamin B12. The procedure of cobalamin separation is done by using 70 cm capillary length with 20 KV voltage supply, and 9.0 pH tris buffer 25 mM that contain 15 mM sodium dodecyl sulfate as electrophoretic buffer [46].

The main disadvantage of this technique is in its low ability to detect the sample (i.e. low sensitivity) due to the wall of the capillary, which is dissolved by coupling system of capillary electrophoresis-inductively coupled plasma mass spectrometry (CE-ICP-MS). It is mainly used in basic research. [46, 47]

Another source of error that is unique to CE but absent in CE-ICP-MS is the electrokinetic sample injection [45]. On the other hand, no problems are unique to the coupled system method mentioned above. In general controlling the column over loading, calibration to prevent sample aging and facilitate analysis, and buffering according to the sample pH, are important aspects that should always be taken care of while separating vitamin B12 [45].

[IV] DISCUSSION AND CONCLUDING REMARKS

The microbiological method remains the routine method for the determination of vitamin B12 concentration, despite the fact that it is time consuming, and has relatively poor precision, and low specificity. This might be because ECL and radioimmunoassay which are simpler and faster are very expensive - since they require pure intrinsic factor and some special reactants. Also, ECL and atomic absorption spectrometry depends on indirect measurement of the cobalt. On the other hand, Capillary electrophoresis and HPLC methods include the use of UV or visible photometry, atomic absorption and ICP-MS.

Determination of the best way of measuring vitamin B12 concentration would require critical consideration of the required/desired sensitivity and specificity, the available time, and the process of preparation of the sample, as well as cost. Some of the important characteristics of the different methods have been summarized in **Table–1**.

Finally, we should say that cases of serious discrepancies between results of vitamin B12 concentration determined by different methods is highly common. We therefore think that it would be important that every laboratory specifies on it reports the method that had been used when reporting the results of vitamin B12 concentration. This might present clear picture to physician and patient. The reasons for deciding to measure vitamin B12 concentration should also play a crucial role in determining the most appropriate method. For example, the investigator might want to use ECL, RIA or atomic absorption spectroscopy if the results would be used for clinical/medical purposes, while ICP-Ms might be preferred for industrial or pharmaceutical needs or for basic research purposes. More importantly, several advantages and disadvantages of each of these methods govern the choosing of the suitable methods. **Table–2** has clearly summarized some of these.

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Table: 1. Comparison of the Sensitivity of different methods used in measuring the concentration of vitamin B12

Procedure	Sample preparation	Sensitivity
ECL	Serum	30 pg/ml
ICP-MS	Need Preparation	50 pg/ml
Atomic absorption	Urine or serum	20 pg/ml
Radioimmunoassay	Serum	200 pg/ml
HPLC	Need Preparation	68000 pg/ml
Capillary electrophoresis	Need preparation	Depending on the attached method

Table: 2. Advantages, disadvantages and applications of each of the methods used in measuring the concentration of vitamin B12

Procedure	Advantages	Disadvantages	Usage
Microbiological and Specrophotometric	Low cost, Acceptable specificity, Considered as reference method.	Tedious, Time consuming, Poor Precision, Low Specificity, False low results (Antibiotic), Need preparation, Low sensitivity.	Not used routinely, only for basic research.
ECL	Very sensitive, Very precise, Low cross reactivity, High reproducibility and good processability, Very specific, Low maintenance cost for machines uses this method.	Expensive, Only uses serum samples	Used in pharmacology, industry, clinical and chemical basic research.
ICP-MS	Fast, High Sensitivity, Easy (Full automated). Linearity (1x10^-10 g/ml – 8x10^-5 g/ml) , Small sample amount (0.05ng/ml), Use Solid and liquid samples.	Destructive technique, Isobaric, molecular and doubly-charged ion interferences	Used in Pharmacology, industry and basic research, not used in clinical field due to its high cost.
Atomic absorption	Very Sensitive, Fast	Hollow Cathode lamp for each element, Expensive	Used in pharmacology, industry, clinical and chemical basic research.
Radioimmunoassay (RIA)	Highly specific: Immune reactions are specific. High sensitivity: Immune reactions are sensitive	Radiation hazards: Use sra diolabelled reagents. Requires specially trained persons. Labs require special license to handle radioactive material. Requires special arrangements for: Requisition, storage of radioactive material. Radioactive waste disposal	Used in pharmacology, industry, clinical and chemical basic research.
HPLC	It can use different kinds of detectors that determine different sensitivity to the method.	Sample needs preparation and certain conditions.	Used in pharmacology, industry and basic research, not used in clinical approach due to its requirements of certain pH and sample preparations.
Capillary electrophoresis	Quantitative and semi quantitative, Recommended use CE-MS-ICP	Ionic strength and protein-rich matrix affect migration happens in low biological samples. Electrokinetic sample injection Controlling the column	Used in basic research

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