

**Addis Ababa University
School of Graduate Studies**



**HPTLC-DENSITOMETRIC DETERMINATION
OF SOME WATER SOLUBLE VITAMINS
PRESENT IN PHARMACEUTICALS**

*A Thesis Presented to the School of Graduate Studies of Addis Ababa
University in Partial Fulfillment of the Requirements of the Degree of
Master of Science in Pharmaceutical Analysis and Quality Assurance*

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Table of contents	page
Acknowledgements	i
Table of contents	ii
List of tables	iv
List of figures	v
Acronyms	vi
Abstract	vii
1. Introduction	1
1.1 High performance thin layer chromatography	1
1.2 Vitamins	4
1.2.1 Water soluble vitamins as supplements	5
1.3 Components of the studied vitamins and their analysis	6
1.3.1 Thiamine hydrochloride	6
1.3.2 Pyridoxine hydrochloride	7
1.3.3 Cyanocobalamine	8
1.4 Analysis of vitamin B-complex supplements	9
2 Objectives	16
2.1 General objective	16
2.2 Specific objectives	16
3. Experimental	17
3.1 Equipment and instruments	17
3.2 Chemicals, solvents and materials	17
3.3 Pharmaceutical preparations	17
3.4 Procedures	18
3.4.1 Standard solutions preparation	18
3.4.2 Samples preparation	18
3.4.3 Instrumentation and chromatographic conditions	19
3.5 Method validation	20
3.5.1 Linearity	20

3.5.2 Accuracy and recovery studies	20
3.5.3 Precision	20
3.5.3.1 System precision	20
3.5.3.2 Method precision	21
3.5.4 Limit of detection and quantification	21
3.5.5 Robustness	21
4 Results and discussion	22
4.1 Method optimization	22
4.1.1 Mobile phase composition	22
4.1.2 Appropriate conditions for validation	23
4.2 Method validation result	27
4.2.1 Linearity	27
4.2.2 Accuracy (% recovery)	30
4.2.3 Precision	33
4.2.4 Limit of detection and quantification	35
4.2.5 Robustness	35
4.2.6 Analysis of marketed formulation	37
4.3 Stability in sample solution	39
4.4 Comparison between the HPTLC and HPLC methods	40
5. Conclusion	42
6. Suggestions for further work	43
Reference	44

List of Tables	page
Table 1.1 Comparison between HPTLC and TLC.	2
Table 4.1 The different mobile phase combinations.	23
Table 4.2 Calibration data of vitamin B ₁ .	27
Table 4.3 Calibration data of vitamin B ₆ .	28
Table 4.4 Calibration data of vitamin B ₁₂ .	29
Table 4.5 Characteristic parameters for the linear regression equation.	30
Table 4.6 Characteristic parameters for the second order polynomial regression equation.	30
Table 4.7 Recovery study for the determination of vitamin B ₁ , B ₆ and B ₁₂ .	31
Table 4.8 System precision of the developed method.	33
Table 4.9 Intra-day and inter-day precision.	34
Table 4.10 Experimental variables used in the robustness study.	36
Table 4.11 Robustness testing of the developed method.	36
Table 4.12 Summary of method validation parameters.	37
Table 4.13 HPTLC Analysis of the marketed formulations. of vitamin B ₁ , B ₆ and B ₁₂ in pharmaceuticals.	38
Table 4.14 Applicability of the proposed methods for the determination of B ₁ , B ₆ and B ₁₂ in commercial pharmaceuticals.	38
Table 4.15 Stability of vitamin B ₁ , B ₆ and B ₁₂ in sample solutions.	40
Table 4.16 Cost comparison between HPTLC and HPLC	40
Table 4.17 Time comparison between HPTLC and HPLC	41

List of Figures	Page
Figure 1.1 Structure of thiamine hydrochloride.	6
Figure 1.2 Structure of pyridoxine hydrochloride.	7
Figure 1.3 Structure of cyanocobalamine.	8
Figure 4.1 UV spectra for vitamins B ₁ , B ₆ and B ₁₂ .	24
Figure 4.2 HPTLC chromatogram of B ₁ , B ₆ and B ₁₂ at 255,290, 361nm.	25
Figure 4.3 Calibration curves for thiamine hydrochloride.	27
Figure 4.4 Calibration curves for pyridoxine hydrochloride.	28
Figure 4.5 Calibration curves for cyanocobalamine.	29
Figure 4.6 Standard addition of vitamin B ₁ , B ₆ and B ₁₂ .	32

ACRONYMS

AU: Absorbance unit

C.V: Coefficient of variation

DACA: Drug administration and control authority

HPTLC: High performance thin layer chromatography

ICH : International conference on harmonization

LOD: Limit of detection

LOQ: Limit of quantification

S.D: Standard deviation

UV : Ultraviolet

Abstract

A simple, accurate and precise high-performance thin-layer chromatographic (HPTLC) method has been developed for the determination of vitamin B₁, B₆ and B₁₂ in tablet and injection. Using methanol: ammonium acetate (5M) (10:1; v/v) as mobile phase and HPTLC plates (0.2mm thickness) precoated with 60F₂₅₄ silica gel on glass plate as stationary phase. Quantification was carried out densitometrically using a UV detector at 255, 290 and 361nm. The retardation factors (R_f) of B₁, B₆ and B₁₂ were 0.20 ± 0.02 , 0.40 ± 0.02 and 0.68 ± 0.02 respectively. Calibration curves were polynomial in the range 250-2050 ng/μl for B₁ and B₆, 50-1050 ng/μl for B₁₂.

The method has been robust for small variation with mobile phase composition, amount of mobile phase and time variation before chromatographic development and scanning.

The stability of sample solution was studied over eight hours and no additional spot was observed on the plate. As a result no evidence was observed for the degradation of the product. In addition the proposed method has been compared statistically (t-test and F-test) with the classical method (HPLC) and there was no difference in terms of accuracy of the dosage analysis between the two methods.

The analysis result of both synthetic mixture and pharmaceutical samples showed the quantitative determination of each component accurately in the presence of the other components and back ground interference.

Assay of vitamin B₁, B₆ and B₁₂ were $99.10 \pm 1.37 \%$, $99.68 \pm 1.50 \%$ and $99.42 \pm 1.26 \%$ for vitamin B₁, B₆ and B₁₂ injection, $99.44 \pm 1.30 \%$ and $99.95 \pm 1.49 \%$ for vitamin B₁ and B₆ tablet respectively.

1. Introduction

1.1 High performance thin layer chromatography (HPTLC)

The basic thin layer chromatography procedure has largely remained unchanged over the last fifty years. In 1973, Halpaap was one of the first to recognize the advantage of using a smaller average particle size of silica gel (about 5–6 μm) in the preparation of chromatographic plates. He compared the effect of particle size on development time, retardation factor (R_f) and plate height. By the mid 1970s high performance thin layer chromatography (HPTLC) added a new dimension to thin layer chromatography, as precision could be improved ten-fold, analysis time could be reduced by a similar factor, less mobile phase was required, and the development distances on the layers could be reduced [1].

The technique could now be made fully instrumental to give accuracy comparable with high performance liquid chromatography (HPLC). In 1977 the first major high performance thin layer chromatography publication appeared, simply called ‘‘HPTLC high performance thin-layer chromatography’’ [2].

The 1980s show improvements in spectrodensitometric scanners with full computer control becoming possible, including options for peak purity and the measurement of full UV/visible spectra for all separated components. Automated multiple development (AMD) made its appearance in 1984. This improvement enabled a marked increase in number and resolution of the separated components [3].

At the present time all steps of thin layer chromatography can be computer controlled. The use of highly sensitive charge coupled device (CCD) cameras which have high resolution has enabled the chromatographer to electronically store images of chromatograms for future use and for direct entry into reports at a later date.

When one considers the latest technical and methodological developments modern high performance thin-layer chromatography (HPTLC), is a reliable and powerful analytical technique, which can be the method of choice when many samples have to be analyzed,

flexibility is of importance and rapid quantitative and semi-quantitative data are needed at low cost per sample [4].

As smaller particles improved efficiencies in liquid chromatography (LC) columns and resulted in high performance liquid chromatography (HPLC). Small particles (about 5-6 μ m) were also tried in thin layer chromatography. As in column in LC, these plates resulted in higher performance thin layer chromatography - HPTLC. Some characteristics of two types of plates are included in table 1.1 [5].

Table1.1 Comparison between high performance thin layer chromatography (HPTLC) and thin layer chromatography (TLC).

	Thin layer chromatography	High performance thin layer chromatography
Mean particle size	10-15 μ m	3-7 μ m
Size distribution	wide	narrow
Layer thickness	\geq 250 μ m	100-200 μ m
Number of samples	12	36-72
Migration distance	100-150mm	30-70 mm
Migration time	30-200min	3-20 min.
Solvent use	\geq 50ml	5-10 ml
Detection limit		
Absorption measurement	100-1000ng	10-100 ng
Fluorescence measurement	1-100ng	0.1-10 ng

Nowadays, HPTLC is involved in a lot of applications, analysis in pharmaceuticals and drugs, clinical chemistry, forensic chemistry, biochemistry, cosmetology, food analysis, environmental analysis and other areas [6].

Unlike the ordinary TLC high performance thin layer chromatography (HPTLC) uses automatic micro syringe for sample application and scanner for quantification.

(i) Sample application:

Successful quantitative thin layer chromatography is strongly dependent on the quality of sample application. Reproducibility of sample amount and spot (band like) size are quite important. To achieve good chromatographic resolution and sensitivity of detection, the shape of the spots of the applied sample is also great importance. Micro syringes are commonly used in high performance thin layer chromatography. They are equipped with a micrometer screw for precise control of the position of the syringe plunger. One advantage of a micro syringe over a pipette is that it delivers the sample solution by displacement rather than by capillary action [7].

(ii) Detection, quantification and documentation:

The simplest method of detecting substances on high performance thin layer chromatography is by visual detection of the spots caused by substances with a color of their own. Inspection of the chromatogram under ultraviolet (UV) light is also a non destructive detection method. Spots of fluorescent compounds can be seen at 254 nm (short wave length) or at 366 nm (long wave length) but spots of non fluorescent compounds require fluorescent stationary phase (silica gel GF) to be seen using UV light. Non UV absorbing compounds can be detected by dipping the plates in iodine vapour. But when individual component does not respond to UV, derivatization (changing the compound in to UV absorbing one by using certain chemical reaction) is required for detection [8]. In situ densitometry offers a simple way of quantifying directly on the plate. A definition of direct densitometry is resolving the compounds to be separated on the chromatoplate and measuring the optical density of the separated spots directly on the plate. The amounts of compounds are determined by comparing them to a standard curve from reference materials chromatographed simultaneously under the same conditions [9]. After an evaluation by scanner, the complete data is recorded in the form of a number of hard-copy pages, representing the main part of the whole documentation. This documentation system is useful to

recall the photo at any time easily and include it in a printed text document, for easy archiving and retrieval and easy reference to previous work [10].

1.2 Vitamins

Vitamins are organic compounds present mostly in food and are needed in very small amounts for various metabolic processes and other functions [11]. The two types of vitamins are named as fat-soluble and water soluble.

Fat soluble vitamins: - are vitamins A, D, E and K. They dissolve in fat before they are absorbed in the blood stream to carry out their functions. Excesses of these vitamins are stored in the liver [12].

Water-soluble vitamins: - are the B-complex group and vitamin C. They dissolve in water and are not stored in the body; if in excess, they are eliminated in urine [13]. Although present in many foods such as meat, vegetables, grains, fish etc, the B-group vitamins are delicate and easily destroyed, particularly by alcohol and cooking. Food processing may remove most of the B-vitamins, for example making white flours, breads and rice make them less nutritious than their whole meal counterparts.

Eight of the water-soluble vitamins are: - thiamine (vitamin B₁), riboflavin (vitamin B₂), niacin, vitamin B₆, folate, vitamin B₁₂, biotin and pantothenic acid. These vitamins are widely distributed in foods. They function as coenzymes that help the body obtain energy from food. They are also important for normal appetite, good vision, healthy skin, healthy nervous system and red blood cell formation.

In addition, studies have shown that simple daily regimen of multivitamins (vitamin B-complex, C and E) can significantly delay the progress of the AIDS virus in infected women [14].

Beriberi, pellagra and pernicious anemia are the three well-known manifestations of B-vitamin deficiencies. All the B-vitamins function as coenzymes or cofactors, assisting in the activity of important enzymes and allowing energy producing reactions to proceed normally. As a result, any lack of water-soluble vitamins mostly affects growing or rapidly metabolizing tissues such as skin, blood, the digestive tract, and the nervous system [15].

1.2.1 Water soluble vitamins as supplements

Vitamin supplements are intended for ingestion in form of pill, capsule, tablet, powder or liquid [16]. The B-complex vitamins act favorably against inflammatory diseases and the degeneration of locomotory organs due to their particular influence on the trophism of nervous and muscular cells. B-vitamins are most commonly used in the treatment of nervous problems, fatigue, stress and in the prevention of alcohol problems [17]. The B vitamins participate in all the most important aspects of food metabolism and in the production of energy. Children require B-vitamins for normal development and these vitamins are also essential to the renewal of cells. Some of the B-vitamins would also appear to contribute to the reinforcement of the immune system and to the prevention of blood clots. In addition to this, most of the B-vitamins are also antioxidants. Those who drink too much alcohol, women who take contraceptive pills, the elderly and children on antibiotics are particularly susceptible to deficiencies of B-vitamins [18]. The binary and ternary mixtures of vitamin-B supplements are available in the market. Vitamin B₁- B₆-B₁₂ injection is a combination of the three essential neurotropic factors of the vitamin B-complex. They are indispensable for a normal and coordinated function of the nervous metabolism. Vitamin B₁-B₆ tablet contains active ingredients of B₁ and B₆ [19]. Basically, this study focused on the determination of a mixture of vitamin B₁, B₆ and B₁₂ in pharmaceutical formulations by HPTLC densitometric technique. Once the method has developed it can be applied for other pharmaceutical formulations containing thiamine hydrochloride (vitamin B₁), pyridoxine hydrochloride (vitamin B₆) and cyanocobalamine (vitamin B₁₂).

1.3 Components of the studied vitamins and their analysis

1.3.1 Thiamine Hydrochloride

Synonyms. Anneurine; Vitamin B₁.

3-(4-Amino-2-methylpyrimidin-5-ylmethyl)-5-(2-hydroxyhydroxyethyl)-4-methylthiazolium chloride.

$C_{12}H_{17}ClN_4OS = 300.8$

Thiamine Hydrochloride

$C_{12}H_{17}ClN_4OS.HCl = 337.3$

Colourless crystals or white crystalline powder. M.p. about 248⁰, with decomposition [20].

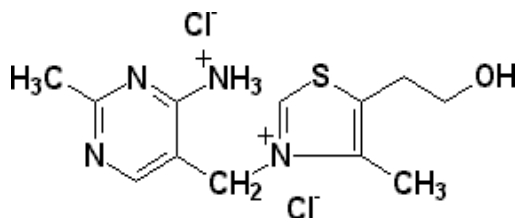


Figure 1.1; Structure of thiamine hydrochloride

Thiamine plays an important role in helping the body convert carbohydrates and fat into energy. It is essential for normal growth and development and helps to maintain proper functioning of the heart and the nervous and digestive systems. Thiamine is concentrated in muscle tissue. Systemic thiamine deficiency can lead to myriad problems including neuro degeneration, wasting, and death [21]. A lack of thiamine can be caused by malnutrition, well-known syndromes caused by thiamine deficiency including Wernicke-Korsakoff syndrome and beriberi, diseases also common in chronic abusers of alcohol. Thiamine functions as the coenzyme thiamine pyrophosphate (TPP) in the metabolism of carbohydrate and in conduction of nerve impulses [22].

1.3.2. Pyridoxine Hydrochloride

Synonyms. Adermine; Pyridoxol; Vitamin B₆.

3-Hydroxy-4,5-bis (hydroxymethyl)-2-methylpyridine

$C_8H_{11}NO_3 = 169.2$

Pyridoxine Hydrochloride

$C_8H_{11}NO_3 \cdot HCl = 205.6$

A white crystalline powder or crystals. M.p. 202^o, with decomposition [23].

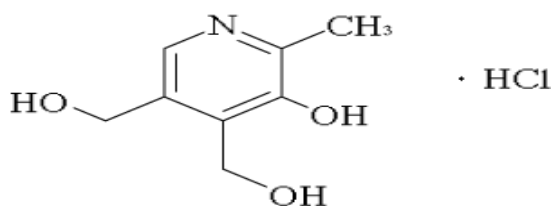


Figure 1.2; Structure of pyridoxine hydrochloride

Pyridoxine along with pyridoxal and pyridoxamine assists in the balancing of sodium and potassium as well as promoting red blood cell production. It is linked to cancer immunity and helps fight the formation of homocysteine. Pyridoxine can help balance hormonal changes in women and aid in immune system. Lack of pyridoxine may cause anemia, nerve damage, seizures, skin problems, and sores in the mouth [24]. Vitamin B₆ is present in three forms: pyridoxal, pyridoxine, and pyridoxamine. All forms can be converted to the active vitamin-B₆ coenzyme in the body. Pyridoxal phosphate (PLP) is the predominant biologically active form. Vitamin B₆ is not stable in heat or in alkaline conditions, so cooking and food processing reduce its content. Both coenzyme and free forms are absorbed in the small intestine and transported to the liver, where they are phosphorylated and released into circulation, bound to albumin for transport to tissues. Vitamin B₆ is stored in the muscle and only excreted in urine when intake is excessive [25]. Vitamin-B₆ deficiency is common in alcoholics and elderly persons who consume an inadequate diet. Individuals taking medication to treat Parkinson's disease or

tuberculosis may take extra vitamin B₆ with physician supervision. A nerve disorder of the wrist has also been treated with large daily doses of B₆ [26].

1.3.3 Vitamin B₁₂ (Cyanocobalamin)

Synonyms. Cobamin; Cycobemin; Vitamin B₁₂.

Co α -[α -(5, 6-Dimethylbenzimidazolyl)]-Co β -cyanocobamide

C₆₃H₈₈CoN₁₄O₁₄P = 1355

Dark red hygroscopic crystals or powder [27].

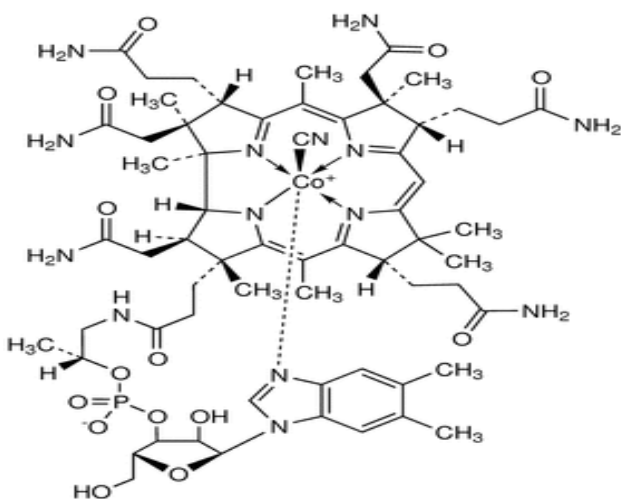


Figure 1.3; Structure of cyanocobalamin

Vitamin B₁₂ is found in its free-vitamin form, called cyanocobalamin, and in two active coenzyme forms. Absorption of vitamin B₁₂ requires the presence of intrinsic factor, a protein synthesized by acid-producing cells of the stomach. The vitamin is absorbed in the terminal portion of the small intestine called the ileum. Most of body's supply of vitamin B₁₂ is stored in the liver [28].

Vitamin B₁₂ is stable when heated and slowly loses its activity when exposed to light, oxygen, and acid or alkaline environments.

Vitamin B₁₂ coenzymes help recycle folate coenzymes involved in the synthesis of DNA and RNA, and in the normal formation of red blood cells. Vitamin B₁₂ prevents degeneration of the myelin sheaths that cover nerves and help maintain normal electrical conductivity through the nerves [29].

Vitamin-B₁₂ deficiency results in pernicious anemia, which is caused by a genetic problem in the production of intrinsic factor. When this occurs, folate function is impaired, leading to macrocytic anemia due to interference in normal DNA synthesis. Unlike folate deficiency, the anemia caused by vitamin-B₁₂ deficiency is accompanied by symptoms of nerve degeneration, which if left untreated can result in paralysis and death [30].

Deficiency is usually observed when B₁₂ absorption is hampered by disease or surgery to the stomach or ileum, damage to gastric mucosa by alcoholism, or prolonged use of anti-ulcer medications that affect secretion of intrinsic factor. Age related decrease in stomach-acid production also reduces absorption of B₁₂ in elderly persons. These groups are advised to take a supplemental form of vitamin B₁₂ [31].

1.4. Analysis of Vitamin B-complex supplements

The determination of water-soluble vitamins has always been a peculiar problem largely because of the instability of these compounds and the complexity of the matrices in which they usually exist [32]. As their chemical structure is not related, a considerable number of publications have been appeared using different physical and chemical methods [33]. Actually there is no official method for determination of a combination of water soluble vitamins. However, British pharmacopoeia (BP) and United States pharmacopoeia (USP) state the identification and assay of vitamins B₁, B₆ and B₁₂ separately.

The determinations of water soluble vitamins by using different techniques have been given below.

1. Thiamine hydrochloride injection [34].

British pharmacopoeia states both the qualitative and quantitative determination of thiamine hydrochloride injection as follows.

To a mixture of 0.1 ml of nitrobenzene and 0.2 ml of sulphuric acid a volume of the injection containing 5 mg of thiamine hydrochloride is added. The solution is allowed to stand for 10 minutes, then is cooled in ice and added slowly with stirring 5 ml of water followed by 5 ml of 10M sodium hydroxide. Five ml of acetone is added and

allowed to stand for 5 minutes. Absence of violet color in the upper layer is an indication for the presence of thiamine hydrochloride.

Assay: Liquid chromatography is used for quantitative determination. Solution (1) contains 0.005% w/v of thiamine mononitrate in 0.005M hydrochloric acid. Solution (2) a volume of the injection containing 0.1 g of thiamine hydrochloride and it is diluted to 100 ml with 0.1M hydrochloric acid and further diluted to 100 ml with water.

2. Pyridoxine hydrochloride tablet [35].

United States pharmacopoeia states the determination of pyridoxine hydrochloride tablet as follows.

A quantity of the powdered tablets containing 20 mg of pyridoxine hydrochloride is shaken with 50 ml of 0.025 M standard phosphate buffer for 15 minutes and diluted to 100 ml with the same solvent. Five ml of the filtrate is mixed, filtered and diluted to 100 ml with the same solvent. The light absorption of the resulting solution, in the range 230 to 350 nm exhibits two maxima, at 254 nm and 324 nm is an indication of the presence of pyridoxine hydrochloride.

Assay: Twenty tablets are weighed and powdered. To a quantity of the powdered tablets containing 25 mg of pyridoxine hydrochloride, 50 ml of 0.1M hydrochloric acid is added and heated on a water bath for 15 minutes. The solution is diluted to 100 ml with 0.1M hydrochloric acid and filtered, discarding the first 20 ml of filtrate. 5 ml of the filtrate is diluted to 100 ml with 0.1M hydrochloric acid and the absorbance of the resulting solution is measured at the maximum at 290 nm, the content of $C_8H_{11}NO_3.HCl$ taking 430 as the value of A (1%, 1 cm) is calculated at the maximum at 290 nm [35].

3. Cyanocobalamine tablet [36].

British pharmacopoeia states the determination of cyanocobalamine tablet as follows.

Ten tablets with 20 ml of chloroform are gently shaken to remove the coating and dry the tablet cores in a current of air. It is transferred to a clean flask and powder with the aid of a glass rod. To a quantity of the powdered tablet cores containing 0.2 mg of cyanocobalamin, 10 ml of a mixture of 1 volume of 2-ethoxyethanol and 3

volumes of water is added and shaken vigorously for 5 minutes then centrifuged at 3000 revolutions per minute for 15 minutes and filtered using a filter with a pore size of 0.45 μm . The light absorption of the filtrate, in the range 345 to 560 nm exhibited maxima at about 361 nm and 550 nm. If no maximum is exhibited at 351 nm is an indication of the presence of cyanocobalamine.

Assay: liquid chromatography is used for quantitative determination. Using 100 μl of the following solutions. Solution (1) one finely-crushed tablet is shaken with 6 ml of water for 5 minutes and diluted to 10 ml, centrifuged for 15 minutes and the supernatant solution is used. Solution (2) contains 0.0005% w/v, or other appropriate concentration, of cyanocobalamin in water.

4. An optimized method for the simultaneous determination of vitamin B₁-B₆-B₁₂ in multivitamin tablets by high performance liquid chromatography [37].

In this study an accurate, precise and selective HPLC-RP method has been developed for the simultaneous determination of B₁ (150mg), B₆ (150mg) and B₁₂ (0.150 mg) in multi vitamin tablets. The method used Hypersil-BDS C₁₈ reversed phase column and gradient elution. The aqueous mobile phase contained 0.015% triethylamine adjusted to pH 2.7 with 1 N sulfuric acid and acetonitrile. Separation and quantitation was achieved by changing the proportion of the system linearly with a time-schedule programme. Detection was carried out using a dual-beam UV detector set at 280, 350 nm. Good linearity was observed between the concentration of the analytes and peak area ($r = 0.9999, 0.9998$). Sample preparation was relatively simple whereas excipients present in the dosage forms did not interfere with the peaks of interest. Recovery of the compound from the B₁, B₆ and B₁₂ was quantitative.

5. A comparison of matrix resolution method, ratio spectra derivative spectrophotometry and HPLC method for the determination of thiamine HCl and pyridoxine HCl in pharmaceutical preparation [39].

A comparison of two spectrophotometric methods and HPLC method were described in this study for the analysis of pyridoxine hydrochloride and thiamine hydrochloride in a vitamin combination. In the first method A (1%, 1cm) values of these two compounds were calculated using absorbances measured at 246.8 and 290.5 nm in

zero order spectra. The matrix was written for A (1%, 1cm) values and the concentration of both compounds were determined using 'Matlab' software. The second method, the measurements in the derivative of the ratio spectra were made at 297.8 and 309.5nm for pyridoxine hydrochloride and at 245.6 and 257.7 nm for thiamine hydrochloride. The calibration graphs were established in the range 8-40µg/ml of both vitamins. In the HPLC method, the separation of these compounds was analyzed on a Nucleosil 100-5C₁₈ column with 0.1M (NH₄)₂CO₃ - water-methanol (5:15:80V/V) as the mobile phase. Results of spectrophotometric and HPLC procedures were compared. These three methods were found to be suitable for the determination of both vitamins in their binary mixtures.

6. Reversed phase ion-pair HPLC determination of some water-soluble vitamins in pharmaceuticals [40].

A reversed-phase ion-pair high-performance liquid chromatographic method (RP-IPC) was developed to assay some water soluble vitamins in solution dosage forms. Vitamins of the B-group B₁, B₂, B₃ and B₆, including vitamin C were determined in Oligiovit coated tablets. In Beviplex coated tablets the vitamins B₁, B₂, B₃, B₆ and p-aminobenzoic acid were analyzed. Hexanesulphonic acid sodium salt and triethanolamine in water-methanol were used as mobile phase with adjusting pH to 2.8 with orthophosphoric acid. Phenol was used as an internal standard for quantitative simultaneous analysis of vitamins in pharmaceutical formulations; the method of internal standard was used. This RP-IPC method is rapid and accurate.

7. HPLC and chemometric methods for the simultaneous determination of Cyproheptadine hydrochloride, multivitamins and sorbic acid [41].

Three methods are presented for the simultaneous determination of cyproheptadine hydrochloride (CP), thiamine hydrochloride (B₁), riboflavin-5-phosphate sodium dihydrate (B₂), nicotinamide (B₃), pyridoxine hydrochloride (B₆), and sorbic acid (SO). The chromatographic method depends on a high performance liquid chromatographic (HPLC) separation on a reversed-phase, RP 18 column. Elution was carried out with 0.1% methanolic hexane sulphonic acid sodium salt (solvent A) and

0.01M phosphate buffer containing 0.1% hexane sulphonic acid sodium salt, adjusted to an apparent pH of 2.7 (solvent B). Gradient HPLC was used with the solvent ratio changed from 20:80 to 70:30 (over 9 minutes), then to 80:20 (over 11 minutes) for solvent A:B, respectively. Quantitation was achieved with UV detection at 220 and 288 nm based on peak area. The other two chemometric methods applied were principal component regression (PCR) and partial least squares (PLS). These approaches were successfully applied to quantify each drug in the mixture using the information included in the UV absorption spectra of appropriate solutions in the range 250–290 nm with the intervals $\Delta\lambda = 0.4$ nm at 100 wavelengths. The chemometric methods do not require any separation step. The three methods were successfully applied to a pharmaceutical formulation.

8. Simultaneous determination and classification of riboflavin, thiamine, nicotinamide and pyridoxine in pharmaceutical formulations, by UV-visible spectrophotometry and multivariate analysis [42].

Partial least square regression method was used in this work for the identification and quantification of thiamine, riboflavin, nicotinamide and pyridoxine by UV-Vis spectrophotometry, without separation or preconcentration steps in the analytical procedure. For quantitative purposes, the working range established was 1-14 mg L⁻¹ for riboflavin, 2-26 mg L⁻¹ for thiamine, 2-30 mg L⁻¹ for nicotinamide, and 2-22 mg L⁻¹ for pyridoxine. Recovery results higher than 95% were obtained in all cases during the analysis of synthetic and commercial samples. Thus, a simple and reliable method is developed for the simultaneous estimation of these compounds.

9. High-performance liquid chromatography/electrospray ionization-mass spectrometry for simultaneous determination of taurine and 10 water-soluble vitamins in multivitamin tablets [43].

An HPLC/ESI-MS method for the simultaneous determination of taurine and 10 water-soluble vitamins including vitamin B₁ (thiamine), B₂ (riboflavin), B₅ (pantothenic acid), B₆ (pyridoxine, and pyridoxal), B₈ (biotin), B₉ (folic acid), C (ascorbic acid) and PP (nicotinamide and nicotinic acid) in multivitamin tablets was developed and validated.

The separation was accomplished on a Johnson Spherigel C₁₈ (250mm×4.6 mm) reversed phase column with methanol in an aqueous solution of heptafluorobutyric acid (5 mM) as mobile phase under gradient elution mode. Detection of target components was by ESI-MS switching continuously from positive ion mode (PI) to negative ion mode (NI). Precision and accuracy were determined.

10. Chemometrics assisted spectroscopic determination of vitamin B₆, vitamin B₁₂ and dexamethasone in injectables [44].

A spectrophotometric method is described and applied to resolve ternary mixtures of the corticosteroid dexamethasone sodium phosphate and the vitamins B₆ and B₁₂. It involves multivariate calibration based on partial least-squares regression.

The model was built with UV- vis absorption spectra, and was evaluated by cross-validation on a number of synthetic mixtures. Satisfactory results for both artificial and commercial samples were obtained. A spectrofluorometric method was also developed for the determination of B₆ in the presence of vitamin B₁₂ and dexamethasone. The results provided by both methods for pharmaceutical formulations were compared successfully. A spectrophotometric method is described and applied to resolve ternary mixtures of the corticosteroid dexamethasone sodium phosphate and the vitamins B₆ and B₁₂. It involves multivariate calibration based on partial least-squares regression.

The quality control of pharmaceutical preparations of mult vitamins require reliable and quick analytical methods. UV-visible spectrophotometry and fluorimetric methods generally involve tedious and lengthy extractions.

Many reversed phase high-performance liquid chromatographic (HPLC) methods have been described that use various ion-pairing reagents with preliminary automated extraction and spectro- photometric or electrochemical detection.

Microbiological and chemical assays are also available for identification and quantification of several water-soluble vitamins from biological samples and pharmaceutical formulations, but most of these methods are complicated.

High performance liquid chromatography has been widely employed for vitamins determination, in spite of the fact that the sample preparation usually requires laborious and time consuming steps [45].

To the knowledge of the investigator, there is no reported evidence for the determination of thiamine hydrochloride (vitamin B₁), pyridoxine hydrochloride (vitamin B₆) and cyanocobalamin (vitamin B₁₂) by using HPTLC- densitometric technique. This study examined the determination of a combination of B-vitamins and provided an alternative to the reported HPLC method.

2. Objectives

2.1 General objective

To develop a simple, inexpensive, accurate and precise method for the analysis of vitamin B-complex formulation by using HPTLC- densitometric technique.

2.2 Specific objectives

- To obtain appropriate mobile phases for the analysis of each component in the vitamin B-complex.
- To obtain the R_f values for each standard reference material and establishment of the calibration data under the applied experimental conditions.
- Quantitative determination of each component accurately in the presence of the other components and the background interferences.
- To provide a considerably efficient procedure for controlling the quality of the studied vitamins.
- Detection and determination of the degradation products for each component if any.
- To compare the cost and time effectiveness of the method to that of the classical HPLC method.

3. Experimental

3.1 Equipment and Instruments:-

The method development was done by using computerized Camag HPTLC system (Camag, Muttenz, Switzerland) consisting of a Camag Linomat V semiautomatic spotting device connected to a nitrogen tank and WinCATS 4 software (version 4.05, Camag), a Camag TLC scanner III densitometer equipped with mercury, tungsten and deuterium lamp driven by the same WinCATS 4 software, a Camag 100 µl HPTLC sample syringe (Hamilton, Bonaduz, Switzerland) and a Camag glass twin-trough (20cm×20cm) development chamber, different size pipettes, volumetric flasks, measuring cylinders, micro-syringes and ruler.

3.2 Chemicals, solvents and materials:-

Reference standards: thiamine hydrochloride (99.9%, Cadila, India), pyridoxine hydrochloride, (99.4%, Cadila, India), cyanocobalamine (99.9%, Cadila, India),

HPLC grade methanol (Fisher Scientific-Leicestershire, UK), ethanol (BDH Laboratory Supplies, England), analytical grade toluene (E.Merck-Darmstadt, Germany), Ammonium acetate 99% (Fisher Scientific-Leicestershire, UK), 1-Butanol, n-propanol (BDH Laboratory Supplies, England), acetic acid (Fisher Scientific-Leicestershire, UK), acetone (Aurangbad, India), acetonitrile (Aurangbad, India).

HPTLC glass backing plates: silica gel 60F₂₅₄, (Mean pore size of 60A⁰ fluorescent excitation wavelength 254 nm).

3.3 Pharmaceutical preparations;

The following pharmaceutical preparations were purchased from the local market and subjected to analysis by the proposed method:-

(a) Neurobion injections: (Merck, Darmstadt, Germany) each ampoule is labeled to contain 100 mg of vitamin B₁, 100 mg vitamin B₆ and 1000 µg of vitamin B₁₂ in 3ml.

(b) Vit-B-Denk tablets: (E-Denk, Germany), each tablet is labeled to contain 100 mg of vitamin B₁ and 200 mg of vitamin B₆.

3.4 Procedures:-

3.4.1 Standard solutions preparation:-

Standard solutions were prepared by dissolving 50 mg of vitamin B₁, B₆ and 10 mg of B₁₂ in 50 ml of deionized water. Further dilution was made by transferring 6.25ml of the solution in 25ml volumetric flask and filled with methanol to the mark. The working concentrations of vitamin B₁, B₆ and B₁₂ were 250, 250 and 50 ng/μl respectively.

The standard solutions were prepared to reach a concentration range 250-2050 ng/μl for vitamin B₁ and B₆, 50-1050 ng/μl for vitamin B₁₂. Different volumes (1, 2.8, 4.6, 6.4 and 8.2 μl) were applied to give a concentration of 250, 700, 1150, 1600 and 2050 ng/μl for vitamin B₁ and B₆ and (1, 6, 11, 16 and 21μl) to give 50, 300, 550, 800 and 1050 ng/μl for vitamin B₁₂. In order to protect the degradation of the vitamins, the working solutions were protected from direct light by using amber colored volumetric flasks at 5⁰C. In addition sample application and chromatographic development were done under subdued light.

3.4.2 Samples preparation

(a) Vitamin B₁, B₆ and B₁₂ injection:-

The contents of ten ampoules were collected and the average volume of one ampoule was calculated. The average volume of one ampoule (3 ml) was measured with analytical balance and transferred to 10 ml volumetric flask. The volume was made up to the mark by using methanol. For analysis of B₁ and B₆ further dilutions were made by transferring 5 ml of the sample solution in 50 ml volumetric flask and diluted with deionized water and again 6.25 ml was transferred to 25 ml volumetric flask. For analysis of B₁₂ the 5 ml sample solution was diluted with methanol in 10ml volumetric flask. The working concentrations of vitamin B₁, B₆ and B₁₂ were 250, 250 and 50 ng/μl respectively.

(b) Vitamin B₁ and B₆ tablet:-

Twenty tablets were weighed and finely powdered. Weighed amount of the powder equivalent to one tablet was transferred to 100 ml volumetric flask. An amount about 80 ml of deionized water was added and the solution was shaken well for at least 10 minutes

and sonicated for additional 5 minutes. The volume was made up to the mark with deionized water and the contents of the flask were then centrifuged for 10 minutes in 3000 revolution per minute (r.p.m.) An aliquot of 6.25ml of the clear supernatant was withdrawn and transferred to 25 ml volumetric flask and diluted with methanol to the mark. The concentrations of vitamin B₁ and B₆ after dilution were 250, 500 ng/μl respectively.

The solvents in the above solutions contain deionized water and methanol. Deionized water is a solvent in which the B-vitamins readily be dissolved. In order to protect the diffusion of spots and also to shorten the development time, methanol was used as a solvent in the dilution process.

3.4.3 Instrumentation and chromatographic conditions

The samples were spotted in the form of bands of width 6 mm with a micro litre syringe on precoated silica gel 60F₂₅₄ plate (10 cm x 10 cm with 200 μm thickness, using a Linomat V. A constant application rate of 0.1μl/s was employed and space between two bands was 15 mm. The slit dimension was kept at 5 mm x 0.45 mm, and 20 mm/s scanning speed was employed. The mobile phase consisted of methanol-5 M ammonium acetate (10:1v/v) [46]. Linear ascending development was carried out in twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 40 minutes at room temperature. The length of chromatogram run was 8 cm. Subsequent to the development; TLC plates were dried in a current of air with the help of an air-dryer.

Densitometric scanning was performed on TLC scanner III in the absorbance mode at 255, 290 and 361nm. The source of radiation utilized was deuterium lamp that emits a continuous UV spectrum between 190 and 400 nm. Concentrations of chromatographed compound were determined from the intensity of diffusely reflected light.

3.5 Method Validation:-

The method was validated in compliance with ICH guidelines [47, 48]. The following parameters were validated.

3.5.1 Linearity

The linearity of responses for vitamins B₁ and B₆ was assessed in the range of 250-2050 ng/μl and for vitamin B₁₂ was in the range 50-1050 ng/μl. Five different concentrations of the standards solutions were applied five times to study the linearity.

3.5.2 Accuracy and recovery studies

Both accuracy and recovery were studied. Accuracy of the method was tested by taking six determinations of each component in analytical concentration. The recovery study was carried out by addition of known amounts of standards to the product. Standards added were 80%, 100% and 120% of the label claim. Three determinations were done to study the recovery. The % recovery of vitamins B₁, B₆ and B₁₂ was compared with the actual amounts.

3.5.3 Precision

3.5.3.1 System precision

The system precision study basically focuses on the exactness of the instrument. Repeatability of sample application and measurement of the peak area were studied. Six determinations at a concentration of 700 ng/μl, for vitamin B₁, B₆ and 300 ng/μl for vitamin B₁₂ were applied. The repeatability of sample application and the repeatability of measurement of the peak area were evaluated by comparing their coefficient of variations which are obtained from the peak area measurements.

3.5.3.2 Method precision

To study the precision of the method, both intra - day and inter - day precision were applied. Intra day precision was studied by taking three different concentrations 700,1150 and 1600 ng/ μ l of vitamin B₁, B₆ and 300, 550 and 800 ng/ μ l of vitamin B₁₂ were applied three times to see variation in their peak areas with in a day. For inter-day precision the same concentrations were applied but their peak area variation was studied for three different days.

3.5.4 Limit of detection and quantification

Detection and quantification limits were calculated from the calibration equations obtained from the experiment. The determinations of the detection and quantitation limits were based on the standard deviation of the response and the slope. The slope was estimated from the calibration curve of the analyte and the estimate of the standard deviation was carried out from the standard deviation of the y-intercept.

3.5.5 Robustness

In order to study the robustness of the method, slight but deliberate changes were made in some parameters. Parameters such as; the mobile phase composition, total mobile phase amount, time from application to development and time from development to scanning were used to study the robustness. Concentrations of 700, 1150 and 1600ng/ μ l for vitamin B₁ and B₆ and 300, 550 and 800 ng/ μ l for vitamin B₁₂ were applied for the analysis.

4. Results and Discussion

4.1 Method optimization

4.1.1 Mobile phase composition

Selection of appropriate mobile phase is a major step in HPTLC method development. Though selection of the most suitable mobile phase system is difficult, literature and monographs of pharmacopoeias offer useful information. Firstly, different mobile phase systems have been tried to separate the binary mixture of vitamin B₁ and B₆. Secondly ternary mixtures of vitamins B₁, B₆ and B₁₂ were evaluated to arrive at an optimum resolution, as shown in table 4.1. Separation of mixtures basically relies on the polarity of the components, mobile phase and the stationary phase. For example thiamine hydrochloride is a polar compound in nature; usually it requires highly polar mobile phase system so as to compete with the polar stationary phase. But unlike thiamine hydrochloride, pyridoxine hydrochloride is less polar and it easily moves with high polar solvent system. Usually it goes up with the solvent front. Due to this reason one of the most polar compound (thiamine hydrochloride) remains at the application point or moves a short distance while the least polar compound (pyridoxine hydrochloride) travel a near distance with the solvent front in the same mobile phase. In order to get the best mobile phase combination, it was necessary to change the polarity and the proportions of the components. By changing the polarity and amount of the mobile phase a number of trials were being made. Mobile phase combination containing methanol: 5 M ammonium acetate (v:v), (10:1) gave best separation of the mixture. The R_f values were 0.20 ± 0.02, 0.40 ± 0.02 and 0.68 ± 0.02 for thiamine hydrochloride, pyridoxine hydrochloride and cyanocobalamine respectively.

In Table 4.1, the polarity of the mobile phase was changed by changing one of the components in the mobile phase and a significant change in the R_f values of the B-vitamins were observed. For example methanol: water (7:3) combination has given a closer R_f value for vitamin B₆ and B₁₂. In order to separate these two compounds ethanol:water (8:4) combination was tested because no significant difference was observed by changing the volume ratio of water-methanol.

Table 4.1 the different mobile phase combinations used for method development [39, 47].

No	Mobile phase	Ratio (v:v)	Vitamin B ₁	Vitamin B ₆	Vitamin B ₁₂
1	Methanol-water	7:3	0.34	0.80	0.76
2	Ethanol-water	8:4	0.09	0.80	0.70
3	1-Butanol-acetic acid- ammonia	7:5:1	0.38	0.81	0.73
4	1-Butanol-acetic acid- ammonia -water	7:5:1:1	0.30	0.59	0.52
5	n-propanol-acetic acid- ammonia -water	7:5:1:1	0.32	0.55	0.58
6	Methanol-(5M)ammonium acetate	10:1	0.20	0.40	0.68
7	Acetonitrile-water	7:3	0.49	0.65	0.50
8	Toluene-methanol-acetone- acetic aid	7:2:0.5:0.5	0.08	0.18	0.08

4.1.2 Appropriate conditions for validation

The following conditions were used for method validation:

- Precoated silica gel glass plate 60 F₂₅₄ (10 cm×10 cm with 200µm thickness).
- Methanol: Ammonium acetate (5M) (10: 1 V/V) as mobile phase.
- Application rate of the micro syringe 0.1 µl/s.
- Chamber saturation time 40 minutes.
- Lengths of chromatogram run 8 cm.
- Detection wave lengths of 255, 290 and 361 nm for vitamin B₁, B₆ and B₁₂, respectively.

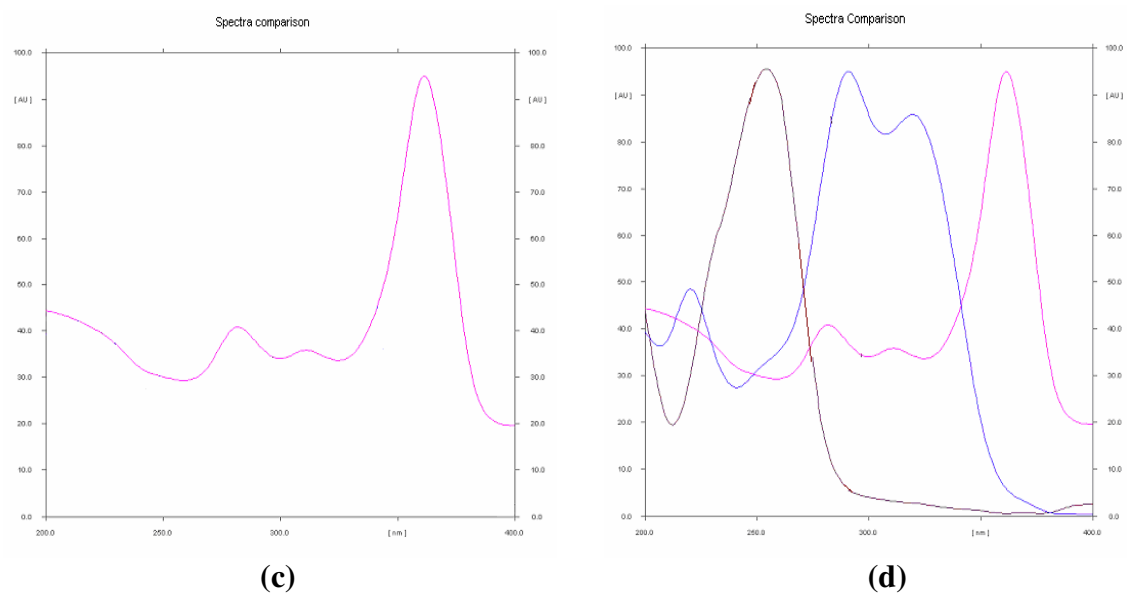
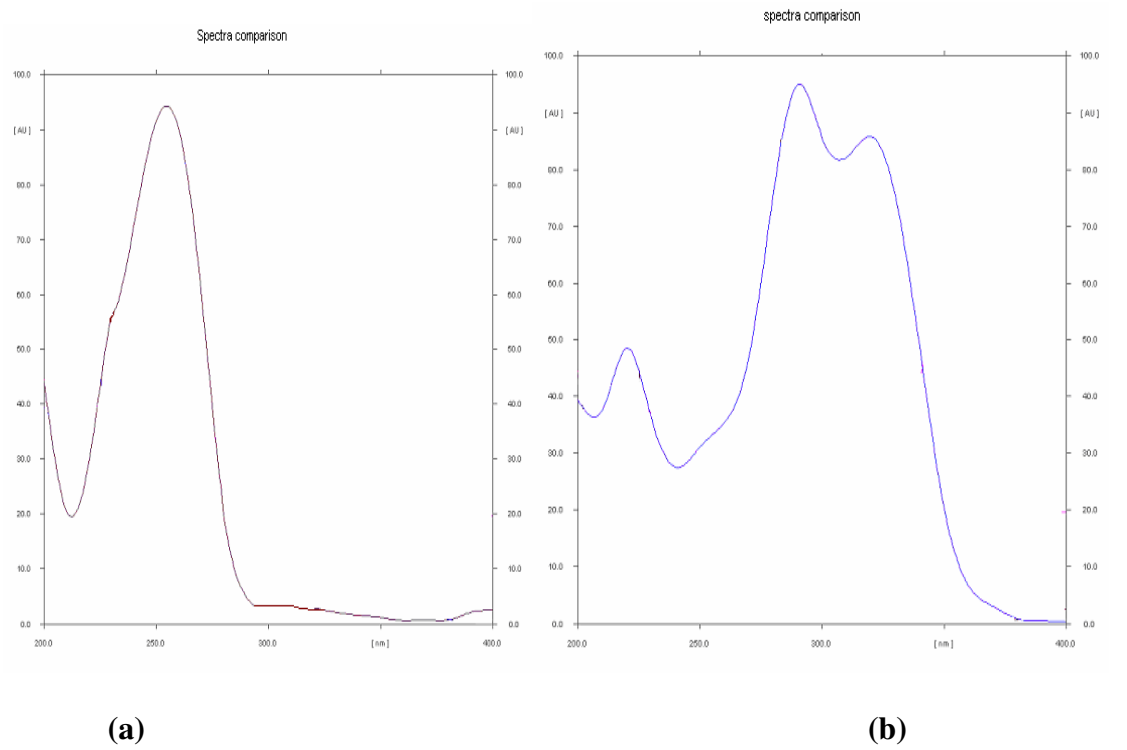
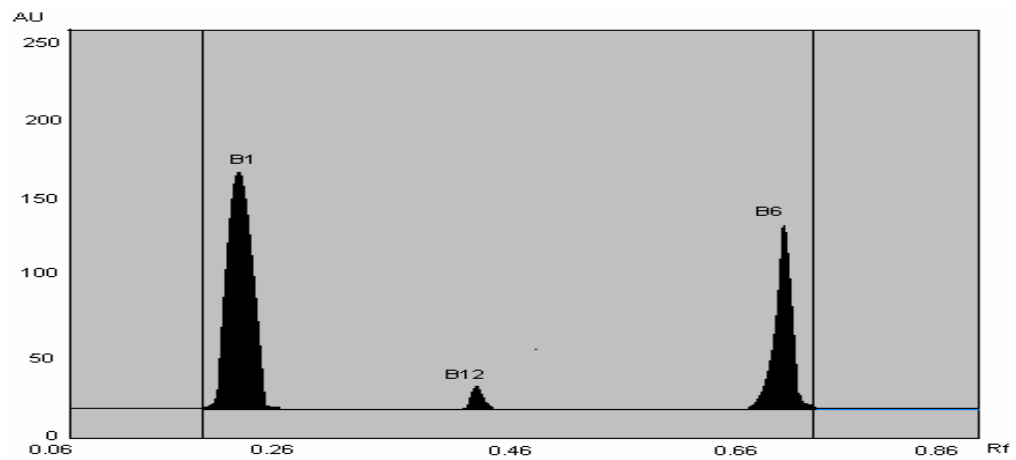
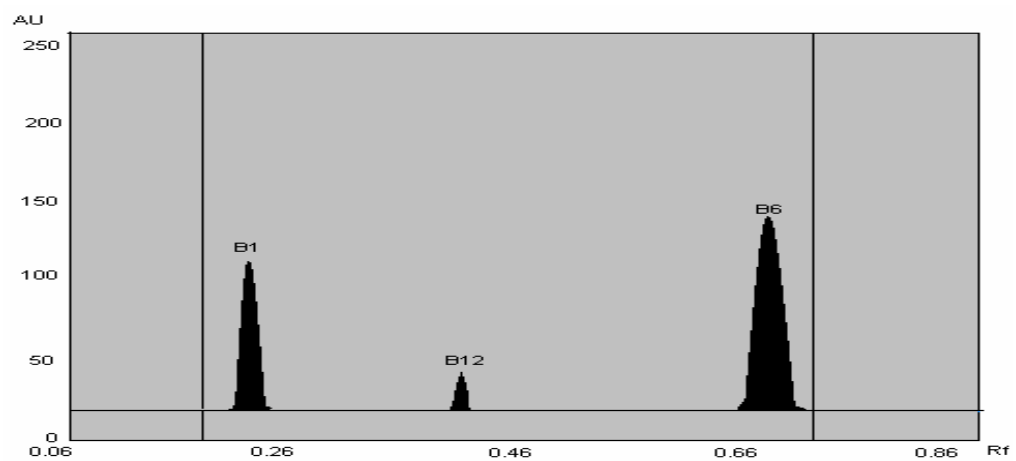


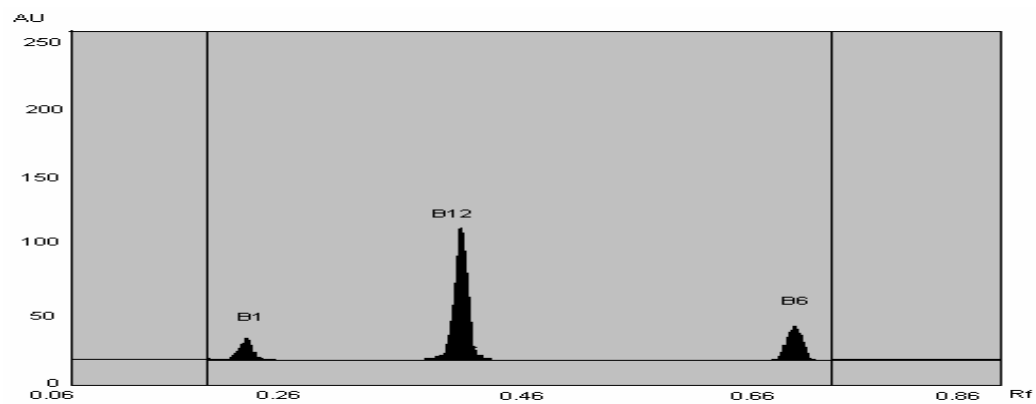
Figure 4.1 UV spectra for of vitamin B₁ ,700 ng/μl (a), vitamin B₆ ,700 ng/μl (b), vitamin B₁₂ ,300 ng/μl (c) and their mixture (d) at 255, 290 and 361nm respectively.



(a)



(b)



(c)

Figure 4.2 HPTLC chromatogram for a mixture vitamin B₁, 700 ng/μl, vitamin B₆, 700 ng/μl and B₁₂, 300 ng/μl measured at 255, 290 and 361nm respectively.

Figure 4.1 shows the UV spectra of vitamin B₁, B₆ and B₁₂ and their maximum absorbance at 255, 290 and 361nm respectively.

One of the advantages of using TLC scanner is not only giving quantitative results, it also involved in qualitative analysis by giving maximum wave length (λ_{\max}) of the given compound. Both the standards and samples gave similar λ_{\max} with the literature.

The λ_{\max} for B₁, B₆ and B₁₂ was 255, 290 and 361nm respectively. The overlapped spectra in Figure 4.1(d) shows similar spectra as in fig 4.1 (a), (b) and (c) this shows that there was no interference in the mixture of B₁, B₆ and B₁₂.

Figure 4.2 shows the absorbance versus R_f graph of vitamin B₁, B₆ and B₁₂ and they have shown maximum absorbance at 255, 290 and 361nm respectively. The TLC scanner read the absorbance (peak area) at a given wavelength. The exact peak area (absorbance) can be obtained at a specific wavelength (λ_{\max}) for each compound. In case of vitamin B₁, B₆ and B₁₂ they have got a specific wave length (λ_{\max}) at 255, 290 and 361nm respectively. One cannot get the exact peak area for vitamin B₁ by reading at 361nm. Because the absorbance of B₁ (at 361nm) is very small as compared with the absorbance at (255nm). Refer to figure 4.2 (a) and (c).

4.2 Method validation result

4.2.1 Linearity

The peak area (response) versus concentration of each vitamin B₁, B₆ and B₁₂ was observed. The linearity was tested and found to be less precise as shown below (figure 4.3, 4.4 and 4.5) and make refer also to table 4.5 and 4.6.

Table 4.2 Calibration data of vitamin B₁.

Concentration (ng)	Vitamin B ₁				
	Peak area (AU)				
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
250	1700.23	1732.58	1688.78	1740.10	1715.42
700	4750.17	4784.06	4722.34	4780.69	4759.32
1150	7800.11	7829.17	7791.42	7850.41	7817.78
1600	10700.41	10799.81	10682.38	10763.74	10736.59
2050	13200.17	13235.46	13179.08	13232.20	13211.73

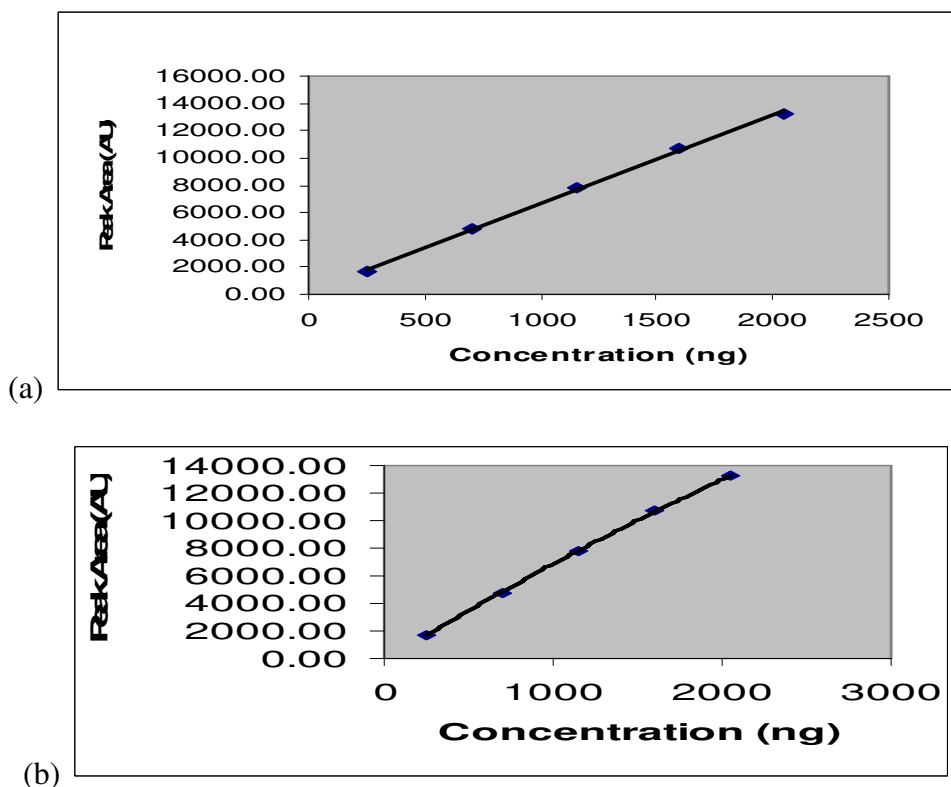


Fig. 4.3 Calibration curves for vitamin B₁ (a) linear and (b) polynomial fits for the same given data.

Table 4.3 Calibration data of vitamin B₆.

Concentration (ng)	Vitamin B ₆				
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
250	1350.23	1341.25	1365.36	1357.48	1353.58
700	4602.25	4645.59	4605.24	4669.91	4630.75
1150	7404.69	7428.02	7434.39	7402.84	7417.49
1600	10050.36	10071.33	10080.47	10044.77	10061.73
2050	11801.87	11827.96	11835.81	11706.96	11793.15

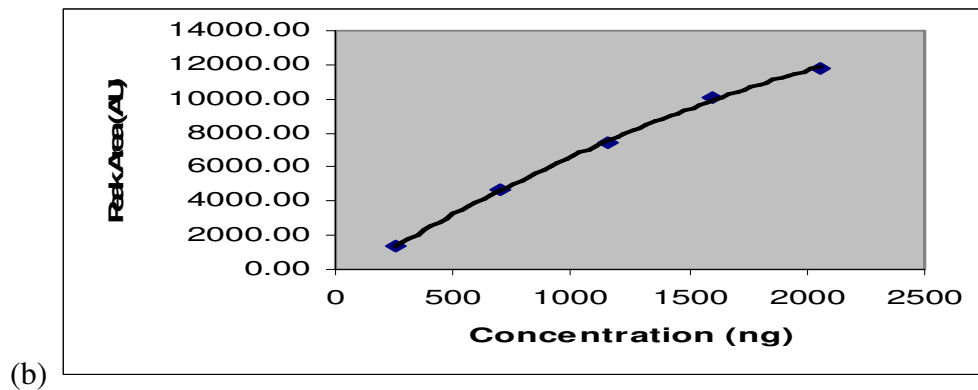
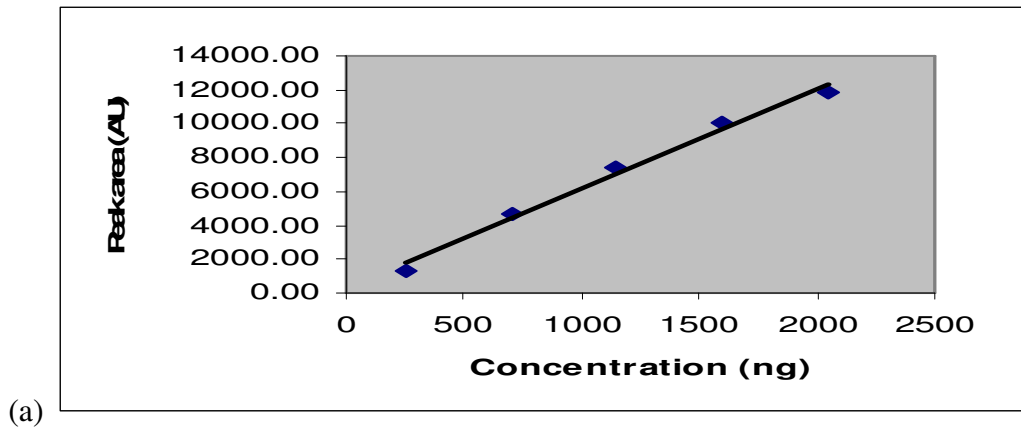


Figure 4.4 Calibration curves for vitamin B₆ (a) linear and (b) polynomial fits for the same given data.

Table 4.4 Calibration data of vitamin B₁₂.

Concentration (ng)	Vitamin B ₁₂				
	Peak Area (AU)				
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
50	505.32	515.34	500.65	530.79	513.02
300	2430.36	2460.39	2430.66	2425.52	2436.73
550	4240.15	4248.74	4248.66	4198.68	4234.05
800	5720.44	5736.15	5728.33	5743.81	5732.18
1050	7200.36	7248.2	7210.94	7130.87	7197.59

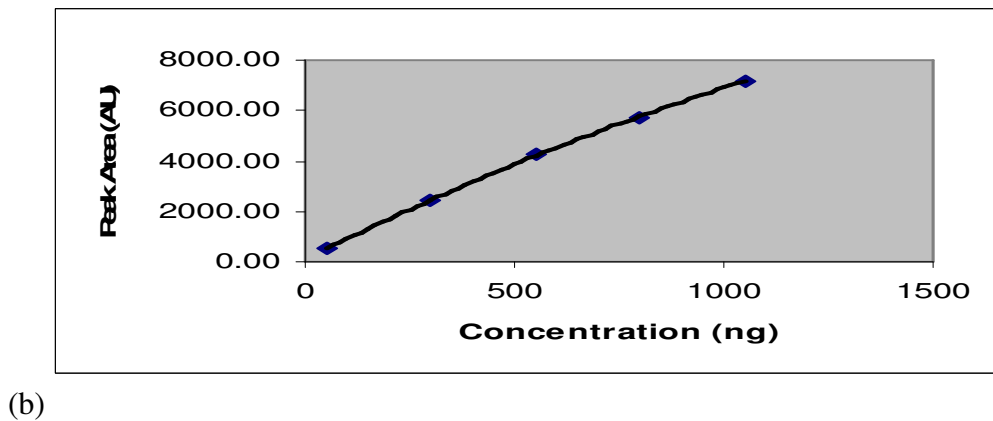
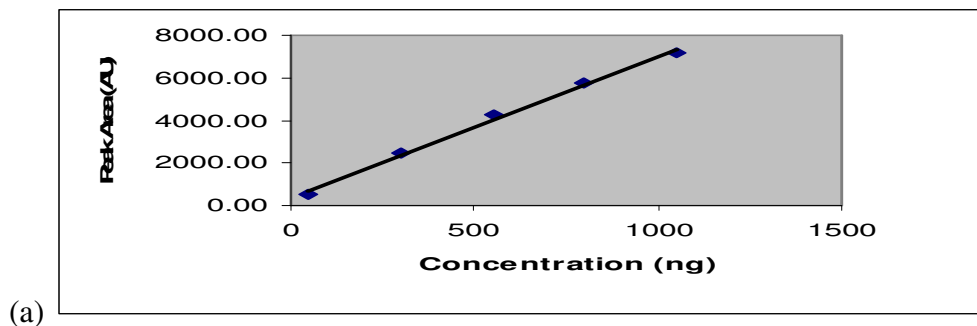


Fig. 4.5 Calibration curves for vitamin B₁₂ (a) linear and (b) polynomial fits for the same given data.

The second order polynomial fit was found to be more suitable and more precise as its residuals plot showed a much better fitting than that for linear model, indicating good correlation (figure 4.3b, 4.4b and 4.5b).

The calibration graphs were constructed in the range of 250–2050 ng/μl for vitamin B₁, B₆ and of 250–1050 ng/μl for vitamin B₁₂. The linear and the second order polynomial regression equation parameters are shown in tables 4.5 and 4.6 respectively.

Table 4.5 Characteristic parameters for the linear regression equation (*n*=5)

Parameters	Vitamin B ₁	Vitamin B ₆	Vitamin B ₁₂
Linearity range (ng/μl)	250-2050	250-2050	50-1050
*Coefficient of X ± S.D	6.4378 ± 0.0079	5.8467 ± 0.0355	6.66584 ± 0.0402
Intercept ± S.D	244.75 ± 24.7483	327.64 ± 30.5138	356.51 ± 11.5664
Determination coefficient <i>r</i> ²	0.9984	0.9888	0.9961
Correlation coefficient <i>r</i>	0.9990	0.9940	0.9980

* Coefficient of X = slope of the graph

Table 4.6 Characteristic parameters for the second order polynomial regression equation of the HPTLC-densitometric method (*n*=5).

Parameters	Vitamin B ₁	Vitamin B ₆	Vitamin B ₁₂
Linearity range (ng/μl)	250-2050	250-2050	50 -1050
Coefficient X ² ± S.D	0.0005 ± 5.7735E- 05	0.0011 ± 5E- 05	0.0014 ± 0.0001
Coefficient X ± S.D	7.4739 ± 0.0315	8.4704 ± 0.0596	8.1943 ± 0.0341
Intercept ± S.D	168 ± 16.0312	718 ± 10.4747	109.88 ± 12.9100
Determination coefficient <i>r</i> ²	0.9997	0.9995	0.9999
Correlation Coefficient <i>r</i>	0.9998	0.9997	0.9999

4.2.2 Accuracy (% recovery)

As indicated in table 4.7, good recoveries of the product in the range of 98.16 to 99.13 %, 97.80 to 99.08 % and 98.44 to 99.69 % were obtained for vitamin B₁, B₆ and B₁₂ respectively of the injection and 97.60 to 99.12 %, 98.03 to 99.14 % for vitamin B₁ and B₆ of the tablet. Figure 4.6 also shows the graph of % added of the standard versus the total amount obtained.

The accuracy graph shows the linear relation ship between the added standards (80, 100, 120 %) and the obtained amount (ng).

Table 4.7 Recovery study (using standard addition technique) for determinations of (a) Vitamin B₁, B₆ and B₁₂ injection and (b) vitamin B₁ and B₆ tablet.

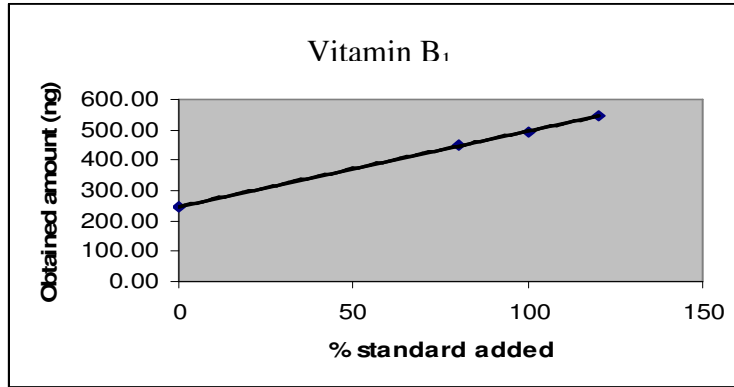
(a)

Vitamin B₁, B₆ and B₁₂ injection				
Vitamin B ₁	Theoretical content (ng)	% recovery	*C.V.	
0	700	98.16	0.89	
80	1260	99.13	0.62	
100	1400	99.11	0.59	
120	1540	99.13	0.66	
Vitamin B ₆				
0	700	97.80	0.94	
80	1260	99.08	0.85	
100	1400	99.92	1.05	
120	1540	98.52	0.62	
Vitamin B ₁₂				
0	300	98.44	0.53	
80	540	99.25	0.65	
100	600	99.55	0.43	
120	660	99.69	0.38	

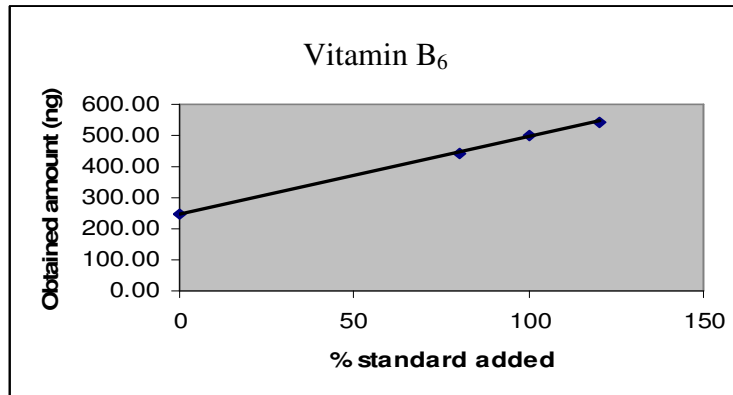
(b)

Vitamin B₁ and B₆ tablet				
Vitamin B ₁	Theoretical content (ng)	% recovery	*C.V.	
0	300	97.60	0.79	
80	540	99.12	0.67	
100	600	99.06	0.66	
120	660	98.86	0.96	
Vitamin B ₆				
0	600	98.83	0.96	
80	1080	99.14	0.57	
100	1200	98.03	0.82	
120	1320	99.14	0.53	

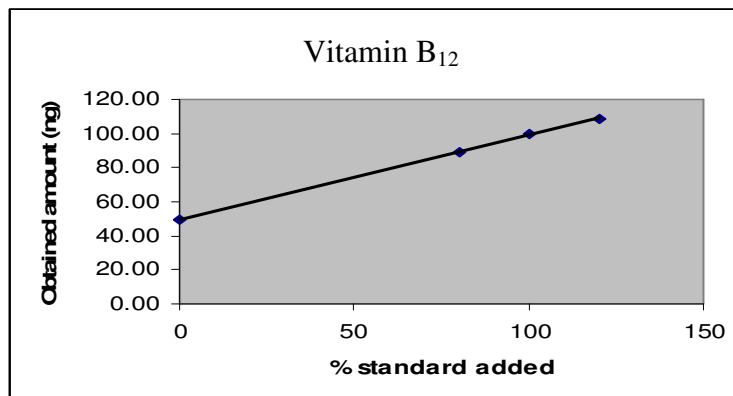
* C.V. = Coefficient of variation



(a)



(b)



(c)

Figure 4.6 Standard addition of vitamin B₁ (a) Vitamin B₆ (b) and Vitamin B₁₂ (c)

4.2.3 Precision

Data obtained from precision experiments for repeatability studies are given in table 4.8. The repeatability of sample application and measurement of peak area were expressed in terms of coefficient of variation. The results show that the repeatability of measurement of area is more precise than the repeatability of sample application. Since their C.V. is less than 2.0 % it is possible to conclude that the instrument has good precision.

The repeatability of the instrument (sample applicator and scanner) was considered in this study.

Table 4.8 System precision studies of the developed method

	Sample application			Measurement of area		
	B ₁ (700ng/μl)	B ₆ (700ng/μl)	B ₁₂ (300ng/μl)	B ₁ (700 ng/μl)	B ₆ (700 ng/μl)	B ₁₂ (300 ng/μl)
1	4761.32	4609.47	2435.91	4725.23	4645.41	2435.17
2	4769.58	4618.48	2445.42	4732.55	4670.66	2431.54
3	4749.37	4628.66	2428.35	4740.64	4596.47	2428.64
4	4758.48	4700.39	2440.24	4711.61	4612.39	2406.98
5	4690.47	4615.11	2452.37	4702.88	4654.34	2445.61
6	4760.11	4664.67	2400.17	4760.37	4630.64	2412.28
Mean*	4748.22	4639.46	2433.74	4727.05	4634.82	2426.87
S.D.**	29.02	35.77	18.37	20.64	27.45	14.51
C.V.***	0.61	0.77	0.75	0.44	0.59	0.6

*Mean = Mean of the peak area; **S.D. = Standard deviation; ***C.V. = Coefficient of variation.

The data in table 4.9 shows the intra and inter day variation for the determination of vitamin B₁, B₆ and B₁₂ at two different concentration levels.

Intraday precision used to describe the variation of the method with in the same day, while inter day precision is for variation between different days. The experimental results show that the method is precise and their coefficient of variations is in the limited range.

Table 4.9 Intra-day and inter-day precision

Intra day precision					Inter day precision								
Amount (ng)	Mean area	S.D.	C.V.	Mean area	Day 1			Day 2			Day 3		
					S.D.	C.V.	Mean area	S.D.	C.V.	Mean area	S.D.	C.V.	
B ₁	700	4741.93	12.63	0.27	4741.93	12.63	0.27	4712.27	31.68	0.67	4763.94	24.94	0.52
	1150	7810.60	14.06	0.18	7810.60	14.06	0.18	7832.05	28.84	0.37	7831.05	32.12	0.41
	1600	10751.34	16.62	0.15	10751.34	16.62	0.15	10758.54	49.02	0.46	10761.57	35.94	0.33
B ₆	700	4667.42	24.65	0.53	4667.42	24.65	0.53	4619.68	26.30	0.57	4660.66	28.49	0.61
	1150	7434.23	26.28	0.35	7434.23	26.28	0.35	7426.44	29.90	0.40	7449.64	29.18	0.39
	1600	10062.90	23.27	0.23	10062.90	23.27	0.23	10064.24	33.04	0.33	10075.36	25.61	0.25
B ₁₂	300	2461.06	16.75	0.68	2461.06	16.75	0.68	2533.96	19.17	0.76	2478.06	19.05	0.76
	550	4230.73	17.91	0.42	4230.73	17.91	0.42	4228.72	19.05	0.45	4237.18	18.99	0.45
	800	5752.60	20.93	0.36	5752.60	20.93	0.36	5732.66	23.82	0.42	5751.61	24.43	0.42

4.2.4 Limit of detection and quantification (LOD and LOQ)

The limit of detection and limit of quantification of the proposed method are shown in table 4.10, which were calculated according to $3.3 \times S.D/S$ and $10 \times S.D/S$ criterions, respectively, where S.D is the standard deviation of the absorbance ($n=5$) of the sample and S is the slope of the corresponding calibration curve. The LOD of vitamin B₁, B₆ and B₁₂ were found to be 7.078, 4.081 and 5.1991 ng/ μ l, respectively. These were the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The LOQ of vitamin B₁, B₆ and B₁₂ were 21.449, 12.366 and 15.755 ng/ μ l, respectively. These were the lowest concentration of drugs, accurately detected and integrated by the instrument.

4.2.5 Robustness

The robustness of analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. In this study influence of variations in mobile phase composition, amount of mobile phase, time from spotting to development and time from development to scanning were considered.

- (i) The mobile phase composition was varied by changing the volume ratio of methanol: ammonium acetate (10:0.5), methanol: ammonium acetate (10:1.5), methanol: ammonium acetate (9:1), methanol: ammonium acetate (11:1).
- (ii) The amount of mobile phase was varied by $\pm 5\%$ of methanol: ammonium acetate (10:1).
- (iii) Time from spotting to development was studied at different time intervals, 0, 20 and 40 minutes.
- (iv) Time from development to scanning was studied at different time intervals, 0, 20 and 40 minutes.

The standard deviation of peak areas was calculated for each parameter and their coefficient of variations (C.V.) were found to be less than 2%. The low values of coefficient of variations as shown in table 4.11 indicated if the proposed method is robust.

Among the parameters chosen to evaluate robustness very little variation was made on mobile phase composition, which was found to be a sensitive parameter during the optimization process.

Table 4.10 Experimental variables used in the robustness study

Variable	Level		
	Lower range	Upper range	Optimized
Mobile phase combination			
Methanol: Ammonium acetate (10: 1)	9.5:0.5	10.5-1.5	10:1
Amount of mobile phase (ml.)	10.0	12.0	11.0
Time from spotting to development (min.)	0	40	5
Time from development to scanning (min.)	0	40	5

Table 4.11 Robustness testing of the developed method ($n=3$).

Parameters	Vitamin B ₁		Vitamin B ₆		Vitamin B ₁₂	
	S.D.*	C.V.**	S.D.	C.V.	S.D.	C.V.
Mobile phase composition	36.00	0.50	37.28	0.56	21.34	0.57
Amount of mobile phase	28.44	0.42	31.61	0.49	16.12	0.44
Time from spotting to development	28.11	0.39	25.09	0.38	13.33	0.34
Time from development to scanning	28.8	0.42	25.01	0.38	12.00	0.33

* S.D = Standard deviation of peak area.

** C.V.= Coefficient of variation (Average of three concentrations 700, 1150 and 1600ng/μl of vitamin B₁, B₆ and 300, 550 and 800 ng/μl of vitamin B₁₂).

Changing some parameters, such as amount of mobile phase, time from spotting to development and time from development to scanning caused a slight change in the result obtained. A significant difference was observed with the change of mobile phase composition.

Table 4.12 Summary of method validation parameters

Parameter	B ₁	B ₆	B ₁₂
Linearity range(ng/spot)	250-2050	250-2050	50-1050
Correlatrion coefficient	0.9998	0.9997	0.9999
Limit of detection (ng/spot) LOD	7.078	4.0809	5.1991
Limit of quantification LOQ	21.4496	12.366	15.755
Recovery (Mean ± S.D.)	98.88 ± 1.72	98.83±1.74	99.23±1.15
<u>Precision (C.V.)</u>			
Repeatability of application (n=6)	1.05	1.01	1.23
Repeatability of measurement (n=6)	0.44	0.59	0.62
Intra-day *	0.24	0.45	0.56
Inter-day **	0.48	0.52	0.78
Robustness	robust	robust	robust

* Mean of three concentrations in the same day.

** Mean of three concentrations in three different days.

4.2.6 Analysis of the marketed formulation

The proposed method was applied to the determination of vitamin B₁, B₆ and B₁₂ in commercial pharmaceuticals. Vitamin B₁, B₆ and B₁₂ injections and vitamin B₁ and B₆ tablets were selected for the analysis. Six replicates of determinations were made and satisfactory results were obtained for both drugs and were in a good agreement with the labeled claims. The low C.V. value indicated the suitability of this method for routine analysis of vitamin B₁, B₆ and B₁₂ in pharmaceutical dosage forms. The results obtained from the HPTLC method were compared with that of the HPLC method [54]. As shown in table 4.14b statistical evaluation was made to compare the accuracy of vitamin B₁, B₆ and B₁₂ in dosage form. Student's t-test was used to compare the means of the two methods and F-value was used to compare their standard deviations. In both statistical tests the calculated values are less than the theoretical or critical values hence there was no significant difference between the two methods.

Table 4.13 HPTLC analysis of the marketed formulations;

(a) Vitamin B₁, B₆ and B₁₂ injection

	Actual mount (ng)	Obtained amount (ng)	% recovery	*S.D.
Vitamin B ₁	700	690.89	98.70	1.91
	1150	1140.88	99.21	1.23
	1600	1590.43	99.40	0.97
				1.37
Vitamin B ₆	700	695.91	99.42	1.61
	1150	1149.58	99.96	1.36
	1600	1594.68	99.67	1.51
				1.50
Vitamin B ₁₂	300	297.42	99.14	1.17
	550	548.03	99.64	1.26
	800	795.75	99.47	1.34
				1.26

(b) Vitamin B₁ and B₆ tablet

	Amount (ng)	Obtained amount (ng)	% recovery	*S.D.
Vitamin B ₁	700	694.22	99.17	1.15
	1150	1141.88	99.29	1.19
	1600	1597.82	99.86	1.56
				1.30
Vitamin B ₆	700	1399.93	100.00	1.47
	1150	2297.34	99.88	1.34
	1600	3199.16	99.97	1.65
				1.49

Table 4.14; Applicability of the proposed methods for the determination of B₁, B₆ and B₁₂ in commercial pharmaceuticals (*n*=6).

(a) Dosage analysis (accuracy).

	Vitamin B ₁		Vitamin B ₆		Vitamin B ₁₂	
	HPTLC (%)	HPLC (%)	HPTLC (%)	HPLC (%)	HPTLC (%)	HPLC (%)
1	97.62	98.86	99.46	97.67	98.77	98.41
2	98.47	97.86	99.17	98.66	99.11	99.34
3	99.01	98.42	99.62	98.66	99.55	99.45
4	99.31	98.66	99.86	98.06	99.40	99.12
5	99.10	98.32	99.17	99.90	99.11	99.20
6	99.22	98.64	100.10	98.19	100.10	99.33

(b) Statistical comparisons between HPTLC and HPLC

	Vitamin B ₁		Vitamin B ₆		Vitamin B ₁₂	
	HPTLC	HPLC	HPTLC	HPLC	HPTLC	HPLC
Label claim(mg)	100	100	100	100	1.00	1.00
Drug content (%)	98.78	98.46	99.56	98.52	99.34	99.14
S.D	1.37	1.22	1.50	1.51	1.26	1.10
*t-stat	1.04		2.47		1.36	
F-value	3.37		4.27		1.48	

. * The theoretical values for *t*- and *F*-values are equal to 2.57 and 5.05, respectively (*P* =0.05).

Results of analysis of the dosage form containing vitamin B₁, B₆ and B₁₂ given in Table 4.13 indicate that the proposed method can be successfully used to estimate vitamin B₁, B₆ and B₁₂ when present in combination in pharmaceuticals. In addition the results of analysis of the commercial formulations and the recovery study suggested that there was no interference from any excipients, which are normally present in tablets and injections.

4.3 Stability in sample solution

The time the sample is left in solution prior to chromatographic development can influence the stability of separated spots and are required to be investigated. Solutions of different concentrations 700ng/μl for B₁, B₆ and 300ng/μl for B₁₂ were prepared and stored at room temperature for 1.0, 4.0 and 8.0 hours respectively. They were then applied on the HPTLC plate, after development the densitogram was evaluated to check out the presence of additional spots. So there was no indication of compound instability during the analysis. Also Table 4.15 shows a C.V. value less than 2% which indicates that the solution is stable for eight hours. However, it can be suggested that, the increasing the value of C.V with time is an indication that the solution will lose its stability after some time.

Table 4.15 Stability of vitamins B₁, B₆ and B₁₂ in sample solutions (*n*=3)

Time (hour)	Amount (ng)	Peak area 1 (AU)	Peak area 2 (AU)	Peak area 3 (AU)	Mean area (AU)	S.D.	C.V
B ₁ 1hr.	700	4750.23	4746.14	4728.00	4741.46	11.83	0.24
		4755.88	4705.36	4739.34	4733.53	25.76	0.54
		4730.57	4701.68	4754.21	4728.82	26.31	0.55
					4735.71		0.44
B ₆ 1hr.	700	4630.31	4618.31	4647.37	4632.00	14.60	0.31
		4649.21	4600.03	4624.28	4624.51	24.59	0.53
		4640.23	4594.23	4578.37	4604.28	32.13	0.69
					4620.26		0.51
B ₁₂ 1hr.	300	2440.21	2456.59	2433.68	2443.49	11.80	0.48
		2420.36	2401.70	2445.36	2422.47	21.91	0.90
		2434.20	2401.32	2382.39	2405.97	26.22	1.09
					2423.98		0.82

4.4 Comparison between the HPTLC and HPLC methods

There are some conditions which make HPTLC more preferable than HPLC.

HPLC usually requires solvents which are more expensive (HPLC grade) as compared with analytical grade solvents in HPTLC. Sample preparation such as filtering the solutions and preparing a series of dilutions make the HPLC more laborious. In HPTLC many samples can be analyzed using a single plate in a given solvent at a time and also the instrument is simpler to manipulate as compared with HPLC.

As shown in table 4.16 and 4.17 It was possible to confirm that the proposed method was both time and cost effective as HPLC method.

Table 4.16 Cost comparison between the HPTLC and HPLC for a single run [50].

Chemicals Used for HPTLC	Cost (Birr)	Chemicals used for HPLC	Cost (Birr)
Methanol (100 ml)	200.00	Triethylamine (150ml)	300.00
5M Ammonium acetate (1ml)	100.00	Acetonitrile (10 ml)	200.00
Total	300.00		500.00

Table 4.17 Time comparison between the HPTLC and HPLC for a single run.

Process	HPTLC (min.)	HPLC (min.)
Degassing and equilibrating the UV detector	-	30
Run (development) time	15	15
Saturation time	40	-
Sample preparation	20	40
Time for scanning	2	-
Application of sample	2	-
Total	81	85

5. Conclusion

In the preceding method, determination of vitamin B₁-B₆-B₁₂ levels in tablets and injections, using HPTLC-densitometric method has been applied. The method is successful for vitamin B₁-B₆-B₁₂ formulations even in the ratios between vitamin B₁₂ and others 1:100.

This method has several advantages. Similar to high performance liquid chromatography, the new method-high performance thin layer chromatography (HPTLC) needs less time and cost. The method requires small amount of mobile phase and the instrument is easier to handle as compared with HPLC.

The method was validated under international conference on harmonization (ICH) guidelines so that it is applicable in laboratories. The proposed method - HPTLC reduces the duration of analysis and appear to be suitable for routine determination of vitamin B₁, B₆ and B₁₂ simultaneously in pharmaceutical preparations.

Since the method provide a simple, accurate and precise quantitative analysis for determination of vitamin B₁, B₆ and B₁₂ in injections and tablets, it can be applied for routine quality control analysis in pharmaceutical laboratories.

6. Suggestions for further work

Vitamin supplements are available in the market in different forms - tablets, injections, pills etc. Due to the resource limitation only three of the water soluble vitamins are considered in this study hence, it will be essential to focus on the determination of the remaining water soluble vitamins (riboflavin, nicotinamide, folic acid and calcium pantothenate etc.) by using HPTLC- densitometric technique.

Since most water soluble B-vitamins are light sensitive, stability indicating study of vitamin B₁, B₆ and B₁₂ can be recommended, photo degradation can be studied by subjecting those vitamins to light for a longer time intervals.

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