SPECTROPHOTOMETRIC AND SPECTROFLUORIMETRIC METHODS FOR THE DETERMINATION OF THIAMINE IN PHARMACEUTICAL FORMULATIONS

By

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DEDICATION

This work is dedicated to:

My parents,

My husband,

My sons: Amro and Ammar

And:

My friends and colleagues.

Declaration

I hereby declare that this thesis is the original work of the author and has not been submitted for a degree in any other university.

Sulafa Tageldin Abdel Rahman

List of publications

1/ Sulafa Tageldin Abdel Rahman, Abdalla Ahmed Elbashir, Mohamed El-Mukhtar and Mohamed Mustafa Ibrahim,

Development and Validation of Spectrophotometric Method for Determination of Thiamine (VB1) in Pharmaceutical Formulations using 1,2-Naphthoquine-4- Sulphonate (NQS)

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2/ Sulafa Tageldin Abdel Rahman, Abdalla Ahmed Elbashir, Mohamed El-Mukhtar and Mohamed Mustafa Ibrahim

Application of Spectrophotometric methods for the determination of thiamine (VB1) in pharmaceutical formulations using 7-Chloro-4-nitrobenzoxadiazole (NBD-Cl)

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ABSTRACT

The objective of this research is to develop and validate simple and cheap method for determination of Thiamine (vitamin B1) in pharmaceutical formulation by means of Spectrophotometry and Spectrofluorimety. This goal has been accomplished through four methods.

The first method (A1) is a direct Spectrophotometric method based on the reaction of Thiamine with 1,2-Naphthoquinone -4-sulphonic acid sodium salt (NQS) reagent in alkaline medium (pH = 11), to produce a brown-colored product exhibiting maximum absorption peak (λ_{max}) at 487 nm. Under the optimized reaction conditions, Beer's law was obeyed in the range of 10 – 40µg/mL. Linear regression equation of the calibration curve is: A= 0.022x + 0.171 with a linear correlation coefficient of 0.997.

The limit of detection (LOD) and limit of quantification (LOQ) were found to be 1.71 µg/mL and 5.18 µg/mL respectively.

The second method (A2) is a direct Spectrophotometric method based on the reaction between Thiamine and 7-Chloro-4-nitrobenzoxadiazole (NBD-Cl) reagent. In alkaline medium of pH 10.5, a yellowish-brown adduct exhibiting maximum absorption peak (λ_{max}) at 434 nm was produced. Under the optimized reaction conditions, Beer's law was obeyed in the range of 5 – 35µg/mL. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.667 µg/ml and 2.020 µg/ml respectively.

The third method (B1) is Spectrofluorimetric method based on the measurement of the fluorescence activity of the product formed between Thiamine and NBD-Cl in alkaline medium. Excitation was carried out at 472 nm, with emission at 562 nm. All variables affecting the reaction were studied and optimized. Beer's law was obeyed in the concentration range of 0.2 – 1.0 µg/mL.

The fourth method (B2) is also Spectrofluorimetric method based on measuring the relative fluorescence intensity of the product formed when Thiamine reacts with NQS in alkaline conditions. The measurement was carried out with excitation at 390 nm, and emission at 460 nm. All variables affecting the reaction were studied and optimized. Beer's law was obeyed in the concentration range of 0.1 – 1.0 µg/mL. The four methods were validated with respect to accuracy, precision, linearity, sensitivity, limit of detection and limit of quantification according to the International Conference of Harmonization (ICH) guidelines for validation of analytical procedures. They successfully applied to the determination of the drug in its pharmaceutical dosage form with a high accuracy and precision, without interferences from the common pharmaceutical additives.

The proposed methods are simple, sensitive, and they are practical and valuable for the routine application in quality control laboratories for the analysis of the Thiamine.

الملخص

الهدف من هذا البحث هو تطوير وتقييم طريقة بسيطة وغير مكلفة لتحديد الثيامين (فايتمين ب1) في المستحضرات الصيدلانية عن طريق التحليل الطيف ضوئي والطيف تألقي، حيث تم الوصول إلى هذا الهدف بواسطة أربعة طرق:

الطريقة الأولى وهي طريقة طيف مضوائية مباشرة تعتمد على التفاعل بين الثيامين و الملح الصوديومي لنافثكوين حمض السلفونيك (NQS) في وسط قاعدي عند الرقم الهيدروجيني 11 لتكوين ناتج بني اللون يظهر امتصاصية قصوى عند الطول الموجي 478 نانومتر. عند ظروف التفاعل المثلى ينطبق قانون بير في مدى التركيز 10 – 40 ميكروجرام/مل حيث كانت معادلة خط المعايرة هي ($A = 0.022x + 0.171$) ومعامل الارتباط الخطي هو 0.997، حد الكشف 1,71 ميكروجرام/مل وحد القياس 5,18 ميكروجرام/مل.

الطريقة الثانية هي أيضاً طريقة طيف مضوائية مباشرة تعتمد على تفاعل الثيامين مع 7- كلورو 4- نيتروبنزوكسادايزول (NBD-Cl) حيث يتكون في الوسط القاعدي عند الاس الهيدروجيني 10,5 ناتج بني مصفر يظهر امتصاصية قصوى عند الطول الموجي 434 نانومتر.

عند ظروف التفاعل المثلى ينطبق قانون بير لامبر في مدى التركيز 5 – 35 ميكروجرام/مل، حد الكشف 0,667 ميكروجرام/مل و حد القياس 2,020 ميكروجرام/مل.

الطريقة الثالثة طريقة طيف تألقية تعتمد على قياس خاصية التألق الضوئي للمركب الناتج عن تفاعل الثيامين مع 7- كلورو 4- نيتروبنزوكسادايزول (NBD-Cl) في الوسط القاعدي. تم الاثارة عند الطول الموجي 472 نانومتر والانبعاث كان عند 562 نانومتر. كل المتغيرات التي تؤثر على التفاعل تمت دراستها وضبطها حيث انطبق قانون بير في مدى التركيز من 0,2 – 1,0 ميكروجرام/مل.

الطريقة الرابعة هي أيضاً طريقة طيف تألقية تعتمد على قياس كثافة التألق النسبي للمركب الناتج عن تفاعل الثيامين مع (NQS) في الوسط القاعدي. تم اجراء الحث عند الطول الموجي 390 نانومتر والانبعاث كان عند 460 نانومتر. جميع المتغيرات التي تؤثر على التفاعل تمت دراستها وضبطها حيث انطبق قانون بير في مدى التركيز من 0,1 – 1,0 ميكروجرام/مل.

كل هذه الطرق الأربع تم تقييمها من حيث الدقة، المصداقية، الخطية، الحساسية، حد الكشف وحد القياس طبقاً للمؤتمر الدولي للتوافق لتصديق طرق القياس التحليلية.

هذه الطرق طبقت بنجاح لتحديد الثيامين في المستحضرات الصيدلانية المحتوية عليه حيث أظهرت دقة ومصداقية عاليتين ودون أي تداخل من المضافات الصيدلانية الشائعة.

تمتاز الطرق المقترحة بالبساطة والحساسية ويمكن تطبيقها بصورة عملية وبشكل روتيني في معامل ضبط وتأكيد الجودة لتحليل الثيامين.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

1.1 Thiamine, an overview

Thiamine is a sulfur-containing vitamin and it is a member of the B complex group. It was discovered in 1926 by two Dutch scientists, Jansen and Donath. In 1934 it was prepared and isolated in pure form for the first time in the laboratory by Williams (James J, 2013, Wooley JA, 2008). Throughout history, thiamine took various names which included: vitamin B_1, aneurin (for the detrimental neurological effects if not present in the diet) and antineuritic vitamin.

It was eventually given the descriptive name vitamin B_1. Thiamine was used by all living organisms, but it is synthesized only by bacteria, fungi, and plants. Animals must obtain it from their diet, and it is considered to be an essential nutrient for human beings (Alternative Medicine, 2003).

The two major sources of thiamine are: dietary intake and bacterial production (Sriram K, 2012). Bacterial production of thiamine is extremely small compared to dietary intake and several food groups contain small amounts of thiamine such as wheat, rice, yeast, beef, pork, poultry, fish, milk, green leafy vegetables, nuts, and seeds (Leslie D, 1996).

Thiamine exists in four forms in the human body: unphosphorylated thiamine, thiamine monophosphate, thiamine diphosphate (80%), and thiamine triphosphate (Saif MW, 2003).

Thiamine acts as a coenzyme for oxidation-reduction reactions in the body. It is necessary for carbohydrate metabolism, especially glucose metabolism, the pentose shunt, and the citric acid cycle (Wooley JA, 2008, Leslie D, 1996, Saif MW, 2003). Thiamine diphosphate, also called thiamine pyrophosphate (TPP), is involved in oxidative decarboxylation in the mitochondrion (Sica DA, 2007) and is involved in more than 24 enzymatic reactions in the body (Alternative Medicine, 2003). Most importantly, it acts as coenzyme in the reactions catalyzed by the enzymes pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase, and transketolase (TK) (Bakker SJL, 1995). Free thiamine and thiamine monophosphate are transported into the central nervous system and the nerves to work in maintaining sodium and potassium gradients required for conducting nerve impulses (Wooley JA, 2008, Sica DA, 2007).

One of the major functions of thiamine is the maintenance of normal neural activity and prevention and treatment of beriberi disease. Pregnant women, infants, adolescents, and especially, elderly people are the groups at risk of avitaminosis of vitamin B1 (Ghazale, 2014, Du J, 2002). Thiamine is very important to the brain, particularly in terms of emotional health and wellbeing, and also is useful for focus and concentration. There have been suggestions that thiamine may have a beneficial effect in treating Alzheimer's disease (Zeeb M, 2010).

Potentially fatal outcomes may emerge as a result of thiamine deficiency if not treated (Mahan L. K, 2000), and in less severe cases, nonspecific signs

including malaise, weight loss, irritability and confusion may appear (Combs, 2008).

1.2 Chemical structure and some physical properties of thiamine:

Thiamine, a pyrimidyl substituted thiazole [3-(4- amino-2-methyl-pyrimidyl-5 methyl)-4-methyl-(β-hydroxyethyl)-thiazole], (Figure 1.1) was first isolated in 1936 by Williams and Cline (Williams R., 1936).

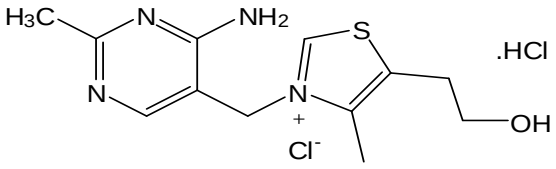


Figure 1.1 Chemical structure of thiamine hydrochloride

It is a colorless organosulfur compound with a chemical formula $C_{12}H_{17}N_4OS$. Its structure consists of an aminopyrimidine and a thiazole ring linked by a methylene bridge. The thiazole is substituted with methyl and hydroxyethyl side chains. Thiamine is soluble in water, methanol, and glycerol and practically insoluble in less polar organic solvents. It is stable in acidic media, but is unstable in alkaline solutions (Mahan L. K, 2000, Tanphaichitr V, 1999). Thiamine, which is an N-heterocyclic carbene, can be used in place of cyanide as a catalyst for benzoin condensation. Thiamine is unstable to heat, but stable during frozen storage. It is unstable when exposed to ultraviolet light (Tanphaichitr V, 1999) and gamma irradiation [Luczak M, 1969, Syunyakova ZM, 1966]. Thiamine reacts strongly in Maillard-type reactions (Mahan L. K, 2000).

1.3 Methods of analysis of Thiamine:

1.3.1 Spectrophotometric methods:

Many spectrophotometric methods have been proposed for determination of thiamine in different samples appeared in the literature.

Barbara Szpikowska-Sroka (Barbara, 2103) proposed a simple and sensitive method for spectrophotometric determination of thiamine with leucocrystal violet. In their method, thiamine was oxidized with potassium iodate (V) to a colorless product, releasing a stoichiometric amount of iodide ions. These ions reacted further with the excess iodate (V) ions in acidic medium, to form free iodine which oxidized leucocrystal violet to the crystal violet dye. The absorbance of the crystal violet dye formed was measured at pH 4.1 and 589 nm. Beer's law was obeyed over the thiamine concentration range 0.4–2.4 μg/ml. The linear regression coefficients were $a = 0.3339$, $b = –0.0001$, and $r^2 = 0.9998$ (for the general form of the linear equation $y = ax + b$). The apparent molar absorptivity of the colored compound was 1.12×10^5 l/mol cm for thiamine. The limit of detection (LOD) and limit of quantification (LOQ) of thiamine were found to be 0.19 and 0.26 μg/ml, respectively. Optimum reaction conditions providing maximum and constant absorbance were optimized. The procedure was successfully used for the determination of vitamin B1 in pharmaceutical formulations.

Najih H. Shekho (Najih H, 2013) introduced another simple, rapid, accurate and precise spectrophotometric method for determination of thiamine indirectly in

both pure form and in its pharmaceutical formulations. The method depends on the oxidation of thiamine by a known amount of chromate (CrO_4^{--}) in acidic medium of 2N H_2SO_4. The excess of chromate is measured via 1,5-diphenylcarbazide which gives a pinkish-violet, water soluble and stable complex exhibiting maximum absorbance at 543 nm, with a molar absorptivity of 1.5×10^4 l/mol cm. Sandell's sensitivity index was found to be 0.02248 $\mu g.cm^{-2}$ and a relative standard deviation of ±0.31 to ± 0.57%, was achieved depending on the concentration level. Thiamine concentration range that obeys Beer's law extends from 0.4 to 40$\mu g\ ml^{-1}$, and the method can be used in the determination of thiamine hydrochloride in the presence of sulphite. It is also being successfully applied to the determination of vitamin B1 in pharmaceutical preparations.

Shaopu Liu and co-workers (Shaopu Liu, 2002) pioneered a highly sensitive colorimetric method based on reacting vitamin B1 with a triphenylmethane acid dye such as thymol blue, bromothymol blue, bromophenol blue, bromocresol green, phenol red or cresol red to form colored ion association complex in a weak-base aqueous solution in the presence of some solubilization agents e.g. polyvinyl alcohol, emulgent OP, Triton X-100 or Tween-20. The wavelengths of maximum absorbance of the six ion-association complexes lie between 420 and 450 nm, and fading reaction appeared at longer wavelengths with the maximum fading wavelengths between 550 and 620 nm. The reactions had high sensitivities and their apparent molar absorptivities of the color reactions were

(0.82–1.65)×10^5 l mol^{-1} cm^{-1} and those of fading reactions were (1.26–3.92)×10^5 l mol^{-1} cm^{-1} depending on the different dye systems. Job's method and equilibrium shift method were used to establish the stoichiometric ratio of the reaction, and it was found to be 1:1 with respect to B1: dye for all six complexes. The method had good selectivity and could be applied to direct spectrophotometric determination of vitamin B1 in aqueous phase without using organic solvent extraction. Furthermore, the method was simple and rapid, and the color reaction mechanism was also explained with the aid of quantum chemical AM1 calculation method.

1.3.2 Flow injection methods:

Mouayed Q. Al Abachi *et al* (Mouayed Q, 2012) reported and optimized normal and reverse flow injection methods for determination of thiamine hydrochloride (THC) in microgram levels. The two methods rely on the reaction between THC and diazotized metoclopramide in alkaline medium. The linear range that obeys Beer's law was 10–300 mg/mL for the normal flow injection method and 2–90 mg/ml for the reverse flow method. At a sampling rate of 80 injections per hour, the normal method can be used to detect thiamine concentrations as low as 2.118 mg/ml, whereas the other method is more sensitive and the limit of detection reaches 0.839 mg/ml at a sampling rate of 95 injections per hour. Acceptable results have been produced when the two methods were applied to commercially available pharmaceuticals. The flow system is suitable for application in quality control laboratories.

Another flow injection method was described by Andrei and Martinez (Andrei F, 1994). The method was based upon the UV photo-degradation of thiamine in a single line flow injection assembly. The irradiation source was placed in the sample loop in such a way that half of the sample was irradiated. The FIA output shows a double peak with two adjoining maxima, the highest one corresponding to the nonirradiated sample, while the smallest corresponds to the irradiated thiamine sample. Irradiation period of three minutes and 500 µl injection volume was selected in order to improve the selectivity of the analytical procedure. The influence of different parameters related to the method was examined, which included the inside diameter (0.8, 0.5 and 0.3 mm) and length of the tubing injection valve-flow cell (in the range of 30-60 cm), and flow-rates from 2.0 to 8.0 ml/min. Results obtained with the 0.3 mm i.d. tubing were found to be better than the others. The calibration graph was constructed by plotting the differences between the two adjoining maxima of the FIA output detected at 264 nm, vs. thiamine hydrochloride concentrations, and it was found to be linear over the range of 1.2-30 µg/ml thiamine hydrochloride. The linear regression equation was: $A = -0.00033 + 0.0074X$, where A is the FIA-output difference and X the concentration of thiamine in µg/ml. The correlation coefficient was found to be 0.997. A series of 10 injections containing 10 µg/ml of thiamine hydrochloride were used to test the reproducibility of the method through which the calculated relative standard deviation was only 1.7%. The influence of commonly occurring excipients or additives with thiamine hydrochloride in

dosage forms was studied by preparing solutions containing 10 µg/ml of the drug and adding various concentrations of the possible interferents up to 3000 µg/ml. Some of the tested interferents like lactose, inositol, glucose and aminoacetic acid, did not interfere even at the highest tested interferent/thiamine hydrochloride ratio due to the lack of absorbance at 264.0 nm; others having absorbance at such wavelength interfered only by increasing the background absorbance.

A continuous flow-through UV spectrophotometric sensor had been initiated for thiamine determination on the basis of transient retention of the analyte in the flow cell and monitoring its intrinsic UV absorbance at 247 nm (Ortega Barrales P, 1998). Three calibration lines were constructed by using 300, 600, and 1000 ml of injected sample volume. Their linear dynamic ranges were 2.0-33.0, 1.0-20.0 and 0.6-12.0 µg/ml and their RSD (%) 0.8, 0.9 and 1.8 for the determination of 28, 15 and 9 µg ml, respectively, with sampling frequency of 18, 16 and 14 h^{-1}. The common excipients associated with the B-complex were tolerated at mass ratios (w/w) higher than 10, thus they did not interfere at the usual thiamine concentrations. The method was successfully applied to the determination of thiamine in pharmaceuticals containing only thiamine or other B vitamins as well. It was also superior to the conventional UV spectrophotometric method in terms of sensitivity, selectivity, simplicity, and speed. This is because the cation exchange nature of the solid support placed in the cell preconcentrates and selectively fixes the cation thiamine in the detection

region (active sensing microzone), thus preventing those non-cationic species present along with thiamine to interfere. Moreover, these features in combination with an FIA system in which the carrier itself regenerates the active microzone, make the sensor a cheap, continuous, simple, and reusable sensing device suitable for determination of thiamine in pharmaceuticals.

J. Martinez Calatayud *et al* [Martinez, 1990] also described a flow-injection fluorimetric determination of thiamine. In the method thiamine was subjected to oxidation by potassium hexacyanoferrate(III) complex immobilized on an anionic exchange resin, and the fluorescence intensity in aqueous basic solution was monitored. The linear range was found to be within 0.1 – 4 ppm thiamine with injection rate of 28 samples/h, and the relative standard deviation was 1.8%. The influence of other substances within the pharmaceutical formulation and the stability of thiamine in different media (acidic or basic) were also studied. It was found that thiamine aqueous solution at pH 2 and 37°C was stable till six months and the oxidation of thiamine with hexacyanoferrate(III) complex was rapid and complete.

Another fluorimetric procedure for the determination of thiamine using flow injection analysis was proposed (Pilar VinÄ AS, 2000). The method is based on the derivatization reaction of the primary amine group with o-phthalaldehyde in the presence of 2-mercaptoethanol using fluorimetric detection. The calibration graph based on peak area was linear in the range 0.2 – 6 ng/ml. The detection

limit was close to 0.1 ng ml^{-1}. The method was successfully applied to the determination of the vitamin in commercial pharmaceutical preparations.

1.3.3 Spectrofluorimetric methods:

A simple and efficient cloud point extraction-spectrofluorimetric method for the determination of thiamine in human urine was described (Ahad Bavili, 2006). The procedure relies on the oxidation of thiamine with ferricyanide to a thiochrome, which in turn extracted to Triton X-114 micelles before spectrofluorimetric determination. The variables affecting oxidation of thiamine, extraction and phase separation were studied and optimized. Under the optimum experimental conditions, the calibration curve was linear over the range 2.5 – 1000 ng ml^{-1}. The limit of detection was found to be 0.78 ng ml^{-1} of thiamine and the relative standard deviation for 5 replicate determinations of thiamine at concentration level of 400 ng ml^{-1} was 2.42%. Recoveries of 93-107% were obtained for spiked samples. The proposed method was utilized in the determination of thiamine in human urine.

A sensitive, selective and rapid spectrofluorimetric method was introduced for the determination of thiamine (Qiu-ying, 1999). The method used mimetic enzyme iron (III) tetrasulfonatophthalocyanine (FeTSPc) as a catalyst for the oxidation of thiamine with hydrogen peroxide. The oxidation was carried out in alkaline medium to give an intensively fluorescent compound, which has an excitation wavelength of 375 nm and an emission wavelength of 440 nm. The determination was found to be activated by fluorogenic substrates with a *p*-

hydroxyphenyl structure such as l-tyrosine, tyramine and *p*-hydroxyphenylpropionic acid. Under optimum conditions, the method was linear over the range from 1.0×10^{-8} to 1.0×10^{-4} mol/l, and the limit of detection was 4.3×10^{-9} mol/l. For six determinations (n = 6), the relative standard deviation was found to be 2.2%. The activation of the *p*-hydroxyphenyl substrates, the effects of some experimental conditions and the influence of foreign substances were also investigated. The method proved to be capable of selectively determining thiamine in commercial vitamin B1, vitamin B complex and rice.

Light has been shed to a combined dispersive liquid-liquid microextraction (DLLME) and spectrofluorimetry method to the extraction, pre-concentration and analysis of thiamine (vitamin B1) (Zeeb M, 2010). The method was carried out in three steps. The first step was the oxidation of thiamine with ferricyanide to form fluorescent thiochrome (TC). The second step involves the extraction of the thiochrome into a microextraction solvent, followed by spectrofluorometric determination in the last step. During the process, microextraction solvent and disperser solvent were directly injected into an aqueous solution containing TC. The fine droplets of the microextraction solvent was separated by centrifuge, and the settled phase was transferred into a fluorometer for the determination of thiamine at excitation/emission wavelengths of 375/438 nm. Under the optimized experimental conditions, the linear dynamic range was found to be 0.2 –100 ng mL^{-1}, the detection limit was 0.06 ng ml^{-1}, and the relative standard deviation was 3.0%. The method was successfully applied to pharmaceutical

formulations and human urine. The results were validated by recovery test and by comparison with other methods, and were found to be highly satisfactory.

1.3.4 Electrochemical methods:

The study led by Chengxiao Zhang *et al* explored highly sensitive electrochemical luminescence method for determination of thiamine (Chengxiao, 1999). The method depends on the electrochemical oxidation of thiamine with rhodamine B as a sensitizer. The rhodamine B sensitizer was strongly enhancing the weakly generated electrochemical luminescence (ECL) signal of thiamine. Under the conditions of: 0.10 mM rhodamine B, 6 mM cetyltrimethylammonium bromide (CTMAB) and 0.36 M sodium hydroxide, the response to the concentration of thiamine was linear over the range 0.1 µg/ml – 2 µg/ml, and the detection limit achieved was 0.08 µg/ml.

A simple, reliable and reproducible adsorptive stripping voltammetry (AdSV) method for determination of vitamin B1 (thiamine) in pharmaceutical preparation and food was adopted (Katarzyna, 2012). The method used in situ plated lead film electrode as a working electrode. Thiamine was accumulated at -1.25 V (vs. Ag/AgCl) on a glassy carbon electrode, then the pre-concentrated thiamine was reduced by scanning the potential of the electrode from -1.25 to -1.55 V using a square-wave technique. The linearity of the method lies within the concentration range 0.0133 – 0.265 mg l^{-1} for vitamin B1, with good regression coefficient of 0.999. The detection limit for vitamin B1 was 0.0053 mg l^{-1} for accumulation time of 120 s.

The method was applied to the determination of thiamine in certified reference material (BCR-485), pharmaceutical formulation and commercially available juices, and the assay results were satisfactory.

Simple and selective argentometric titration method for determination of thiamine (vitamin B1) was described (Saad S, 1989). The method was based on direct potentiometric titration of thiamine in alkaline medium and normal temperature using silver/silver sulphide ion selective electrode for end point detection. Potentiometric titration curves with two consecutive potential peaks specific for thiamine were created. The second peak is reproducible and indicated a 2:1 reaction ratio of silver to thiamine. No interference was observed by other vitamins, or other ingredients normally present in multivitamin preparations. The results obtained for determination of thiamine in pure powders, pharmaceutical tablets and ampoules showed an average recovery of 98.2% of the nominal values and a mean standard deviation of 0.5%. These results agreed fairly well with data obtained by the British Pharmacopoeia procedure (Saad S, 1989).

A capillary zone electrophoresis method with high-sensitivity cell (Z-cell) has been developed for the determination of thiamine in biological media (plasma, urine, saliva) (Yahya, 2000). In the determination of thiamine level in urine, the samples were diluted in water (1:1 by volume), then directly injected into the apparatus whereas in case of plasma it is necessary to precipitate the protein component first. The detection limit was improved nine-fold by means of the

capillary cell and four-fold compared to capillary with bubble cell. The biological media samples were analyzed for thiamine concentrations between 0.1 – 200 mg/ml, detection limits were found to lie within the range 0.80 – 0.05 µg/ml for all samples. High sensitivity, low amounts of samples and short analysis time were the main advantages associated with the method, and the method was successfully applied in the determination of thiamine levels in clinical and medical research.

1.3.5 Methods using High Performance Liquid Chromatography (HPLC):

High performance liquid chromatography is used extensively for determination of thiamine in different forms. The methods are often similar and interchangeable with sample extraction and clean up procedures being the major differences. Most of the methods used either ultraviolet or fluorescence detection. Fluorescence detection requires either pre-column or post-column oxidation of thiamine to thiochrome [Lynch P.L.M, 2000].

Tomas Perez and co-workers (Tomas, 2009) outlined a method for simultaneous determination of thiamine and its phosphate esters by a liquid chromatographic method based on post-column photolysis and chemiluminescence detection. On-line photolysis of the analytes was carried out to produce products having a strong enhancing effect on the chemiluminescence permanganate–luminol reaction. The complete separation of the thiamine was achieved by utilizing a stationary phase involving a ligand with amide groups known as RP-amide C16,

and phosphate buffer of pH 7 as mobile phase in isocratic elution with an analysis time of less than 7 min.

Under the optimum conditions, analytical results were linear over the range 10 – 1000 nM for thiamine and 100 –2000 nM for its mono- and di-phosphate esters. The method has satisfactory inter-day precision, relative standard deviation over the range 1.52 – 1.86%, and the limit of detection span of 0.6nM – 4.8 nM. The method was successfully applied to the determination of the thiamine in pharmaceutical preparations and baby foods (Tomas, 2009).

A reverse phase high performance liquid chromatographic method has been developed for the determination of a number of water-soluble vitamins, including thiamine, and fat-soluble vitamins in multivitamin pharmaceutical formulations. Fat-soluble vitamins were trapped using solid phase extraction with C18 AR cartridge, then the water-soluble vitamins were analyzed by HPLC on a Nova-Pack C18 (150×3.9 mm, 4 µm) analytical column, using CH_3OH/0.05*M* CH_3COONH_4 as mobile phase. The chromatographic analysis of the fat-soluble vitamins was carried out after their sequential elution with methanol and chloroform from C18 sorbent in the same column. The mobile phase employed was MeOH/CH_3CN (95:5, v/v) working at a flow-rate of 2 ml/min in isocratic mode. Various experimental variables were studied, among them: application volume, elution solvents and cleaning solutions. The UV–Vis detection of vitamins, with the exception of vitamin B 12, was measured at 270 nm for all water-soluble vitamins and 285 nm for the water-soluble and fat-

soluble vitamins present in real samples at different concentration levels. The method shows accuracy with average recovery ranging between 78 and 116%. (Moreno P, 2000).

Alaa El-Gindy and coworkers (Alaa El-Gindy, 2004) used HPLC and chemometric methods for the simultaneous determination of cyproheptadine hydrochloride, multivitamins – including thiamine – and sorbic acid. Separation was made in a reverse phase RP 18 column with two different elution solvents. Solvent (A) was composed of 0.1% methanolic hexane sulphonic acid sodium salt, and the second solvent (B) was a phosphate buffer of pH 2.7. Gradient HPLC was used with the solvent ratio changed from 20:80 to 70:30 (over 9 min), then to 80:20 (over 11 min) for solvent A:B respectively. The results of their method revealed that linearity was achieved at thiamine concentration range 1.3 – 3.4 µg/ml. The limit of detection (LOD) and limit of quantitation (LOQ) were found to be 0.06 µg/ml and 0.20 µg/ml respectively. The techniques were applied successfully to commercial pharmaceutical syrup and the results obtained were satisfactory.

C. K. Markopoulou and co-workers (Markopoulou C.K, 2002) also developed a simple, precise, rapid and selective HPLC-RP method for simultaneous determination of thiamine hydrochloride (B1), pyridoxine hydrochloride (B6) and hydroxocobalamine chloride (B12) in multivitamin tablets. The mobile phase used was 0.015% triethylamine in acetonitrile adjusted to pH 2.7 with 1 N sulfuric acid. Separation and quantitation was attained by changing the

proportion of the system linearly with a time-schedule program. Isocratic conditions could not be used mainly because the chromatographic peaks were not well resolved. Dual-beam UV detector was used in the wavelengths of 280 and 350 nm. The results showed good correlation coefficients (r^2 = 0.9999, 0.9998) and linearity, and no interferences with the peaks of interest was observed from common excipients. The method is inexpensive, simple and rapid with a high degree of accuracy and precision and applied successfully to the routine analysis of B1- B2 - B6 in B-complex tablets. Furthermore, the method has the advantage of measuring the analytes and their possible degradation products with high sensitivity and selectivity.

1.4 1,2-Naphthoquinone -4-sulphonic acid sodium salt (NQS) as analytical reagent for pharmaceutical amines:

1,2-Naphthoquinone -4-sulphonic acid sodium salt (NQS) (Figure 1.2) is a derivatization agent widely used for the determination of pharmaceutical amines. It improves the detection limits by introducing chromophores and/or fluorophores for chromatographic, spectrophotometric, and spectrofluorimetric detection. It was firstly introduced by Folin in 1922 (Folin, O, 1922) to calorimetric determination of amino acids and then it was widely used for detection of amino group-containing drugs. The compound is stable, commercially available and forms suitable adducts with a variety of amino acid-containing pharmaceuticals that enable precise and quantitative determination.

NQS is able to react in basic medium and moderate temperatures with both primary and secondary amino groups to produce spectrophotometrically detectable derivatives. The applications of NQS as an analytical reagent for the determination of pharmaceutical amines have been reviewed recently by Abdalla Elbashir [Abdalla Ahmed Elbashir, 2012].

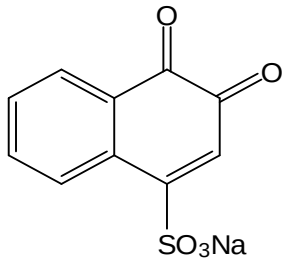


Figure 1.2 Chemical structure of NQS

1.5 7-chloro-4-nitrobenzoxadizole (NBD-Cl) as analytical reagent in pharmaceutical analysis:

NBD-Cl (Figure 1.3) is a derivatization reagent widely used in determination of many amines in pharmaceutical formulations due to its ability to give a fluorescent adduct that improves the detection limit. It was firstly introduced by Ghosh and Whitehouse (Ghosh, P.B, 1968) by nitrating 4-chlorobenzofurazan with dichloronitrobenzene. NBD-Cl-amine compounds have a strong fluorescence best observed in low polar solvents. Both primary and secondary amines can be analyzed by the reagent and the reagent has the advantage of low cost but low reactivity and longer reaction time is usually required. The application of NBD-Cl in determination of pharmaceutical amine was a subject

of a recent review written by Abdalla Elbashir and his coworkers (Abdalla A. Elbashir, 2011).

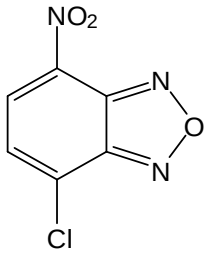


Figure 1.3 Chemical structure of NBD-Cl

1.6 The objectives of this research:

The general objective of this research is to develop direct inexpensive, simple and rapid spectrophotometric and spectrofluorometric methods for the analysis of thiamine in pharmaceutical preparations in order to overcome the drawbacks associated with the previously reported methods. This will be accomplished by the use of both 1,2-Naphthoquinone-4-sulphonic acid sodium salt (NQS) and 7-chloro-4-nitrobenzoxadizole (NBD-Cl) as derivatizing reagents, since no reports were recorded in the literature using these reagents in pharmaceutical determination of thiamine so far.

Reaction conditions including pH, reagent concentration, temperature and reaction time will be optimized and the validation of analytical methods with respect to linearity, accuracy, precision, LOD and LOQ will be assessed according to the International Conference of Harmonization (ICH) guidelines for validation of analytical procedures (International Conference, 2005). Then the methods will be applied to real samples collected from local pharmacies.

CHAPTER TWO

EXPERIMENTAL

2. Materials and methods

2.1 Instrumentation

The absorbance measurements in this study were performed using Mini 1240 spectrophotometer equipped with matched 1 cm Quartz cell.

Spectrofluorimetric measurements were recorded using Shimatzu, Japan Spectrofluorimeter. pH meter model HI 255 (Hanna Instruments, Mumbai, India) was used for pH measurements.

2.2 Reagents and solutions

All chemicals used are of analytical grade. Distilled water and methanol were used for preparing solutions. Thiamine hydrochloride (99.15%), Sodium-1,2 naphthoquinone-4-sulphonate (NQS) and 4-Choro-7-nitrobenzo-2-oxa-1,3 diazole (NBD-Cl) 98% were obtained from Sigma Aldrich, and were used as received without further purification.

2.3 Preparation of standard solutions

2.3.1 Preparation of Thiamine Hydrochloride (THC) [V B1]

An accurately weighed 250 mg of Thiamine Hydrochloride standard was dissolved in distilled water, transferred into 250 ml volumetric flask, diluted to the mark and mixed well.

2.3.2 NQS standard solution

It is prepared by dissolving 0.6g in distilled water, transferred to a 100 ml volumetric flask, diluted to the mark with distilled water and mixed well. The solution was always freshly prepared and protected from light during use.

2.3.3 NBD-Cl standard solution

An accurately weighted 0.2g of NBD-Cl was dissolved in methanol (HPLC grade), transferred into a 100 ml volumetric flask, and diluted to the mark with the same solvent.

2.3.4 Sample solutions

Five capsules (Thiamine 100 mg capsules) were weighted and finely-grinded. A portion of the powder equivalent to 25 mg of the drug was weighed and dissolved in distilled water, filtered and then transferred into 250 ml volumetric flask, completed to the mark with distilled water to give a solution of 100µg/mL

2.3.5 Buffer solutions

Buffer solution of pH 11.0 was prepared by mixing 100 ml of 0.1M aqueous solution of sodium bicarbonate with 50 ml of 0.1M solution of sodium carbonate and adjusted to pH 11.0 with 1M Sodium hydroxide.

An optimum buffer solution of pH 10.5 was prepared by mixing 100 ml of 0.025M aqueous solution of Borax with 36.5 ml of 1 M solution of sodium hydroxide and adjusted to pH 10.5 with 1M Sodium hydroxide.

2.4 Assay procedures:

2.4.1 Method A (Spectrophotometric):

2.4.1.1 Thiamine (THC) with NQS

Aliquots of 100 µg/ml of thiamine solution were transferred into 10 ml volumetric flask, 2.0 ml of 0.6% NQS (w/v) was added, followed by 2.0 ml of Buffer pH 11.0. The reaction was completed to volume with distilled water, and the absorbance was measured at 487 nm against reagent blank treated similarly.

2.4.1.2 Thiamine (THC) with NBD-Cl

The same procedure was followed using 1.0 ml NBD-Cl (0.2%) and appropriate volume of the standard solution to obtain a final concentration range of 5 to 35 µg/ml. The relative absorbance of the reaction product was measured at 434 nm against reagent blank.

2.4.2 Method B (Spectrofluorimetric)

2.4.2.1 Thiamine (THC) with NBD-Cl

Aliquots of standard thiamine solution was transferred into a 10 ml volumetric flask, then 1.5 ml of buffer solution (pH 10.5) was added followed by 1.0 ml of NBD-Cl solution (0.2%, w/v). The reaction was allowed to stand at room temperature for 25 min. The fluorescence intensity of the resulting solution was measured at 562 nm (λ_{ex} 472 nm) against reagent blank treated similarly.

2.4.2.2 Thiamine (THC) with NQS

The same procedure above was followed using 2 ml of buffer solution (pH 11.0) and 2 mls of NQS solution (0.6% w/v), and reaction time of 15 min at room temperature. Excitation was performed at 390 nm where the emission was being at 460 nm.

2.4.3 Job's method

The Job's method of continuous variation was employed to determine the stoichiometric ratio of the reaction between thiamine and NQS or NBD-Cl in the Spectrophotometric method. Molar equimolar (5.0×10^{-3}M) aqueous solution of thiamine hydrochloride and NQS or NBD-Cl were prepared. Series of 10 ml portions of the master solution of thiamine and NQS/NBD-Cl were made up comprising different complementary proportions (0:10, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 and 10:0) in 10 ml volumetric flask. The absorbance of each solution was measured and plotted against the volume fraction of the reagent (NQS or NBD-Cl).

2.4.4 Limiting logarithmic method

The limiting logarithmic method was used to determine the stoichiometric ratio of the reaction between thiamine and NQS or NBD-Cl in the Spectrofluorimetric method. Two sets of experiments were carried out employing the general recommended conditions described above. The first set of experiments was carried out using increasing NQS concentrations (from 6.25×10^{-5}M – 5.0×10^{-4}M) at fixed thiamine concentration (5.0×10^{-4}M), or increasing NBD-Cl

concentration (from 6.13×10^{-5}M – 4.91×10^{-4}M) at fixed thiamine concentration (5.0×10^{-4}M). The second set of experiments was carried out using increasing thiamine concentration (from 1.85×10^{-5}M – 1.48×10^{-4}M) at fixed NQS concentration (1.0×10^{-3}M) or NBD-Cl concentration (1.5×10^{-3}M). The logarithms of the obtained absorbances were plotted as function of the logarithms of the NQS/NBD-Cl concentrations in the first set, and as a function of thiamine concentrations in the second set. The slopes of the fitting lines in both sets of experiments were calculated and compared.

CHAPTER THREE

RESULTS AND DISCUSSION

3. Results and discussion

3.1 Method A: Spectrophotometric method for determination of thiamine (THC) with NQS

3.1.1 Absorption spectrum

The absorption spectrum of thiamine was recorded against water. It was found that thiamine exhibits a maximum absorption peak (λ_{max}) at 232 nm. Because of highly blue shifted λ_{max} of thiamine, its determination in the dosage form based on direct measurements of its absorption in the ultraviolet is susceptible to potential interferences from common excipients. Therefore, derivatization of thiamine to a red-shifted light absorption derivative was necessary. The reaction between thiamine and NQS was performed, and the absorption spectrum of the brown colored product exhibit λ_{max} at 487 nm, and the λ_{max} of NQS was 361 nm. The λ_{max} of thiamine-NQS derivative was red shifted eliminating any potential interference. Therefore the measurements were carried out at 487 nm.

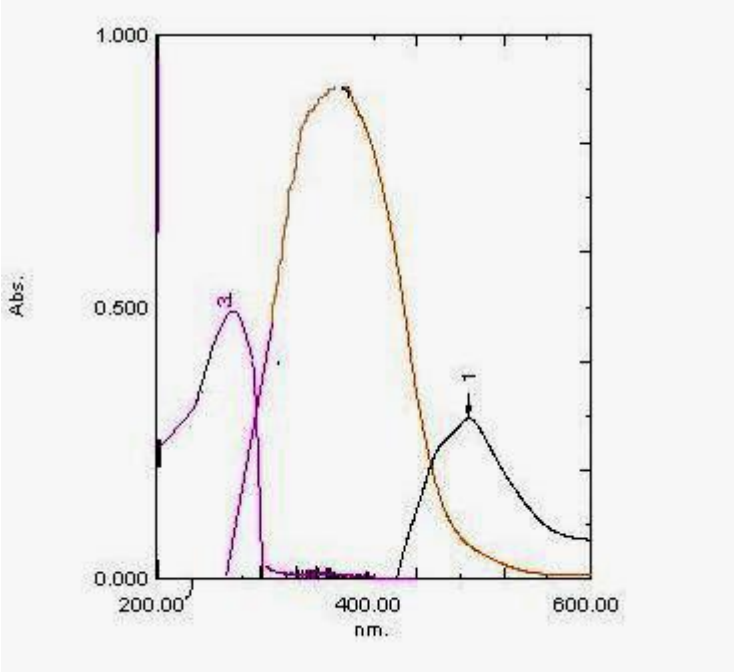


Figure 3.1 Absorption spectra of (1) The reaction product of Thiamine with NQS against reagent blank, (2) NQS (0.06% (w/v) against water blank, (3) Thiamine (15 µg/ml) against water blank.

3.1.2 Optimization of reaction conditions

The optimum conditions for the developed method were established by varying the parameters one at a time while keeping the others fixed and observing the effect produced on the absorbance of the colored product. In order to establish experimental conditions, the effect of various parameters such as pH, reaction time, buffer volume and concentration of NQS were investigated.

3.1.2.1 Effect of pH

The effect of pH on the reaction between thiamine and NQS was examined by varying pH from 7.0 to 13.0. As shown in Figure 3.2, the absorbance of the product is low at pH 7.0, indicating that thiamine has difficulty to react with (NQS) in neutral media. This was possibly due to the existence of the amino group of thiamine in the form of hydrochloride salt, thus it loses its nucleophilic substitution capability. As the pH increased from 7 to 13, the readings increased rapidly, and the amino group of thiamine turns into the free amino group, thus facilitating the nucleophilic substitution. The maximum readings were attained at pH value of 11.0. At pH values more than 11.0 decrease in the readings occurred. This was attributed probably to the increase in the amount of hydroxide ion that holds back the reaction of thiamine with NQS. Reaction of NQS with compound bearing primary amines at pH 11.0 was reported (Sara A.M., 2011).

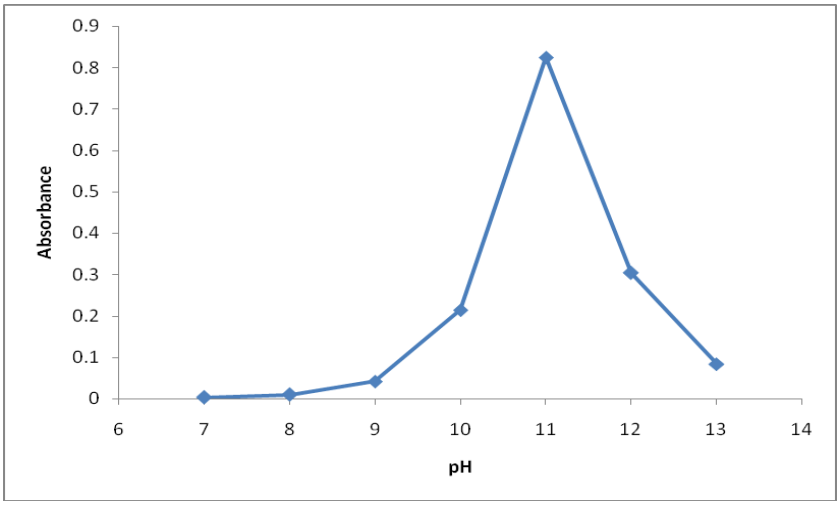


Figure 3.2 Effect of pH on the reaction of thiamine with NQS, 1.0 ml of thiamine (100 µg/ml), 2.0 ml buffer solution (pH 11) , 2.0 ml NQS (0.6 w/v %), reaction time:15 min.

3.1.2.2 Effect of reaction time

The absorbance of the reaction product was determined at different time (Figure 3). Keeping other conditions unchanged, the absorbance of the reaction product was measured after standing for different time periods at 25°C. The results show that thiamine react with NQS at 25°C and the absorbance begins to increase instantly and becomes decrease after 15 min.

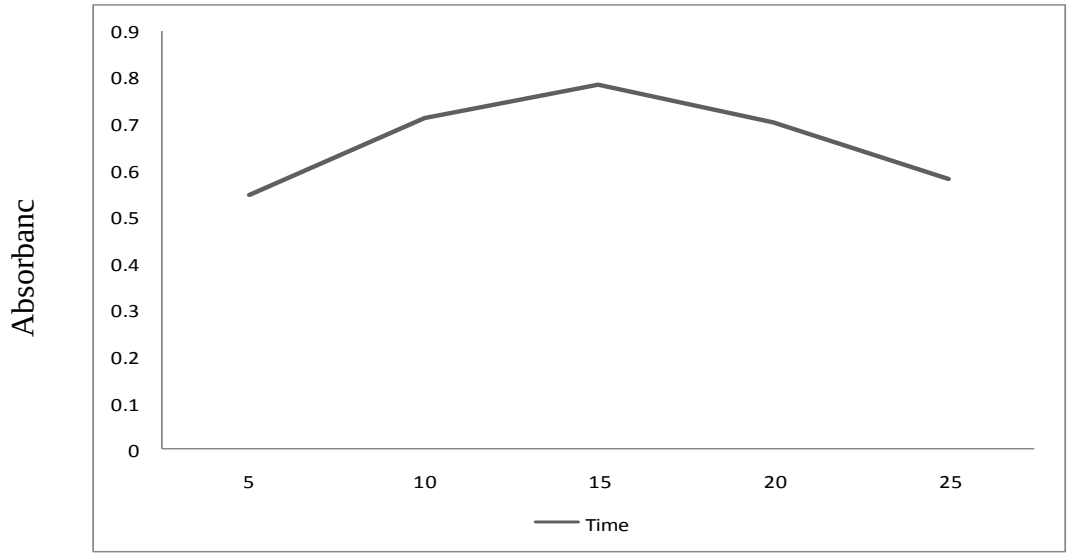


Figure 3.3 Effect of standing time on the reaction of thiamine with NQS, 1.0 ml of thiamine (100 μg/ml), 2.0 ml buffer solution (pH 11), 2.0 ml NQS (0.6 w/v %), reaction time:25 min.

3.1.2.3 The effect of buffer volume

Keeping pH at 11.0, the effect of amount of buffer solution on the absorbance of reaction product was also studied (Figure 3.4). It shows that the absorbance of the reaction product enhances rapidly with the rise of amount of buffer solution, and becomes maximal when the amount of buffer solution is 2.0 ml. Therefore, the amount of 2.0 ml buffer solution was selected to ensure the highest absorbance.

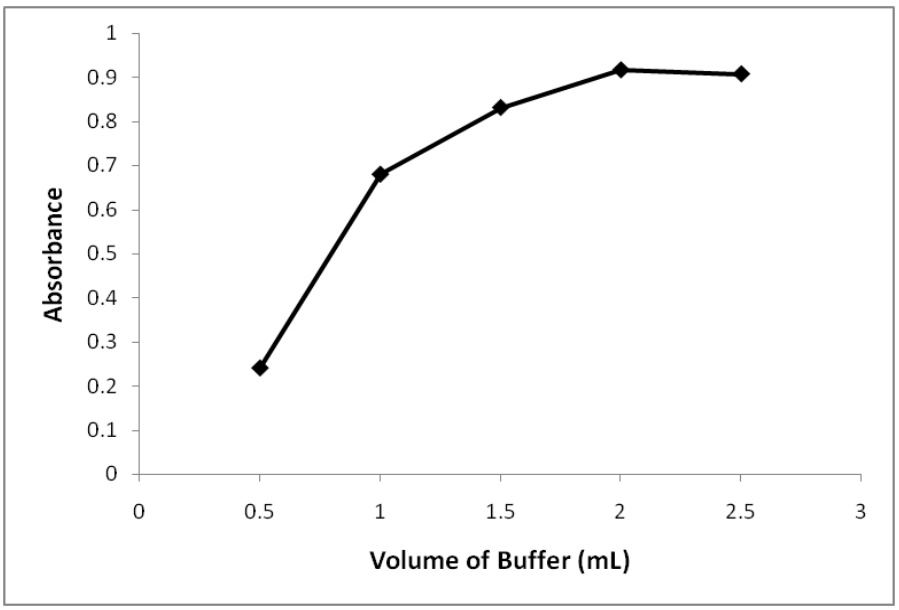


Figure 3.4 Effect of Buffer volume in the reaction of Thiamine with NQS.

Conditions: 1.0 ml of thiamine (100 µg/ml), 2.0 mL NQS (0.6 w/v %), reaction time: 15 min.

3.1.2.4 Effect of temperature

Keeping all conditions constant, the influence of temperature on the absorbance of the reaction mixture of THC with NQS was studied. It was found that the absorbance of the solution was maximum at room temperature.

3.1.2.5 Effect of NQS concentration

The studying of varying the effect of NQS concentrations revealed that the reaction was dependent on NQS reagent. The highest absorption intensity was attained at NQS concentration of 0.6% (w/v), and higher

concentration of NQS 0.7% (w/v) the absorption values decrease, as shown in Figure 3.5.

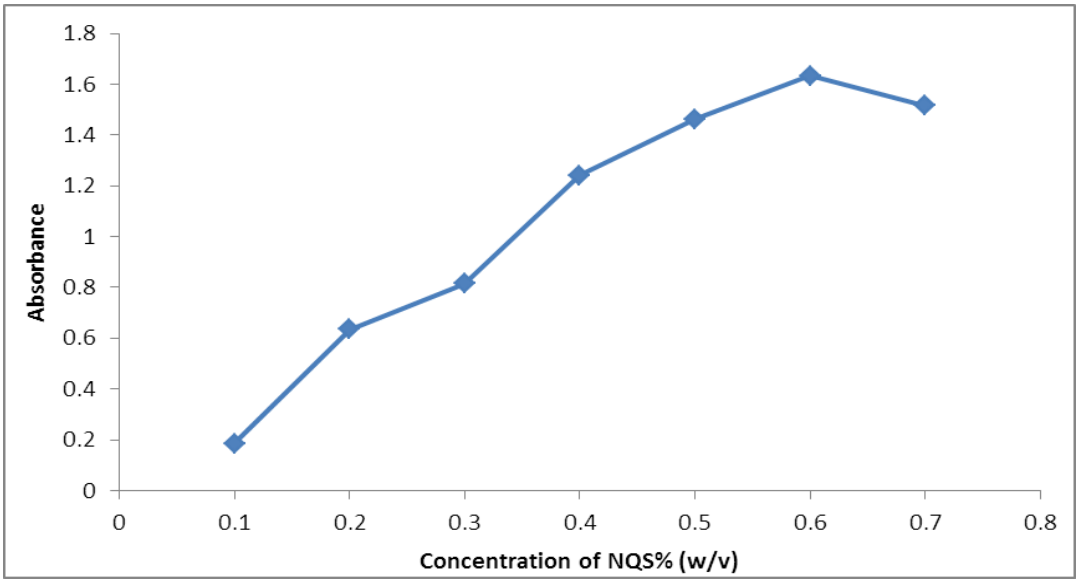


Figure 3.5 Effect of NQS concentration on the reaction of thiamine with NQS, 1.0 ml of thiamine (100 µg/ml), 2.0 ml buffer solution (pH 11), 2.0 ml NQS (0.1 - 0.7 w/v %), reaction time,15 min.

3.1.3 Stoichiometry of the reaction (Job's method)

The Job's method of continuous variation was employed to determine the stoichiometric ratio of the reaction. Master equimolar (5×10^{-3} M) aqueous solutions of thiamine hyrochloride and NQS were prepared. Series of 10 ml portions of the master solution of thiamine and NQS were made up comprising different complementary proportions (1:9,…9:1, inclusive) in 10 mL volumetric flask containing 2 ml of buffer solution (pH=11.0). The solution was further manipulated as described under the general recommended procedures.

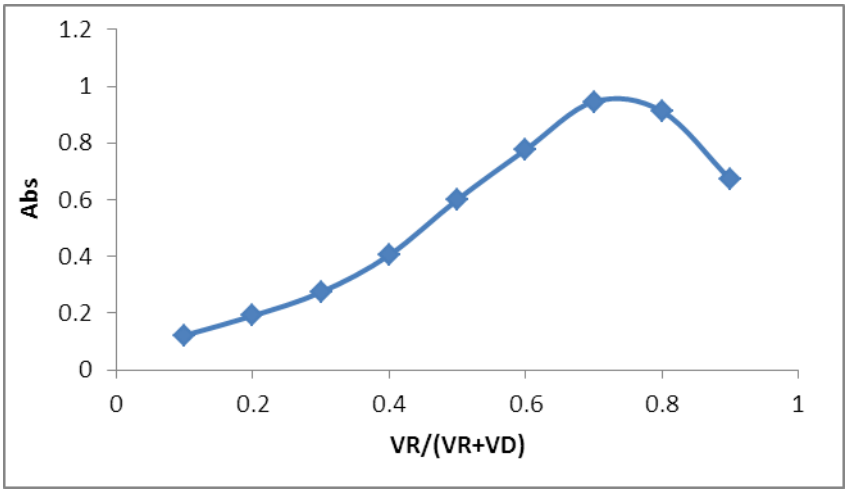


Figure 3.6 The continuous variation plot for the stoichiometry of the reaction of thiamine with NQS.

3.1.4 Method validation

The method was validated for the following parameters: linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness according to the International Conference on Harmonization (ICH) guidelines (International Conference, 2005).

3.1.4.1 Calibration curve and linearity

The linearity was evaluated by linear regression analysis determined by constructing seven concentrations of thiamine, in the range of 10–40µg/ml, which was calculated by the least square regression method to calculate the calibration equation and the correlation coefficient. The calibration curves were constructed by plotting concentration versus absorbance, using linear regression analysis. The regression equation for the results was $A=0.022x + 0.171$ ($r^2=0.997$), where A is the absorbance

at 487 nm, x is the concentration of thiamine in μg/ml in the range of 10-40 μg/mL, and r is correlation coefficient (Table 3.1) and Figure 3.7.

Table 3.1 Parameters for the performance of the proposed method

Parameter	value
Measurement wavelength (nm)	487
Linear range (μg/ml)	10-40
Intercept	0.171643
Standard deviation of the intercept	0.011495
Slope	0.022155
Correlation coefficient (r^2)	0.997
Limit of detection, LOD (μg/ml)	1.71
Limit of quant., LOQ (μg/ml)	5.18
Molar absorptivity	9.308×10^3

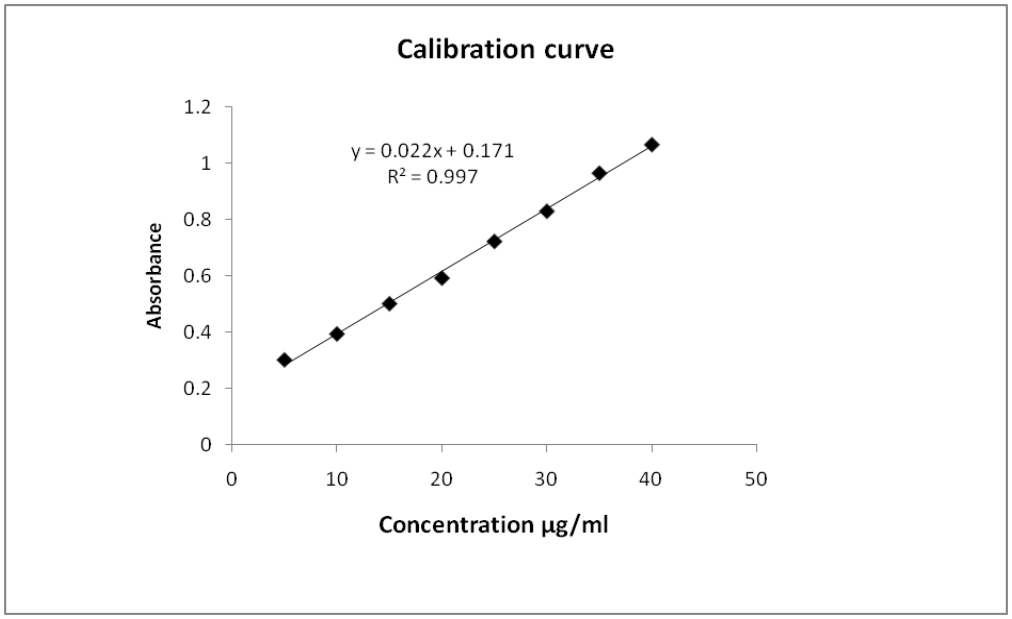


Figure 3.7 Calibration curve for determination of Thiamine with NQS

3.1.4.2 Molar absorptivity

Molar absorptivity is defined as a measure of a chemical's ability to absorb light at a specified wave length. The molar absorptivity depends on a chemical species; actual absorption depends on chemical concentration and bath length. These variables are used in the Beer's Lambert law. Molar absortivity also is known as the molar extinction coefficient and the molar absorption coefficient. The value of ε is given in Table 3.1

3.1.4.3 Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) were determined by establishing the maximum level at which the analyte can be reliably detected and determined by establishing the lowest concentration that can be measured according to ICH. The limit of detection (LOD) and limit of quantitation were determined according to the following formula LOD=3.3×SDa/b, and LOQ=10×SDa/b, SDa is the standard deviation of the blank, b is the slope under the ICH guidelines. The LOD and LOQ were found to be 1.71 and 5.18 µg/ml, respectively (Table 3.1).

3.1.4.4. Precision and Accuracy of the method

The accuracy of the proposed method was carried out by applying 3 different concentrations 10, 25, and 40 µg/ml of thiamine drug within the

linear range and calculated as the percentage of the drug recovered from the samples (Table 3.2).

Table 3.2 Accuracy and precision of the method

Concentration µg/ml	Concentration found µg/ml	Recovery % ± SD	Relative error (%)
10	9.81	98.1±0.012	-1.52
25	26.0	104.0±0.025	3.05
40	39.63	99.07±0.021	-1.97

3.1.4.5 Recovery of the method

The recovery of the proposed method was carried out by applying standard addition technique. A different amount of standard solution was added to a known concentration of the drug sample. The average percent recoveries obtained were given in Table 3.3

Table 3.3 Recovery studies for the determination of thiamine by the proposed method.

Sample No.	Sample content (µg/ml)	Thiamine standard amount (µg/ml)	Amount found (Total) (µg/ml)	Recovery ± SD*
1	5	10	14.95	99.0% ± 0.01
2	5	20	25.05	100.2% ± 0.01
3	5	30	35.00	100.0% ± 0.01

*Values are mean of three determinations.

3.1.4.6 Robustness

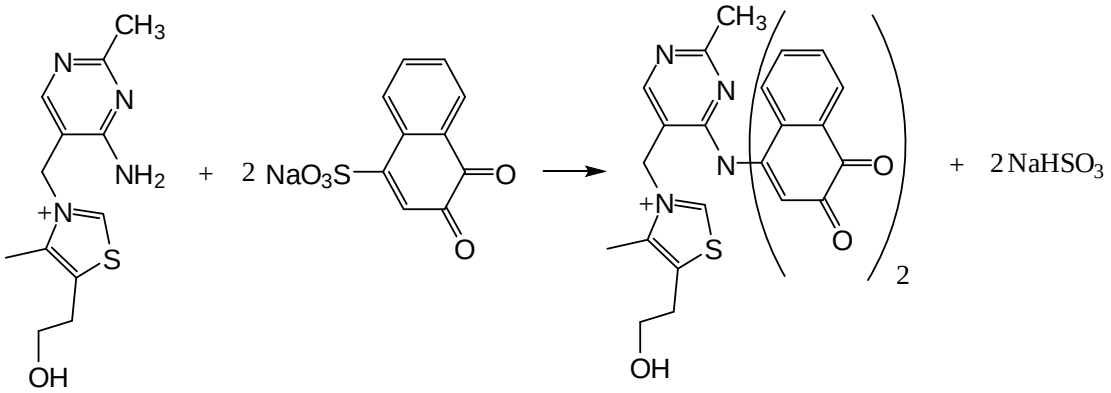
Robustness was examined by evaluating the influence of small variations in the method variables on its analytical performance. In these experiments, one parameter was changed whereas the others were kept unchanged, and the recovery percentage was calculated each time. It was found that small variations in the method variables did not significantly affect the procedures; recovery values were recorded in Table 3.4. This indicated the reliability of the proposed method to routine application for the analysis of thiamine.

Table 3.4 Robustness of the proposed method

Parameter	**Recovery (% ± SD)**
Recommended conditions	100.6±0.04
NQS concentration (%, w/v) 0.58	102.37±0.016
NQS concentration (%, w/v) 0.62	97.82±0.013
Buffer solution (pH) 10.8	96.01±0.029
Buffer solution (pH) 11.2	93.63±0.044
Reaction time (min) 13	98.63±0.009
Reaction time (min) 17	96.56±0.055

3.1.5 Reaction mechanism

It has been reported that NQS could react with amino groups in primary or secondary amine derivatives [Abdalla Ahmed Elbashir, 2012]. Similarly, amino group of thiamine, taking on nucleophilicity due to lone electron pair of nitrogen atom, tend to attack on the electron–deficient center in NQS, namely: carbon atom No.4 (3,4-C=C carbon bond conjugate with 2-C=O, as a result 4-C of NQS becomes electron lacking center). At the same time, it has been proved that, through the Job's method, the composition of the product is 1:2 of thiamine to NQS. So it is concluded that amino group of thiamine react with 4-sodium sulphonate of NQS molecule to form brown N-alkyl-amino-naphthoquinone. The reaction equation is shown in Scheme 3.1.



Scheme 3.8 The reaction pathway of thiamine with NQS

3.1.6 Application of the proposed method to analysis of thiamine dosage form

Thiamine tablets were subjected to analysis by the proposed method and the label claim agrees well with our new method as shown in Table 3.5. The proposed method has the advantage of being virtually free from interferences by excipients.

Table 3.5 Analysis of Thiamine -containing dosage form by the proposed method.

Brand name of label claim (mg)	**Amount found (mg)**	**(% found ± SD)[a]**
Thiamine tablets (100 mg)	95.55	95.33±0.006

a: values are mean of five determinations

3.2 Method A THC with NBD-Cl

3.2.1 Absorption spectra

The absorption spectrum of thiamine was recorded against water (Figure 3.8). It was found that thiamine exhibits a maximum absorption peak (λ_{max}) at 235 nm. Because of highly blue-shifted λ_{max} of thiamine, its determination in the dosage form based on the direct measurement of its absorption in the ultraviolet is susceptible to potential interferences from the common excipients. Therefore, derivatization of thiamine to attain visible-range absorbing species was undoubtedly necessary. Thus, derivatization of thiamine with NBD-Cl was performed, and the absorption spectrum of the product was recorded against reagent blank (Figure 3.8). It was found that the product is brown-colored exhibiting

λ_{max} at 434 nm, and the λ_{max} of NBD-Cl was 342 nm. The λ_{max} of thiamine-NBD-Cl derivative was red-shifted, eliminating any potential interference. The wavelength 434 nm therefore was fixed as optimum.

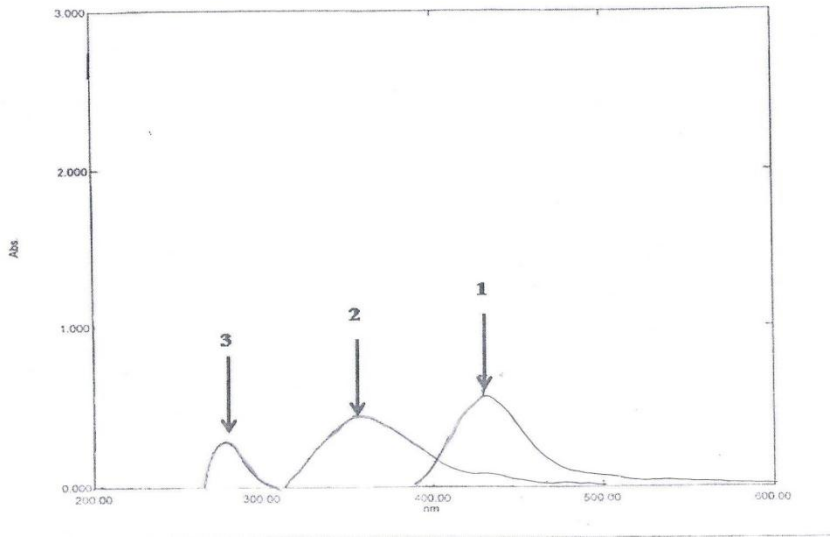


Figure 3.9: Absorption spectra of Thiamine (3), NBD-Cl (2) and the complex between them (1)

3.2.2 Optimization of reaction conditions

3.2.2.1 Effect of pH

The effect of pH on the reaction between thiamine and NBD-Cl was tested by varying the pH form 7.0 to 12.0. As shown in Figure 3.9, the absorbance of the product is low at pH 7.0, indicating that thiamine cannot react with (NBD-Cl) in neutral media. This was possibly due to

the existence of the amino group of thiamine in the form of hydrochloride salt, which hampers nucleophilic substitution capability. As the pH increases from 7 to 12, the absorbance increased dramatically, releasing the amino group of thiamine and facilitates the nucleophilic substitution. The maximum absorption was attained at pH value of 10.5. At pH values more than 10.5, a decrease in the absorption occurred. This was attributed probably to the increase in the amount of hydroxide ion that increases the rate of the backward reaction of thiamine with NBD-Cl.

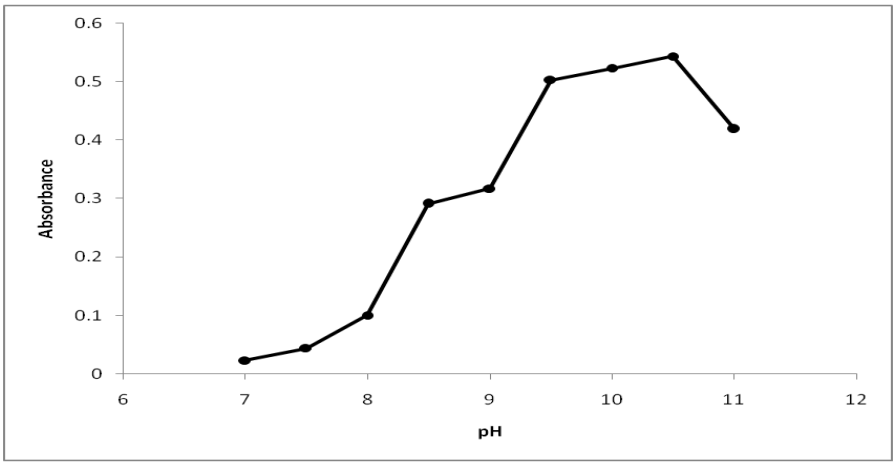


Figure 3.10: Effect of pH on the reaction of Thiamine with NBD-Cl

Thiamine (20µg/ml): 1 ml, NBD-Cl conc. 0.2% (w/v), reaction time 20 min.

3.2.2.2 Effect of reaction time

The absorbance of the reaction product was monitored at different times (Figure 3.10). Keeping other conditions intact, the absorbance of the reaction product was followed after standing for different time spans at 25°C. The results showed that thiamine reacts with NBD-Cl at 25°C and

the absorbance begins to increase gradually and reached a maximum after 25 min. For longer reaction times, a slight drop in the absorbance was observed. Accordingly 25 min was set as the convenient reaction time for determination.

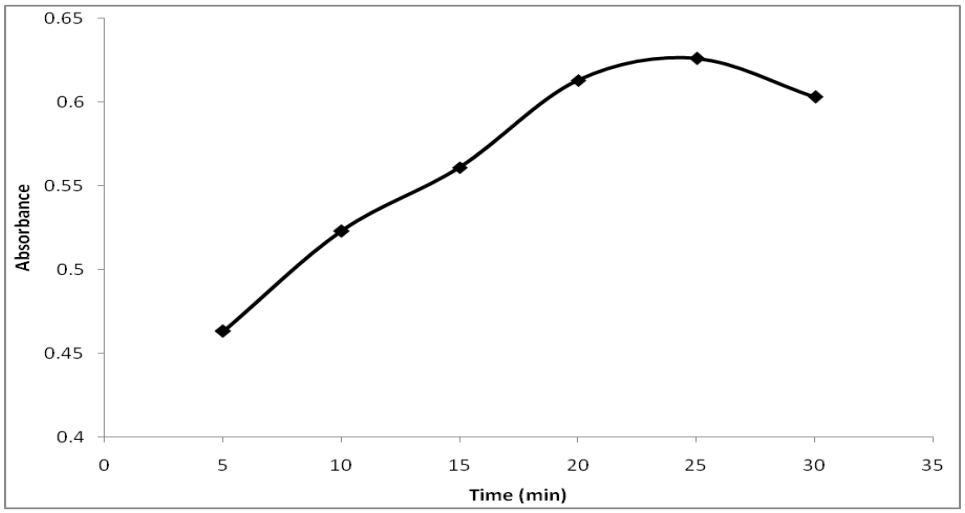


Figure 3.11: Effect of reaction time on the reaction of Thiamine with NBD-Cl

Thiamine (20µg/ml): 1 ml, Buffer (pH 10.5): 1.5 ml, NBD-Cl conc. 0.2% (w/v)

3.2.2.3 Effect of Buffer volume

Keeping pH at 10.5, the effect of amount of buffer solution on the absorbance of reaction product was also studied (Figure 3.11). The result reveals that the absorbance of the reaction product enhances rapidly with the rise of amount of buffer solution, and becomes maximal when the

amount of buffer solution reaches 1.5 ml. Therefore, 1.5 ml buffer solution was selected to ensure the highest absorbance.

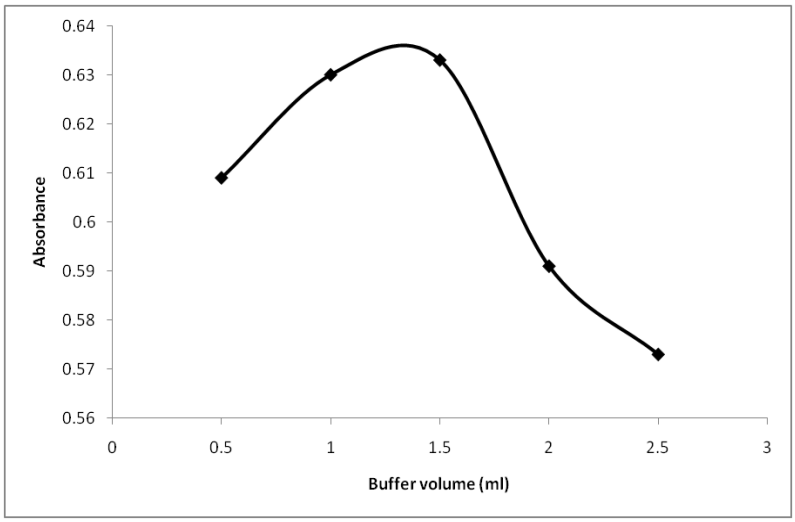


Figure 3.12: Effect of Buffer amount (ml) on the reaction of thiamine with NBD-Cl, at Thiamine (20µg/ml): 1 ml, Buffer pH: 10.5, NBD-Cl conc. 0.2% (w/v), reaction time 25 min.

3.2.2.4 Effect of temperature

Keeping all conditions constant, the influence of temperature on the absorbance of the reaction mixture of VB1 with NBD-Cl was studied. It was found that the absorbance of the solution was maximal at room temperature.

3.2.2.5 Effect of NBD-Cl concentration

The effect of varying NBD-Cl concentrations was investigated, the result showed that the reaction was dependent on the reagent concentration. The highest absorption intensity was attained at NBD-Cl concentration of 0.2% (w/v), and higher concentration of NBD-Cl leads to a decrease in the absorbance (Figure 3.12).

From the above parameters-adjusting experiments, the optimized conditions used for the assay were: pH 10.5, NBD-Cl concentration 0.2% (w/v), volume of the buffer 1.5 ml, reaction time 25 min and temperature 25ºC.

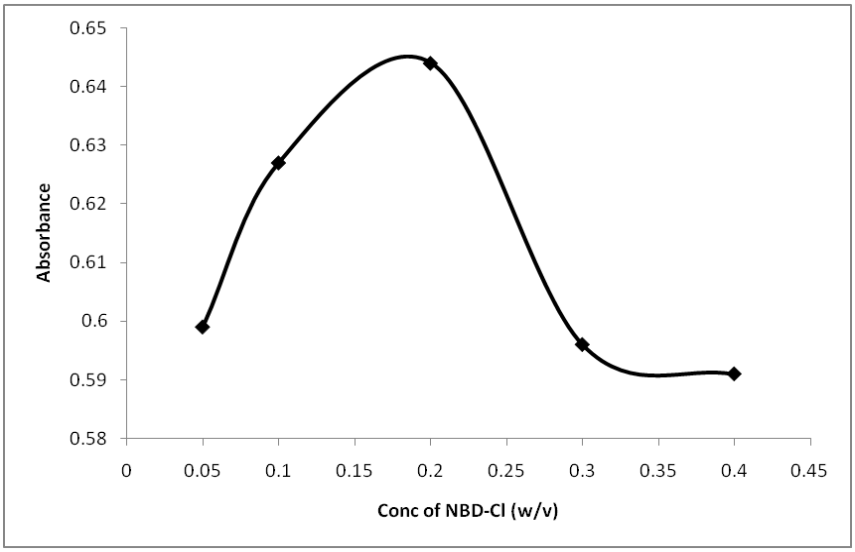


Figure 3.13: Effect of NBD-Cl concentration on its reaction with thiamine

Thiamine (20µg/ml): 1 ml, Buffer (pH 10.5): 1.5 ml, reaction time 25 min.

3.2.3 Validation of the method

The method was validated for the following parameters: linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness according to the International Conference on Harmonization (ICH) guidelines (International Conference, 2005).

3.2.3.1 Linearity, limit of detection (LOD) and limit of quantification (LOQ)

The linearity was evaluated by linear regression analysis determined by constructing seven concentrations of thiamine, in the range of 05–35μg/ml, which was calculated by the least square regression method to calculate the calibration equation and the correlation coefficient. The calibration curves were constructed by plotting concentration versus absorbance, using linear regression analysis. The regression equation for the results was A=0.033x - 0.009 (r^2=0.999), where A is the absorbance at 434 nm, x is the concentration of thiamine in μg/ml in the range of 05-35 μg/ml, and r is correlation coefficient (Table 1 and Figure 3.13). It was found that the linear concentration range is comparable with the previous method using NQS. The limit of detection (LOD) and limit of quantification (LOQ) were determined according to the following formula LOD=3.3×SDa/b, and LOQ=10×SDa/b, SDa is the standard deviation of the intercept; b is the slope under the ICH guidelines. The LOD and LOQ were found to be 0.667 and 2.020 μg/ml, respectively

Table 3.6 Parameters for the performance of the proposed method

Parameter	value
Measurement wavelength (nm)	434
Linear range (µg/ml)	5-35
Regression equation	y = 0.033x - 0.009
Intercept	-0.00986
Standard deviation of the intercept	0.00667
Slope	0.0330
Standard deviation of the slope	0.000298
Correlation coefficient (r^2)	0.9995
Limit of detection, LOD (µg/ml)	0.667
Limit of quant., LOQ (µg/ml)	2.0207

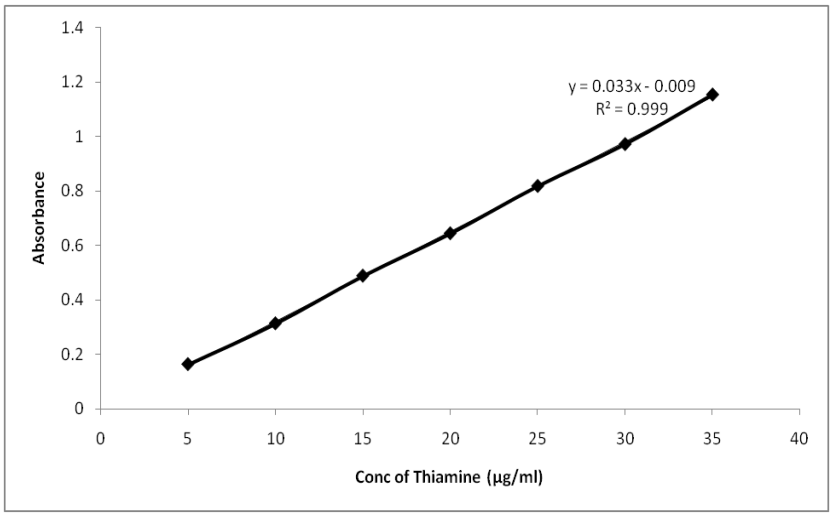


Figure 3.14 Calibration curve of standard thiamine in the range of 5 - 40 µg/ml

3.2.3.2 Accuracy

The accuracy of the proposed method was carried out by applying 3 different concentrations 5, 20, and 30 μg/mL of thiamine drug within the linear range and calculated as the percentage of the drug recovered from the samples.

Relative error (RE) was within 0.24% with corresponding standard deviation within 0.004 for three different determinations (Table 3.7)

Table 3.7 Evaluation of accuracy and precision

Sample No.	Concentation (μg/ml)	Concentration found (μg/ml)	Recovery (% + SD)	Relative error (%)
1	5	4.70	95.5±0.004	0.224
2	20	19.29	96.46±0.002	0.15
3	30	29.25	97.50±0.003	0.24

3.2.3.3 Robustness

Robustness was examined by evaluating the influence of small variations in the method variables on its analytical performance. In these experiments, one parameter was changed whereas the others were kept constant, and the recovery percentage was calculated each time. It was found that small variations in the method variables did not significantly affect the procedures; recovery values were recorded in Table 3.8. This

indicated the reliability of the proposed method to routine application for the analysis of thiamine.

Table 3.8 Influence of small variation in the assay conditions on the analytical performance of the proposed Spectrophotometric method for determination of thiamine using NBD-Cl reagent

Parameter	**Recovery (% ± SD)**
Recommended condition	97.50±0.003
NBD-Cl concentration (0.22%)	98.75±0.002
NBD-Cl concentration (0.180%)	96.76%±0.003
Buffer PH(10.7)	96.90±0.002
Buffer PH(10.3)	95.79±0.22
Reaction Time min (23)	96.09±0.009
Reaction Time min (27)	98.01±0.003

3.2.3.4 Recovery

The recovery of the proposed method was carried out by applying standard addition technique. A different amount of standard solution was added to a known concentration of the drug sample. The average percent recoveries obtained were given in Table 3.9

Table 3.9 Recovery studies for the determination of thiamine by the proposed method.

Sample No.	Sample content (μg/mL)	Thiamine standard amount (μg/mL)	Amount found (Total) (μg/mL)	Recovery ± SD*
1	5	10	14.50	96.29±0.015
2	5	20	24.72	98.90±0.018
3	5	30	35.50	101.47±0.148

*Values are mean of three determinations.

3.2.4 Application of the proposed method to analysis of thiamine dosage form

Thiamine tablets were subjected to analysis by the proposed method and the label claim agrees well with our new method as shown in Table 3.10. The proposed method has the advantage of being virtually free from interferences by excipients.

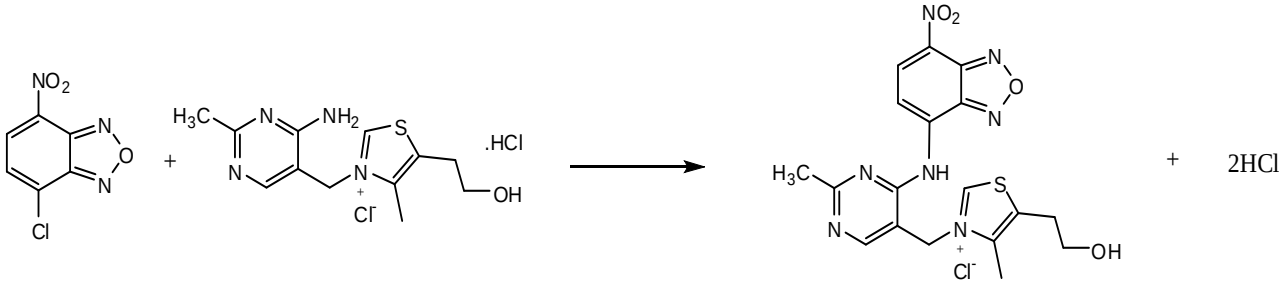
Table 3.10 Analysis of thiamine-containing dosage form by the proposed method.

Brand name of label claim (mg)	**Amount found (mg)**	**(% found ± SD)[a]**
Thiamine tablets (100 mg)	99.96	99.9±0.025

a: values are mean of five determinations

3.2.5 Reaction mechanism

It has been reported that NBD-Cl reacts with amino group of primary or secondary amine derivatives (Klimisch, 1974, Murray, 1983, Tosunoglu S, 1995, Pesez M, 1974). Similarly, amino group of thiamine can act as a nucleophile due to the lone pair of electrons on the nitrogen atom, tending to attack on the electron–deficient center in NBD-Cl. At the same time, it has been proved that the composition of the product is 1:1 of thiamine to NBD-Cl (Figure 3.14). So it is concluded that amino group of thiamine react with NBD-Cl to form a brown adduct. The reaction equation is shown in Scheme 3.2.



Scheme 3.15: Reaction of thiamine with NBD-Cl showing 1:1 stoichiometry

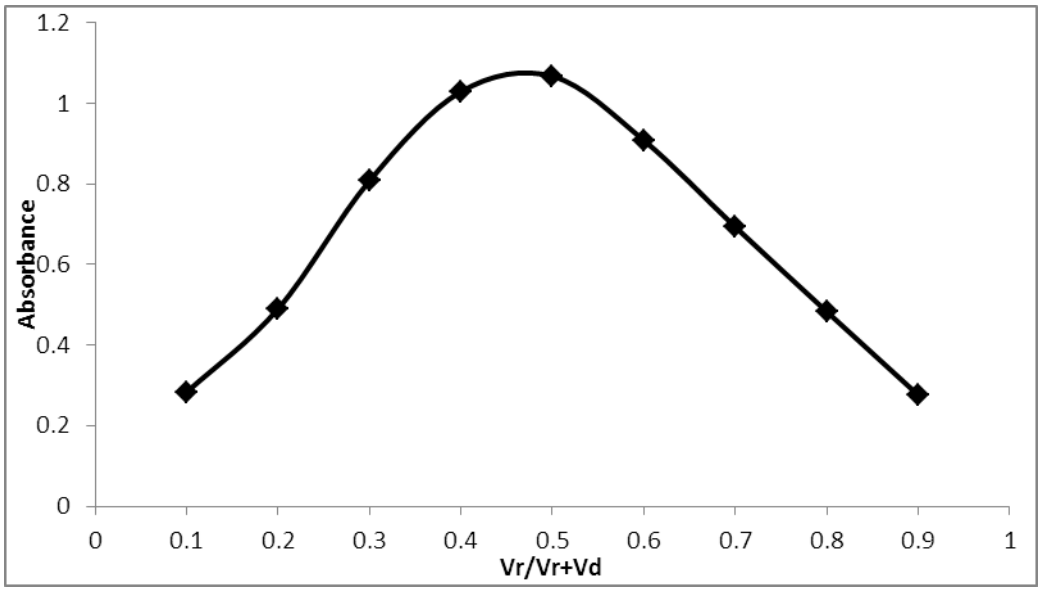


Figure 3.16: The Job's method plot for the stoichiometry of the reaction of thiamine with NBD-Cl

Vr: Volume of NBD-Cl (7.5×10^{-4} mol/l), Vd: Volume of thiamine (7.5×10^{-4} mol/l), Vr + Vd=10 ml.

3.3 Method B: Spectrofluorimetric determination of thiamine (THC) with NBD-Cl

Since the optimum conditions used in the Spectrophotometric determination of thiamine described in method A above are arising mainly from the complex adduct that formed between thiamine and each of NQS or NBD-Cl, the same optimum parameters were selected in the spectrofluorimetric assays. Those conditions show good results when the relative fluorescence intensity was measured with excitation at 472 nm and emission at 562 for the reaction of thiamine with NBD-Cl. Beer's law was obeyed in the concentration range of 0.2 – 1.0 µg/ml. The relative fluorescence intensity (RFI) was increased linearly with increasing

concentration of thiamine. The linear regression was a = 328.78, b = 357.38 and r^2 = 0.9997 calculated for the general equation of the calibration curve (y = ax + b).

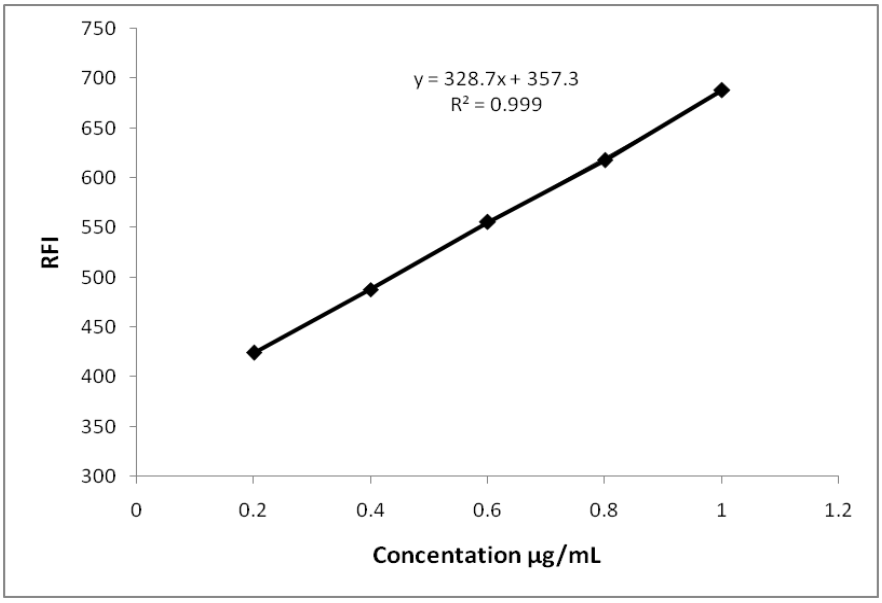


Figure 3.17 Calibration curve of thiamine with NBD-Cl using Spectrofluorimetric method B

3.3.1 Validation of the method B

Calibration curve for the determination of thiamine with NBD-Cl was constructed by plotting the fluorescence intensity as a function of concentration. Five concentrations of thiamine in the range (0.2 – 1.0 µg/ml) were selected in which a proportional increase in fluorescence intensity was observed and a linear plot with good correlation coefficient was obtained. The limit of detection (LOD) was calculated based on the

standard deviation and the slope of the calibration curve. (Figure 3.15 and Table 3.11).

Table 3.11 Parameters for the performance of Method B on analytical determination of Thiamine with NBD-Cl

Parameter	Value
Excitation (nm)	472
Emission (nm)	562
Concentration range (µg/ml)	0.2 – 1.0
Limit of detection (µg/ml)	0.02
Limit of quantitation (µg/ml)	0.06
Regression equation	y = 382.78x + 357.38
Correlation coefficient (r^2)	0.9997
Standard deviation of the intercept (S_a)	2.32
Standard deviation of the slope (S_b)	3.50

3.3.1.1 Accuracy and precision of method B

The accuracy and precision of the method was estimated by three replicate analytical samples of thiamine. The assays gave satisfactory results and the relative standard deviation (RSD) was less than 2. The

level of precision of the proposed method was adequate for analysis of thiamine in pharmaceutical dosage forms.

Table 3.12 Accuracy of the method

Concentration taken µg/ml	**Concentration found µg/ml**	**Recovery % ± RSD**
0.2	0.19	97.89±0.61
0.4	0.39	98.89±0.51
0.8	0.80	100.14±0.58

3.3.1.2 Robustness

Robustness of the procedure was assessed by evaluating the influence of small variations in the experimental variables, concentration of NDB-Cl reagent, reaction time and pH on the analytical performance of the method. In this experiment, one experimental parameter was changed while the other parameters were kept constant and the recovery percentage was calculated each time (Table 3.13). The small variation in any of the variables did not significantly affect the results. This gave an indication of the reliability of the proposed method during routine work.

Table 3.13 The effect of the variation of analytical parameters on Method B

Variation	Recovery %±SD
pH	
10.3	95.01±1.25
10.7	97.74±1.04
NBD-Cl concentration (w/v)	
0.22	105.26±2.71
0.18	97.99±1.79
Reaction time (min)	
23	95.5±1.42
27	102.8±2.24

3.3.1.3 Recovery of the method

The recovery of the proposed method was carried out by applying standard addition technique. A different amount of standard solution was added to known concentration of the drug sample. The recovery percentages obtained were tabulated in Table 3.14

Table 3.14 Recovery studies for the determination of thiamine by NBD-Cl (Method B)

Sample Concentration	Concentration added (STD) (µg/ml)	Concentration found (µg/ml)	Recovery±SD
0.2	0.2	0.38	96.36±4.10
0.2	0.4	0.60	100.35±5.76
0.2	0.6	0.79	99.57±1.17

3.3.2 Application of the method to analysis of thiamine dosage form

Thiamine tablets were subjected to analysis by the proposed method and the label claim agrees well with the method as shown in Table 3.15. The proposed method has the privilege of being virtually free from interferences by excipients.

Table 3.15 Analysis of thiamine-containing dosage form by the proposed method.

Dosage form	Amount found	%found ± RSD
100 mg	98.90	98.90±0.49

3.3.3 Stoichiometry of the reaction

The stoichiometry of the reaction between VB1 and NBD-Cl was studied utilizing the limiting logarithmic method (Rose J, 1964). Plots of log RFI versus log[NBD-Cl] and log [VB1] gave a straight lines, with the slopes of 0.913 and 0.932 respectively. Hence it was concluded that the reaction proceeds in the molar ratio of 1:1 which is consistent with what was found in the spectrophotometric method A described earlier.

3.4 Method B: Spectrofluorimetric determination of thiamine (THC) with NQS

Once again the optimum conditions used in the spectrophotometric determination of thiamine with NQS were examined in the fluorimetric study of the reaction of Thiamine with NQS. Good results were obtained when the measurement was done at 460 nm after excitation at 390 nm. The calibration curve was linear over the concentration range 0.1 – 1.0 µg/ml and the linear regression was a = 445.98 b = 408.83 and r^2 = 0.9996 as shown in figure 3.16. The proposed method was applied to the determination of thiamine in its tablets and the results were in agreement with those obtained using the other methods.

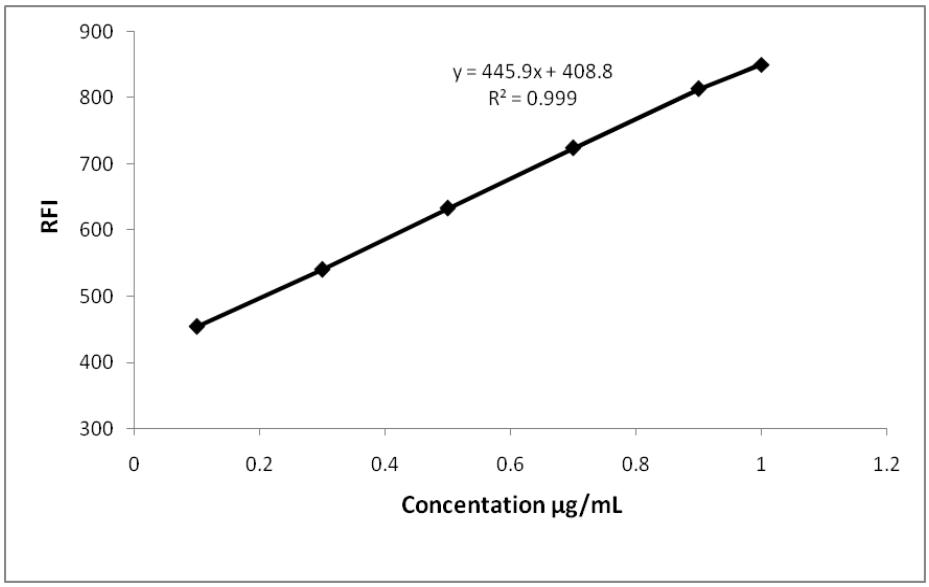


Figure 3.18 Calibration curve of thiamine with NQS using Spectrofluorimetric method B

3.4.1 Validation of the method

Calibration curve for the determination of thiamine with NQS was obtained by plotting the fluorescence intensity as a function of concentration. Six concentrations of thiamine in the range (0.1 – 1.0 µg/ml) were selected in which a proportional increase in fluorescence intensity was observed and a linear plot with good correlation coefficient results. The limit of detection (LOD) was calculated based on the standard deviation and the slope of the calibration curve. (Figure 3.16 and Table 3.16).

Table 3.16 Parameters for the performance of Method B on analytical determination of Thiamine with NQS

Parameter	Value
Excitation (nm)	390
Emission (nm)	460
Concentration range (µg/ml)	0.1 – 1.0
Limit of detection (µg/ml)	0.020
Limit of quantitation (µg/ml)	0.067
Regression equation	y = 445.98x + 408.83
Correlation coefficient (r^2)	0.9996
Standard deviation of the intercept (S_a)	3.02
Standard deviation of the slope (S_b)	4.55

3.4.1.1 Precision and Accuracy Precision of the Method

The accuracy and precision of the method was evaluated by three replicate analytical samples of thiamine. The assays gave satisfactory results and the relative standard deviation (RSD) was less than 2. The level of precision of the proposed method was adequate for analysis of thiamine in pharmaceutical dosage forms. The results were tabulated in table 3.17

Table 3.17 Accuracy of the method

Concentration taken µg/ml	Concentration found µg/ml	Recovery % ± RSD	Relative error
0.2	0.208	104.136±0.34	0.001
0.6	0.626	104.355±0.18	0.002
0.8	0.837	104.678±0.14	0.001

3.4.1.2 Robustness

The robustness of the method adopted is demonstrated by the constancy of the fluorescence intensity with the deliberated minor changes in the experimental parameters such as pH, concentration of NQS reagent, and reaction time. These minor changes that may take place during the experimental operation did not affect the fluorescence intensity of the reaction products, giving an indication for the reliability of the method.

Table 3.18 The effect of the variation of analytical parameters on Method B: Fluorimetric determination of thiamine with NQS.

Variation	Recovery %±SD
pH	
10.8	100.5±1.464
11.2	104.5±0.731
NQS concentration % (w/v)	
0.062	105.99±1.97
0.058	98.55±1.57
Time (min)	
13	99.916±1.06
17	105.116±0.86

3.4.1.3 Recovery of the method

The recovery of the proposed method was carried out by applying standard addition technique. A different amount of standard solution was added to known concentration of the drug sample. The recovery percentages obtained were tabulated in Table 3.19

Table 3.19 Recovery studies for the determination of thiamine by NQS (Method B)

Sample Concentration	Concentration added (STD) (µg/ml)	Concentration found (µg/ml)	Recovery±RSD
0.2	0.2	0.421	103.196±0.24
0.2	0.4	0.621	103.64±0.15
0.2	0.6	0.846	105.809±0.14

3.4.2 Application of the method to analysis of thiamine dosage form

The proposed method was applied to the determination of thiamine in its real tablets and the label claim agrees well with the method. The results were summarized in Table 3.20.

Table 3.20 Analysis of thiamine dosage form by spectrofluorimetric method.

Dosage form	Amount found	%found ± RSD
100 mg	101.00	101.39±1.02

3.4.3 Stoichiometry of the reaction

The stoichiometry of the reaction between VB1 and NQS was studied utilizing the limiting logarithmic method (Rose J, 1964). Plots of log RFI versus log [NQS] and log [VB1] gave straight lines, with the slopes of 0.336 and 0.598 respectively. Hence it was concluded that the reaction proceeds in the molar ratio thiamine:NQS of 1:2 which is consistent to what was found in the spectrophotometric method A described earlier.

CHAPTER FOUR

CONCLUSION

Conclusion

Four methods of analytical determination of thiamine in pharmaceutical formulations have been developed. Two of them are direct Spectrophotmetric methods based on the derivatization of thiamine with 1,2-naphthoquine-4-sulphonate (NQS), and 7-Chloro-4-nitrobenzoxadiazole (NBD-Cl) in alkaline medium. The other two methods are Spectrofluorimetric based on measuring the fluorescence intensity of the adduct formed between thiamine and each of NQS or NBD-Cl.

The proposed methods are simple, reliable, accurate, reproducible, and highly sensitive, for the determination of thiamine in commercially available dosage forms. The analytical procedures doesn't need any expensive apparatus, or tedious and time-consuming preliminary steps before measurement, and can be used advantageously as a routine methods for the determination of thiamine in quality control labs and industry.

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APPENDICES

Research article

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Enliven: Bio Analytical Techniques

1. Development and Validation of Spectrophotometric Method for Determination of Thiamine (VB1) in Pharmaceutical Formulations using 1, 2-Naphthoquine-4- Sulphonate (NQS) (2015)
2. Application of Spectrophotometric methods for the determination of thiamine (VB1) in pharmaceutical formulations using 7-Chloro-4-nitrobenzoxadiazole (NBD-Cl) (2016)