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TOPIC

DESIGN OF HPLC METHOD AND STABILITY STUDIES OF CYANOCOBALAMIN INJECTION

BY

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DESIGN OF HPLC METHOD AND STABILITY STUDIES OF CYANOCOBALAMIN INJECTION

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DECLARATION

Hereby declare that this submission is my own work towards the Mphil and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the university, except where due acknowledgement has been made in the text.

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DEDICATION

This beautiful and blissful handy work is solely dedicated to the entire VIGBEDOR family for their support and kind gesture displayed in diverse ways to the success of this script.

This script is also dedicated to both loved ones and enemies who have kept me vigilant and a sense of consciousness of the task ahead.

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I am very most grateful to the ALMIGHTY GOD for his favour and glory in the form of wisdom, good health and understanding to the success of this beautiful piece of formulated knowledge. I will forever honour you with my life GOD.

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ABSTRACT

An accurate and selective HPLC with ultraviolet absorbance detection was developed for the quantification of vitamin B_{12} (cyanocobalamin) in vitamin B_{12} injections.

The method developed involves an isocratic mode of elution of cyanocobalamin from a reversed-phase 150mm, 4.6mm, 5µm hypersil base-deactivated (BDS) C-18 column.

Cyanocobalamin was quantitatively analysed using the HPLC method comprising methanol/ phosphate buffer pH7 (30 / 70 $^{v}/_{v}$) using ascorbic acid as an internal standard.

Separation for quantitative determination was achieved within 6 minutes at a flow rate of 1.4ml/min .The absorbance of the drugs was monitored at 278nm at a detector sensitivity of 0.03. The standard calibration curves were linear with correlation coefficient (R^2) of 0.996. The limit of quantification (LOQ) and detection (LOD) were 0.069 ug/ml and 0.0228ug/ml respectively.

Four brands of cyanocobalamin injections sampled from some pharmacies and health facilities were analysed with the method and then compared with the standard UV method.

The mean percentage recovery of the stated content of four batches of three brands of the injections were 99.00 ± 3.95, 96.05 ± 0.85, 96.00 ± 5.23, 94.36 ±8.73 as against a standard UV method va1ues of 100.33 ± 4.67, 96.97 ± 0.50, 96.22 ± 6.20, 94.47 ± 10.64 respectively.

Moreover, within and between run relative standard deviations were 1.682% and 1.656% respectively for cyanocobalamin. These values were all less than the maximum threshold value of 2% for a method to be good as a precise method according to the ICH guidelines . All the validation parameters were assessed statistically and proven to be valid.

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LIST OF ABBREVIATIONS

RDA	**Reference dietary allowance**
UIL	**Upper intake limit**
PA	**Pernicious anemia**
WHO	**World health organization**
MPAR	**Mean peak area ratio**
MPA	**Mean peak area**
IS	**internal standard**
MC	**Mean concentration**
RSD	**Relative standard deviation**
S	**Standard deviation**
X	**Mean**
UV	**Ultra violet**
HPLC	**High performance liquid chromatography**
LOQ	**Limit of quantization**
LOD	**Limit of detection**
API	**Active pharmaceutical ingredient**
BP	**British pharmacopoeia**
N	**Number of determinations**

CHAPTER ONE

1.0 INTRODUCTION

Vitamin B_{12} is a water-soluble vitamin of the “B complex vitamins which exist in several forms and contains the mineral cobalt; so compounds with vitamin B_{12} activity are collectively called "cobalamins". Methylcobalamin and 5-deoxyadenosylcobalamin are the forms of vitamin B_{12} that are active in human metabolism.

Vitamin B_{12} is naturally present in some foods, added to others, available as a dietary supplement and a prescription medication. Vitamin B_{12} plays roles in red blood cell formation, nerve cell maintenance, and methyl donation in DNA synthesis. [Institute of medicine, 1998]

The water-soluble vitamin is constantly excreted from the body system and must therefore be replenished in the diet or else results in its deficiency. Deficiency of vitamin B_{12} affects immunologic and hematologic parameter in the body and can potentially cause severe and irreversible damage, especially to the brain and nervous system. At levels only slightly lower than normal, a range of symptoms such as fatigue, depression, mania, psychosis and poor memory may be experienced. Vitamin B_{12} deficiency affects people of all ages, but children under six years of age, pregnant women, vegetarians, the terminally ill and the aged are the most affected usually due to low levels of vitamin B_{12} in diet or malabsorbtion. [Davis et al., 1973]

Vitamin B_{12} status is typically assessed via serum or plasma vitamin B_{12} levels and values below approximately 170–250 pg/ml (120–180 pmol/l) for adults indicate a vitamin B_{12} deficiency.
Vitamin B_{12} deficiency and it’s associated symptoms are normally treated by administering vitamin B_{12} capsules, tablets or injections in large dose over a period of time depending upon severity of the deficiency condition. However, the most effective way of treating vitamin B_{12} deficiency and its associated symptoms is by injecting intramuscularly or intravenously due to gastro-intestinal absorption impairment when administered orally.[Carmel, 1992]

The quality of these vitamin B_{12} injections are sometimes questionable due to the upsurge of fake and low quality B_{12} injections on the market and due to high demand for certain brands of the vitamin coupled with the emergence of some dishonest and profit making oriented manufacturers, one is bound to come across imitation or fake formulations on the market.

However, there are several standard procedures for assessing the quality of Vitamin B_{12} in formulations. This is to ensure that the various formulations meet the required standard of quality. Some of these methods are microbiological, spectrophotometric methods (UV/visible), electroluminescence, inductive-coupled plasma (ICP) - mass spectrometry (MS) (ICP-MS), atomic absorption spectroscopy, radioimmunoassay (RIA), high-performance liquid chromatography (Hplc), capillary electrophoresis etc. [Glick, 2006]

Determination of the best way of measuring vitamin B_{12} 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.

The project seeks to design an accurate and validated selective high performance liquid chromatography with ultraviolet absorbance detection (hplc-uv) for the quantification of vitamin B_{12} (cyanocobalamin) in vitamin B_{12} injections using ascorbic acid as internal standard.

1.1.0 STATEMENT OF THE PROBLEM

Quality of vitamin B_{12} injections in relation to their percentage composition is always under dispute due to instability issues arising from photodegradation, hydrolysis and oxidation.

The project seeks to design a highly sensitive, low cost, faster hplc method to quantify the levels of cyanocobalamin in the injections using ascorbic acid as internal standard.

1.2.0 AIMS AND OBJECTIVES

1.2.1 General objective

- To develop a simple, sensitive, low cost and validated hplc method for the quantification of cyanocobalamin injection using ascorbic acid as internal standard.

1.2.2 Specific objectives

- To develop an isocratic hplc method that can elute cyanocobalamin and ascorbic acid
- To check the quality of the injections with respect to required pharmacopoeia reference range

- To study the temperature effect on cyanocobalamin injections.
- To verify the effect of pH and sunlight on cyanocobalamin solutions.

1.3.0 JUSTIFICATION OF THE PROJECT

Cyanocobalamin deficiency is the main cause of pernicious anaemia among infants, adults and pregnant woman due to the fact that sources of these vitamins from food are not easily absorbed by the stomach due to gastro-intestinal absorption impairment.

Therefore, this peculiar problem requires that the vitamin is injected intravenously or intramuscularly for it to be bioavailable for treatment of this medical condition.

The study would help address issues of quality of these injections in the field of quantitative assay by introducing a new method for assessing the quality of cyanocobalamin injections.

CHA PTER TWO

LITERATURE REVIEW

2.1.0 VITAMINS

Vitamins are classified as either water-soluble or fat-soluble. In humans there are 13 vitamins: 4 fat-soluble (A, D, E, and K) and 9 water-soluble (8 B vitamins and vitamin C). Water-soluble vitamins dissolve easily in water and, in general, are readily excreted from the body, to the degree that urinary output can be used to predict vitamin consumption.[Fukuwatari and Shibata, 2008] Because they are not readily stored, consistent daily intake is important.[Mohammed and Said, 2006]. Many types of water-soluble vitamins are synthesized by bacteria.[Maqbool and Stallings, 2008]. Fat-soluble vitamins are absorbed through the intestinal tract with the help of lipids . Because they are more likely to accumulate in the body, they are more likely to lead to hypervitaminosis than are water-soluble vitamins. Fat-soluble vitamin regulation is of particular significance in cystic fibrosis.[Kutsky, 1973]

For the most part, vitamins are obtained from food, but a few are obtained by other means. For example, microorganisms in the intestine commonly known as "gut flora" produce vitamin K and biotin, while one form of vitamin D is synthesized in the skin with the help of the natural ultraviolet wavelength of sunlight. Humans can produce some vitamins from precursors they consume. Examples include vitamin A, produced from beta carotene, and niacin, from the amino acid tryptophan.[Bender, 2003]

Once growth and development are completed, vitamins remain essential nutrients for the healthy maintenance of the cells, tissues, and organs that make up a multicellular organism; they also enable a multicellular life form to efficiently use chemical energy provided by food intake and to help process the proteins, carbohydrates, and fats required for respiration. [Victor, 1988]

Table 2.1.0 Vitamins showing their RDA, UIL, effects of deficiency and overdose [Kutsky, 1973]

Vitamin	RDA	Upper intake level (UIL)	Deficiency disease	Overdose disease
A (Retinol)	900ug	300ug	Night blindness	Hypervitaminosis A
B_1(Thiamin)	1.2mg	-	Beriberi	
B_2(Riboflavin)	1.3mg	-	Ariboflavinosis	Hypersnsitivity reaction
B_3 (Niacin)	16.0mg	35mg	Pellagra	Drowsiness
B_5 (Panthotenic acid)	5.0mg	-	Paresthesia	Liver disease
B_6 (Pyridoxine)	1.3-1.7mg	100mg	Anaemia	-
B_7 (Biotin)	30ug	-	Dermatitis,enteristis	Nerve damage
B_9 (Folic acid)	400ug	1000ug	Neural tube defects during birth	Decrease in seizure threshold
B_{12} (Cynocobalamin)	2.4ug	-	Megaloblastic anaemia	-
C(Ascorbic acid)	90.0mg	2000mg	Scurvy	Hypervitaminosis C
D (Calciferol)	5ug-10ug	50ug	Ricket	Hypervitaminosis D, Hypercalcaemia
E (Tocopherol)	15mg	1000mg	Mild hymolitic anaemia in infants	Congestive heart failure
K	120ug	-	Bleeding diathes	Increased coagulation in patience taking warfarin

2.1.2 Vitamin B_{12}

Cyanocobalamin is an especially common vitamin of the vitamin B_{12} family. It is the most famous vitamin of the family, because it is, in chemical terms, the most air-stable. It is the easiest to crystallize and, therefore, easiest to purify after it is produced by bacterial fermentation, or synthesized in vitro. A form of vitamin B_{12} called hydroxocobalamin is produced by bacteria and then changed to cyanocobalamin in the process of being purified in activated charcoal columns after being separated from the bacterial cultures. Cyanide is naturally present in activated

charcoal, and hydroxocobalamin, which has great affinity for cyanide, picks it up, and is changed to cyanocobalamin. Thus, the cyanocobalamin form of B_{12} is the most widespread in the food industry. [Victor, 1988]

In fact, vitamin B_{12} is the name for a whole class of chemicals with vitamin B_{12} activity, and cyanocobalamin is only one of these. Cyanocobalamin usually does not even occur in nature, and is not one of the forms of the vitamin that are directly used in the human body (or that of any other animal). However, animals and humans can convert cyanocobalamin to active (cofactor) forms of the vitamin, such as methylcobalamin.

This process happens by equilibration, as cyanocobalamin slowly loses its cyanide in surroundings that contain no cyanide.Cyanocobalamin in an atmosphere of hydrogen with a platinum or Raney nickel is reduced to a red crystalline compound with slightly changed UV absorbtion maxima and a reduced stability to heat.

Cyanide is present in almost every type of smoke produced by burning organic materials including tobacco and cannabis; therefore, there is some concern that vitamin B_{12}-deficient smokers should not be given cyanocobalamin, as it will have more difficulty being broken down. In such cases, other forms of vitamin B_{12} for injection (such as hydroxocobalamin itself) are commonly available as pharmaceuticals, and are actually the most commonly used injectable forms of vitamin B_{12} in many countries.[Victor et al, 1988]

2.1.2.1 Physicochemical properties of B_{12}

Molecular formulae **:** $C_{63}H_{88}CoN_{14}O_{14}P$

Molecular weight : 1355.38g/mol

Appearance : Dark red solid

Melting point : > $300^{o}C$

Boiling point : >$300^{o}C$

Solubility : Soluble at $20^{O}C$ in 80 parts of water ,soluble in alcohol(96% ethanol) but practically insoluble in either ,in chloroform and ether.

Specific absorbance **:** 207 at 361nm using water as solvent.[Genaro, 1990]

2.1.2.2 Stability and reactivity of cyanocobalamin [Kutsky, 1973]

Cyanocobalamin is very hygroscopic in the anhydrous form. It is stable to autoclaving for short periods at 121 °C. The vitamin B_{12} coenzymes are very unstable in light.

Table 2.1.2.2 Stability and reactivity of cyanocobalamin

Parameter	Product
Stability	Stable
Conditions to avoid	Do not mix with other drugs; avoid heat, light and humidity. Keep away from flames , thermally decompose to form toxic vapours
Incompatible materials	Reactive with strong acids and strong oxidizers
Hazardous decomposing products	Carbon monoxide , carbon dioxide , nitrogen oxides phosphorus Oxides and cobalt oxides may be released by thermal decomposition
Possibility of hazardous reactions	Hazardous polymerization will not occur

2.1.2.3 Synthesis and commercial production of vitamin B_{12}

Commercially, cyanocobalamin is obtained from the fermentation of *streptomyces griseus*.The vitamin is precipitated from the aqueous solution saturated with ammonium sulphate by n-butanol. Purification is achieved by chromatography using aluminium silicate as the adsorbent. Sharply defined red bands are formed during the development of the chromatograms indicating the location of the vitamin. The red band is separated mechanically and eluted with water .The concentrated water solution on addition of acetone gives the crystalline vitamin which can be further purified by recrystallization from aqueous acetone .Cyanocobalamin may be in the form of powder, tablets or injections.

Cyanocobalamin injection is a stable solution of cyanocobalamin in water for injections containing sufficient acetic acid or hydrochloric acid to adjust the PH of the solution to about 4.5 It may contain a suitable buffering agent and / or a preservative such as benzyl alcohol or phenylmercuric nitrate at a concentration of 0.001%w/v. Cyanocobalamin injections are

sterilized by heating in an autoclave or by filteration. The injection is reddish –pink in colour.[Sciele pharmaceuticals inc., 2009]

Structure formulae:

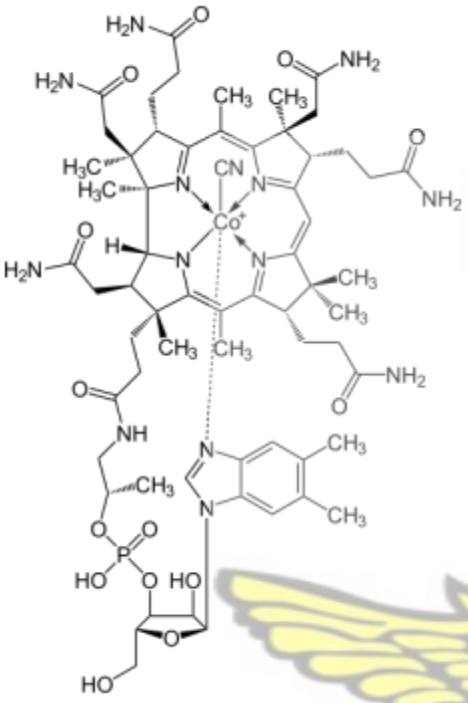


Fig 2.1.2.3 Chemical structure of cyanocobalamin

After structural elucidation of cyanocobalamin,it became obvious that structural adaptations are possible such that derivatives of vitamin B_{12} can be obtained by replacing the cyanide ligand with specific groups like hydroxide(OH), water(H_2O), nitro(NO_2),methyl(CH_3), adenosyl, sulphite and thiocyanates. This led to derivatives such as hydroxocobalamin, aquobalamin, nitroocobalamin, methylcobalamin and thiocyanocobalamin respectively.

Hydroxocobalamin and cyanocobalamin are the principal vitamin B_{12} forms in clinical use. Mecobalamin, adenosylcobalamin and hydroxocobalamin are the most stable vitamin B_{12} derivatives which are both co-enzymes nucleic acid synthesis. [Genaro, 1990]

Hydroxocobalamin is generally preferred to cyanocobalamin for vitamin B_{12} injection because it binds firmly to plasma proteins and is retained longer in the body than cyanocobalamin. Cyanocobalamin and hydoxocobalamin are more stable outside the body, and are therefore administered by intramuscular route though cyanocobalamin can also be administered orally or intranasally.

Hydroxocobalamin is formulated as injections whiles cyanocobalamin may be formulated as tablet or injections. A deep subcutaneous injection of hydroxocobalamin or cyanocobalamin is also possible. Cyanocobalamin powder can be formulated into different dosage forms. Cyanocobalamin can be found in various forms in the market as capsule, tablets and injection. [Kutsky, 1973]

2.1.2.4 Clinical pharmacology of vitamin B_{12}

Vitamin B_{12} is essential to growth, cell reproduction, haematopoietic, and nucleoprotein and myelin synthesis.

Cyanocobalamin is quantitatively and rapidly absorbed from intramuscular and subcutaneous sites of injection; the plasma level of the compound reaches its peak within 1 hour after intramuscular injection. Absorbed vitamin B_{12} is transported via specific B_{12} binding proteins, transcobalamin I and II to the various tissues. The liver is the main organ for vitamin B_{12} storage.

Within 48 hours after injection of 100 or 1000 ug of vitamin B_{12}, 50 to 98% of the injected dose may appear in the urine. The major portion is excreted within the first eight hours. Intravenous administration results in even more rapid excretion with little opportunity for liver storage.

Gastrointestinal absorption of vitamin B_{12} depends on the presence of sufficient intrinsic factor and calcium ions. Intrinsic factor deficiency causes pernicious anemia, which may be associated with subacute combined degeneration of the spinal cord. Prompt parenteral administration of vitamin B_{12} prevents progression of neurologic damage.

The average diet supplies about 5 to 15ug/day of vitamin B_{12} in a protein-bound form that is available for absorption after normal digestion. Vitamin B_{12} is not present in foods of plant origin, but is abundant in foods of animal origin. In people with normal absorption, deficiencies have been reported only in strict vegetarians who consume no products of animal origin (including no milk products or eggs).

Vitamin B_{12} is bound to intrinsic factor during transit through the stomach; separation occurs in the terminal ileum in the presence of calcium, and vitamin B_{12} enters the mucosal cell for absorption. It is then transported by the transcobalamin binding proteins. A small amount (approximately 1% of the total amount ingested) is absorbed by simple diffusion, but this

mechanism is adequate only with very large doses. Oral absorption is considered too undependable to rely on in patients with pernicious anemia or other conditions resulting in malabsorption of vitamin B_{12}.

Cyanocobalamin is the most widely used form of vitamin B_{12}, and has hematopoietic activity apparently identical to that of the anti-anaemia factor in purified liver extract. Hydroxycobalamin is equally as effective as cyanocobalamin, and they share the cobalamin molecular structure. [Institute of medicine, 1998]

2.1.2.5 Recommended intake

Intake recommendations for vitamin B_{12} and other nutrients are provided in the Dietary Reference Intakes (DRIs) developed by the Food and Nutrition Board (FNB) at the Institute of Medicine (IOM) of the National Academies (formerly National Academy of Sciences) . DRI is the general term for a set of reference values used for planning and assessing nutrient intakes of healthy people. These values, which vary by age and gender include:

- Recommended Dietary Allowance (RDA): average daily level of intake sufficient to meet the nutrient requirements of nearly all (97%–98%) healthy individuals.
- Adequate Intake (AI): established when evidence is insufficient to develop an RDA and is set at a level assumed to ensure nutritional adequacy.
- Tolerable Upper Intake Level (UL): maximum daily intake unlikely to cause adverse health effects [Institute of medicine, 1998].

Table 2.1.2.5 lists the current RDAs for vitamin B_{12} in micrograms (ug). For infants aged 0 to 12 months, the FNB established an AI for vitamin B_{12} that is equivalent to the mean intake of vitamin B_{12} in healthy, breastfed infants.

Table 2.1.2.5 Recommended dietary allowances (RDAs) for vitamin B_{12} [Institute of medicine, 1998]

Age	Male	Female	Pregnancy	Lactation
0-6 months	0.4mcg	0.4mcg	Not applicable	Not applicable
7-12 months	0.5mcg	0.5mcg	Not applicable	Not applicable
1-3 years	0.9mcg	0.9mcg	Not applicable	Not applicable
4-8 years	1.2mcg	1.2mcg	Not applicable	Not applicable
9-13 years	1.8mcg	1.8mcg	Not applicable	Not applicable
14+ years	2.4mcg	2.4mcg	2.6mcg	2.8mcg

2.1.2.6 Sources of vitamin B_{12}

Vitamin B_{12} is naturally found in animal products, including fish, meat, poultry, eggs, milk, and milk products. Vitamin B_{12} is generally not present in plant foods but fortified breakfast cereals are a readily available source of vitamin B_{12} with high bioavailability for vegetarians [Butler et al, 2006; Institute of medicine, 1998]. Some nutritional yeast products also contain vitamin B_{12}. Fortified foods vary in formulation, so it is important to read product labels to determine which added nutrients they contain.

2.1.2.7 General precautions about usage

Patients with early Leber's disease (hereditary optic nerve atrophy) who were treated with cyanocobalamin suffered severe and swift optic atrophy. Hypokalemia and sudden death may occur in severe megaloblastic anaemia which is treated intensely.

Anaphylactic shock and death have been reported after parenteral vitamin B_{12} administration. An intradermal test dose is recommended before cyanocobalamin Injection USP is administered to patients suspected of being sensitive to this drug.

This product contains Benzyl Alcohol. Benzyl Alcohol has been reported to be associated with a fatal "Gasping Syndrome" in premature infants.

This product contains aluminum that may be toxic. Aluminum may reach toxic levels with prolonged parenteral administration if kidney function is impaired. Premature neonates are particularly at risk because their kidneys are immature, and they require large amounts of calcium and phosphate solutions, which contain aluminum.

Research indicates that patients with impaired kidney function, including premature neonates, who receive parenteral levels of aluminum at greater than 4 to 5mcg/kg/day, accumulate aluminum at levels associated with central nervous system and bone toxicity. Tissue loading may occur at even lower rates of administration.

Doses of cyanocobalamin exceeding 10 mcg daily may produce haematologic response in patients with folate deficiency. Indiscriminate administration may mask the true diagnosis.

Long term studies in animals to evaluate carcinogenic potential have not been done. There is no evidence from long-term use in patients with pernicious anemia that cyanocobalamin is carcinogenic. Pernicious anemia is associated with an increased incidence of carcinoma of the stomach, but this is believed to be related to the underlying pathology and not to treatment with cyanocobalamin. [Institute of medicine, 1998]

Table 2.1.2.8 Toxicology information of vitamin B_{12}

Acute effects	**Threshold value /Effect**
Oral LD_{50}	5000mg/Kg Oral
Subcutaneous(LD_{50})	2.727mg/Kg subcutaneous
Intreperitoeol(LD_{50})	1.364mg/Kg intraperiteol
Intravenous (LD_{50})	LD_{50}: 2000 mg/kg oral – mouse
Dermal (LD_{50})	Not available
Inhalation LD_{Lo}:	1.364 mg/kg intraperitoneal – mouse
Eye Irritation	Eye irritation is possible.
Skin Irritation	Skin irritation is possible.
Sensitization	Allergic reactions may result in anaphylactic

2.1.2.9 Chemical composition of vitamin B_{12} injection

The main chemical composition of a typical pharmaceutical ingredient in cyanocobalamin injection is tabulated in the table below.

Table 2.1.2.9 Active ingredients and exipients in B_{12} injection

Component	% By weight	Use
Cyanocobalamin	0.1	Active ingredient
Benzyl alcohol/Phenylmercuric iodide	1.5	Preservative
Sodium chloride	0.9	
Sodium hydroxide	-	For PH adjustment
Hydrochloric acid	-	For PH adjustment
Water for injection	97.5	Solvent for injection

2.1.3 Uses of vitamin B_{12}

Cyanocobalamin is indicated for Vitamin B_{12} deficiencies due to malabsorption which may be associated with the following conditions:

Addisonian (pernicious) anaemia:

Gastrointestinal pathology, dysfunction, or surgery, including gluten enteropathy or sprue, small bowel bacteria overgrowth, total or partial gastrectomy, fish tapeworm infestation, malignancy of pancreas or bowel, folic acid deficiency. [Herbert et al, 1996]

It may be possible to treat the underlying disease by surgical correction of anatomic lesions leading to small bowel bacterial overgrowth, expulsion of fish tapeworm, discontinuation of drugs leading to vitamin malabsorption, use of a gluten-free diet in nontropical sprue, or administration of antibiotics in tropical sprue. Such measures remove the need for long-term administration of cyanocobalamin.

Requirements of Vitamin B_{12} in excess of normal (due to pregnancy, thyrotoxicosis, hemolytic anemia, hemorrhage, malignancy, hepatic and renal disease) can usually be met with oral supplementation. [Institute of medicine, 1998].

Cyanocobalamin injection USP is also suitable for the vitamin B_{12} absorption test (Schilling test).

2.1.4 Signs and symptoms of overdose and deficiency of vitamin B_{12}

Vitamin B_{12} deficiency is characterized by megaloblastic anaemia, fatigue, weakness, constipation, loss of appetite, and weight loss [Herbert et al, 1996]. Neurological changes, such as numbness and tingling in the hands and feet, can also occur [Sciele pharmaceuticals inc., 2009]. Additional symptoms of vitamin B_{12} deficiency include difficulty maintaining balance, depression, confusion, dementia, poor memory, and soreness of the mouth or tongue [Davis et al, 1973]. The neurological symptoms of vitamin B_{12} deficiency can occur without anemia, so early diagnosis and intervention is important to avoid irreversible damage [Institute of medicine, 1998]. During infancy, signs of a vitamin B_{12} deficiency include failure to thrive, movement disorders, developmental delays, and megaloblastic anemia [Kelleher and O'Brion, 1992].

Many of these symptoms are general and can result from a variety of other medical conditions. Typically, vitamin B_{12} deficiency is treated with vitamin B_{12} injections, since this method bypasses potential barriers to absorption. However, high doses of oral vitamin B_{12} may also be effective. The authors of a review of randomized controlled trials comparing oral with intramuscular vitamin B_{12} concluded that 2,000 mcg of oral vitamin B_{12} daily, followed by a decreased daily dose of 1,000 mcg and then 1,000 mcg weekly and finally, monthly might be as effective as intramuscular administration [Mohammed and Said, 2006].

Overall, an individual patient's ability to absorb vitamin B_{12} is the most important factor in determining whether vitamin B_{12} should be administered orally or via injection [British national formulary,2007].

Large amounts of folic acid can mask the damaging effects of vitamin B_{12} deficiency by correcting the megaloblastic anemia caused by vitamin B_{12} deficiency [Sciele pharmaceuticals inc., 2009] without correcting the neurological damage that also occurs.[21]. Moreover, preliminary evidence suggests that high serum folate levels might not only mask vitamin B_{12} deficiency, but could also exacerbate the anemia and worsen the cognitive symptoms associated with vitamin B_{12} deficiency [Barutca et al, 2003].

Permanent nerve damage can occur if vitamin B_{12} deficiency is not treated. For these reasons, folic acid intake from fortified food and supplements should not exceed 1,000 mcg daily in healthy adults [Institute of medicine, 1998].

2.1.5 Preservatives in B_{12} injection

The main preservatives normally used in vitamin B_{12} injection are benzyl alcohol and phenylmercuric iodide.

Benzyl alcohol

Benzyl alcohol is an organic compound with the formula $C_6H_5CH_2OH$. Benzyl alcohol is a colourless liquid with a mild pleasant aromatic odor. It is a useful solvent due to its polarity, low toxicity, and low vapor pressure. Benzyl alcohol is partially soluble in water (4 g/100 mL) and completely miscible in alcohols and diethyl ether.

It is very stable with no occurrence of hazardous polymerization. Benzyl Alcohol can react violently in contact with strong oxidizing agents, isocyanates, acetaldehyde, $LiAlH_4$ (lithium aluminum hydride), aluminum alkyl compounds and strong mineral acids.
Incomplete combustion will produce carbon monoxide and other potentially toxic and/or poisonous vapors.

Benzyl alcohol is used as a bacteriostatic preservative at low concentration in intravenous medications. It is oxidized rapidly in healthy individuals to benzoic acid, conjugated with glycine in the liver, and excreted as hippuric acid.

High concentrations can result in toxic effects including respiratory failure, vasodilation, hypotension, convulsions, and paralysis. Newborns, especially if critically ill, may not metabolize benzyl alcohol as readily as adults. Reports in the early 1980s of sixteen neonatal deaths associated with the use of saline flush solutions containing benzyl alcohol preservative led to recommendations to avoid its use in neonates.[Barutca et al, 2003]

The use of benzyl alcohol as a 5% solution has been approved by the U.S. FDA in the treatment of head lice in children older than 6 months and in adults. The product is sold in the United States (prescription-only) as ulesfia lotion. The benzyl alcohol prevents the lice from closing

their respiratory spiracles: the spiracles become blocked with the other products in the lotion, and the lice asphyxiate. The lotion is not effective against louse eggs, and so two applications with an interval of seven days are required to treat a case of louse infestation.[British national formulary, 2007]

Table 2.1.6 Acute health hazards of Vitamin B_{12}

Inhalation:	Inhalation of concentrated vapor may irritate the nose and throat. Overexposure to vapors causes headache, vertigo, nausea, vomiting, and diarrhea. Over-exposure can cause central nervous system depression.
Eye Contact:	Causes eye irritation. Is a severe eye irritant.
Skin Contact:	May cause skin irritation.
Ingestion:	Harmful if swallowed. LD_{50} =1230mg/.Kg

2.2.0 PERNICIOUS ANAEMIA (PA)

Pernicious anaemia is one of many types of the larger family of megaloblastic anaemias. It is caused by loss of gastric parietal cells, and subsequent inability to absorb vitamin B_{12}.

Usually seated in an atrophic gastritis, the autoimmune destruction of gastric parietal cells leads to a lack of intrinsic factor. Since the absorption from the gut of normal dietary amounts of vitamin B_{12} is dependent on intrinsic factor, the loss of intrinsic factor leads to vitamin B_{12} deficiency. [Robilotti et al, 2005; Kumar et al, 2007]

The loss of ability to absorb vitamin B_{12} is the most common cause of adult vitamin B_{12} deficiency. Such a loss may be due to pernicious anemia (with loss of intrinsic factor) or to a number of other conditions which decrease production of gastric acid, which also plays a part in absorption of vitamin B_{12} from foods. [Domenech et al, 2007]

Replacement of vitamin stores does not correct the defect in absorption from loss of intrinsic factor that technically defines the disease. A person, who has pernicious anemia defined by inability to absorb vitamin B_{12} in this way, will have it for the remainder of his or her life. However, unless the patient has sustained permanent peripheral nerve damage before treatment, regular B_{12} replacement will keep pernicious anemia in check, with no anemia and no further symptoms. [Annibale and Lahner, 2009]

2.2.1 Signs and symptoms of pernicious anaemia

The patient may complain of fatigue, depression, forgetfulness, difficulty concentrating, low-grade fevers, nausea and gastrointestinal symptoms (heartburn), weight loss. Because PA may affect the spinal cord, the patient may also complain of impaired urination, loss of sensation in the feet, unsteady gait, weakness and clumsiness. Anemia may cause tachycardia (rapid heartbeat) and cardiac murmurs, along with a waxy pallor. In severe cases, the anemia may cause evidence of congestive heart failure. Long term complications may include gastric cancer and carcinoids. [Annibale and Lahner, 2009]

Many signs and symptoms are attributed to pernicious anaemia:

- Fatigue, low blood pressure, rapid heart rate, high blood pressure, pallor, depression, muscle weakness and shortness of breath (known as 'the sighs')
- Difficulty in proprioception
- Mild cognitive impairment, including difficulty concentrating and sluggish responses, colloquially referred to as brain fog
- Neuropathic pain
- Frequent diarrhea
- Paresthesias, such as pins and needles sensations or numbness in fingers or toes, due to B_{12} deficiency affecting nerve function
- Jaundice due to impaired formation of blood cells
- Glossitis (swollen red tongue) due to B_{12} deficiency
- May present with hyperthyroidism or hypothyroidism
- Personality or memory changes [Anthony, 2009]

2.2.2 Causes of pernicious anaemia

Most commonly (in temperate climates), the cause for impaired binding of vitamin B_{12} by intrinsic factor is autoimmune atrophic gastritis, in which autoantibodies are directed against parietal cells (resulting in their loss), as well as against the intrinsic factor itself (rendering it unable to bind vitamin B_{12}). [Dosias et al, 2007]

A similar disorder involving impaired B_{12} absorption can also occur following gastric removal (gastrectomy) or gastric bypass surgery, especially the Roux-en-Y bypass. In this procedure, the stomach is separated into two sections, one a very small pouch for holding small amounts of food, and the other, the remainder of the stomach, which is resultingly nonfunctional. Therefore, the mucosal cells are no longer available, nor are the required intrinsic factor. This results in inadequate GI absorption of B_{12}, and may result in a syndrome indistinguishable from pernicious anemia. Gastric bypass or gastrectomy patients must take B_{12} as in treatment of pernicious anemia: either oral megadoses or B_{12} by injection. [Dosias et al, 2007]

2.2.3 Pathophysiology

Vitamin B_{12} cannot be produced by the human body, and must be obtained from the diet. Normally, dietary vitamin B_{12} is absorbed by the body in the small bowel only when it is bound by the intrinsic factor (IF) produced by parietal cells of the gastric mucosa. Pernicious anaemia is thought to occur when the body's immune system mistakenly targets the intrinsic factor, with a loss of parietal cells. Insufficient IF results in insufficient absorption of the vitamin. Although the normal body stores three to five years' worth of vitamin B_{12} in the liver, the usually undetected autoimmune activity in one's gut over a prolonged period of time leads to vitamin B_{12} depletion and the resulting anaemia. Inhibition of DNA synthesis in red blood cells results in the formation of large, fragile megaloblastic erythrocytes. [Dosias et al, 2007]

2.2.4 Diagnosis of pernicious anaemia

Pernicious anemia is suspected when the patient's blood smear shows large, fragile, immature erythrocytes (megaloblasts). The Schilling test is no longer widely available, and the other main diagnostic signpost of low levels of serum B_{12} cannot be relied upon, as sufferers can have high levels of serum B_{12} and still have pernicious anemia. [Dosias et al, 2007]

Blood and urine tests for methylmalonic acid may indicate a B_{12} deficiency, even though serum B_{12} is within the normally-acceptable range. Serum B_{12} is not necessarily an indicator of efficient use by the body, in the muscles, for example. [Annibale and Lahner, 2009]

A diagnosis of pernicious anemia first requires demonstration of megaloblastic anemia (through a full blood count) which evaluates the mean corpuscular volume (MCV), as well the mean corpuscular hemoglobin concentration (MCHC). Pernicious anemia is identified with a high MCV and a normal MCHC (that is, it is a macrocytic, normochromic anemia). Ovalocytes are also typically seen on the blood smear, and a pathognomonic feature of megaloblastic anemias (which include pernicious anemia and others) is hypersegmented neutrophils. [Devalia, 2006]

Pernicious anemia can also be diagnosed by evaluating its direct cause, vitamin B_{12} deficiency, by measuring B_{12} levels in serum. A Schilling test can then be used to distinguish pernicious anemia from other causes of vitamin B_{12} deficiency (notably malabsorption). [Cohen et al, 2003]

Table 2.2.4 WHO heamoglobin thresholds used to define anemia

Age or gender group	Hb threshold(g/dl)	Hb threshold (mmol/l)
Children(0.5-5yrs)	11.0	6.8
Children(5-12yrs)	11.5	7.1
Teens(12-15yrs)	12.0	7.4
Women, non-pregnant >15yrs	12.0	7.4
Women, pregnant	11.0	6.8
Men (> 15yrs)	13.0	8.1

2.2.5 Treatment of pernicious anaemia

The treatment of pernicious anaemia varies from country to country and from area to area. There is no permanent cure for pernicious anaemia, although repletion of vitamin B_{12} should be expected to result in a cessation of anaemia-related symptoms, a halt in neurological deterioration, and (in cases where neurological problems not advanced) neurological recovery and a complete and permanent remission of all symptoms, so long as B_{12} is supplemented. Repletion of B_{12} can be accomplished in a variety of ways. [Cohen et al, 2003]

The most accessible and inexpensive method of repletion is through dietary supplementation, in the form of oral or sublingual B_{12} tablets. B_{12} supplements are widely available at supermarkets, health food stores, and drug stores, though quality and cost may vary. In some countries, the cobalamin preparation may be available only via prescription. Doctors can prescribe cobalamin tablets that contain doses higher than what is commercially available. [Lederle, 1998]

A 2003 study found that oral and sublingual B_{12} were absorbed equally well in a group of patients with very low B_{12}. In this study, 22% of the subjects that agreed to undergo the test (5 of 23), had abnormal Schilling tests, but showed no difference in treatment levels from the other subjects. When oral tablets are used to treat PA, higher-than-normal doses may be needed. The efficacy of using high dose B_{12} tablets to treat ordinary PA (i.e. anaemia due to atrophic gastritis) is well established. Oral supplementation allows B_{12} to be absorbed in places other than the

terminal ileum (where B_{12} absorption usually takes place). A 2006 study found that oral B_{12} repletion has the potential to be as effective as injections. [De Parz and Henandez, 2005]

However, if oral and sublingual repletion of B_{12} is inadequate, the patient may require B_{12} injections, which are usually given once a month, bypassing the need for gastrointestinal absorption altogether. Eventually, the patient may be able to do this at home. Cobalamin (one of the forms of B_{12}) is usually injected into the patient's muscle (intramuscular or IM) using cyanocobalamin (the United States, Canada and most European countries) or hydroxocobalamin (Australia and the U.K.). The injections will typically need to be given for the remainder of the patient's life. The frequency of injections varies by country and health care practitioner, and may be as infrequent as once every three months in some countries. The most common complaint by members of the pernicious anaemia society is that patients have different needs, with some patients needing more frequent injections than others. Some medical professionals believe that subcutaneous injections are more effective than intramuscular injections, but the evidence for this is currently unclear. [Carmel, 1992; Markus et al, 1997]

There are other methods of administering B_{12}, including nasal sprays and behind-the-ear patches. One small study from 1997, with six participants, found that intranasal delivery of B_{12} led to increases in plasma cobalamin as high as eight times a given patient's baseline measurement. [Markus et al, 1997].

2.3.0 METHODS OF MEASURING VITAMIN B_{12} CONCENTRATION

2.3.1 Qualitative methods

In the first qualitative test, about 1mg of the vitamin B_{12} sample is mixed with about 10mg of potassium sulphate and two drops of sulphuric acid is added in a porcelain dish.

The mixture is heated carefully to red hot and then allowed to cool. The residue is then dissolved in two drops of water, ten drops of saturated ammonium thiocyanate solution and 0.5ml of benzyl alcohol with shaking. A blue colour formed which is taken up in the benzyl alcohol layer shows that vitamin B_{12} is present in the sample. [Davis et al, 1973]

In UV and visible spectrophotometry, aqueous solutions of cyanocobalamin exhibit maximum UV and visible region at 278nm, 361nm, and 550 nm [Kelleher and O'Brion, 1992]. The ratios of the absorbance at 361nm to that at 550nm ranges from 3.15 – 3.45 whilst the ratios of the absorbance at 361nm to that at 278nm ranges from 1.70 to 1.90.

In other qualitative test, about 1mg of the sample is fused with 50mg of potassium hydrogen sulphate and cooled. The mass is broken up and 3.0ml of water is added and boiled until dissolved. Sufficient sodium hydroxide solution is added to make the solution just alkaline to phenolphthalein solution. 500mg of sodium acetate, 0.5ml of dilute acetic acid and 0.5ml of a 2% solution of disodium 1-nitroso-2-naphthol-3,6-disodium is added. A red or orange-red colour immediately produced such that upon addition of 0.5ml of HCl with boiling for 1 minute the red colour persists shows that vitamin B_{12} is present in the sample. [Genaro, 1990; Bruno, 2005]

2.3.2 Quantitative methods

Determination of the best way of measuring vitamin B_{12} concentration would require critical consideration of the required sensitivity and specificity, the available time, and the process of preparation of the sample, as well as cost. Some of the methods of measuring vitamin B_{12} are reviewed below.

- ***Microbiological method***

Microbiological method is one of the oldest methods for measuring the concentration of vitamin B_{12}.Ross (1950) was the first scientist to describe microbiological method using *Euglena gracilis var-bacillaris* as test organism.

Further experiments on measurement of vitamin B_{12} 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 B_{12} to the active free form.

This method solved the challenge of how to dissociate vitamin B_{12} from its protein carrier. Automated microbiological method 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 Microbiological methods are facing difficulties in the assay of vitamin B_{12}, mainly because they are tedious, and time consuming; they have poor precision, and relatively low specificity [Xu et al, 1988].

- ***Spectrophotometric methods***

Spectrophotomery for vitamin B_{12} measuring was very diverse according to the use of many light spectra like gamma-ray counter spectrophotometer. Ultraviolet-visible spectrophotometer of different types

In UV and visible spectrophotometry, aqueous solutions of cyanocobalamin exhibit maximum UV and visible region at 278nm, 361nm, and 550nm. However, several factors such as changes in solvent, temperature, and pH can affect the spectrum.

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 B_{12} 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) Spectophotometric application to injections containing vitamin B_{12} gave results closer to the results obtained by capillary electrophoresis.

Spectrophotometric method has low cost and acceptable specificity in comparison with radio ligand assay. However, it is not suitable for complex samples, and the sensitivity is relatively low in such cases. [Kelleher and O'Brion, 1992; Bratu et al, 2009].

- ***Atomic absorption spectroscopic method***

Atomic absorption spectroscopy deals 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

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 electrothermal atomic absorption correctly and optimally measures cobalt (and thus, vitamin B_{12}) 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%.

The main advantages of this method are 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.

Another method that is used is the flame atomic absorption spectrometry. The lowest concentration for quantitative recovery is 4ng/cm^3 of vitamin B_{12}. The method is used for vitamin B12 determination in pharmaceutical sample [Haswell, 1991; Leeming et al, 1992].

- ***Radioimmunoassay (RIA) method***

Radioimmunoassay is a primer immunoassay techniques developed for detecting extremely small concentrations. This method is so sensitive that it can measure one trillionth of grams of substance per milliliter of blood and only small samples are required. These (among other reasons) made RIA to quickly become a standard laboratory tool.

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.

For vitamin B_{12} assay by this method, 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 B_{12}. [Chen et al, 2006; Bratu et al, 2009].

- ***High-performance liquid chromatography (HPLC)***

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 [4

The 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.

HPLC results are often precise, and it is very sensitive with detection limits of 50nmol/L. An example of this Instrument is Kontron HPLC-system 400. This method is frequently used in pharmacology, industry and basic research [Honda et al, 2001].

- ***Inductive-coupled plasma (ICP) - mass spectrometry (MS) (ICP-MS)***

One of the best methods for the determination of vitamin B_{12} 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.

MS actually measures mass to charge ratio and not just the mass. 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.

MS can be combined 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 $1.0 \times 10{-}10$ g /mL− to $8.0 \times 10{-}5$ g /mL and the limit of detection is 0.05ng/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 [Biji et al, 2005].

- ***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 platinum electrode producing light.

This method employs various test principles (such as competitive principle, sandwich and bridging) for the measurement. The most important method in measuring vitamin B_{12} concentration is the competitive principle. The competitive principle is applied to low molecular weight molecules. It uses antibodies (intrinsic factor) for vitamin B_{12} labeled with ruthenium complex. These antibodies are incubated with the sample, then biotinylated vitamin B_{12} 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 B_{12} that is measured. [Antonello et al, 2009]

Table 2.3.0 Sensitivity of methods used in measuring vitamin B_{12} concentration

Procedure	Sample preparation	Sensitivity
ECL	Serum	30pg/ml
ICP-MS	Need preparation	50pg/ml
Atomic absorbtion	Urine and Serum	20pg/ml
Radioimmunoassyay	Serum	200pg/ml
HPLC	Need preparation	6800pg/ml
Cappillary electrophoresis	Need preparation	Depends on the attached method

2.4.0 REVIEW OF SOME METHODS OF CHEMICAL ANALYSIS

A given compound can often be measured by several methods. Specific and sensitive methods of analysis are in a wide ranging concept, and the choice of specific method depends on a number of factors taking into consideration:

- The state and the chemistry of the drug in question
- Which concentrations of the compound will be present in the samples?
- What is the sample matrix?
- Will the measurement be qualitative or quantitative?
- What precisions are required?
- What is the cost of the analysis?
- What are the cost and equipment requirements?
- Where will the method be implanted?
- Are qualified personnel available to perform the analysis?
- What are the requirements of the throughput?

Reliable methods have been developed extensively to undertake the identity, purity, stability and efficacy studies of unformulated and formulated drugs. In the achievement of overall quality assessment of drugs and their dosage forms, methods typically used are grouped as:

- Classical or Chemical Methods
- Instrumental or Physicochemical Methods
- Biological Methods
- Biopharmaceutical methods

2.4.1 Classical Methods

This method of analysis has been the earliest approach to solving laboratory problems, in determining the identity, purity, efficacy, stability and content of drugs. Classical methods are in two categories: titrimetry and gravimetry. These methods are still widely used in official compendial assays, because of their robustness, cheapness and capability of high precision. They involve the use of volumetric flasks, burettes, conical flasks, beakers and pipettes.

The chemical methods of analysis are sometimes referred to as stoichiometric methods, since an analyte is chemically reacted with a standard amount of solution of a reagent of precisely known concentration or with a concentration that can provide data for calculating the precise amount of

the compound. The amount of a standard solution required to completely react with all the sample is used to estimate the purity of the sample.

The choice of titration for analysis depends on the sensitivity required. This method has limitations:

• Non-selective

• Time-consuming if not automated and require a greater level of operator skill than routine instrumental methods.

• Require large amounts of sample and reagents

• Reactions of standard solutions with the analyte should be rapid and complete.

With the fast growing modernisation of measurement methods, titrimetric analysis can now be automated [David, 1999; Moffat, 1986; United state pharmacopoeia 27, 2004; Olaniyi et al, 2000].

2.4.2 Biological Methods

Biological methods used in pharmaceutical analysis involve both qualitative biological tests and quantitative assay of pharmaceutical preparations. These processes employ intact animals (whole, *in vivo*), animal tissues or organs as preparations, isolated living tissues (*in vitro*) (in pharmacology) or micro-organisms (in microbiology).

With the advent of more improved separation methods and better alternate assay techniques bioassay have become less significant in quality control of pharmaceuticals. Nevertheless, bioassay and microbiological assays still remain the standard procedures for resolving doubt in view of a possible loss of bioactivity in a product [United state pharmacopoeia 27, 2004; Olaniyi et al, 2000].

2.4.3 Biopharmaceutical Methods.

Drugs manufactured into different dosage forms and raw materials needs to be specifically controlled for certain physical properties. Formulated drugs contain certain amounts of active ingredients that have to be efficacious when used. Nonetheless, the potency of the drug depends primarily on the rate of release of the active ingredients from the formulation into active sites and also its biological availability. Various biopharmaceutical methods have been designed to ensure the standardisation of raw materials and drug products, and these include: particle size

measurement, dissolution rate, disintegration time, friability, tablet hardness, viscosity of liquid dosage forms and bioavailability/bioequivalency assessments [David et al, 1999; Mofat et al, 1986; Olaniyi et al, 2000].

2.4.4 Instrumental Methods

Over the last few decades Instrumental Methods of analysis has found a more useful application in pharmaceutical analysis. It is the methods of analysis which factor in a great deal of sensitivity and selectivity concurrently. It has an added advantage over the classical methods by being more accurate and precise, less time in analysis, and measures in the concentration range of nano- (10-9) and below.

This method of analysis can then be sub-divided into:

- Spectroscopic Methods
- Chromatographic Methods
- Electrochemical Methods
- Other Dispersive Methods (Polarimetry, Refractometry etc)

The methods can also be inter-coupled for use to achieve greater results. For instance chromatographic separations often at times require other specific detectors to elucidate detection. These include HPLC-MS, HPLC-EC, GC-MS, HPLC-FTIR and GC-FTIR. Instances where a sufficiently larger number of similar units are to be subjected routinely to the same type of examination, automated methods of analysis may be far more efficient and precise than manual methods. Automated methods have been found useful in testing the content uniformity of tablets and capsules. It has now become more convenient for establishments and well organized laboratories to utilize automated methods as alternatives to pharmacopoeial methods. Additionally, the detection system and calculation of results are often computerised. Even though automated methods are extensively used as an alternative to pharmacopoeial methods, the need for precision and accuracy has to be ascertained before their usage. Continuous monitoring of the performance of the automated analytical system is of crucial importance, by assaying standard preparations of known concentration intermittently interspersed the test preparation [David et al,1999; Mofat et al,1986; Olaniyi et al, 2000].

2.4.4.1 UV/Visible method analysis

UV/Visible spectroscopy is often used to determine the concentration of a light absorbing species in solution .Provided the concentration is not too high, the measured absorbance, A obeys the Beer lamberts law

The absorbance (*A*) of a solution is defined as the logarithm to base 10 of the reciprocal of the transmittance (*T*) for monochromatic radiation:

$$\boldsymbol{A = \log_{10} \frac{1}{T} = \log_{10} \frac{I_0}{I}}$$

$$\boldsymbol{T = \frac{I}{I_0}}$$

I_0 = intensity of incident monochromatic radiation,

I = intensity of transmitted monochromatic radiation.

In the absence of other physico-chemical factors, the absorbance (A) is proportional to the path length (b) through which the radiation passes and to the concentration (c) of the substance in solution in accordance with the equation:

$$\boldsymbol{A = ecb}$$

e = molar absorptivity, if *b* is expressed in centimetres and *c* in moles per litre.

The expression ${}^{1\%}_{1cm}A$ representing the specific absorbance of a dissolved substance refers to the absorbance of a 10 g/l solution in a 1 cm cell and measured at a defined wavelength so that:

$$\boldsymbol{{}^{1\%}_{1cm}A = \frac{10e}{M_r}}$$

UV/Visible spectrophotometers

Spectrometers for UV/visible absorbtion spectrosocopy can either be a single beam or double beam. In single beam instrument all the light passes through the sample cell, so the sample must be removed in order to measure the incident intensity, Io. In double beam instrument, the light split into two beams, one beam passes through the sample and the other is used as a reference.The transparent cell used to hold the sample, called the cuvette, may be a quartze, glass or plastic. Glass and most plastics absorb in the UV, so can only be used for visible wavelengths. [United state pharmacopoeia 27, 2004]

Chromophores

Chromophore is a part of a molecule that gives rise to an optical absorption. The most common chromores in organic compounds are associated with $\pi \longrightarrow \pi^*$ transitions

A $\pi \longrightarrow \pi^*$ transition is observed in molecules with double bonds (π bonds). An unconjugated double bond abosorbes in the UV (180nm) but conjugated systems absorb at longer wavelengths (200-800nm).

2.4.4.2 Chromatographic methods

Chromatography is the most frequently used analytical technique in pharmaceutical analysis.
Chromatographic techniques can be classified into the following basic categories; liquid chromatography LC, gas chromatography (GC), supercritical fluid chromatography (SFC) and capillary electrophoresis (CE).
Chromatography involves a sample (or sample extract) being dissolved in a *mobile phase* (which may be a gas, a liquid or a supercritical fluid). The mobile phase is then forced through an immobile, immiscible *stationary phase*. The phases are chosen such that components of the sample have differing solubilities in each phase. A component which is quite soluble in the stationary phase will take longer to travel through it than a component which is not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary phase.

Techniques such as H.P.L.C. (High performance liquid chromatography) and G.C. (Gas chromatography) use *columns* - narrow tubes packed with stationary phase, through which the mobile phase is forced through. The sample is transported through the column by continuous addition of mobile phase. This process is called *elution*. The average rate at which an analyte moves through the column is determined by the time it spends in the mobile phase.

The distribution of analytes between phases can often be described quite simply. An analyte is in equilibrium between the two phases.

The equilibrium constant, *K*, is termed the *partition coefficient*; defined as the molar concentration of analyte in the stationary phase divided by the molar concentration of the analyte in the mobile phase.

The time between sample injection and an analyte peak reaching a detector at the end of the column is termed the *retention time* (t_r). Each analyte in a sample will have a different retention time. The time taken for the mobile phase to pass through the column is called the *void volume* (*to*), a compound which does not partition appreciably into the mobile phase also elutes at this time A term called the *retention or capacity factor, k'*, is often used to describe the migration rate of an analyte on a column. The retention factor for analyte *A* is defined as;

$$k'A = (tr - to)/to$$

t_r and *to* are easily obtained from a chromatogram. When an analytes retention factor is less than one, elution is so fast that accurate determination of the retention time is very difficult. High retention factors (greater than 20) mean that elution takes a very long time. Ideally, the retention factor for an analyte is between one and five.

We define a quantity called the *selectivity factor*, which describes the separation of two species (A and B), on the column;

$$\alpha = k'A/k'B$$

When calculating the selectivity factor, species A elutes faster than species B. The selectivity factor is always greater than one.

Band Broadening and Column Efficiency

To obtain optimal separations, sharp, symmetrical chromatographic peaks must be observed.

This means that band broadening must be limited. It is also beneficial to measure the efficiency of the column.

Column Efficiency

Column efficiency refers to the performance of the stationary phase to accomplish particular separations. This entails how well the column is packed and its kinetic performance. The efficiency of a column can be measured by several methods which may or may not be affected by chromatographic anomalies, such as "tailing" or appearance of a "front."This is important because many chromatographic peaks do not appear in the preferred shape of normal Gaussian distribution. For this reason efficiency can be an enigmatic value since manufacturers may use different methods in determining the efficiency of their columns.

The plate model supposes that the chromatographic column is contains a large number of separate layers, called *theoretical plates.* Separate equilibrations of the sample between the stationary and mobile phase occur in these "plates". The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next.

It is important to remember that the plates do not really exist; they are a figment of the imagination that helps us understand the processes at work in the column. They also serve as a way of measuring column efficiency, either by stating the number of theoretical plates in a column, *N* (the more plates the better), or by stating the plate height; the *Height Equivalent to a Theoretical Plate* (the smaller the better).

If the length of the column is *L*, required for one partition step to occur, then the HETP is

HETP =L/N

Calculation of Column Efficiency value

Half-peak height Method

As the name suggests, the measurement is based upon the width at 50% of peak height. For the same reason as inflection method, this measurement is not affected by asymmetry; however, this method is more reproducible from person to person since width at 50% peak height is less prone to be varied. The number of theoretical plates that a real column possesses can be found by examining a chromatographic peak after elution. Figure 2.4.4.2 shows a chromatographic peak

emerging at a time *t*r after injection; the efficiency of the column is most readily assessed from the width of the peak at half its height $W_{½}$ and its retention time using the equation below:

$$n = 5.54\left[\frac{t_r}{W_{1/2}}\right]^2$$

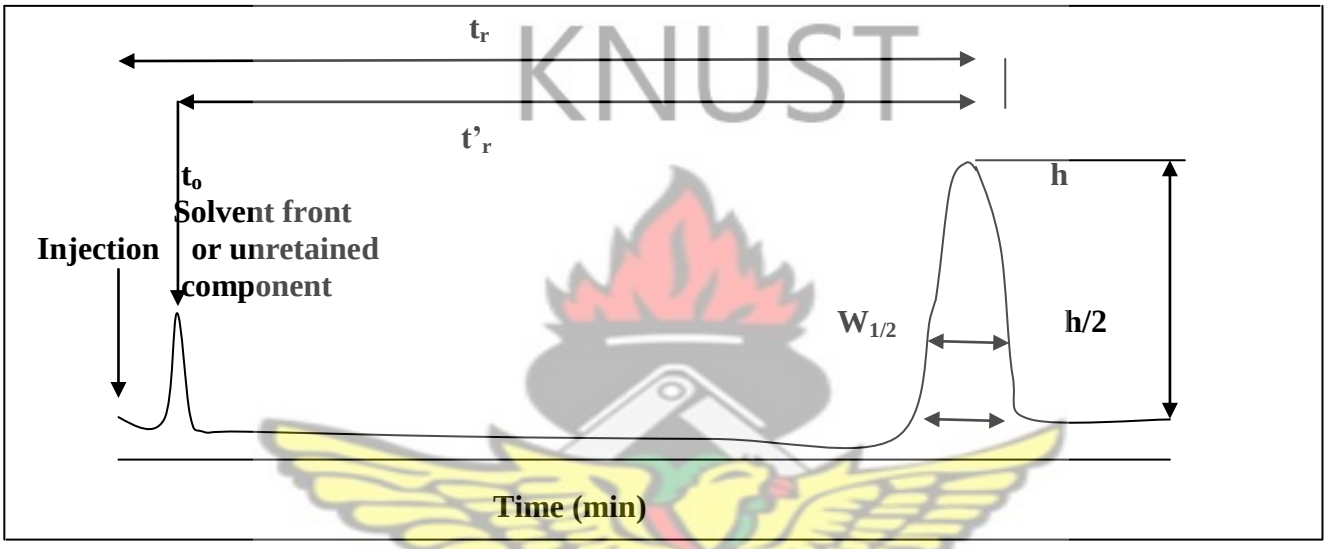


Figure 2.4.4.2 Determination of retention time and peak width at half height

Column efficiency is usually measured in theoretical plates per metre:

Nx100/L

Where L is column length in cm.

A stricter measure of column efficiency, especially if the retention time of the analyte is short, is given by equation

$$N_{eff} = 5.54\left[\frac{t_r}{W_{1/2}}\right]^2$$

where *Neff* is the number of effective plates and reflects the number of times the analyte partitions between the stationary phase and the mobile phase during its passage through the column and $t'_r = t_r - t_o$

The Rate Theory of Chromatography

A more realistic description of the processes at work inside a column takes account of the time taken for the solute to equilibrate between the stationary and mobile phase (unlike the plate model, which assumes that equilibration is infinitely fast). The resulting band shape of a chromatographic peak is therefore affected by the rate of elution. It is also affected by the different paths available to solute molecules as they travel between particles of stationary phase. If we consider the various mechanisms which contribute to band broadening, we arrive at the Van Deemter equation for plate height;

In gas chromatography, the Van Deemter equation is written as:

$$HETP = A + B/u + Cu$$

In HPLC the equation is given as:

$$H = A\,(1 + Cm/u^{1/2} + B/u + C_s u + Cmu^{1/2}$$

where u is the average velocity of the mobile phase. A, B, and C are factors which contribute to band broadening.

***A* - Eddy diffusion**

The mobile phase moves through the column which is packed with stationary phase. Solute molecules will take different paths through the stationary phase at random. This will cause broadening of the solute band, because different paths are of different lengths.

***B* - Longitudinal diffusion**

The concentration of analyte is less at the edges of the band than at the centre. Analyte diffuses out from the centre to the edges. This causes band broadening. If the velocity of the mobile phase is high then the analyte spends less time on the column, which decreases the effects of longitudinal diffusion.

***C* - Resistance to mass transfer**

The analyte takes a certain amount of time to equilibrate between the stationary and mobile phase. If the velocity of the mobile phase is high, and the analyte has a strong affinity for the stationary phase, then the analyte in the mobile phase will move ahead of the analyte in the stationary phase. The band of analyte is broadened. The higher the velocity of mobile phase, the worse the broadening becomes. *Cs* is resistance to mass transfer of a molecule in the stationary phase and is dependent on its diffusion coefficient in the stationary phase and upon the thickness of the stationary phase coated onto the silica gel:

$$Cs = d^2\ thickness/Ds$$

Where $d^2\ thickness$ is the square of the stationary phase film thickness and *Ds* is the diffusion coefficient of the analyte in the stationary phase. *Cm* is resistance to mass transfer brought about by the diameter and shape of the particles of stationary phase and the rate of diffusion of a molecule in the mobile phase.

$$Cm = d^2\ packing/D_m$$

Where d^2 packing is the square of the stationary phase particle diameter and D_m is the diffusion coefficient of the analyte in the mobile phase.

Parameters used in evaluating column performance

Resolution

Although the selectivity factor, α, describes the separation of band centres, it does not take into account peak widths. Another measure of how well species have been separated is provided by measurement of the *resolution*. The resolution of two species, A and B, is defined as

$$R = 2\ \{(t_{rB} - t_{rA})/(\ W_{bA} - W_{bB})\ \}$$

Baseline resolution is achieved when R = 1.5

It is useful to relate the resolution to the number of plates in the column, the selectivity factor and the retention factors of the two solutes; To obtain high resolution, the three terms must be maximised. An increase in *N*, the number of theoretical plates, by lengthening the column leads to an increase in retention time and increased band broadening - which may not be desirable.

Instead, to increase the number of plates, the height equivalent to a theoretical plate can be reduced by reducing the size of the stationary phase particles.

It is often found that by controlling the capacity factor, *k'*, separations can be greatly improved. This can be achieved by changing the temperature (in Gas Chromatography) or the composition of the mobile phase (in Liquid Chromatography).

The selectivity factor, α, can also be manipulated to improve separations. When α is close to unity, optimising *k'* and increasing *N* is not sufficient to give good separation in a reasonable time. In these cases, *k'* is optimised first, and then α is increased by one of the following procedures:

• Changing mobile phase composition

• Changing column temperature

• Changing composition of stationary phase

• Using special chemical effects (such as incorporating a species which complexes with one of the solutes into the stationary phase, or pH value)

Peak asymmetry

Figure 2.4.4.3 shows another situation which may lead to poor integrator performance is where peaks are tailing or fronting and this have high element of asymmetry. The expression used to assess this is:

Asymmetry Factor (AF) = b/a

Where a is the leading half of the peak measured at 10% of the peak height and b is the trailing half of the peak measured at 10% of the peak height (Figure 2.4.4.3). This value should have a range of 0.95 – 1.15. Poor symmetry may be caused through: loading too much sample onto column, sample decomposition, the analyte absorbing strongly onto active sites in the stationary phase, poor trapping of the analyte when it is loaded onto the column or too much "dead volume" in the chromatographic system.

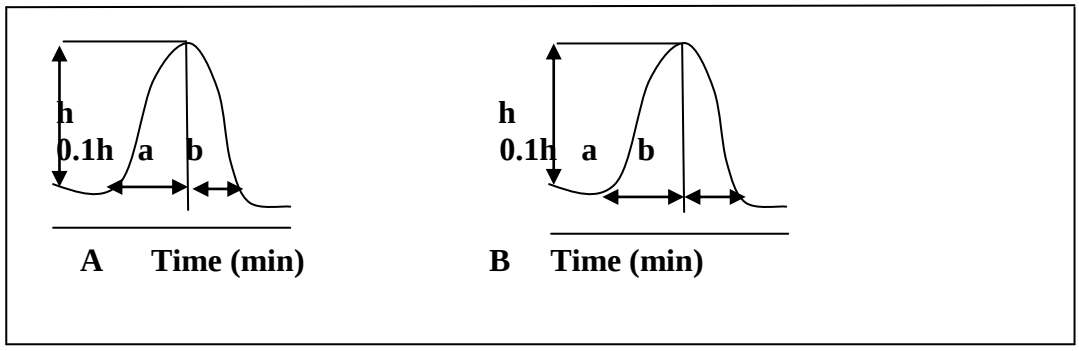


Figure 2.4.4.3 Determination of peak asymmetry

The peak in Figure 2.4.4.3 A will have a asymmetry factor less than 1, due to peak tailing slightly at

the front edge; this may be due to insufficient trapping of the sample at the head of the A Time (min) B Time (min) column as it is loaded. The peak in Figure B will have asymmetry factor greater than 1 [David et al, 1999; Moffat et al,1986 ;United state pharmacopeia 27,2004; Dolan et al, 2009].

2.4.4 Thin-layer chromatography (TLC)

Thin-layer chromatography is a separation technique in which a stationary phase consisting of an appropriate material is spread in a uniform thin layer on a support (plate) of glass, metal or plastic. Solutions of analytes are deposited on the plate prior to development. The separation is based on adsorption, partition, ion-exchange or on combinations of these mechanisms and is carried out by migration (development) of solutes (solutions of analytes) in a solvent or a suitable mixture of solvents (mobile phase) through the thin-layer (stationary phase).

Apparatus in TLC

Plates:

The chromatography is carried out using pre-coated plates. It may be necessary to wash the plates prior to separation. This can be done by migration of an appropriate solvent. The plates may also be impregnated by procedures such as development, immersion or spraying. At the time of use, the plates may be activated, if necessary, by heating in an oven at 120 °C for 20 min.

Chromatographic tank:

A chromatographic tank with a flat bottom or twin trough, of inert, transparent material, of a size suitable for the plates used and provided with a tightly fitting lid is required. For horizontal development the tank is provided with a trough for the mobile phase and it additionally contains a device for directing the mobile phase to the stationary phase.

Micropipettes, micro syringes, calibrated disposable capillaries or other application devices suitable for the proper application of the solutions.

Fluorescence detection device: To measure direct fluorescence or the inhibition of fluorescence.

Visualisation reagents:

Suitable devices are used for derivatisation to transfer to the plate reagents by spraying, immersion or exposure to vapour and, where applicable, to facilitate heating for visualization of separated components.

A device may be used to provide documentation of the visualized chromatogram, for example a photograph or a computer file.

Sample application

Apply the prescribed volume of the solutions at a suitable distance from the lower edge and from the sides of the plate and on a line parallel to the lower edge; allow an interval of at least 10 mm (5 mm on high-performance plates) between the centres of circular spots and 5 mm (2 mm on high-performance plates) between the edges of bands. Apply the solutions in sufficiently small portions to obtain circular spots 2-5 mm in diameter (1-2 mm on high-performance plates) or bands 10-20 mm (5-10 mm on high-performance plates) by 1-2 mm.

Vertical development:

Line the walls of the chromatographic tank with filter paper. Pour into the chromatographic tank a sufficient quantity of the mobile phase for the size of the tank to give after impregnation of the

filter paper a layer of appropriate depth related to the dimension of the plate to be used. For saturation of the chromatographic tank, replace the lid and allow to stand at 20-25 °C for 1 h. Unless otherwise indicated in the monograph, the chromatographic separation is performed in a saturated tank. Apply the prescribed volume of solutions as described above. When the solvent has evaporated from the applied solutions, place the plate in the chromatographic tank, ensuring that the plate is as vertical as possible and that the spots or bands are above the surface of the mobile phase. Close the chromatographic tank, maintain it at 20-25 °C and protect from sunlight. Remove the plate when the mobile phase has moved over the prescribed distance, measured between the points of application and the solvent front. Dry the plate and visualize the chromatograms as prescribed. For two-dimensional chromatography, dry the plates after the first development and carry out a second development in a direction perpendicular to that of the first development.

Horizontal development:

Apply the prescribed volume of the solutions as described above. When the solvent has evaporated from the applied solutions, introduce a sufficient quantity of the mobile phase into the trough of the chamber using a syringe or pipette, place the plate in the chamber after verifying that the latter is horizontal and connect the mobile phase direction device according to the manufacturer's instructions. If prescribed, develop the plate starting simultaneously at both ends. Close the chamber and maintain it at 20-25 °C. Remove the plate when the mobile phase has moved over the distance prescribed in the monograph. Dry the plate and visualize the chromatograms as prescribed.

For two-dimensional chromatography, dry the plates after the first development and carry out a second development in a direction perpendicular to that of the first development.

Identification and verification

The principal spot in the chromatogram obtained with the test solution is visually compared to the corresponding spot in the chromatogram obtained with the reference solution by comparing the colour, the size and the retention factor (R_f) of both spots.

The retardation factor (R_F) is defined as the ratio of the distance from the point of application to the centre of the spot and the distance travelled by the solvent front from the point of application.

2.4.5 Gas chromatography

Gas chromatography is a chromatographic technique that can be used to separate volatile organic compounds. A gas chromatograph consists of a flowing mobile phase, an injection port, a separation column containing the stationary phase, and a detector. The organic compounds are separated due to differences in their partitioning behaviour between the mobile gas phase and the stationary phase in the column. Mobile phases are generally inert gases such as helium, argon, or nitrogen. The injection port consists of a rubber septum through which a syringe needle is inserted to inject the sample.

The injection port is maintained at a higher temperature than the boiling point of the least volatile component in the sample mixture. Since the partitioning behaviour is dependant on temperature, the separation column is usually contained in a thermostat-controlled oven.

Separating components with a wide range of boiling points is accomplished by starting at a low oven temperature and increasing the temperature over time to elute the high-boiling point components. Most columns contain a liquid stationary phase on a solid support.

Separation of low-molecular weight gases is accomplished with solid adsorbents. Since separation in GC occurs in the gas phase, liquid samples have to be vaporized. This represents the main constraint of the technique since the analytes have to be thermostable and sufficiently volatile. Dramatization can be used to convert the analytes to a more volatile form. Other disadvantages include the unsuitability of water or salt solutions and the small injection volumes. The strength of GC is its high efficiency and the high separation capability. GC can be divided into two categories: gas-solid chromatography (GSC) and gas liquid chromatography (GLC).

In GSC, the mobile phase is a gas and the stationary phase is a solid that retains the analytes by adsorption. GSC is most suitable for low molecular weight gaseous species like nitrogen oxides and carbon dioxide.

GLC is based upon partition of the analytes between an immobilized liquid and a gas phase. Several different liquid phases exist for GLC with a wide range of applications. This technique is suitable for all kinds of volatile analytes, such as steroids, alcohols, amino acids, fatty acids and sugars.

Efficient separation of compounds in GC is dependent on the compounds travelling through the column at different rates. The rate at which a compound travels through a particular GC system depends on the factors listed below:

• *Volatility of compound*: Low boiling (volatile) components will travel faster through the column than will high boiling components

• *Polarity of compounds*: Polar compounds will move more slowly, especially if the column is polar.

• *Column temperature*: Raising the column temperature speeds up all the compounds in a mixture.

• *Column packing polarity*: Usually, all compounds will move slower on polar columns, but polar compounds will show a larger effect.

• *Flow rate of the gas through the column*: Speeding up the carrier gas flow increases the speed with which all compounds move through the column.

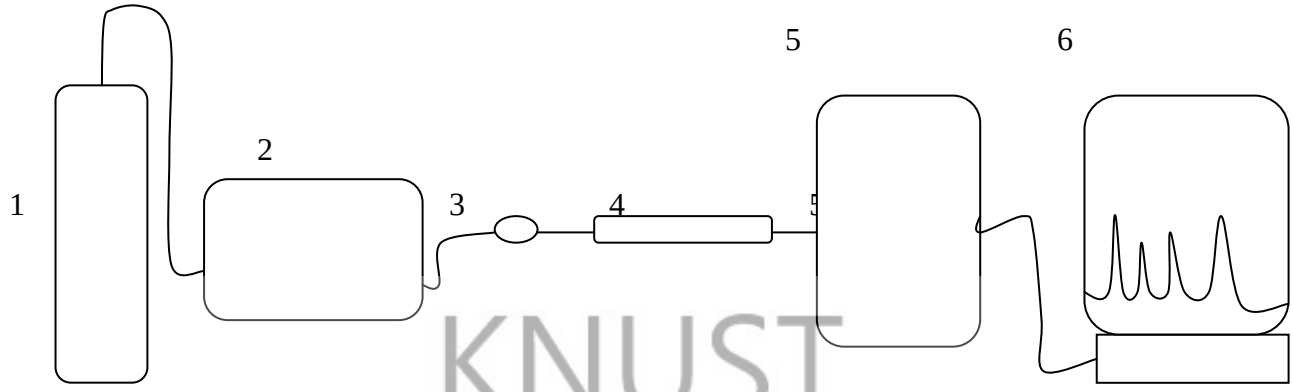
• *Length of the column*: The longer the column, the longer it will take all compounds to elute. Longer columns are employed to obtain better separation.

Generally the number one factor to consider in separation of compounds on the GCs is the boiling points of the different components. Column temperature, the polarity of the column, flow rate, and length of a column are supposed to be constant during analysis. For each planned GC experiment, these factors have been optimized to separate compounds [David et al, 1999; Mofat et al, 1986; Shabir et al, 2003].

2.4.6 High performance liquid chromatography

High-performance liquid chromatography (or High pressure liquid chromatography, HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used.

Figure 2.4.6 shows a simple schematic representation of a liquid chromatographic system consisting of a solvent reservoir (1) containing the mobile phase, a pump (2) to compel the mobile phase through the system at high pressure, an injector (3) to introduce the sample into the mobile phase, a chromatographic column (4), a detector (5), and a data collection device (6) such as computer, integrator, or recorder.



`Figure 2.4.6 A scheme of liquid chromatography system

Partition chromatography

Partition chromatography uses a retained solvent, on the surface or within the grains or fibres of an "inert" solid supporting matrix as with paper chromatography; or takes advantage of some additional coulombic and/or hydrogen donor interaction with the solid support. Molecules equilibrate (partition) between a liquid stationary phase and the eluent. Known as Hydrophilic Interaction Chromatography (HILIC) in HPLC, this method separates analytes based on polar differences. HILIC most often uses a bonded polar stationary phase and a non-polar, water miscible, mobile phase. Partition HPLC has been used historically on unbonded silica or alumina supports. Each works effectively for separating analytes by relative polar differences, however, HILIC has the advantage of separating acidic, basic and neutral solutes in a single chromatogram.

The polar analytes diffuse into a stationary water layer associated with the polar stationary phase and are thus retained. Retention strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength depends on the functional groups in the analyte molecule which promote partitioning but can also include coulombic (electrostatic) interaction and hydrogen donor capability. Use of more polar solvents in the mobile phase will decrease the retention time of the analytes, whereas more hydrophobic solvents tend to increase retention times.

Partition and NP-HPLC had fallen out of favor in the 1970s with the development of reversed-phase HPLC because of a lack of reproducibility of retention times as water or protic organic solvents changed the hydration state of the silica or alumina chromatographic media. Recently it has become useful again with the development of HILIC bonded phases which improve reproducibility.

Normal phase chromatography

Also known as Normal phase HPLC (NP-HPLC), or adsorption chromatography, this method separates analytes based on adsorption to a stationary surface chemistry and by polarity. It was one of the first kinds of HPLC that chemists developed. NP-HPLC uses a polar stationary phase and a non-polar, non-aqueous mobile phase, and works effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength depends not only on the functional groups in the analyte molecule, but also on steric factors. The effect of sterics on interaction strength allows this method to resolve (separate) structural isomers.

The use of more polar solvents in the mobile phase will decrease the retention time of the analytes, whereas more hydrophobic solvents tend to increase retention times. Very polar solvents in a mixture tend to deactivate the stationary phase by creating a stationary bound water layer on the stationary phase surface. This behavior is somewhat peculiar to normal phase because it is most purely an adsorptive mechanism (the interactions are with a hard surface rather than a soft layer on a surface).

NP-HPLC fell out of favor in the 1970s with the development of reversed-phase HPLC because of a lack of reproducibility of retention times as water or protic organic solvents changed the hydration state of the silica or alumina chromatographic media. Recently it has become useful again with the development of HILIC bonded phases which improve reproducibility.

Displacement Chromatography

The basic principle of displacement chromatography is: A molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus

displace all molecules with lesser affinities. There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds, and thereby be resolved, there must be substantial differences in some interaction between the biomolecules and the chromatography matrix. Operating parameters are adjusted to maximize the effect of this difference. In many cases, baseline separation of the peaks can be achieved only with gradient elution and low column loadings. Thus, two drawbacks to elution mode chromatography, especially at the preparative scale, are operational complexity, due to gradient solvent pumping, and low throughput, due to low column loadings. Displacement chromatography has advantages over elution chromatography in that components are resolved into consecutive zones of pure substances rather than "peaks". Because the process takes advantage of the nonlinearity of the isotherms, a larger column feed can be separated on a given column with the purified components recovered at significantly higher concentrations.

Reverse Phase Chromatography (RPC)

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is a silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. With these stationary phases, retention time is longer for molecules which are more non-polar, while polar molecules elute more readily. An investigator can increase retention time by adding more water to the mobile phase; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding more organic solvent to the eluent. RPC is so commonly used that it is often incorrectly referred to as "HPLC" without further specification. The pharmaceutical industry regularly employs RPC to qualify drugs before their release.

RPC operates on the principle of hydrophobic forces, which originate from the high symmetry in the dipolar water structure and play the most important role in all processes in life science. RPC is allowing the measurement of these interactive forces. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the

analyte molecule upon association with the ligand in the aqueous eluent. This solvophobic effect is dominated by the force of water for "cavity-reduction" around the analyte and the C_{18}-chain versus the complex of both. The energy released in this process is proportional to the surface tension of the eluent (water: 7.3×10^{-6} J/cm², methanol: 2.2×10^{-6} J/cm²) and to the hydrophobic surface of the analyte and the ligand respectively. The retention can be decreased by adding a less polar solvent (methanol, acetonitrile) into the mobile phase to reduce the surface tension of water. Gradient elution uses this effect by automatically reducing the polarity and the surface tension of the aqueous mobile phase during the course of the analysis.

Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area (C-H, C-C, and generally non-polar atomic bonds, such as S-S and others) results in a longer retention time because it increases the molecule's non-polar surface area, which is non-interacting with the water structure. On the other hand, polar groups, such as -OH, $-NH_2$, COO^- or $-NH_3^+$ reduce retention as they are well integrated into water. Very large molecules, however, can result in an incomplete interaction between the large analyte surface and the ligand's alkyl chains and can have problems entering the pores of the stationary phase.

Retention time increases with hydrophobic (non-polar) surface area. Branched chain compounds elute more rapidly than their corresponding linear isomers because the overall surface area is decreased. Similarly organic compounds with single C-C-bonds elute later than those with a C=C or C-C-triple bond, as the double or triple bond is shorter than a single C-C-bond.

Aside from mobile phase surface tension (organizational strength in eluent structure), other mobile phase modifiers can affect analyte retention. For example, the addition of inorganic salts causes a moderate linear increase in the surface tension of aqueous solutions (ca. 1.5×10^{-7} J/cm² per Mol for NaCl, 2.5×10^{-7} J/cm² per Mol for $(NH_4)_2SO_4$), and because the entropy of the analyte-solvent interface is controlled by surface tension, the addition of salts tend to increase the retention time. This technique is used for mild separation and recovery of proteins and protection of their biological activity in protein analysis (hydrophobic interaction chromatography, HIC).

Another important component is the influence of the pH since this can change the hydrophobicity of the analyte. For this reason most methods use a buffering agent, such as sodium phosphate, to control the pH. The buffers serve multiple purposes: they control pH, neutralize the charge on any residual exposed silica on the stationary phase and act as ion pairing

agents to neutralize charge on the analyte. Ammonium formate is commonly added in mass spectrometry to improve detection of certain analytes by the formation of ammonium adducts. A volatile organic acid such as acetic acid, or most commonly formic acid, is often added to the mobile phase if mass spectrometry is used to analyze the column eluent. Trifluoroacetic acid is used infrequently in mass spectrometry applications due to its persistence in the detector and solvent delivery system, but can be effective in improving retention of analytes such as carboxylic acids in applications utilizing other detectors, as it is one of the strongest organic acids. The effects of acids and buffers vary by application but generally improve the chromatography.

Reversed phase columns are quite difficult to damage compared with normal silica columns; however, many reversed phase columns consist of alkyl derivatized silica particles and should never be used with aqueous bases as these will destroy the underlying silica particle. They can be used with aqueous acid, but the column should not be exposed to the acid for too long, as it can corrode the metal parts of the HPLC equipment. RP-HPLC columns should be flushed with clean solvent after use to remove residual acids or buffers, and stored in an appropriate composition of solvent. The metal content of HPLC columns must be kept low if the best possible ability to separate substances is to be retained. A good test for the metal content of a column is to inject a sample which is a mixture of 2,2'- and 4,4'- bipyridine. Because the 2,2'-bipy can chelate the metal, the shape of the peak for the 2,2'-bipy will be distorted (tailed) when metal ions are present on the surface of the silica.

Size exclusion chromatography

Size exclusion chromatography (SEC), also known as gel permeation chromatography or gel filtration chromatography, separates particles on the basis of size. It is generally a low resolution chromatography and thus it is often reserved for the final, "polishing" step of purification. It is also useful for determining the tertiary structure and quaternary structure of purified proteins. SEC is used primarily for the analysis of large molecules such as proteins or polysaccharides. SEC works by trapping these smaller molecules in the pores of a particle. The larger molecules simply pass by the pores as they are too large to enter the pores. Larger molecules therefore flow through the column quicker than smaller molecules, that is, the smaller the molecule, the longer the retention time.

This technique is widely used for the molecular weight determination of polysaccharides. SEC is the official technique (suggested by European pharmacopeia) for the molecular weight comparison of different commercially available low-molecular weight heparins.

Ion exchange chromatography

In ion-exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded. Types of ion exchangers include:

- Polystyrene resins – These allow cross linkage which increases the stability of the chain. Higher cross linkage reduces swerving, which increases the equilibration time and ultimately improves selectivity.
- Cellulose and dextran ion exchangers (gels) – These possess larger pore sizes and low charge densities making them suitable for protein separation.
- Controlled-pore glass or porous silica

In general, ion exchangers favor the binding of ions of higher charge and smaller radius.

An increase in counter ion (with respect to the functional groups in resins) concentration reduces the retention time. An increase in pH reduces the retention time in cation exchange while a decrease in pH reduces the retention time in anion exchange.

This form of chromatography is widely used in the following applications: water purification, preconcentration of trace components, ligand-exchange chromatography, ion-exchange chromatography of proteins, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, and others.

Bioaffinity chromatography

This chromatographic process relies on the property of biologically active substances to form stable, specific, and reversible complexes. The formation of these complexes involves the participation of common molecular forces such as the Vander Waals interaction, electrostatic interaction, dipole-dipole interaction, hydrophobic interaction, and the hydrogen bond. An efficient, biospecific bond is formed by a simultaneous and concerted action of several of these forces in the complementary binding sites.

Aqueous Normal Phase Chromatography

Aqueous normal phase chromatography (ANP) is a chromatographic technique which encompasses the mobile phase region between reversed-phase chromatography (RP) and organic normal phase chromatography (ONP).

This technique is used to achieve unique selectivity for hydrophilic compounds, showing normal phase elution using reverse-phase solvents. [David et al,1999; Mofat et al,1986; United state pharmacopoeia 27,2004; Olaniyi et al, 2000].

Aqueous normal Phase operates on the basis of hydrophilicity and lipophilicity by using a polar stationary phase and a less polar mobile phase. Silica gel is the most commonly parking material used,) and there are vast varieties of normal phase column parkings.

The extent to which a compound is retained depends primarily upon its polarity. Thus hydrophobic compounds elute more quickly than do hydrophilic compounds.

The mobile phase often consists of relatively hazardous solvents like chloroform, ethyl acetate, and hexane. In pharmaceutical analysis where the sample contains two or more highly polar drugs that needs to be separated, straight phase stationary phase is used

2.5.0 HPLC ANALYTICAL METHOD DESIGN

High performance liquid chromatography was developed in the mid-1970's and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. Later new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds.

With time hplc was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification far above previous techniques. Computers and automation added to the convenience of hplc.

Hplc is widely considered a technique mainly for biotechnological, biomedical, and biochemical research as well as for the pharmaceutical industry. Currently hplc is used by a variety of fields including cosmetics, energy, food, and environmental industries.

HPLC Pumps

There are three types of pumps available for use with hplc analysis to achieve flow rates, they are: Reciprocating Piston Pumps, Syringe Type Pumps, and Constant Pressure Pumps.

The pumping systems deliver metered amounts of the mobile phase from the solvent reservoirs to the column through high pressure tubings and fittings. Typical hplc pumps operate with pressures of 4000psi or higher, with delivery rates in the range of 0.05 to 10 mL per minute.

Reciprocating Piston Pumps consist of a small motor driven piston which moves rapidly back and forth in a hydraulic chamber that may vary from 35-400 µL in volume. A wide range of flow rates can be attained by altering the piston stroke volume during each cycle, or by altering the stroke frequency. Dual and triple head pumps consist of identical piston chamber units which operate at 180 or 120 degrees out of phase. This type of pump system is significantly smoother because one pump is filling while the other is in the delivery cycle and is the most widely used.

Syringe Type Pumps are most suitable for small bore columns because this pump delivers only a finite volume of mobile phase before it has to be refilled. These pumps have a volume between 250 to 500 mL. The pump operates by a motorized lead screw that delivers mobile phase to the column at a constant rate. The rate of solvent delivery is controlled by changing the voltage on the motor.

In ***Constant Pressure Pumps*** the mobile phase is driven through the column with the use of pressure from a gas cylinder. A low-pressure gas source is needed to generate high liquid

pressures. The valving arrangement allows the rapid refill of the solvent chamber whose capacity is about 70 mL. This provides continuous mobile phase flow rates.

Injectors for HPLC

Samples are injected into the HPLC via an injection port. The injection port of an HPLC commonly consists of an injection valve and the sample loop. The sample is typically dissolved in the mobile phase. The sample is then drawn into a syringe and injected into the loop via the injection valve. A rotation of the valve rotor closes the valve and opens the loop in order to inject the sample into the stream of the mobile phase. Loop volumes can range between 10 µl to over 500µl. In modern HPLC systems, the sample injection is typically automated, where every thing is controlled by computer applications.

Columns, Stationary phase, and Column length

For most pharmaceutical analyses, separation on the column is achieved by partition of compounds in the test solution between the mobile and the stationary phases. The stationary phase in HPLC refers to the solid support contained within the column over which the mobile phase continuously flows. The sample solution is injected into the mobile phase of the assay through the injector port. As the sample solution flows with the mobile phase through the stationary phase, the components of that solution will migrate according to the non-covalent interactions of the compounds with the stationary phase. The chemical interactions of the stationary phase and the analyte with the mobile phase determine the degree of migration and separation of the components contained in the sample. For example, those samples which have stronger interactions with the stationary phase than with the mobile phase will elute from the column less quickly and thus have a longer retention time, while the reverse is also true. The common particle size for RPLC is 5µm. Ion exchange and size exclusion can be larger, 7-12µm. However there are smaller particle sizes ranging from 3µm and below. The lengths of column typically used are the 250mm and the 150mm types. Shorter columns with vast variety of stationary phases are now being invented for RPLC.

Hypersil BDS Columns

In order to improve symmetry of peaks and the avoidance of fronting and tailing of drugs, reverse phase stationary phases are being modified to enhance peak properties. For the purpose of this thesis, *base deactivated silanol stationary phase* (BDS) is discussed. The BDS parking material is a base deactivated RP18 or RP8 materials for the analysis of basic compounds which tails on a RP18 or RP8 column and also give superior peak shape for analysis of neutral and acidic compounds Hypersil BDS phases use 120Å - 130Å spherical silica which has been specially end-capped for added stability. End capping reagents are applied to block active silanol sites. A common end-capping reagent is dimethyldichlorosilane, which provides dimethyl groups on the surface. En-capping is often called base deactivation [Olaniyi et al, 2000; Michael et al, 2006; Dolan et al, 2009].

Secondary Columns

There are various columns that are secondary to the separating column or stationary phase. They are: *Guard, derivatizing, capillary, fast, and Preparatory columns*.

Guard Columns are placed anterior to the separating column. This serves as a protective factor that prolongs the life and usefulness of the separation column. They are dependable columns designed to filter or remove: 1) particles that clog the separation column; 2) compounds and ions that could ultimately cause "baseline drift", decreased resolution, decreased sensitivity, and create false peaks; 3) compounds that may cause precipitation upon contact with the stationary or mobile phase; and 4) compounds that might co-elute and cause extraneous peaks and interfere with detection and/or quantification. These columns must be changed on a regular basis in order to optimize their protective function. Size of the packing varies with the type of protection needed.

Derivatizing Columns- Pre- or post-primary column derivatization can be an important aspect of the sample analysis. Reducing or altering the parent compound to a chemically related daughter molecule or fragment elicits potentially tangible data which may complement other results or prior analysis. In few cases, the derivatization step can serve to cause data to become questionable, which is one reason why HPLC was advantageous over gas chromatography, or GC. Because GC requires volatile, thermally stabile, or nonpolar analytes, derivatization was

usually required for those samples which did not contain these properties. Acetylation, silylation, or concentrated acid hydrolysis are few derivatization techniques.

Capillary Columns- Advances in HPLC led to smaller analytical columns. Also known as microcolumns, capillary columns have a diameter much less than a millimeter and there are three types: open-tubular, partially packed, and tightly packed. They allow the user to work with nanolitre sample volumes, decreased flow rate, and decreased solvent volume usage which may lead to cost effectiveness. However, most conditions and instrumentation must be miniaturized, flow rate can be difficult to reproduce, gradient elution is not as efficient, and care must be taken when loading minute sample volumes.

Microbore and ***small-bore*** columns are also used for analytical and small volumes assays. A typical diameter for a small-bore column is 1-2 mm. Like capillary columns, instruments must usually be modified to accommodate these smaller capacity columns (i.e., decreased flow rate). However, besides the advantage of smaller sample and mobile phase volume, there is a noted increase in mass sensitivity without significant loss in resolution capillary Electrophoresis.

Fast Columns- One of the primary reasons for using these columns is to obtain improved sample throughput (amount of compound per unit time). For many columns, increasing the flow or migration rate through the stationary phase will adversely affect the resolution and separation. Therefore, fast columns are designed to decrease time of the chromatographic analysis without forsaking significant deviations in results. These columns have the same internal diameter but much shorter length than most other columns, and they are packed with smaller particles that are typically 3 μm in diameter. Advantages include increased sensitivity, decreased analysis time, decreased mobile phase usage, and increased reproducibility.

Preparatory Columns- These columns are utilized when the objective is to prepare bulk (milligrams) of sample for laboratory preparatory applications. A preparatory column usually has a large column diameter which is designed to facilitate large volume injections into the HPLC system. Accessories important to mention are the back-pressure regulator and the fraction collector. The back-pressure regulator is placed immediately posterior to the HPLC detector. It is designed to apply constant pressure to the detector outlet which prevents the formation of air

bubbles within the system. This, in turn, improves chromatographic baseline stability. It is usually devised to operate regardless of flow rate, mobile phase, or viscosity.

The fraction collector is an automated device that collects uniform increments of the HPLC output. Vials are placed in the carousel and the user programs the time interval in which the machine is to collect each fraction. Each vial contains mobile phase and sample fractions at the corresponding time of elution. Packings for columns are diverse since there are many modes of HPLC. They are available in different sizes, diameters, pore sizes, or they can have special materials attached (such as an antigen or antibody for immunoaffinity chromatography). Packings available range from those needed for specific applications (affinity, immunoaffinity, chiral, biological, etc.) to those for all-purpose applications. The packings are attached to the internal column hull by resins or supports, which include oxides, polymers, carbon, hydroxyapatite beads, and silica, the most common type.

Mobile Phase

The mobile phase in HPLC refers to the solvent being continuously applied to the column, or stationary phase. The mobile phase acts as a carrier for the sample solution. A sample solution is injected into the mobile phase of an assay through the injector port. As a sample solution flows through a column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of the compound with the column. The chemical interactions of the mobile phase and sample, with the column, determine the degree of migration and separation of components contained in the sample. For example, those samples which have stronger interactions with the mobile phase than with the stationary phase will elute from the column faster and thus have a shorter retention time, while the reverse is also true. The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase. There are two main types of mobile phases, these include: Isocratic and gradient.

In ***isocratic elution*** compounds are eluted using constant mobile phase composition. All compounds begin migration through the column at onset. However, each migrates at a different rate, resulting in faster or slower elution rate. This type of elution is both simple and inexpensive, but resolution of some compounds is questionable and elution may not be obtained in a reasonable amount of time and it involves a lot of try and error approach based on knowledge of compound to be analysed.

In ***gradient elution*** different compounds are eluted by increasing the strength of the organic solvent. The sample is injected while a weaker mobile phase is being applied to the system. The strength of the mobile phase is later increased in increments by raising the organic solvent fraction, which subsequently results in elution of retained components. This is usually done in a stepwise or linear fashion.

At the onset of sample introduction, the compounds are initially retained at the inlet of the column. As the solute capacity, or *k*', for the compound decreases, the compound begins to migrate through the stationary phase. Each of the other compounds in the sample subsequently migrate as their *k*' values decrease. Compared with isocratic elution, resolution and separation are improved, and bandwidths are nearly equal

Detectors and Detection Limits

The detector for an HPLC is the component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is positioned immediately posterior to the stationary phase in order to detect the compounds as they elute from the column. The bandwidth and height of the peaks may usually be adjusted using the coarse and fine tuning controls, and the detection and sensitivity parameters may also be controlled (in most cases). There are many types of detectors that can be used with HPLC.

Some of the more common detectors include: Ultra-Violet (UV), Refractive Index (RI), Fluorescent, Radiochemical, Electrochemical, Near-Infra Red (Near-IR), Mass Spectroscopy (MS), Nuclear Magnetic Resonance (NMR), and Light Scattering (LS).

Ultra-Violet (UV) detectors measure the ability of a sample to absorb light. This can be accomplished at one or several wavelengths:

• **Fixed Wavelength** measures at one wavelength, usually 254 nm

• **Variable Wavelength** measures at one wavelength at a time, but can detect over a wide range of wavelengths

• **Diode Array** measures a spectrum of wavelengths simultaneously (multiwavelengths) UV detectors have a sensitivity to approximately 10-8 or 10 -9 g/mL or 1 to 10 ng/mL. Many compendia HPLC methods require the use of spectrophotometric detectors [Kantor et al, 2003]

Refractive Index (RI) detectors measure the ability of sample molecules to bend or refract light. This property for each molecule or compound is called its refractive index. For most RI detectors, light proceeds through a bi-modular flow-cell to a photodetector. One channel of the flow-cell directs the mobile phase passing through the column while the other directs only the mobile phase. Detection occurs when the light is bent due to samples eluting from the column, and this is read as a disparity between the two channels. Refractive index detectors are used to detect non-UV absorbing compounds, but they are less sensitive than UV detectors.

Fluorescent detectors measure the ability of a compound to absorb then re-emit light at given wavelengths. Each compound has a characteristic fluorescence. The excitation source passes through the flow-cell to a photodetector while a monochromator measures the emission wavelengths. Has sensitivity limit of 10-9 to 10-11 g/mL.

Radiochemical detection involves the use of radio-labelled material, usually tritium (3H) or carbon-14 (14C). It operates by detection of fluorescence associated with beta-particle ionization, and it is most popular in metabolite research. Two detector types:

• **Homogeneous**- Where addition of scintillation fluid to column effluent causes fluorescence.

• **Heterogeneous**- Where lithium silicate and fluorescence caused by beta-particle emission interact with the detector cell. Has sensitivity limit up to 10-9 to 10-10 g/mL.

Electrochemical detectors measure compounds that undergo oxidation or reduction reactions. Usually accomplished by measuring gain or loss of electrons from migrating samples, as they pass between electrodes at a given difference in electrical potential. Has sensitivity of 10-12 to 10-13 g/mL

Mass spectroscopy (MS) detectors- The sample compound or molecule is ionized, it is passed through a mass analyzer, and the ion current is detected. There are various methods for ionization:

• **Electron impact (EI)** - An electron current or beam created under high electric potential is used to ionize the sample migrating off the column.

• **Chemical ionization**- A less aggressive method which utilizes ionized gas to remove electrons from the compounds eluting from the column.

• **Fast atom bombardment (FAB)** - Xenon atoms are propelled at high speed in order to ionize the eluents from the column. Has detection it of 10-8 to 10-10 gm/ml.

Nuclear magnetic resonance (NMR) detectors- Certain nuclei with odd- numbered masses, including H and 13C, spin about an axis in a random fashion. However, when placed between poles of a strong magnet, the spins are aligned either parallel or anti-parallel to the magnetic field, with the parallel orientation favoured since it is slightly lower in energy. The nuclei are then irradiated with electromagnetic radiation which is absorbed and places the parallel nuclei into a higher energy state; consequently, they are now in "resonance" with the radiation. Each H or C will produce different spectra depending on their location and adjacent molecules, or elements in the compound, because all nuclei in molecules are surrounded by electron clouds which change the encompassing magnetic field and thereby alter the absorption frequency.

Light-scattering (LS) detectors- When a source emits a parallel beam of light which strikes particles in solution, some light is reflected, absorbed, transmitted, or scattered. Two forms of LS detection may be used to measure the two latter occurrences:

(a.) Nephelometry- This is defined as the measurement of light scattered by a particulate solution. This method enables the detection of the portion of light scattered at a multitude of angles. The sensitivity depends on the absence of background light or scatter since the detection occurs at a black or null background.

(b.) Turbidimetry- This is defined as the measure of the reduction of light transmitted due to particles in solution. It measures the light scatter as a decrease in the light that is transmitted through the particulate solution. Therefore, it quantifies the residual light transmitted. Sensitivity of this method depends on the sensitivity of the machine employed, which can range from a simple spectrophotometer to a sophisticated discrete analyzer. Thus, the measurement of a decrease in transmitted light from a large signal of transmitted light is limited to the photometric accuracy and limitations of the instrument employed.

Near-infrared detectors- Operates by scanning compounds in a spectrum from 700 to 1100 nm. Stretching and bending vibrations of particular chemical bonds in each molecule are detected at certain wavelengths. This is a fast growing method which offers several advantages: speed (sometimes less than 1 second), simplicity of preparation of sample, multiple analyses from single spectrum, and non-consumption of the sample.

Recorders

Two devices are used to record the areas under peaks:

• integrating recorders

• Computer program

Each type of device records the messages sent to them by the detector as peaks, calculates the retention time, and calculates the area under each peak; all of this information is included in the printout. For similar compounds, the area under a peak is roughly proportional to the amount of compound injected.

Applications for HPLC

Preparative HPLC refers to the process of isolation and purification of compounds. Important is the degree of solute purity and the throughput, which is the amount of compound produced perunit time. This differs from analytical HPLC, where the focus is to obtain information about the sample compound. The information that can be obtained includes identification, quantification, and resolution of a compound

Chemical Separations can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. Thus, the chromatographer can separate compounds from each other using HPLC; the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.

Quantification of compounds by HPLC is the process of determining the unknown concentration of a compound in a known solution. It involves injecting a series of known concentrations of the standard compound solution onto the HPLC for detection. The chromatograph of these known concentrations will give a series of peaks that correlate to the concentration of the compound injected. scientist injects a sample of unknown concentration x (x-axis of calibration curve) onto the HPLC; the chromatograph gives a peak output of area y (y-axis of the calibration curve). The area, y, is then in the equation of a line $y=mx + b$ from the calibration curve, and the concentration is found bysolving the equation for x .

2.6.0 HPLC METHOD VALIDATIONS AND PERFORMANCE

Validation is an essential or indispensable part in analytical quality control which basically forms part of good manufacturing practices (GMP). It is, therefore, an element of the quality assurance programme associated with a particular product or process.

Analytical Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use Validation of processes and systems is fundamental to achieving quality assurance goals. It is by design and validation that a manufacturer can establish confidence that the manufactured products will consistently meet their product specifications.

Methods need to be validated or revalidated

• before their introduction into routine use

• whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics.

• whenever the method is changed, and the change is outside the original scope of the method.

Method validation has received considerable attention over decades and from industrial committees and regulatory agencies [David et al, 1999; United state pharmacopoeia 27, 2004; Kantor et al, 2003].

2.6.1 Strategy for Validation of Methods

The validity of a specific method should be demonstrated in laboratory experiments using samples or standards that are similar to the unknown samples analyzed in the routine. Possible steps for a complete method validation are listed below.

• Develop a validation protocol or operating procedure for the validation

• Define the application, purpose and scope of the method

• Define the performance parameters and acceptance criteria

• Define validation experiments

• Verify relevant performance characteristics of equipment

• Qualify materials, e.g. standards and reagents

• Perform pre-validation experiments

• Adjust method parameters or/and acceptance criteria if necessary

• Perform full internal (and external) validation experiments

• Develop SOPs for executing the method in the routine

• Define criteria for revalidation

• Define type and frequency of system suitability tests and/or analytical quality control (AQC) checks for the routine

• Document validation experiments and results in the validation report first the scope of the method and its validation criteria should be defined. These include:

• compounds,

• matrices,

• type of information: qualitative or quantitative,

• detection and quantitation limits,

• linear range,

• precision and accuracy

• type of equipment and location

The method's performance characteristics should be based on the intended use of the method. For example, if the method will be used for qualitative trace level analysis, there is no need to test and validate the method's linearity over the full dynamic range of the equipment.

Initial parameters should be chosen according to the analyst's best judgment. Finally, parameters should be agreed between the lab generating the data and the client using the data.

The scope of the method should include the different types of equipment and the locations where the method will be run. For example, if the method is to be run on one specific instrument in one specific laboratory, there is no need to use instruments from other vendors or to include other laboratories in the validation experiments. In this way the experiments can be limited to what is really necessary.

Before an instrument is used to validate a method, its performance should be verified using generic standards. Satisfactory results for a method can only be obtained with well performing equipment. Special attention should be paid to the equipment characteristics that are critical for the method. Any material used to determine critical validation parameters, such as reagents and reference standards, should be checked for accurate composition and purity. If there is no or little information on the method's performance characteristics, it is recommended to prove the methods suitability for its intended use in initial experiments.

These studies should include the approximate precision, working range and detection limits. If the preliminary validation data appear to be inappropriate, the method itself, the equipment, the analysis technique or the acceptance limits should be changed. In this way method development and validation is an interactive process. For example, in liquid chromatography selectivity is achieved through selection of mobile phase composition. For quantitative measurements the resolution factor between two peaks should be 2.5 or higher. If this value is not achieved, the mobile phase composition needs further optimization.

There are no official guidelines on the sequence of validation experiments and the optimal sequence can depend on the method itself. For a high performance liquid chromatographic method the following sequence has been proven to be useful:

System suitability (performance check)

- System precision (precision of retention times and peak areas or heights)
- Column efficiency
- Asymmetry factor
- Capacity factor

2.6.2 Parameters for method validation

The main parameters for method validation include the following

Selectivity of standards (optimizing separation and detection of standard mixtures)

The terms selectivity and specificity are often used interchangeably. The term specific generally refers to a method that produces a response for a single analyte only while the term selective refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate. The USP monograph defines selectivity of an analytical method as its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers and known (or likely) degradation products that may be expected to be present in the sample matrix. Selectivity in liquid chromatography is obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, pH, column temperature and detector wavelength.

It is a difficult task in chromatography to ascertain whether the peaks within a sample chromatogram are pure or consist of more than one compound.

Precision

The precision of a method is the extent to which the individual test results of multiple injections of a series of standards agree. The measured standard deviation can be subdivided into three categories: repeatability, intermediate precision and reproducibility. Repeatability is obtained when the analysis is carried out in one laboratory by one operator using one piece of equipment over a relatively short time span. At least 5 or 6 determinations of three different matrices at two or three different concentrations should be done and the relative standard deviation calculated. The acceptance criteria for precision depend very much on the type of analysis. While for compound analysis in pharmaceutical quality control precision of better than 1 % RSD is easily achieved and not more than 10% RSD, for biological samples the precision is more like 15% at the concentration limits and 10% at other concentration levels. For environmental and food samples, the precision is very much dependent on the sample matrix, the concentration of the analyte and on the analysis technique. It can vary between 2% and more than 20%. Repeatability should be assessed using:

(a) a minimum of 9 determinations covering the specified range for the procedure (e.g. 3 Concentrations /3 replicate each)

(b) a minimum of 6 determinations at 100% of the test concentration.

Intermediate precision is a term that has been defined as the long-term variability of the measurement process and is determined by comparing the results of a method run within a single laboratory over a number of weeks. A method's intermediate precision may reflect discrepancies in results obtained by different operators, from different instruments, with standards and reagents from different suppliers, with columns from different batches or a combination of these. The objective of intermediate precision validation is to verify that in the same laboratory the method will provide the same results once the development phase is over. If reproducibility is assessed, a measure of intermediate precision is not required.

Reproducibility as defined by ICH represents the precision obtained between laboratories. The objective is to verify that the method will provide the same results in different laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from homogeneous lots in different laboratories with different analysts and by using operational and environmental conditions that may differ from but are still within the specified parameters of the method (inter-laboratory tests). Validation of reproducibility is important if the method will used in different laboratories, because in different laboratories analysis will be affected by these factors:

• Differences in room temperature and humidity

• Operators with different experience and thoroughness

• Equipment with different characteristics, e.g. delay volume of an HPLC system

• Variations in material and instrument conditions, e.g. in HPLC, mobile phases composition, pH, flow rate of mobile phase

• Equipment and consumables of different ages.

• Columns from different suppliers or different batches

• Solvents, reagents and other material with different quality

• linearity

Limit of detection and quantitation

The limit of detection (LOD) is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified.

The limit of quantitation (LOQ) is the minimum injected amount that gives precise measurements, in chromatography typically requiring peak heights 10 to 20 times higher than baseline noise.

Several approaches for determining detection and quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

• **Based on Visual Evaluation**

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. In chromatography the LOD is the injected amount that results in a peak

with a height at least twice or three times as high as the baseline noise level. The LOQ is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

• **Based on Signal-to-Noise Approach**

This mostly applies to LOQ. This approach can only be applied to analytical procedures that exhibit baseline noise.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

• **Based on the Standard Deviation of the Response**

The quantization limit (LOQ) may be expressed as:

LOQ = 10 x SD/Slop

Where *SD* is the residual standard deviation. [ICH guidelines, 1996]

Range

The range of an analytical method is the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as the test results (e.g. percentage, parts per million) obtained by the analytical method.

Robustness

Robustness tests examine the effect operational parameters have on the analysis results. For the determination of a method's robustness a number of chromatographic parameters, for example, flow rate, column temperature, injection volume, detection wavelength or mobile phase composition are varied within a realistic range and the quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method's robustness range. Obtaining data on these effects will

allow to judge whether a method needs to be revalidated when one or more of parameters are changed, for example to compensate for column performance over time. In the ICH document it is recommended to consider the evaluation a methods robustness during the development phase, but it is not required to be included as part of a registration application.

Simplicity

Simplicity of a method is carrying out an analysis in a minimum number of steps, and using easily available cheap reagents and equipment. More steps in analysis introduce errors to some degree. Simple methods are needed for the adoption by small laboratories of limited.

Selectivity with real samples

The terms selectivity and specificity are often used interchangeably. The term specific generally refers to a method that produces a response for a single analyte only while the term selective refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate. The USP monograph defines selectivity of an analytical method as its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers and known (or likely) degradation products that may be expected to be present in the sample matrix. Selectivity in liquid chromatography is obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, pH, column temperature and detector wavelength. It is a difficult task in chromatography to ascertain whether the peaks within a sample chromatogram are pure or consist of more than one compound.

Trueness/accuracy, at different concentrations

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. The true value for accuracy assessment can be obtained in several ways. One alternative is to compare results of the method with results from an established reference method. This approach assumes that the uncertainty of the reference method is known.

Secondly, accuracy can be assessed by analyzing a sample with known concentrations, for example, a certified reference material, and comparing the measured value with the true value as supplied with the material. If such certified reference material is not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume. After injection of analyte into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent. Because this accuracy assessment measures the effectiveness of sample preparation, care should be taken to mimic the actual sample preparation as closely as possible.

The concentration should cover the range of concern and should particularly include one concentration close to the quantization limit. The expected recovery depends on the sample matrix, the sample processing procedure and on the analyte concentration.

Linearity and Calibration Curve

The linearity of an analytical method is its ability to elicit test results that are directly, or by means of well-defined mathematical transformations, proportional to the concentration of analytes in samples within a given range.

Linearity is determined by a series of three to six injections of five or more standards whose concentrations span 80-120 percent of the expected concentration range. The response should be directly or by means of a well defined mathematical calculation proportional to the concentrations of the analytes. A linear regression equation applied to the results should have an intercept not significantly different from zero. If a significant nonzero intercept is obtained, it should be demonstrated that there is no effect on the accuracy of the method.

Frequently the linearity is evaluated graphically in addition or alternatively to mathematical evaluation. The evaluation is made by visual inspection of a plot of signal height or peak area as a function of analyte concentration. Because deviations from linearity are sometimes difficult to detect two additional graphical procedures can be used. The first one is to plot the deviations from the regression line versus the concentration or versus the logarithm of the concentration, if the concentration range covers several decades. For linear ranges the deviations should be equally distributed between positive and negative values.

An instance where precision is questionable in terms of peak height, retention time shape etc, an internal standard is used in the analysis to serve as a guard to high level of precision.

Internal standard must have certain properties that include:

• Resolved completely from all components of analysis.

• Elute closely to the drug in question

• Reproducibly recovered in any extraction procedures

• Stable enough to prevailing conditions (PH changes, temperature, and humidity)

• Desirable to be commercially available in high purity.

Ruggedness (inter-laboratory studies)

Some of the parameters can be measured in combined experiments. For example, when the Precision of peak areas is measured over the full concentration range, the data can be used to validate the linearity. [Kantor et al, 2003]

During method validation the parameters, acceptance limits and frequency of ongoing system Suitability tests or quality control checks should be defined. Criteria should be defined to indicate when the method and system are out of statistical control. The goal is to optimize these experiments such that with a minimum number of control analyses the method and the complete analytical system will provide long-term results that will meet the objectives defined in the scope of the method.

Time cycle is the time needed for the successful completion of analysis. Time cycle has been a very pertinent issue for industries.

Cost of analysis of all the characters mentioned above working to perfection reduces cost. These factors affect productivity very much. Even though reduction in cost is very crucial, it should not be compromised for quality. [Glajch et al, 1997; ICH guidelines, 1996; Jirat et al, 2006]

CHAPTER THREE

3.1.0 METHOD DEVELOPMENT, REAGENTS AND APPARATUS

Samples:

Cyanocobalamin powder

Ascorbic acid powder

Source: Department of Pharmaceutical chemistry, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST- Kumasi.

Vitamin B_{12} (Cyanocobalamin) injection BP ampoules

Reagents:

Distilled water

Sulphuric acid BDH

Methanol (HPLC grade) BDH

Orthophosphoric acid BDH

Sodium metaphosphate BDH

Pottassium metaphosphate BDH

Apparatus and Equipments:

pH meter

Water bath

CECIL CE UV Spectrophotometer (Double beam)

Electronic balance

Melting point apparatus

Sonicator

Vacuum filter

HPLC facility with the following components:

Pump: Shimadzu, LC-10AT Liquid chromatograph pump.
Sample injector: Rheodyne Inc, Syringe loading injector, 20µL sample loop.
Stationary phase (column): Keystone, Hypersil BDS C-18, 150 x 4.6 mm, 5µm, 120Å.
Detector: Applied Biosystems, 783A programmable absorbance detector.
Integrator: Shimadzu, CR501 chromatopac.

Glassware

Graduated pipette (25ml, 10ml, 5ml, 2ml, 1ml, 0.5ml)

Volumetric flask (100ml, 50ml, 25ml, 10ml, 5ml)

10ml Beaker

Thistles funnel (100ml)

Measuring cylinder (1000ml, 500ml, 100ml, 10ml)

3.2.0 SURVEY

A survey was carried out in the Kumasi metropolis to determine the different formulations of cyanocobalamin as well as different brands of cyanocobalamin injections sold in the Kumasi metropolis and their country of origin.

Table 3.2(a) Vitamin B_{12} (cyanocobalamin) formulations sold in the Kumasi metropolis and their country of origin.

Cyanocobalamin formulation	Country of Origin
Tablet	UK , Ghana , Nigeria
Capsule	UK
Injection	China, Lichensteine

Table 3.2(b) Brands of vitamin B_{12} injections sold in the Kumasi metropolis

Brand	Country of Origin	Batch no	Strength (%)	Man. Date	Exp. Date
Cyanamin-12	China	0910119	0.005	10/2009	10/2012
Binox-12	Lichensteine	1550351	0.005	02/2010	02/2013
Bena^{R}	China	100602	0.005	06/2010	06/2013

3.3.0 PHARMACOPOEIA ASSESSMENTS

3.3.1 Qualitative and quantitative assay of cyanocobalamin samples

- **Melting point determination**

A capillary tube closed at one end was filled with pure cyanocobalamin powder and then inserted into the sample tube of the melting point apparatus. The plateu temperature was then set and then heated. The melting point was then read when the sample melted.

This procedure was repeated two more times and the mean melting point calculated.

- **UV spectrum determination of cyanocobalamin pure powder**

2.5mg of cyanocobalamin powder was weighed and dissolved in a beaker using distilled water. It was then transferred into a 100ml volumetric flask and then made up to the mark with the same solvent.

A UV light absorption spectrum was obtained with the solution prepared between 200nm - 600nm.

The peak bands, their respective absorbances and the ratios of their absorbances were noted and compared with literature values.

The ratio of the absorbance at the maximum at 361 nm to that at the maximum at 547 nm to 559 nm is 3.15 to 3.45.

The ratio of the absorbance at the maximum at 361 nm to that at the maximum at 278 nm is 1.70 to 1.90.

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3.3.2 Quantitative analysis of reference sample and commercial injections

- **Assay of cyanocobalamin pure powder.**

25mg of cyanocobalamin powder was weighed into a 1000ml volumetric flask and distilled water was added to dissolve it.

It was then made up to the mark with the same solvent. The absorbance was then taken at 361nm using 1cm fused silica cuvette. The percentage purity was then calculated taking the specific absorbance of 207.

- **Calibration curve for assay of cyanocobalamin injections**

0.01g of cyanocobalamin powder was weighed and transferred quantitatively into a clean, dry pre-washed 50ml volumetric flask. 15ml of distilled water was added while swirling to dissolve the powder. More distilled water was added to make the solution up to the 50ml mark. The solution was labeled as 0.02% stock solution A.

Six different standard solutions of 0.001%,0.0015%,0.002%,0.0025%,0.003%,0.0035%w/v respectively were prepared from the cyanocobalamin standard stock solution above by serial dilutions using distilled water.

The absorbance of each solution was obtained with the CECIL UV spectrophotometer (double beam) at a wavelength of 361nm using 1cm fused silica cuvette.

A calibration curve was obtained using the concentration of the six solutions prepared and their respective absorbances measured.

The calibration curve was then used to analyse the commercials samples obtained from the market after measuring their absorbances. Concentration amount prepared was 0.002% for each brand of cyanocobalamin.

3.3.3 Qualitative and quantitative assay of ascorbic acid

- **Melting point determination of ascorbic acid**

A capillary tube closed at one end was filled with pure Ascorbic acid powder and then inserted into the sample tube of the melting point apparatus .The plateu temperature was then set and then heated .The melting point was then read when the sample started melting.

- **UV spectrum of ascorbic acid**

0.10 g of pure ascorbic acid powder was dissolved in distilled water and dilute immediately to 100.0 ml with the same solvent. 1.0ml of the solution was taken and 10ml of 0.1 M Hydrochloric acid added to the solution and then diluted to 100.0 ml with distilled water. The absorbance was then measured at the maximum at 243 nm immediately after dissolution. The specific absorbance was taken at the maximum as 545nm to 585nm.

- **Quantitative assay of ascorbic acid pure powder**

0.150 g was dissolved in a mixture of 10 ml of dilute sulphuric acid R and 80 ml of carbon dioxide-free water R.1 ml of starch solution R was added and then titrated with 0.05 M iodine until a persistent violet-blue colour was obtained. The percentage purity of the ascorbic acid was then calculated taking 1 ml of 0.05 M iodine as equivalent to 8.81 mg of $C_6H_8O_6$.

3.3.4 Determination of pH and volume of ampoule

Ampoule was broken at the mouth and the content was drawn with a 5ml graduated pipette and it's volume recorded. This procedure was repeated for six ampoules each of the brands of cyanocobalamin injections bought from the market.

The content was then drawn into a 10ml beaker and the pH determined with the pH meter. The mean and the standard deviations were then calculated for each brand.

3.4.0 STABILITY STUDIES OF CYANOCOBALAMIN SAMPLES

The stability of cyanocobalamin solutions were studied under the conditions of pH, sunlight and temperature.

3.4.1 pH effect on cyanocobalamin solutions

0.002g of cyanocobalamin pure sample was weighed and then dissolved in 100ml of distilled water and the absorbance was measured at 361nm.

Other solutions were also prepared with different pH between 4 to 8) and their absorbances measured and then compared with each other.

3.4.2 pH effect on cyanocobalamin solutions exposed to sunlight.

0.002 g of cyanocobalamin was weighed and then dissolved in phosphate buffers of various pH between (4-8) to make 100ml of solutions. The solutions were exposed to sunlight at two hours interval over six hours period and their absorbances were taken at 361nm wavelength and the trend of effect assessed with a plot of Absorbance against Time of exposure.

3.4.3 Temperature effects on the stability of commercial samples

Ampoule was broken at the mouth and a concentration of 0.002% prepared from it by measuring 0.8ml of the injection in 100ml distilled water and then subjected to temperatures ranging between 15 to 60^{o}C in an oven at 6 hours intervals over 12 hours period.

A control concentration was also prepared from the same ampoule of the same concentration of 0.002% but was not subjected to the temperature range chosen.

The absorbance was then recorded at 361nm wavelength for each preparation. The trend of change in absorbance was then monitored and compared to the control concentration.

3.5.0 DEVELOPMENT OF HPLC METHOD OF ANALYSIS

3.5.1 Method development strategy

Cyanocobalamin and ascorbic acid were evaluated for their physicochemical properties as well as their interaction in the injection.

Upon critical examination on the physicochemical properties of these drugs, the choice of mode of HPLC was RPLC. A number of mobile phase combinations as well as column types were considered, with the prime aim of achieving better resolution for drugs for quantitative purposes. Mobile phase combinations included methanol/water, methanol/phosphate buffer in various combinations and at different pH-values.

Preparation of phosphate buffers

50ml of monobasic potassium phosphate was poured into a 200ml volumetric flask and the specified volume of 0.2M sodium hydroxide added and then made up to the mark with distilled water according to the table shown below.

Table 3.6.2 Volume of 0.20M NaOH required for preparing various pH

PH	5.8	6.0	6.2	6.4	6.6	6.8	7.0	7.2	7.8	8.0
0.2M NaOH / ml	3.6	5.6	8.1	11.6	16.4	22.4	29.1	34.7	44.5	46.1

Preparation of standard solutions of reference cyanocobalamin

0.05g cyanocobalamin was dissolved in mobile phase. Standard solutions were prepared by further dilution with the mobile phase to the required concentrations. Concentration ranging from 0.03% to 0.005% were prepared and used for the calibration curve.

Selection of column.

A number of RPLC columns exist for the simultaneous separation of acidic, basic and neutral drugs using isocratic mode of elution. Amongst these columns, ODS C-18, 250mm, 4.6mm; C8, 250mm, 4.6mm; Hypersil BDS C8, 150mm, 4.6mm .

Hypersil BDS C-18, 150mm, 4.6mm was chosen for the method design due to the fact that cyanocobalamin and ascorbic acid are more polar and therefore easily eluted from the coloumn

The effect of mobile phase pH on the retention time on reverse phase HPLC columns

In reverse phase chromatography, solvent strength increases with increase in the organic portion of the mobile phase. Solvent strength can be controlled by adjustment of the pH of the mobile phase. For ionisable acidic or basic drugs, the pH of the mobile phase affects their elution rates in RPLC.

Using the effect of pH on partition coefficient of a base and by knowing the pH of the mobile phase and the pKa of the analyte, it is possible to predict approximately the retention time of the analyte upon change in pH, whilst all other parameters of the HPLC system remains fixed. This is achievable when a first chromatogram is obtained.

In the case of accuracy in the calculations, pH of the mobile phase was used, because this was the actual pH environment in which the drug was being analysed, and also ensuring that the pH was set in the pH range of 2 to 8 units because of the tendency of extremes of pH to dissolve silica gel.

Selection of pH of mobile phase

Various proportions of mobile phase of (methanol /water/phosphate buffer) were prepared and a specified concentration of cyanocobalamin and Ascorbic acid added and then injected into the HPLC system.

The pH of the aqueous phase ranging from 4 to 8 was adjusted upwards with the phosphate buffer solutions prepared.

The pH of the aqueous portion as well as that of the mobile phase was monitored and peaks obtained after injection into the HPLC system were accessed with the one with the best separation and resolution.

Selection of UV detection wavelength and detector sensitivity

A suitable wavelength of maximum absorption was obtained for the analysis by scanning with UV spectrophotometer of solution containing the sample dissolved in the mobile phase using the mobile phase in the reference cell. With careful investigation of the UV spectrum of the sample,

wavelength of maximum absorption of 278nm was most suitable for both cyanocobalamin and ascorbic acid. After varying the sensitivity ranging from 0.001 to 0.1, 0.030 was most suitable for the detection.

Internal Standard

Ascorbic acid was chosen as the internal standard for the method design. Mobile phase pH and compositions were varied until there was a resolution between the test drugs and the proposed internal standard.

Ascorbic acid worked best for cyanocobalamin owing to the fact that it gave the best resolution with it under the established conditions without interfering with others and also meets all the requirements for the selection of an internal standard.

In the method design, a constant amount (0.04mg) of the ascorbic acid was used.

HPLC Pump flow rate

Flow rates were carefully chosen in order to ensure pumps reproducibility of results. Upon several adjustments, 1.40mL/min (column back pressure; 111-115bar) was chosen for cyanocobalamin analysis.

Chart recorder speed

The speed of the recorder was of great concern because it helped in the elucidation of resolved peaks and shapes of peaks. The appropriate speed employed was 5mm/min at attenuation 0.

3.5.2 Method performance

0.002g of pure cyanocobalamin powder was dissolved in 100ml of the mobile phase and then subjected to the conditions established for the method design and 0.04mg of ascorbic acid added and then analysed.

In the determination of limit of quantitation (LOQ) and limit of detection (LOD), six replicate solutions were analysed differently for the reference solutions of cyanocobalamin.

Resultant peaks were then analysed for their mean peak area ratios (MPAR), mean peak areas (MPA) and concentrations.

Mean and standard deviation values were calculated and then deduced LOQ and LOD values for cyanocobalamin using specific equations.

The relative standard deviation (RSD %) as a fractional error expressed as a percentage was deduced for the analysis. RSD% value of less than 2% is achievable for HPLC method for analysis.

Intermediate precision was assessed with the developed method in three different days after the development phase was over and standard deviations and RSD% values compared.

CHAPTER FOUR

4.0.0 RESULTS AND CALCULATION

4.1.0 QUALITATIVE ASSAY OF SAMPLES

4.1.1 UV spectrum of pure and commercial samples of cyanocobalamin

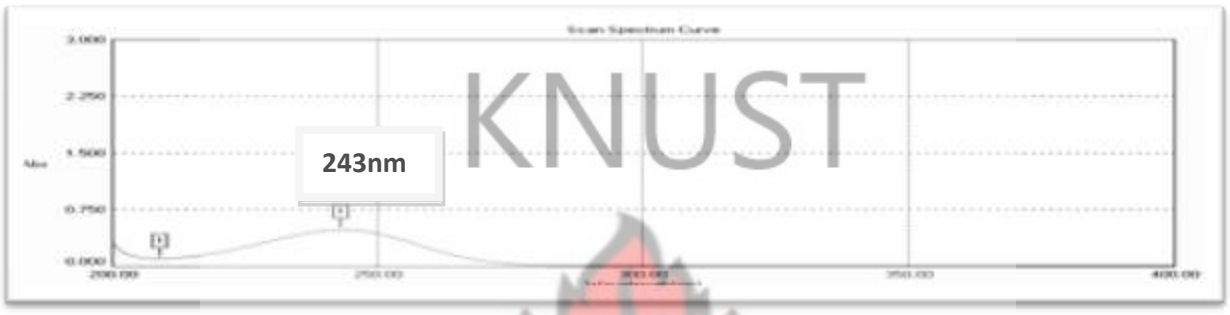

Fig. 4.1.1.1 UV spectrum of pure ascorbic acid

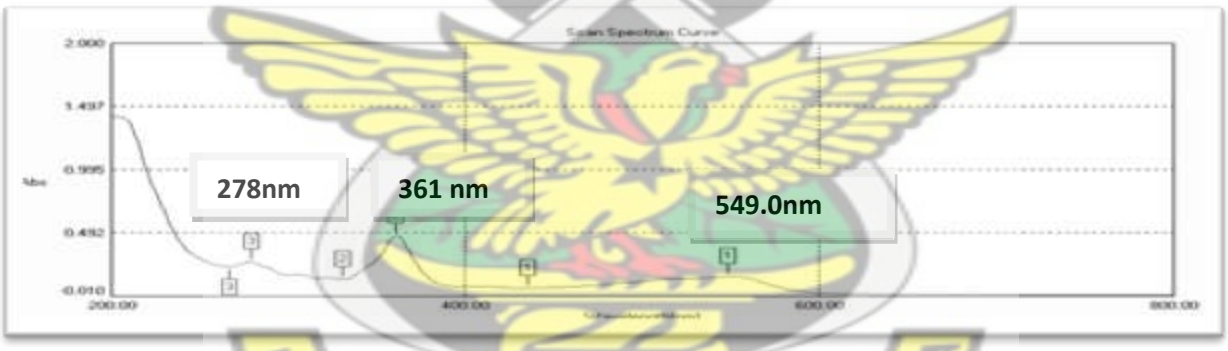

Fig. 4.1.1.2 UV spectrum of pure cyanocobalamin

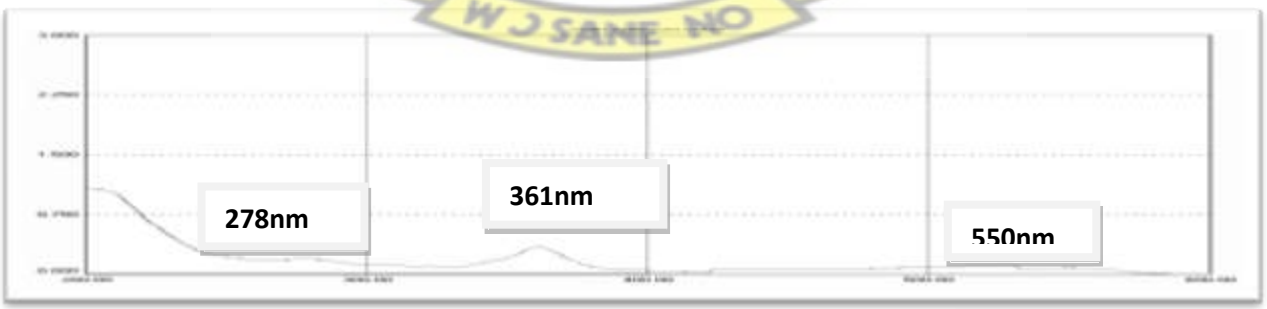

Fig.4.1.1.3 UV spectrum of cyanamin-12

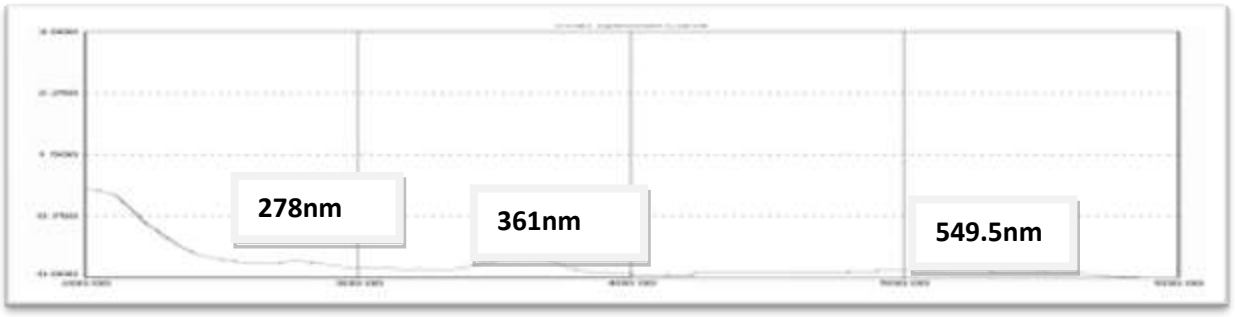

Fig.4.1.1.4 UV spectrum of binox-12

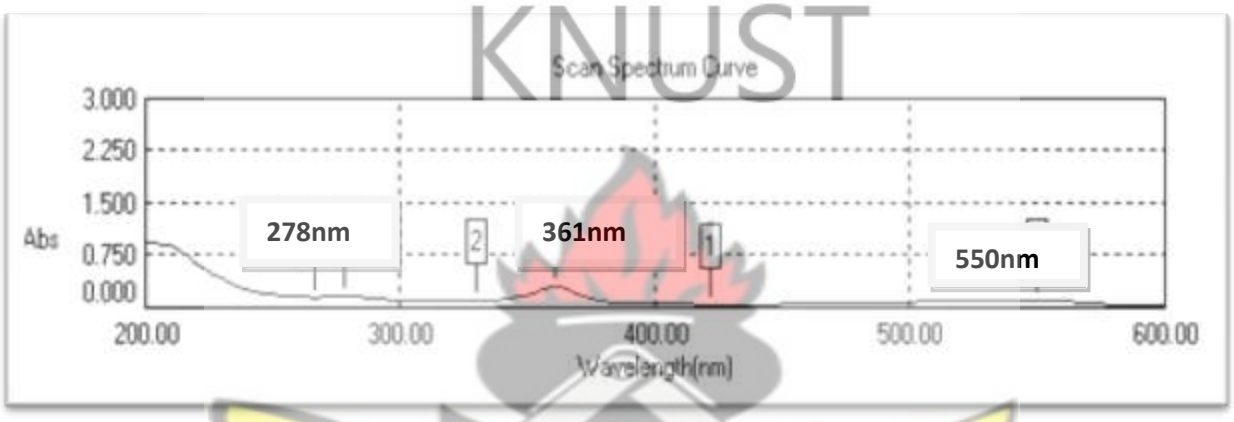

Fig.4.1.1.5 UV spectrum of bena^{R}

4.1.2 Data for comparison of qualitative assay of samples with reference range

Table 4.1.2 Data for comparison of qualitative assay of samples with reference range

Sample	Peaks	Wavelenght/nm	Reference range	Absorbance	Comment
Ascorbic acid	1	243.0	243.0	0.480	Passed
Cyanocobalamin pure powder	1	549.0	547-559	0.128	Passed
	2	361.0	361-361.5	0.410	Passed
	3	278.5	278-278.5	0.241	Passed
Bena^{R}	1	550.0	547-559	0.088	Passed
	2	361	361-361.5	0.291	Passed
	3	278	278-278.5	0.157	Passed
Cyanamine-12	1	550.0	547-559	0.097	Passed
	2	361	361-361.5	0.321	Passed
	3	278	278-278.5	0.188	Passed
Binox-12	1	549.5	547-559	0.102	Passed
	2	361	361-361.5	0.340	Passed
	3	278	278-278.5	0.196	Passed

4.1.3 Data for comparison of ratios of absorbances of samples with reference range

Table 4.1.3 Data for comparison of ratios of absorbances of samples with reference range

Brand of B_{12}	Ratio of Absorbance	Reference range	Ratio of Absorbance	Reference range	Comment
	361.5/549.5	3.15 - 3.45	361.5/278.5	1.70 – 1.90	
Pure B_{12}	3.20	Within range	1.70	Within range	Passed
Cyanamin-12	3.31	Within range	1.71	Within range	Passed
Binox -12	3.33	Within range	1.73	Within range	Passed
Bena^{R}	3.31	Within range	1.85	Within range	Paeesd

4.1.4 Data for melting point of pure samples

Table 4.1.4 Melting point results for pure samples

Sample	Melting point /^{o}C	Literature value / ^{o}C	Comment
Ascorbic acid	190 -191	190 -192	Passed
Cyanocobalamin	300 -305	< 300	Passed

4.1.5 Volume and pH uniformity of the vitamin B_{12} brands

All the various brands of cynocobalamin injections were neither broken nor leaking upon inspection.They had no growth on them and there were no particles found on their content.
Their colour, acidity (pH) and volume of the contents of each is tabulated below.

Table 4.1.5.1 pH and volume uniformity of bena^{R} batch 1

Ampoule	Colour	Volume/ml	pH
1	Red	2.10	4.60
2	Red	2.10	4.59
3	Red	2.00	4.60
4	Red	2.10	4.60
5	Red	2.00	4.59
6	Red	2.00	4.60
		Mean = 2.05ml	Mean = 4.598

Table 4.1.5.2 pH and volume uniformity for bena^{R} batch 2

Ampoule	Colour	Volume/ml	pH
1	Red	2.00	4.60
2	Red	2.10	4.59
3	Red	2.00	4.60
4	Red	2.10	4.61
5	Red	2.00	4.59
6	Red	2.00	4.60
		Mean = 2.03ml	Mean = 4.598

Table 4.1.5.3 pH and volume uniformity of binox-12

Ampoule	Colour	Volume/ml	pH
1	Dark red	1.30	4.80
2	Dark red	1.30	4.79
3	Dark red	1.30	4.80
4	Dark red	1.30	4.78
5	Dark red	1.30	4.79
6	Dark red	1.30	4.80
		Mean = 1.30ml	Mean = 4.793

Table 4.1.5.4 pH and volume uniformity for cyanamin-12

Ampoule	Colour	Volume/ml	pH
1	Red	2.00	4.50
2	Red	2.10	4.49
3	Red	2.00	4.50
4	Red	2.10	4.50
5	Red	2.00	4.48
6	Red	2.00	4.49
		Mean = 2.30ml	Mean = 4.493

Table 4.1.5.5 Data for comparison of mean pH and volume with reference ranges

Brand	Mean volume/ml	Colour	Colour range	Mean PH of content	pH range	Comment
Bena^{R} 1	2.05±0.06	Red	Dark red-pink	4.60±0.005	4.5 - 7.0	Passed
Bena^{R} 2	2.03±0.03	Red	Dark red-pink	4.60±0.01	4.5 - 7.0	Passed
Cyanamin-12	1.30±0.05	Red	Dark red-pink	4.50±0.01	4.5 - 7.0	Passed
Binox-12	2.03±0.00	Dark red	Dark red-pink	4.80±0.01	4.5 - 7.0	Passed

4.2.0 QUANTITATIVE ASSAY OF SAMPLES

4.2.1 Quantitative assay of ascorbic acid pure powder

- **Standardization of 0.05M I_2 using sodium thiosulphate**

Amount of $Na_2S_2O_3$ weighed = 2.4800g

$$\text{Factor of } Na_2S_2O_3 = \left[\frac{\text{Actual weight}}{\text{Nominal weight}}\right]$$

$$= \frac{2.4800}{2.4800} = 1.0000$$

Volume of I_2 = 25.50ml

Volume of $Na_2S_2O_3$ = 25ml

$$\text{Factor } (I_2) = \left[\frac{\text{Factor } (Na_2S_2O_3) \times \text{Volume } (Na_2S_2O_3)}{\text{Volume } (I_2)}\right]$$

$$= \left[\frac{1.0000 \times 25}{25.5}\right] = 0.9804$$

Table 4.2.1 Data for quantitative assay of ascorbic acid pure powder

Weight obtained = Factor (I_2) × Titre × 0.00881mg

Sample	Weight taken/mg	End point/ml	Titre/ml	Weight obtained/mg	% Content
1	0.1504	7.50	17.40	0.15029	100.19
2	0.1502	17.50	17.40	0.15049	100.19
3	0.1501	17.40	17.30	0.14953	99.62
Blank	-	0.1			Average = 100.00

4.2.2 Data for quantitative assay of cyanocobalamin pure powder and injections

Table 4.2.2 Data for quantitative assay of cyanocobalamin pure powder

Concentration obtained = Absorbance / 207

Sample no	Concentration prepared (%)	Absorbance	Concenttration obtained (%)	% Content
1	0.002	0.400	0.00193	96.62
2	0.002	0.410	0.00198	99.03
3	0.002	0.411	0.00199	99.28
Average				Average = 98.31

4.2.3 UV quantitative assay of commercial cyanocobalamin injections

Table 4.2.3 Absorbance data for calibration

Conc. (%)	Absorbance 1	Absorbance 2	Mean absorbance
0.0001	0.014	0.014	0.0140
0.00025	0.040	0.039	0.0396
0.005	0.034	0.031	0.0323
0.001	0.173	0.163	0.1680
0.002	0.333	0.343	0.3380
0.003	0.510	0.507	0.5085

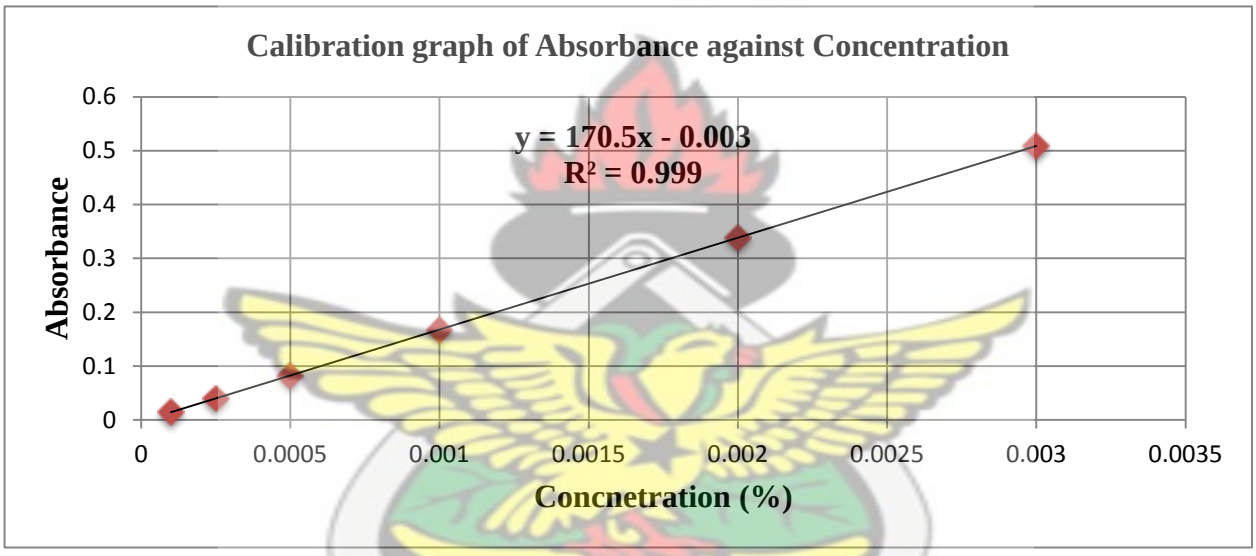

Fig. 4.2.3 Calibration graph for UV analysis

Table 4.2.4 UV quantitative assay of commercial cyanocobalamin injections

Sample	Ampoule	Concentration prepared (%)	Absorbance	Concentration obtained (%)	% Purity
Bena^{R} 1	1	0.005	0.832	0.00486	97.20
	2	0.005	0.904	0.00529	105.7
	3	0.005	0.870	0.00491	98.10
Bena^{R} 2	1	0.005	0.832	0.00486	97.20
	2	0.005	0.825	0.00482	96.40
	3	0.005	0.806	0.00487	97.32
Cyanamine-12	1	0.005	0.884	0.00517	103.40
	2	0.005	0.789	0.00461	92.15
	3	0.005	0.797	0.00466	93.10
Binox-12	1	0.005	0.714	0.00417	83.44
	2	0.005	0.895	0.00523	104.67
	3	0.005	0.815	0.00477	95.30

Table 4.2.5 Comparison of quantitative assay of samples with reference range

Sample	Sample no	% Assay	Reference range	Comment
Ascorbic acid powder	1	100.00	99.00 – 100.5	Passed
Cyanocobalamin powder	1	98.31	96.00 – 102.0	Passed
Bena^{R} 1	1	97.20	95.00 – 115.0	Passed
	2	105.7	95.00 – 115.0	Passed
	3	98.10	95.00 – 115.0	Passed
Bena^{R} 2	1	97.20	95.00 – 115.0	Passed
	2	96.40	95.00 – 115.0	Passed
	3	97.32	95.00 – 115.0	Passed
Cyanamine-12	1	103.40	95.00 – 115.0	Passed
	2	92.15	95.00 – 115.0	Failed
	3	93.10	95.00 – 115.0	Failed
Binox-12	1	83.44	95.00 – 115.0	Failed
	2	104.67	95.00 – 115.0	Passed
	3	95.30	95.00 – 115.0	Passed

4.3.0 STABILITY STUDIES OF CYANOCOBALAMIN SOLUTIONS

Table 4.3.1 Stability of cyanocobalamin in water at various pH values

Concentration (%) w/v	Absorbance in water	pH of medium	Absorbance in pH medium	Concentration (%) w/v obtained
0.002	0.414	4	0.394	0.00190
0.002	0.414	6	0.372	0.00179
0.002	0.414	7	0.398	0.00192
0.002	0.414	8	0.384	0.001855

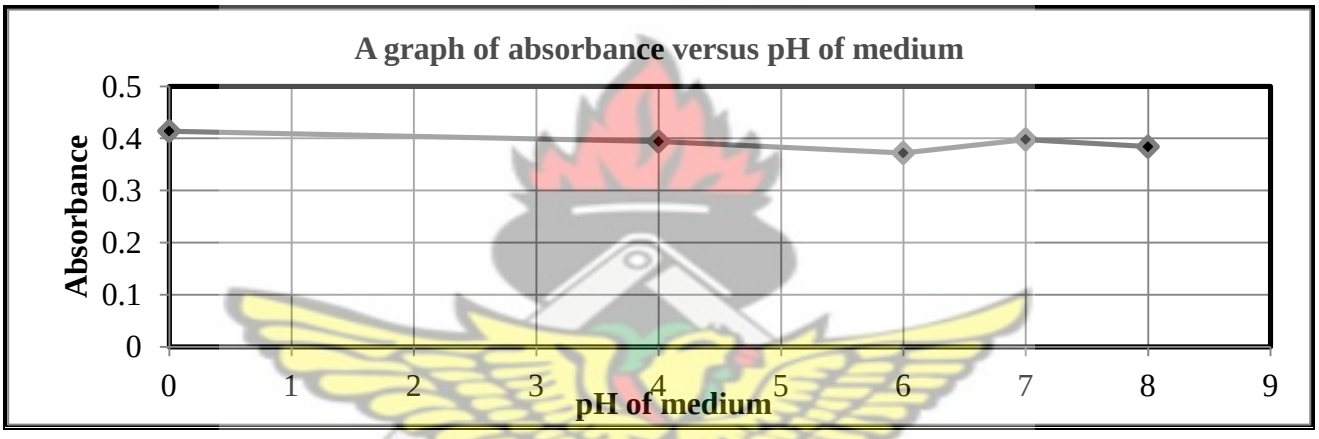

Fig.4.3.1 A graph of absorbance vrs pH of medium

Table 4.3.2 pH and Sunlight effect of stability of cyanocobalamin solutions

pH	Concentration (%) $^{w}/_{v}$	Absorbance	Absorbance after exposure to sunlight		
			2hrs	4hrs	6hrs
4.0	0.002	0.384	0.239	0.236	0.230
4.7	0.002	0.385	0.350	0.291	0.250
6.0	0.002	0.362	0.322	0.306	0.294
7.0	0.002	0.389	0.350	0.338	0.331
8.0	0.002	0.374	0.355	0.313	0.300

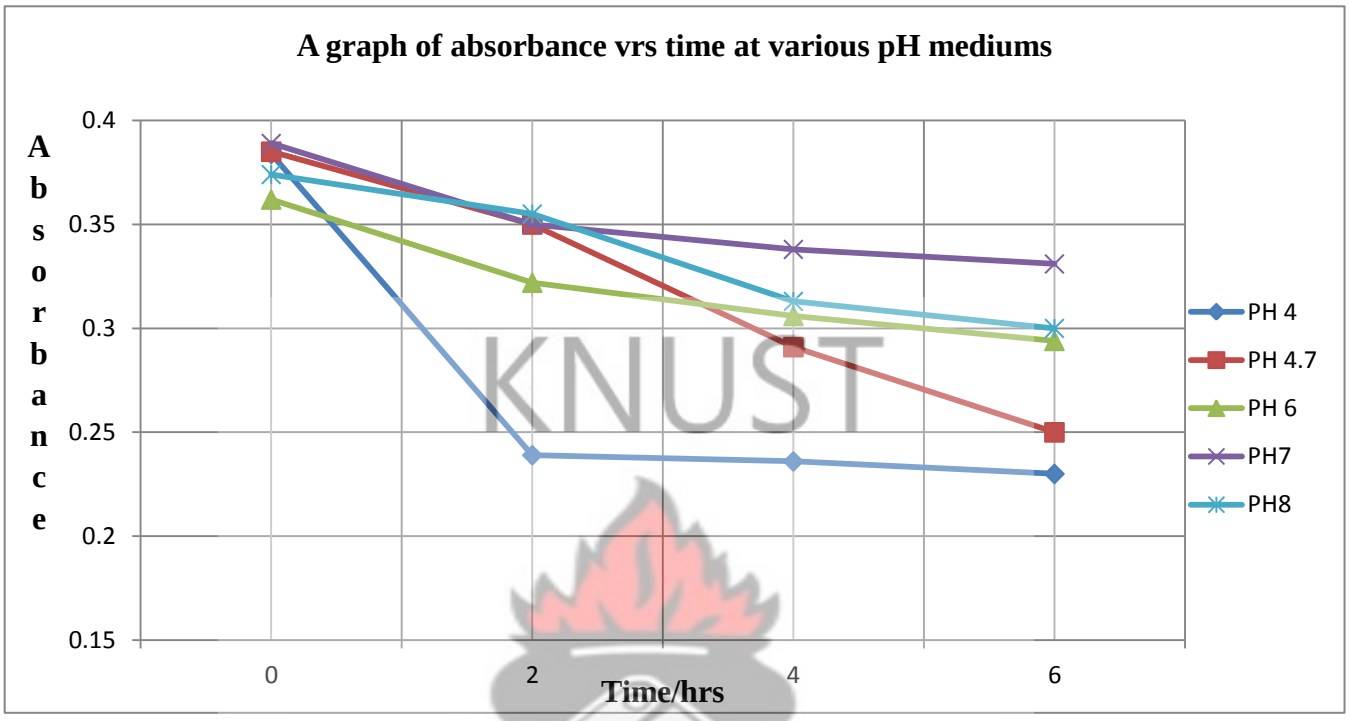

Fig. 4.3.2.1 A graph of absorbance vrs time at various pH mediums

Table 4.3.3 Temperature effect on the stability of commercial samples

Sample	Ampoule No	pH of ampoule	Conc. Prepared	Absorbance	Temperature ^{o}C	Absorbance	
						6hrs	12hr
Bena^{R}	1	4.60	0.002	0.580	15	0.580	0.579
	2	4.59	0.002	0.570	28	0.569	0.566
	3	4.60	0.002	0.572	36	0.573	0.571
	4	4.61	0.002	0.570	45	0.560	0.555
	5	4.60	0.002	0.555	60	0.557	0.554
Cyanamin - 12	1	4.50	0.002	0.548	15	0.548	0.548
	2	4.49	0.002	0.548	28	0.548	0.547
	3	4.50	0.002	0.555	36	0.455	0.453
	4	4.49	0.002	0.549	45	0.541	0.540
	5	4.49	0.002	0.555	60	0.553	0.553

4.4.0 CHROMATOGRAMS AND HPLC CONDITION FOR ANALYSIS

4.4.1 Chromatograms of cyanocobalamin and ascorbic acid in methanol/phosphate buffer (30/70 $^{v}/_{v}$)

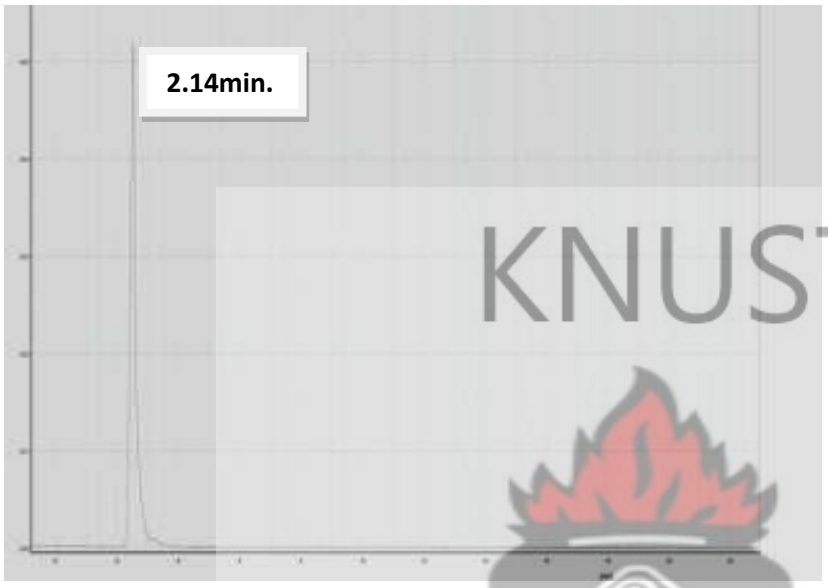

Fig. 4.4.1.1 Chromatogram of ascorbic acid

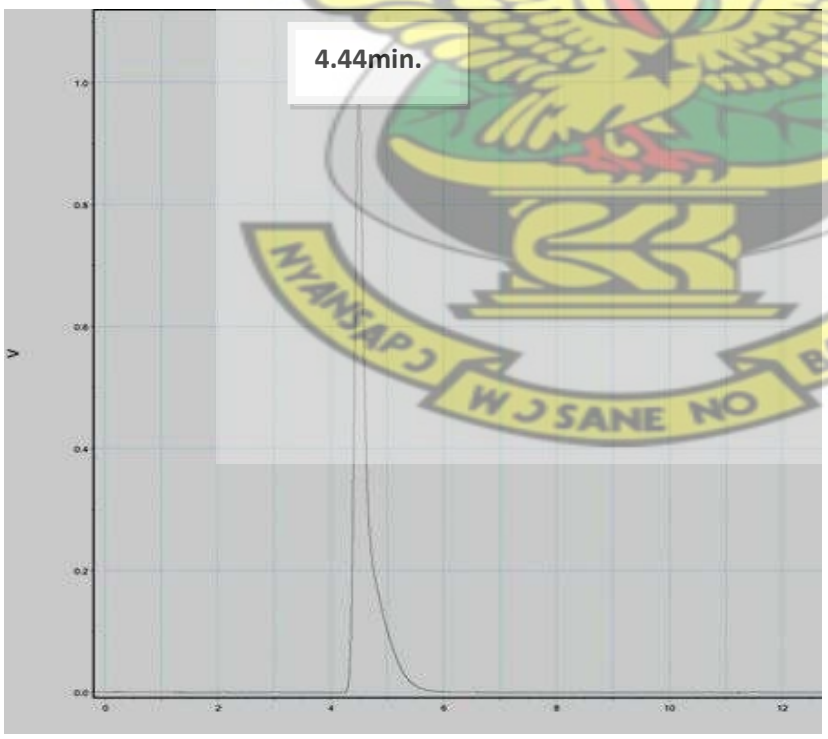

Fig.4.4.1.2 Chromatogram of cyanocobalamin

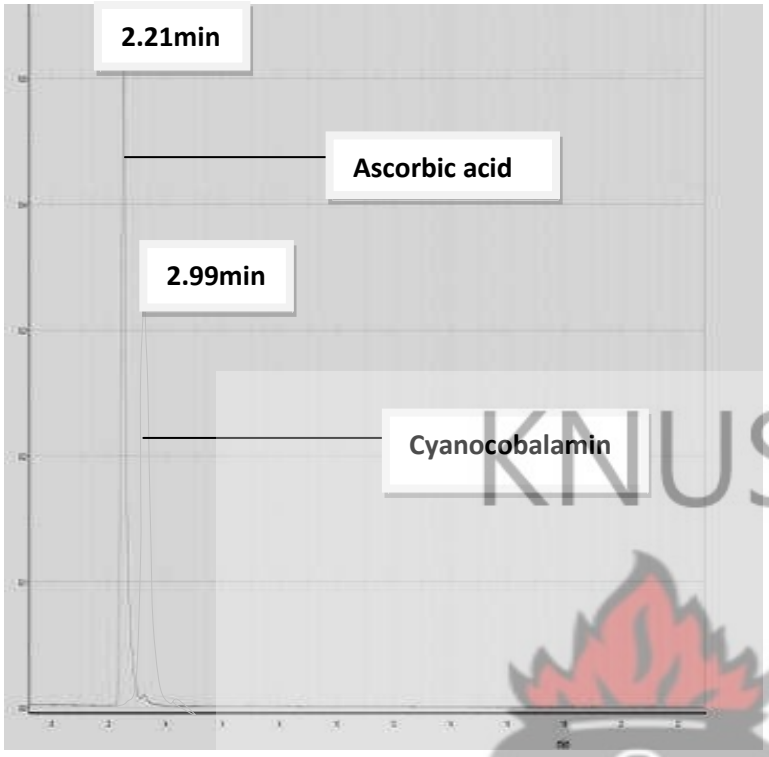

Fig 4.4.1.3 Chromatogram of cyanocobalamin and ascorbic acid at pH 6

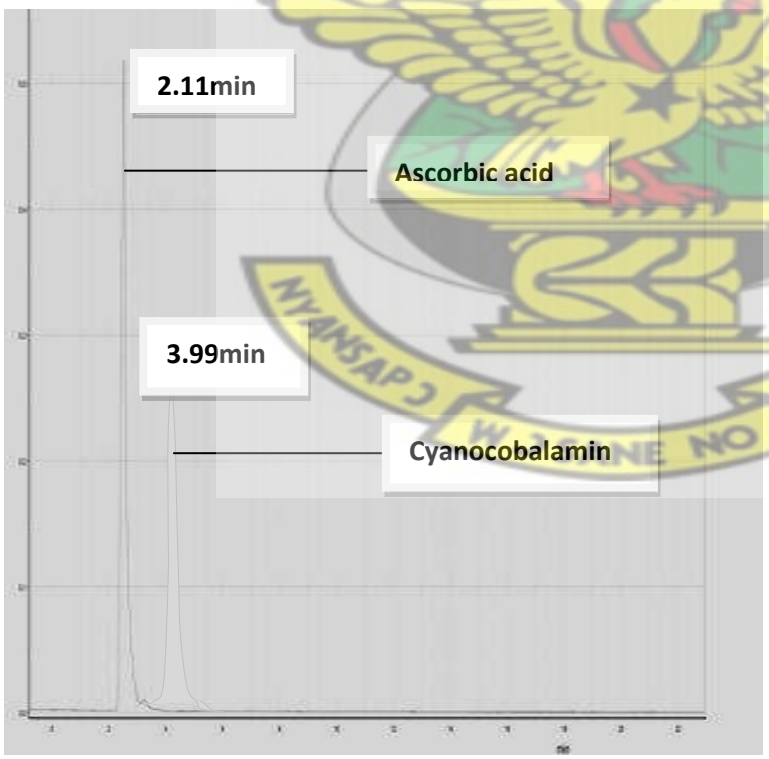

Fig 4.4.1.4 Chromatogram of cyanocobalamin and ascorbic acid at pH 6.5

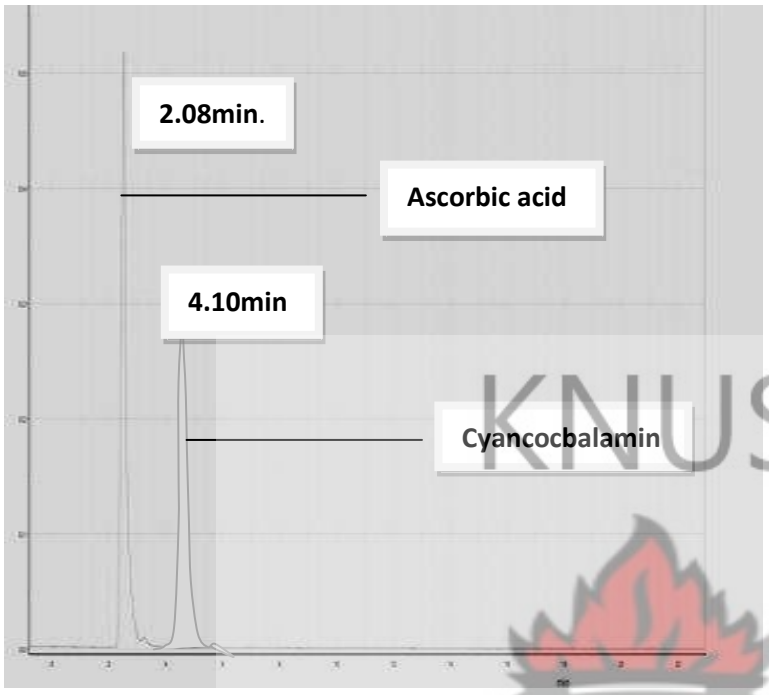

Fig 4.4.1.5 Chromatogram of cyanocobalamin and ascorbic acid at pH 6.8

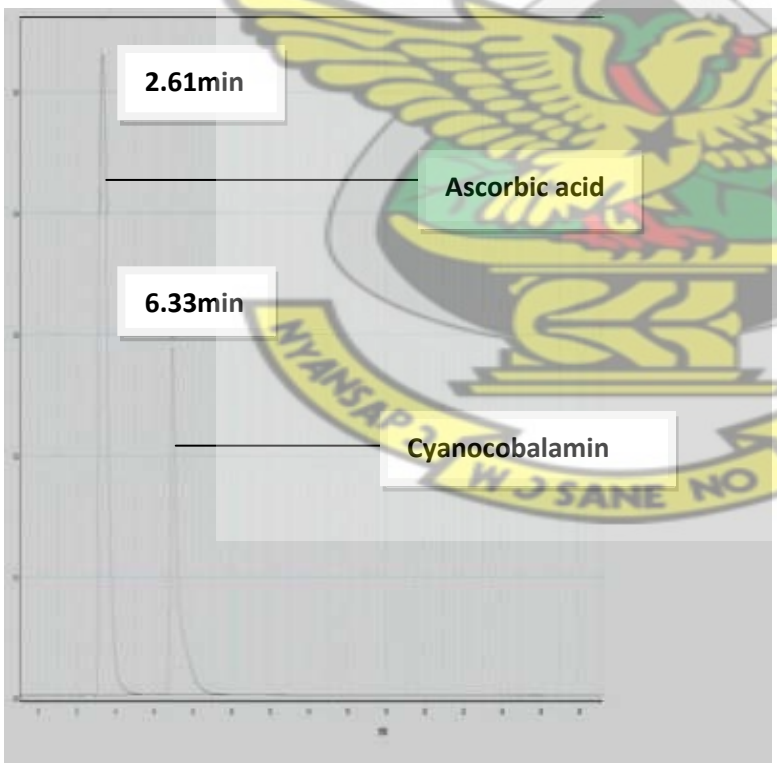

Fig 4.4.1.6 Chromatogram of cyanocobalamin and ascorbic acid at pH 7

Table 4.4.1 Data for chromatograms showing their retention times and peak areas

Sample mixture	pH of mobile phase	Peaks	Peak name	Retention time / min.
Cyanocobalamin/Ascorbic acid	6.0	1	Ascorbic acid	2.21
		2	Cyanocobalamin	2.99
Cyanocobalamin/Ascorbic acid	6.5	1	Ascorbic acid	2.11
		2	Cyanocobalamin	3.99
Cyanocobalamin/Ascorbic acid	6.8	1	Ascorbic acid	2.08
		2	Cyanocobalamin	4.10
Cyanocobalamin/Ascorbic acid	7.0	1	Ascorbic acid	2.61
		2	Cyanocobalamin	6.33

4.4.2 Hplc condition for assay of commercial samples

Table 4.4.2.1 Hplc condition for analysis

Parameter	Condition
Mobile phase	Phosphate buffer PH7/Methanol (70/30 $^{v}/_{v}$)
Stationary phase	150mm, 4.6mm, 5µm Hypersil base-deactivated C-18 Coloumn
Wavelenght of analysis	278nm
Sensitivity	0.03
Flow rate:	1.40ml/min.

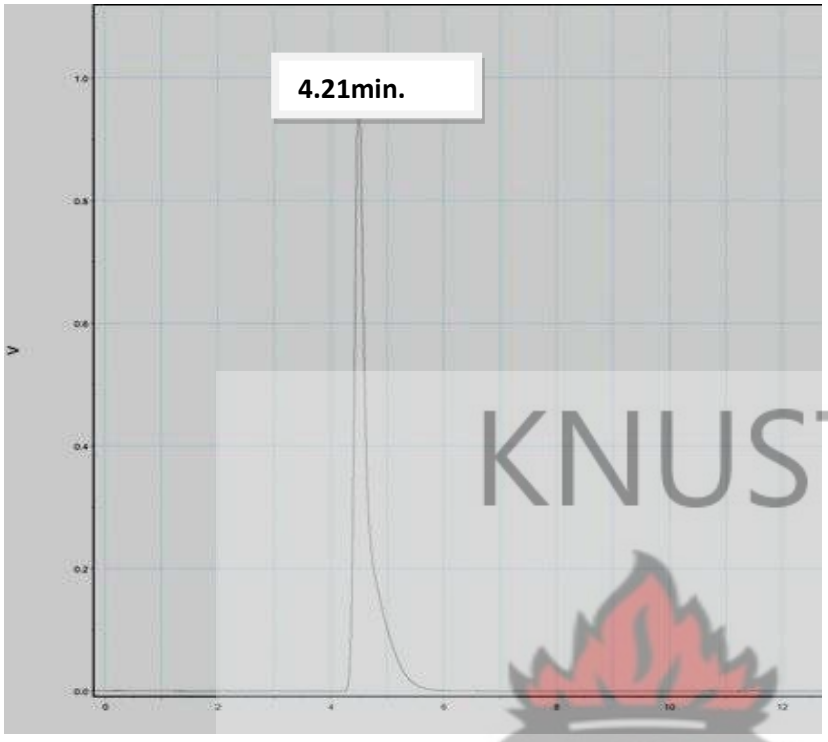

Fig. 4.4.2.1 Chromatogram of cyanocobalamin

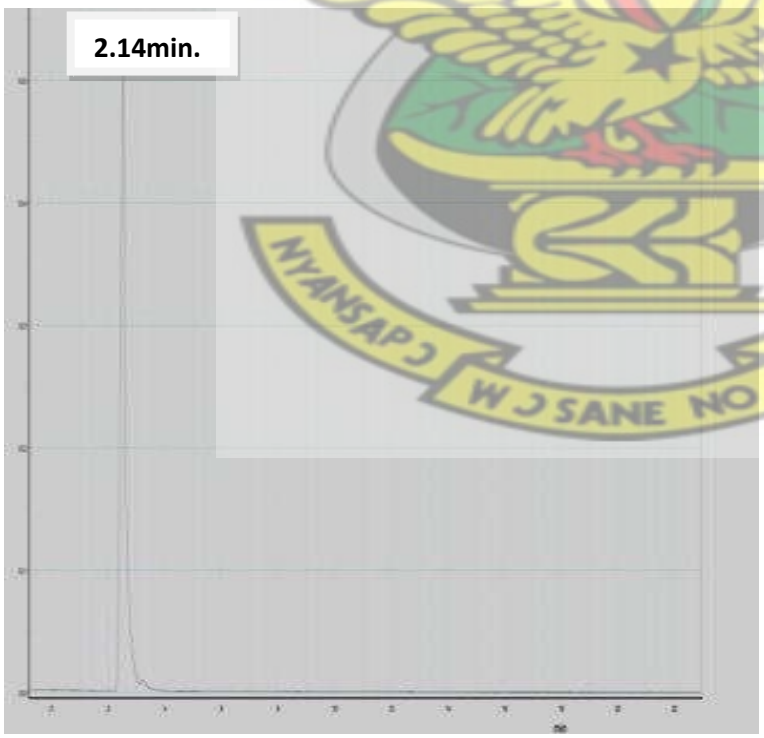

Fig.4.4.2.2 Chromatogram of ascorbic acid

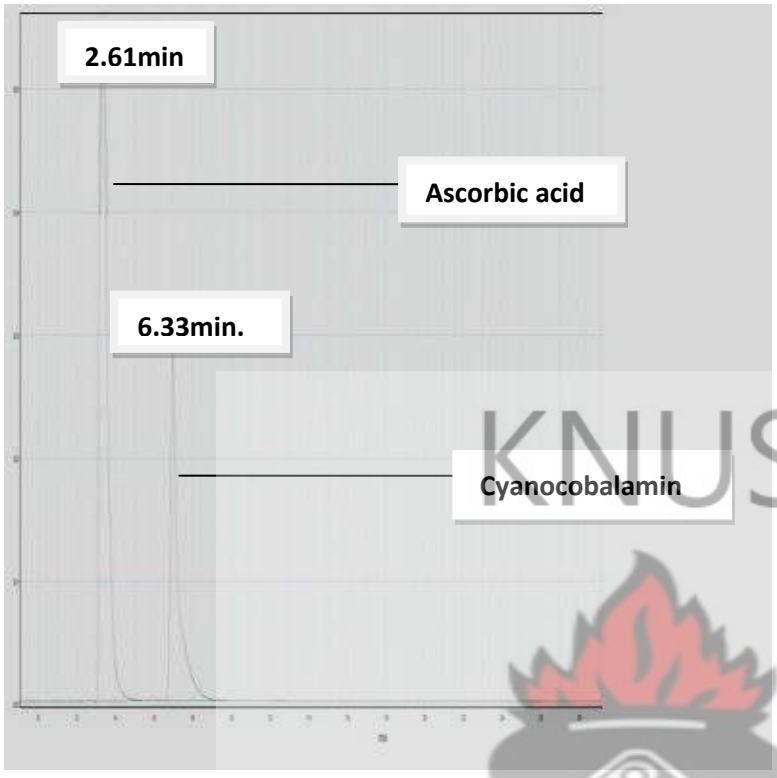

Fig. 4.4.2.3 Chromatogram of cyanocobalamin and ascorbic acid

Table 4.4.2.2 Hplc data for calibration graph

Conc./%	Peak area (mm^2)				MPA-B_{12}	MPA-IS	MPAR
	Cyanocobalamin		Internal standard				
	No of determinations		No of determinations				
	1	2	1	2			
0.005	3.09	3.09	7.47	7.47	3.09	7.47	0.41
0.010	5.16	5.63	7.33	7.52	5.40	7.43	0.73
0.015	7.65	7.55	7.24	7.12	7.62	7.37	1.03
0.020	10.49	10.49	7.41	7.41	10.49	7.41	1.42
0.025	12.47	12.20	7.49	7.51	12.30	7.50	1.64
0.030	15.52	15.52	7.68	7.68	15.52	7.68	2.02

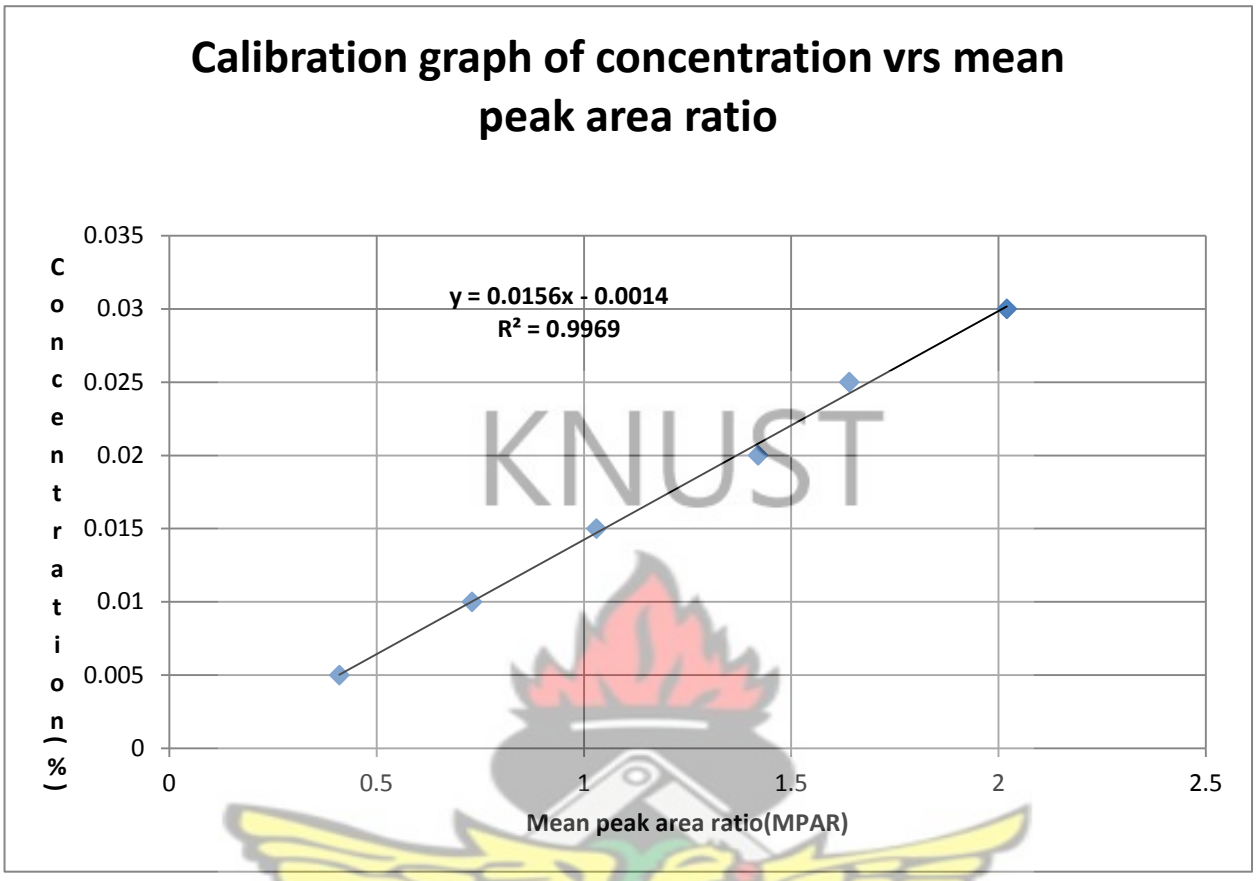

Fig.4.4.2.4 Calibration graph of concentration against mean peak area ratio (MPAR)

Table 4.4.2.3 Data for hplc assay of cyanocobalamin injections

Brand	Ampoule No	Peak areas No of determinations				MPAR	Conc.	%Conc. ($^{v}/_{v}$)
		Cyanocobalamin		Ascorbic acid				
		1	2	1	2			
Bena^{R} 1	1	2.76	2.74	7.30	7.10	0.382	0.00475	95.00
	2	3.01	3.00	7.22	7.20	0.418	0.00529	104.80
	3	2.77	2.75	6.99	7.14	0.391	0.00486	97.20
Bena^{R} 2	1	2.75	2.74	7.09	7.05	0.388	0.00482	96.30
	2	2.72	2.70	7.08	7.04	0.384	0.00476	95.10
	3	2.79	2.76	7.16	7.12	0.389	0.00484	96.74
Cyanamin-12	1	2.99	2.97	7.37	7.31	0.407	0.00510	102.00
	2	2.78	2.74	7.35	7.21	0.379	0.00468	93.60
	3	2.71	2.69	7.317	7.09	0.375	0.00462	92.40
Binox-12	1	2.55	2.55	7.30	7.24	0.351	0.00427	85.34
	2	3.03	3.04	7.45	7.39	0.409	0.00514	102.77
	3	2.80	2.82	7.40	7.28	0.383	0.00475	94.97

Table 4.4.2.4 Data for comparison of hplc method to standard BP method

Sample	Ampoule	Method of of analysis (%)			
		Hplc method	Comment	Uv method	Comment
Bena^{R} 1	1	95.00	Passed	97.20	Passed
	2	104.80	Passed	105.7	Passed
	3	97.20	Passed	98.10	Passed
Bena^{R} 2	1	96.30	Passed	97.20	Passed
	2	95.10	Passed	96.40	Passed
	3	96.74	Passed	97.32	Passed
Cyanamin -12	1	102.00	Passed	103.40	Passed
	2	93.60	Failed	92.15	Failed
	3	92.40	Failed	93.10	Failed
Binox -12	1	85.34	Failed	83.44	Failed
	2	102.77	Passed	104.67	Passed
	3	94.97	Failed	95.30	Passed

Table 4.4.2.5 Data for mean % content of brands of injections

Sample	Method of analysis			
	Mean % content (Hplc)	Comment	Mean % content (UV)	Comment
Bena 1	99.00 ± 3.95	Passed	100.33 ± 4.67	Passed
Bena 2	96.05 ± 0.85	Passed	96.97 ± 0.50	Passed
Cyanamin-12	96.00 ± 5.23	Passed	96.22 ± 6.20	Passed
Binox-12	94.36 ± 8.73	Failed	94.47 ± 10.64	Failed

4.5.0 METHOD PERFORMANCE ASSESSMENT

4.5.1 Within run precision or repeatability

Table 4.5.1 Data for repeated measurement of cyanocobalamin

No of runs	Peak area (mm^2)				MPAR	Mean concentration
	Cyanocobalamin		Internal standard			
	1	2	1	2		
1	10.02	10.32	7.32	7.30	1.391245	0.019869
2	10.64	10.54	7.61	7.56	1.396177	0.019943
3	10.14	10.24	7.15	7.20	1.420206	0.020203
4	11.13	10.24	7.63	7.60	1.403152	0.020047
5	10.39	10.55	7.25	7.22	1.447132	0.020707
6	10.24	10.60	7.52	7.50	1.387483	0.019812

4.5.2 Between run precision (Intermediate precision)

Table 4.5.2 Data for between run precision (Intermediate precision)

No of run	1st day		2nd day		3rd day	
	MPAR	Mean conc.	MPAR	Mean conc.	MPAR	Mean Conc.
1	1.391245	0.019869	1.458716	0.020881	1.458571	0.020879
2	1.396177	0.019943	1.433103	0.020497	1.469761	0.021046
3	1.420206	0.020203	1.365563	0.019483	1.419944	0.021046
4	1.403152	0.020047	1.499293	0.021489	1.478992	0.021185
5	1.447132	0.020707	1.479167	0.021188	1.50000	0.021500
6	1.387483	0.019812	1.470994	0.021265	1.49786	0.021468

4.5.3 Statistical analysis of data

Statistical tools for analysing data

- **Mean**

$$\bar{X} = \left[\frac{\sum_{i=1}^{n} Xi}{N}\right]$$

- **Standard deviation (S)**

$$S = \left[\sqrt{\frac{1}{N}\sum_{i=1}^{N}(X_i - \bar{X})^2}\right]$$

- **Relative standard deviation (RSD %)**

$$RSD\% = \left[\frac{S \times 100\%}{\bar{X}}\right]$$ Where $\bar{X}$ is the mean and S is the standard deviation

- **F – Test (Analysis of variance (ANOVA))**

$$F = \frac{[(\sum n (X_{ii} - Y)2/(K-1))}{[\sum (X_i - X_w)2/(N - K))]}$$

Where X_w is the mean concentration for day 1 result as indicated in Table 4.6.3 coloumn 2

N is the overall sample size = 18

n is the number of determinations per day = 6

Y is the overall mean of the data

K is the number of days used = 3

X_{ii} is the mean concentration of the mean concentration a set of datas

X_i is the concentrations obtained for a particular set of data

- **Student t- test**

$$t = \left[\frac{\bar{X} - \mu_o}{S/\sqrt{N}}\right]$$ Where t is the student t test, μ_o is the specified value.

Table 4.5.3.1 Data for test for accuracy of the method (% recovery)

Concentration prepared	Concentration recovered	% Concentration recovered
0.0200	0.019869	99.35
0.0200	0.019943	99.97
0.0200	0.020203	100.10
0.0200	0.020047	100.24
0.0200	0.020707	103.54
0.0200	0.019812	99.06

Table 4.5.3.2 Data for test of precision of the method (Student t-test)

Days	Statistical parameter					
	Mean concentration	Standard deviation	RSD (%)	t_r	t_o	Comment
1st	0.02011	0.000338	1.682	0.133	3.365	$t_r < t_o$ hence precise
2nd	0.02087	0.000756	1.707	0.470	3.365	$t_r < t_o$ hence precise
3rd	0.02089	0.000420	1.580	0.865	3.365	$t_r < t_o$ hence precise

t_o is the theoretical student t value for (N-1) degrees of freedom at 98.0% confidence interval

t_r is the experimental t value for N = 6

Table 4.5.3.3 Data for test for robustness of the method (F-Test)

Within run variance		Between run variance		F	F_o	Comments
$\overline{X_w}$	$\sum (X_i - \overline{X_w})^2$	Y	$\sum (\overline{X_{ii}} - Y)^2$			
0.02011	6.48×10^{-7}	0.02062	3.955×10^{-7}	27.46	49.432	F < Fo hence precise

$$F = \frac{[(\sum n(\overline{X_{ii}} - Y)^2/(K-1))}{[\sum (X_i - \overline{X_w})^2/(N-K))]}$$

Where $\overline{X_w}$ is the mean concentration for day I result as indicated in Table 4.6.3 coloumn 2
N = 18, n = 6, Y = 0.02062, K = 3

$\overline{X_{ii}}$ is the mean concentration of the mean concentration for each day,
X_i is the concentrations obtained for day 1 as indicated in table 4.6.2 coloumn 3,

Where F_o is the F value at 98.0% confidence interval for 2 against 15.F is the experimental value and since F is less than F_o, the null hypothesis is not rejected and therefore the experimental concentrations obtained do not differ significantly from each other even though they were carried out on three different days.The method is therefore robust.

4.5.4 Determination of limit of quantitation (LOQ) and limit of detection (LOD)

From the calibration graph from fig.4.5.2

$Y = 0.015\,\overline{X_i} - 0.001$

Where $\overline{X}_i$ is the Mean peak area ratio (MPAR), Y is the concentration

Table 4.5.4 Data for LOD and LOQ determination

Y	$\overline{X}_e$	$\overline{X}_i$	$(X_e - \overline{X}_i)$	$(X_e - \overline{X}_i)^2$
0.005	0.414	0.410	0.004	1.6×10^{-5}
0.010	0.727	0.730	-0.003	9.0×10^{-6}
0.015	1.034	1.030	0.004	0.000016
0.020	1.417	1.420	-0.003	9.0×10^{-6}
0.025	1.640	1.640	0	0
0.030	2.021	2.020	0.001	1.0×10^{-6}
				$\sum(\overline{X}_e - \overline{X}_i)^2 = 5.10\times10^{-5}$

$$SD = \left[\frac{\sum(\overline{X}_e - \overline{X}_i)2}{(N-1)}\right]^2$$

Where SD is the residual standard deviation

X_e is the estimated mean peak area ratio from the calibration graph

Y_i is experimental mean peak area ratio from table 4.5.2(b) last coloumn

N is the number of coencentration prepared

$SD = \left[\frac{\sum(5.10\times10-5)2}{(6-1)}\right]^2 = 1.040\times10^{-10}$

According to the ICH guidelines for method validation, $LOQ = \left[\frac{10SD}{S}\right]$

Where S is the slope from the calibration in fig.4.5.2

But S = 0.015, Therefore, $LOQ = \left[\frac{10\times1.04\times10-10}{0.015}\right] = 6.90\times10^{-8}$ % = 0.069 ug/ml

Similary, $LOD = \left[\frac{3.3SD}{S}\right] = \left[\frac{3.3\ x\ 1.04\times10-10}{0.015}\right] = 2.20\times10^{-8}$ % = 0.0228ug/ml

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1.1 Pharmacopoiea assesment

The qualities of pharmaceutical products are assessed through certain quality control parameters. The quality assurances of these products are indispensable in maintaining a drugs intergrity and quality over its shelf-life.The three main samples employed for the project were cyanocobalamin and ascorbic acid pure powders and cyanocobalamin injections.

It was necessary to check the quality of both cyanocobalamin and ascorbic acid pure powders using the pharmacopoeia prescribed methods.

This involves checking the quality by both the identification and quantitative assay for percentage content.

Ascorbi4c acid was identified by its UV spectrum with maximum wavelength of absorbtion at 243nm as indicated on the spectrum in fig.4.1.1.1. The melting point was also found to be between 190 to 191 as against a reference range of 190 to 192 ^{o}C.

The main methods employed for the identification of cyanocobalamin pure powder also involved a UV scan of cyanocobalamin solution between 200nm to 600nm, melting point determination and thin-layer chromatography.

A UV scan between 200nm to 600nm produced a spectrum indicating absorptions at 361nm, 278nm and 549.5nm as shown in table 4.1.1.2.

The ratios of the absorbance at 361nm to 549.5nm was found to be 3.20 and that between 361.5nm to 278.5nm was 1.701.These values therefore conform to the range specified by the British pharmacopoeia as shown in table 4.1.3.

Moreover, melting point determination was found to be between 303 to 305 ^{o}C which was near to the specified value of not less than 300^{o}C.

Four brands of commercial cyanocobalamin injections were subjected to the standard methods of assessing their quality and were found to comform to established standards. All the four brands

of the injections had their wavelength of maximum absorbances at 361.5nm, 278nm and 545 – 549.5nm range as indicated in table 4.1.2.

A typical cyanocobalamin injection has colour ranging from dark red to pink and pH ranging between 4.5 to 7.

Ampoules were found to be having colour ranging between dark–red to pink and pH ranging between 4.5 to 7.

The pH test of content showed that the injections had mean pH ranging from 4.50±0.01, 4.60 ±0.01, 4.60±0.005 and 4.80±0.01 for all the four brands of the injections.

Volumes of content test were respectively found to be ranging between 1.30±0.05, 2.05±0.06, 2.03±0.03 and 2.03±0.00ml. This showed that all the four brands of the injection passed the pH, Colour and volume of content test as indicated in table 4.1.5.5.

Cyanocobalamin pure powder and commercial samples were also assayed for their purity before being used for the method development to ensure that they conform to established quality control parameters by the British Pharmacopoiea.

In the quantitative assay of both the pure powder and the commercial samples, percentage purity of pure cyanocobalamin sample was found to be 98.31% as against a reference range of not less than 96.0% and not more than 102% of the dried substance.

The pure cyanocobalamin, ascorbic acid and most of the commercial samples had their percentage content falling within the reference range specified by the British Pharmacopoeia as indicated in table 4.2.5.

Only three of the ampoules failed the percentage content of the dried substance and this could be due to poor storage conditions such as exposure to light which could cause photodegradation, manufacturing errors such erros in weighing, volume inconsistencies etc.

Moreover, two of the failed ampoules were coming from the same batch of injection (cyanamine-12) and therefore prepared under the same conditions.

Other factors could be due to the fact that the injection had almost spent two thirds of its shelf life at the time of the analysis and therefore the product could be degrading gradually with time.

5.1.2 Stability studies of cyanocobalamin solutions

The three main parameters employed for the stability studies were pH effect (hydrolysis), sunlight (radiation) and temperature.

The effect of pH on the stability of cyanocobalamin in aqueous medium was monitored over a pH ranging from 4 to 8 .The pH range employed revealed that at pH7 there was substantial quantity of cyanocobalamin in solution as indicated in the absorbance versus pH graph (Fig. 4.3.1). This is due to the fact that at pH7 cyanocobalamim is fully unionized and undegradable into other B_{12} forms. It has been shown from table 4.3.1 that at pH7 cyanocobalamin has the highest concentration in solution.

The effect of sunlight on the stability of aqueous solutions of cyanocobalamin was also monitored over a six hour period. In this studies, aqueous solutions of cyanocobalamin of varying pH range on exposure to sunlight over six hour period showed slight decrease in absorbance as indicated in table 4.3.2 and fig.4.3.2 (1-5).The decrease in absorbance was more pronounced at pH 4. The photodegradation of B_{12} was greater at pH 4 compared to that at pH 7 and this appears to be due to greater susceptibility of the protonated form of B_{12} i.e., the 5,6-dimethylbenzimidazole moiety to photolysis than the neutral form of the molecule. [Ahmad et al., 1992]

This decrease is due to the fact that cyanocobalamin (vitamin B_{12}) on exposure to daylight, sunlight, and artificial light is degraded to hydroxocobalamin (vitamin B_{12}b) in weak acid solutions as indicated by the slight decline in the absorbance of degraded solutions. Figs.4.3.2 (1-5). [Anderson et al., 1991]

This proved that sunlight (UV, visible) is more effective in degrading B_{12} solutions and the formation of irreversible oxidation products .Commercial samples were therefore stored in a way that protected them from light to avoid degradation.

Thermal stability of cyanocobalamin was also studied using the commercial samples over a temperature ranging from 15 to 60^{o}C. It was observed that the injections were quite stable to heat over the entire temperature range employed (Table 4.3.3).

The maximum tropical temperature is between 40 to 50^{0}C which therefore makes the product stable under normal tropical temperature conditions.

Mostly, the end products of thermal degradation of cyanocobalamin are toxic gases such as carbon monoxides, nitrogen dioxide, oxides of cobalt and phosphorus; however, these products are likely to be released at high temperatures. The melting point of pure cyanocobalamin is greater than 300^{o}C and hence these products are not likely to be released under normal tropical temperatures.

No substantial decrease in absorbance was therefore obtained during the studies. This could be due to the fact that the injections were kept in heat proof ampoules coupled with the fact that the temperature range employed could not have a substantial conductive effect on the ampoules. Heat was therefore not conducted and radiated through the product and hence restricted from degrading the cyanocobalamin.

5.1.3 HPLC method design

During the method development, a number of factors were considered to arrive at the mobile phase that could resolve both the target drug and the chosen internal standard at a reasonable time for quantitation. Some of these factors include the mobile phase composition, chemistry of the drug (e.g pKa, solubility, spectroscopic properties and it's interactions with other compounds and surfaces).

The design of an hplc method of analysis is normally carried out by considering the sample matrix (e.g biological, pharmaceutical etc.), the mobile phase in which the analyte is soluble and most stable, stationary phase which will elute the drug faster as well as resolving it from other drugs in the sample matrix, the method of detection of the sample (e.g UV, refractive properties, conductive properties) and flow rate of the mobile phase etc.

A reverse phase hplc coloumn was chosen because cyanocobalamin has a lot of polar groups on its structure and therefore does not retain much longer on the column.

UV detection was chosen for the method because of the presence of extensive chromophoric system in cyanacobalamin.

The choice of mobile phase was important for successful liquid chromatography. In this work, the organic phase was initially changed while the aqueous medium was fixed.

Various mobile phase compositions comprising of water/methanol at (90/10, 80/20, 70/30, 60/40, 50/50) $^{v}/_{v}$ employed. In each composition, a quantity of the cyanocobalamin and a constant amount of internal standard (0.04mg) were added. The composition was injected whilst varying the conditions (e.g wavelength, sensitivity and the flow rate).

Mobile phase composition of 70/30$^{v}/_{v}$ was found most suitable because it gave the best resolution; however, the separation of the drug and the internal standard was very poor.

A buffer was therefore employed to generate ionic species which could have different affinities for the coloumn and hence separation of the drug and internal standard being achieved.

The organic phase composition was then fixed whilst the aqueous phase (conducting species) was varied. That is, pH was increased thereby decreasing amount of phosphate buffer in solution and ionic strength.

Mobile phase composition was fixed at 70/30 $^{v}/_{v}$ phosphate buffer/methanol whilst varying pH of the aqueous medium, it could be observed that in acidic mobile phase of pH 4 cyanocobalamin tends to ionise and shows little retention, thereby eluting in the void. This behaviour of cyanocobalamin stems from the fact that it is a basic ionic drug and also posses other functional groups for ion transfer.

With pH values towards neutral medium (pH 6 – 7) of the mobile phase, cyanocobalamin retains better thus resolving from solvent front.

At pH 6.8 cyanocobalamin was partially ionised, retained and well resolved from solvent front and ascorbic acid (internal standard).

Further variation of pH of the medium towards neutral medium (pH 7) was more suitable for the elution of peaks.The various pH values of mobile phase employed revealed that at pH7 there was substantial elution and resolution of peaks than the others Figure 4.4.1(1-6). This is due to the fact that at pH 7 cyanocobalamin is fully unionized and undegradable into other B_{12} forms. It has been shown from table 4.3.1 that at pH 7 cyanocobalamin has the highest concentration in

mobile phase. pH 7 was therefore most suitable for establishing the mobile phase condition for the method design.

Upon varying the various parameters (wavelenght of absorption, pH of mobile phase, detector sensitivity and flow rate), the final condition for analysis was obtained. The conditions for the analysis consisted of a reverse stationary phase base-deactivated C-18 material with a mobile phase comprising of phosphate buffer pH 7/mehtanol (70/30 $^{v}/_{v}$). The cyanocobalamin and ascorbic acid were detected at 278nm with detector sensitivity of 0.03% using a flow rate of 1.40ml/min.

The retention time was 4.9 ±1.5 minutes for cyanocobalamin and the mode of elution was isocratic. (Table 4.4.2.1).

Chromatograms obtained from the analysis of cyanocobalamin with ascorbic acid as internal standard were evaluated for their concentrations using peak areas to obtain the mean peak area ratios (MPAR) as shown in table 4.4.2.2. Peak areas were used based on the fact that peaks were symmetrically shaped and narrow.

The data obtained from the conditions for the analysis was then used to prepare a calibration graph of mean peak area ratio against the concentration ranging from 0.005 to 0.03$^{v}/_{v}$ % as indicated in figure 4.4.2.

5.1.4 Method performance assessment

A designed method for drug analysis must meet all the requirements specified by the ICH guidelines. The method must be specific for the analyte, accurate, reproducible or repeatable precise, linear with respect to varying parameters (e.g peak area ratio against concentration etc.), specified range, limit of detection and limit of quantitation, robust etc.

Reproducibility of results were assessed by repeated runs of the samples several times and at different days and data analysed statistically and were found to be reproducible. (Tables 4.5.1 and 4.5.2).

Accuracy of results were also assessed by comparing experimental concentrations to expected concentration by computing percentage recovery against expected concentration and were found to be very accurate as indicated in table 4.5.3.1

Between run precision and intermediate precision were carried out to access the methods performance in the same laboratory. Reapeated measurements were compared and then tested for their precision using the student t- test and were all found to be precise (table 4.5.3.2).

The standard deviations as well as relative standard deviations were compared, also nature of peaks were compared.

Cyanocobalamin analysis produced relative standard deviations of 1.68%, 1.71% and 1.58% respectively for first and second and third occasions. These statistical parameters obtained on the three different occasions were all less than 2% as the ICH guidelines upper limit. (Table 4.5.3)

The linearity of the method was assessed by preparing a calibration graph of concentrations ranging from 0.03 to 0.005 % against mean peak area ratios with a linear correlation coefficient (R^2) of 0.996. (Figure 4.4.2).

In the assessment of sensitivity of the method, the LOD and LOQ were calculated and cyanocobalamin produced LOQ of 0.069ug/ml and LOD of 0.0228ug/ml.This parameter explains the sensitivity of the method and was found to be very sensitive for the detection and quantification of such small concentrations.

Robustness of the method was also assessed by comparing experimental values of two other days and their varations compared using analysis of variance (ANOVA) and were all found to be insignificant (table 4.5.3.3) and hence the various values obtained for other days do not differ significantly from each other.

The designed method was applied for the assay of cyanocobalamin in the injection and then compared with standard UV method.

When the cyanocobalamin was analysed using the newly developed hplc method, it produced percentage content ranging from 94% to 105% for the injections , with most of the ampoules meeting the requirements of the BP percentage of the stated content requirement of not less than 95% and not more than 105% of the labelled amount . (Table 4.4.2 (1-5)). The percentage content obtained from the two methods for most of the ampoules were found not to be significantly different from each other.

5.2.0 CONCLUSION

Hplc with ultraviolet detection method for the analysis of cyanocobalamin in injections formulation has been developed. This consisted of phosphate buffer pH 7/mehtanol (70/30 $^{v}/_{v}$) for the determination of cyanocobalamin using ascorbic acid as an internal standard.

The retention time was 4.9 ±1.5 minutes for cyanocobalamin and the mode of elution was isocratic with experiment conducted at room temperature.The stationary phase was a base-deactivated C-18 column with a mobile phase flow rate of 1.40ml/min. Cyanocobalamin detection and analysis worked best with a mobile phase composition of phosphate buffer pH 7/methanol (70/30) $^{v}/_{v}$ at 278nm with detector sensitivity of 0.03%.

In assessing the method performance (validation), the method was found to be specific for the cyanocobalamin, accurate and sensitive. The method was sensitive with an LOQ and LOD found to be 0.069ug/ml and 0.0228ug/ml respectively.

Moreover, the method was precise, robust, less time consuming for assessing the quantity of cyanocobalamin in the injection. The maximum elution time on the coloumn was found to be six minutes after injection.

The mean percentage recovery of the stated content of four batches of three brands of the injections categories were 99.00 ± 3.95, 96.05 ± 0.85, 96.00 ± 5.23, 94.36 ± 8.73 as against a standard UV method of 100.33 ± 4.67, 96.97 ± 0.50, 96.22 ± 6.20, 94.47 ± 10.64 respectively.

This shows that most of the injections passed the percentage content test as shown in table (4.5.2 .5) when the experimental values were compared with the BP reference range for percentage content of the dried cyanocobalamin in the injections.

The effect of pH variations on cyanocobalamin solutions revealed that it is most stable at pH 7 followed by pH 4, pH 8 and less stable at pH 6 as indicated in table and figure 4.3.1.

Cyanocobalamin solutions exposed to sunlight catalysed by variations in pH (table 4.3.2) revealed that its solution degrades in the presence of these conditions and the degradation is more pronounced at pH 4. Figures 4.3.2 (1-4).

Temperature effect on the cyanocobalamin injections also showed that the temperature range chosen for the stability studies had no significant effect on the strength of the active ingredient (cyanocobalamin) as indicated in table 4.3.3.

5.3.0 RECOMMENDATION

This method was not used for the analysis of cyanocobalamin in any biological fluid, thus further studies can be carried out to access cyanocobalamin and its metabolites in biological systems.

More work should be done to optimize this method to concurrently assay the other B_{12} vitamins as impurities when cyanocobalamin injection undergoes functional group transformations. e.g hydroxocobalamin.

This method should also be modified to aid in the assay of cyanocobalamin and ascorbic acid in vitamin formulations since ascorbic acid and cyanocobalamin elution is highly resolved in this method.

REFERENCES

- Ahmad, I; Fareedi, A.A; Huasseine, W. (1992). Photolysis of cyanocobalamin in aqueous solution, j. pharm biomed anal., 10: 9 -15.

- Anderson, N.H; Johnson D; McLellan, M.A; and Munden, P. (1991). Photostability of drug substances and drug products in UK pharmaceutical laboratory, N., j.pharm.biomed anal., 6: 443 – 449.

- Annibale, B; Lahner, E; (November 2009). "Pernicious anemia: new insights from a gastroenterological point of view". *World J. Gastroenterol.* 15 (41): 5121–8.

- Antonello, J; Green, T; Hammond, O; Marchese, R; Miller, P; Puchalski D.(2009). Optimization and validation of a multiplex. Electrochemiluminescence–Based detection assay for the quantitation of immunoglobulin G. *Clinical and Vaccine Immunology* 16: 387–396.

- Antony, A. C. (2008) Megaloblastic anemias. In: Hoffman R, Benz EJ, Shattil SS, et al., eds. *Hematology: Basic principles and practice*. 5th ed. Philadelphia, Pa: Elsevier Churchill Livingstone: chap 39.
- Barutca, S; Bolaman, Z; Kadikoylu, G; Senturk, T; Yavasoglu, I; Yukselen, V. (2003). "Oral versus intramuscular cobalamin treatment in megaloblastic anemia: a single-center, prospective, randomized open-label study". *Clin Ther* 25 (12): 3124–34.
- Bender, A. (2003). *Nutritional biochemistry of the vitamins*. Cambridge, U.K.: Cambridge university press. 978-0-521-80388-5.

- Biji, S; Mathew, B; Thapalia N (2005). An overview of electrochemiluminscent (ECL) technology in laboratory invistigations. *Kathmandu University Medical Journal* 3: 91–93.

- Bratu, I; Dragan, F; Hincu, L. (2009). Determination of cobalt in human biology liquids from electrothermal atomic absorption spectrometry. *Journal of physics: Conference Series* 1:182–187.

- British national formulary (September, 2007) 54th edition, BMJ publishing group limited, London, UK, pages 264-272.
- Bruno, K. (2005), "Vitamins: 9. Vitamin B12 (cyanocobalamin)", *Ullmann's Encyclopedia of Industrial Chemistry*, Weinheim: Wiley-VCH.
- Butler, C (2006). "Oral vitamin B12 versus intramuscular vitamin B12 for vitamin B_{12} deficiency: a systematic review of randomized controlled trials". *Fam Pract* 23 (3): 279–85. doi:10.1093/fampra/cml008.
- Carmel, R. (1992). Reassessment of the relative prevalences of antibodies to gastric parietal cell and to intrinsic factor in patients with pernicious anaemia: influence of patient age and race. Clin Exp Immunol; 89:74.
- Chen, B; Ding, L; Luo, X; Tang, F; Yao, S. (2006). HPLC–ESI–MS analysis of vitamin B12 in food products and in multivitamins–multimineral tablets. *Analytica Chemical Acta* 562:185–189.
- Christian, G. (2001). Chapter 17 in: Analytical chemistry. 5th ed. John Wiley & Sons, Inc, India. Pp537–545.
- Cohen, E; Garty, M; Sharabi, A; Sulkes, J; (December 2003). "Replacement therapy for vitamin B_{12} deficiency: comparison between the sublingual and oral route". *British Journal of Clinical Pharmacology* 56 (6): 635–8.
- David, G; Watson. (1999). *Pharmaceutical Analysis*. 21-29, chapters 10 &13. Harcourt
- Davis, R; Kelly, A; Moulton, J; (1973) An automated microbiological method for the measurement of vitamin B12. *J Clinical Path* 26: 494–498.
- De Paz, R; Hernández-Navarro, F (2005). "[Management, prevention and control of pernicious anemia]" (in Spanish). *Nutrición Hospitalaria : Organo Oficial De La Sociedad Española De Nutrición Parenteral Y Enteral* 20 (6): 433–5.
- Demeret, S; Gervais, D; Roze, E. (2003). "Neuropsychiatric disturbances in presumed late-onset cobalamin C disease". *Arch. Neurol.* 60 (10): 1457–62.

- Devalia, V. (August 2006). "Diagnosing vitamin B_{12} deficiency on the basis of serum B_{12} assay". *BMJ* 333 (7564): 385–6.
- Dolan, J.W; Kirkland, J.J; Snyder L.R. (2009). Introduction to modern liquid chromatography, John Wiley & Sons, New York.
- Domènech, E; Masnou, H; Navarro-L lavat, M (December 2007). "Pernicious anaemia in triplets. A case report and literature review". *Gastroenterología y Hepatología* 30 (10): 580–2.
- Dozias, D; Girard, C. L; Micol, D; Ortigues-Marty, I. (2005). Lack of correlation between vitamin B_{12} content in bovine muscles and indicators of the body stores in vitamin B_{12}, CAB Abstracts.
- Fukuwatari, T; Shibata, K (2008). "Urinary water-soluble vitamins and their metabolite contents as nutritional markers for evaluating vitamin intakes in young Japanese women". *J. Nutr. Sci. Vitaminol.* 54 (3): 223–9.
- Genaro, A.H. (1990), Remington's pharmaceutical sciences, 18th Edition, Mark printing company, Easton, Pennsylvania, USA, pages 1002-1022.
- Glajch, J. L; Kirkland, J.J; and Snyder, L. R. (1997). Practical HPLC method development, John.
- Glick, D. (2006). Methods of biochemical analysis. *Wiley, UK*, pp81–113.
- Haswell, S. J. (1991) Atomic absorption spectrometry; Theory, design and applications. *Analytical spectroscopy library Ed* 5. *Elsevier*. p529.
- Herbert, V. (1996). Vitamin B_{12} in present knowledge in nutrition. 17th ed. Washington, DC: International life sciences institute press.
- Honda, K; Imanishi, M; Kimura, M; Takeda, T. (2001) Determination of vitamin B_{12} in serum by HPLC/ICP–MS. *Analytical Science* 17:1983–1985.
- Institute of Medicine (1998). "Vitamin B12". *Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline*. Washington, DC: The national academies press. pp. 340–342.

- International conference on harmonisation (ICH) of technical requirements for registration of pharmaceuticals for human use. (1994). *Test on validation of analytical procedures*, Geneva.
- International conference on harmonisation (ICH) of technical requirements for registration of pharmaceuticals for human use. 1996. *Validation of analytical procedures: Methodology*. ICH-

- Jirat, J; Kosata, B; Nic, M eds. (2006). "Detection limit". *IUPAC Compendium of Chemical Terminology* (Online ed.).

- Kantor, E. A.; Vozhdaeva, M. Y.; Tsypysheva, L. G.; Kantor, L. I. Journal of analytical chemistry 62, 11, 1069-1074.
- Kelleher, B; O'Broin S. (1992). Microbiological assay on microtiter plates of folate in serum and red cells. *J Clin Pathol* 45: 344–347.
- Kumar, V. (February 2007). "Pernicious anaemia". MLO 39 (2): 28, 30–1.
- Kutsky, R. J. (1973). Handbook of vitamins and hormones. New York: Van nostrand reinhold, ISBN 0442245491.
- Lederle, F. A. (September 1998). "Oral cobalamin for pernicious anemia: back from the verge of extinction". *Journal of the American Geriatrics Society* 46 (9): 1125–7.

- Leeming, R; Lynch, S; O'Sullivan, J; Pollock A. (1992). Radioimmunoassay that measures serum vitamin B12. *Journal of Clinical Pathology* 45:328–331.

- Maqbool, A; Stallings, V. A (2008). "Update on fat-soluble vitamins in cystic fibrosis". *Curr Opin Pulm Med* 14 (6): 574–81.

- Measurement of quantitation limits for vitamin B12 and caffeine in aqueous solution using UV–1800 UV–VIS spectrophotometer. Application news No.A403. [2010] *Shimadzu*. www.globalspec.com/MyGlobalSpec.

- Merkus, F. W; Slot, W. B; Tytgat, G. N; Van Deventer, S. J. (August 1997). "Normalization of plasma vitamin B12 concentration by intranasal hydroxocobalamin in vitamin B12-deficient patients". *Gastroenterology* 113 (2): 430–3.
- Michael, W. Dong. (2006). *Modern HPLC for practicing scientist*. Chapters 3, 6 & 8. John
- Moffat, A.C. (1986). *Clarke's isolation and identification of drugs*. Second edition.160- 8

- Mohammed, Z. M; Said, H. M (2006). "Intestinal absorption of water-soluble vitamins: an update". *Curr. Opin. Gastroenterol.* 22 (2): 140–6.
- Olaniyi A.A. (2000). *Principles of drug quality assurance and pharmaceutical analysis*. Chapter Mosuro publishers, Nigeria,page 170 -202
- *Prescribing information for ulesfia lotion,* Sciele pharmaceuticals, Inc., April 2009, http://www.ulesfialotion.com/pdf/Ulesfia_pi.pdf, retrieved 2009-08-29. Publishers Limited Q2B, Geneva.
- Robilotti, E; Sadan, Y; Sethi, N. K (2005). "Neurological manifestations of vitamin B-12 deficiency".*The internet journal of nutrition and wellness*.
- Shabir, G. A. (2003) "Validation of HPLC chromatography methods for pharmaceutical analysis. Understanding the differences and similarities between validation requirements of FDA, the US Pharmacopeia and the ICH," *J. Chromatogr. A.* 987(1-2), 57-66.

- United State Pharmacopoeia 27, National formulary 22. 2004. Rockville, M D, the United State Pharmacopoeial Convention, Inc.

- Victor, H. (1988). "Vitamin B-12: plant sources, requirements, and assay". *American Journal of Clinical Nutrition* 48 (3 Suppl): 852–8. http://www.ajcn.org/cgi/reprint/48/3/852.pdf.Wiley & Sons, New York.

- Xu, Q; Yuan, C; Yuan, S. (1988). A quantitative structure-reactivity study of mono-basic organophosphorus acids in cobalt and nickel extraction. *Solvent Extr Ion Exch* 6(3):393–416.