 doi:10.20944/preprints202106.0379.v1

Design of Experiment approach for development and validation of UV Spectrophotometry method for determination of Thiamine in Biological samples and tablet formulation

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Abstract

UV-Visible spectroscopy method has been developed for the analysis of Thiamine in biological and pharmaceutical product, based on a chromogenic derivatizing reaction using PDAB (P-dimethyl amino benzaldehyde) reagent. A Central Composite Design (CCD) design with response surface methodology was executed for optimization of experimental conditions of drug with a smaller number of experimental trials. Three independent factors, the concentration of PDAB, the volume of PDAB reagent, and volume of HCl, were used to construct a mathematical model and study the effects of these independent factors on responses as absorbance. The colored complex exhibits a Red shift with absorption maximum λ_{max} at 425 nm, which was selected as the analytical wavelength. The drug seems to be linear, which was established via the regression analysis from 5-30 $\mu g mL^{-1}$, with an R^2 value of 0.998.The % RSD for intraday and interday precision was < 2%, with good recovery ranging from 95.02 to 101.43% with biological and pharmaceutical samples. LOD and LOQ of the developed method were found to be 1.51 $\mu g\ mL^{-1}$ and 4.57 $\mu g\ mL^{-1}$.This method can be used in routine analysis of pharmaceutical products containing aromatic primary amines along with an estimation of biological samples like urine, blood, sweat, faeces.

Keywords: Thiamine; PDAB reagent; UV Spectrophotometry; Design of experiment; Central composite design

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1 Introduction

Thiamine Hydrochloride is also known as vitaminB_1 is a water-soluble vitamin of the vitamin B complex, with a chemical name 3-[(4-Amino–2 –methyl-5–pyrimidinyl)–methyl]–5-(2–hydroxyl ethyl)–4-methyl thiazolium chloride monohydrochloride [1]. It is found as colorless crystals or white crystalline powder having a characteristic meat-like odour with a bitter taste [2]. Clinically it is used in the treatment of Wernicke-Korsakoff syndrome, optic neuropathy, Beri-Beri and other disorders [3].

The accomplishment of the Design of experiments (DOE) is to optimize the analytical methods for its application, like a decrease in the total number of attempts needed to experiment, less reagent utilization with very less laboratory work. Moreover, DOE adds the sequential framework of a statistical model that allows evaluation of the significant factors along with their statistical acceptance on the responses being examined. The most important aspect of DOE is that it reveals the mutual interactions between the factors for analysis [4]. Some design methodologies to check the robustness of method include; full factorial design, Asymmetrical Factorial Designs (AFD), fractional factorial designs, Central Composite Design (CCD), Plackett–Burman Design (PBD), Doehlert Designs, Box–Behnken Design (BBD), Star Designs[5, 6]. CCD is widely employed for robustness testing if fast and few factors need to be tested because it has high efficiency concerning the number of trials required for analysis [7] . A literature review showed that very few analytical methods have been reported for optimization of spectroscopy conditions in UV-visible Spectrophotometry methods using two-level full factorial design [8] Central Composite Face Centered Design [9] Box-Behnken design [10].

A few UV-visible Spectrophotometry methods have been formerly reported for the estimation of Thiamine Hydrochloride in pharmaceuticals [11-14]. Other techniques reported for the assay of Thiamine Hydrochloride in pharmaceuticals include Fluorimetry [15, 16], HPLC [17-22], Polarography method [23,24] and Voltammetry [25]. The reagent PDAB was used as a derivatizing reaction for the analysis of several drugs like Ambroxol hydrochloride, metronidazole, ganciclovir, etodolac and aceclofenac [26-29].

This research aims to develop a sensitive and cost-effective method for the estimation of Thiamine in Biological material like urine, faecal, blood and pharmaceutical dosage form like tablet using the UV-visible spectrophotometric technique. In general, a one-variable-at-a time approach is carried out to set the optimized parameters but the method has a demerit of a huge experimental trial has to be performed to achieve the target, effects of inter-variable interactions, extended experimental time and massive experimental costs. The proposed method has the merit of large sensitivity and simplicity with excellent accuracy and precision. The applicability of the developed method has been established successfully without the interference of interfering substances. The colour developed was stable for a long period hence this method can be extended for the routine assay of Thiamine containing drugs and other biological materials that are not covered by this study.

2 Experimental

2.1 Drug and reagents

Thiamine was supplied by (Sigma-Aldrich), PDAB from spectrum chemicals. Hydrochloric Acid (HCl) was purchased from Merck Ltd., India. Distilled water was used throughout the analysis.

2.2 Materials and Methods

LT-2100 Double beam UV-visual spectrophotometer with a 10 mm quartz cuvette was used to record the absorbance. The structure of drug and reagent was drawn by the help of Chem Draw

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Ultra 8.0. The experimental design was carried out by using Design Expert® software 11. (Trail version).

2.3 Preparation of Standard solutions

A Standard stock solution of Thiamine was prepared in water (1000 μgmL^{-1}). 1mL of primary stock solution was pipette and transferred into 10 mL volumetric flask using water. 0.5-3 mL of the above solution was transferred into a series of 10 mL volumetric flasks, followed by the addition of 3.75 mL of PDAB reagent and 2.5 mL of concentrated HCl. The final volumes of the above solutions were diluted up to the mark using water and absorbance of each solution was measured at 425 nm against the blank solution.

2.4 Central composite design

CCD was employed to examine the effects of three factors on the response function (absorbance). The volume of PDAB (mL), the concentration of PDAB (%), the volume of HCl (mL) was selected as independent factors. For three independent factors, a total of 18 experiments have been carried out to study the effects of factors on the absorbance. DOE with CCD was applied to study the simultaneous variations of the factors on the response (Absorbance). For the evaluation of mutual relationships between the input variations and output responses, a second-order polynomial equation was quoted as:

$y=b_0+b_1x_1+b_2x_2+b_3x_3+b_{12}x_1x_2+b_{13}x_1x_3+b_{23}x_2x_3+b_{11}x_1^2+b_{22}x_2^2+b_{33}x_3^2$

where, 'y' is the measured response (dependent variable), 'b_0' represents the polynomial equation intercept representing average arithmetic mean of all quantitative outcomes of eighteen runs and 'b_1–b_{33}' are regression coefficients computed from the observed experimental values of 'y'. 'x_1', 'x_2' and 'x_3' correspond to the coded levels of independent variables where x_1: Volume of PDAB (mL), x_2: concentration of PDAB (%) and x_3: volume of conc. HCl (mL); ranging from ; (0.5–7.5), (1–7) and (0.5–4.5), respectively for x_1, x_2, and x_3. The optimized values obtained for x_1, x_2, and x_3 were 3.75 mL, 4% and 2.5 mL, respectively. The x_1x_2, x_1x_3 and x_2x_3 represent the interaction terms. Polynomial terms x^2_1, x^2_2 and x^2_3 are included to investigate the type of model.

2.5 Method of Thiamine Determination on Urine

The elimination of interfering substance and analysis of 100 mL of urine sample was carried out by [30] with some modification. Finally, after centrifugation, the supernatant solution was discarded and thiamine thus obtained was concentrated and extracted with 50 mL water, followed by 3.75 mL of PDAB reagent and 2.5 mL of concentrated HCl. Drug-free urine samples were obtained from healthy volunteers. A known amount of thiamine was added to each urine sample. 0.5 mL of the urine sample was used in subsequent experiments. Sample solutions were withdrawn at different time intervals i.e. (1, 4, 8, 12, and 24) hours, centrifuged for 20 min at 3000 Xg and stored at -20°C. 0.5 mL of the urine solution was used for analysis.

2.6 Thiamine in Faeces-

The analysis of human faeces sample (100gm) was carried out by Alexander, 1943 [31] with some modification. Human faeces were collected in a jar containing 40 mL of 10% hydrochloric acid and 10mL of toluene. The total 24-hour stool was transferred to a mortar and was thoroughly dispersed in 500 mL of water. The mixture was weighed and stirred, and an adequate aliquot (by weight) was taken for analysis. The mixture after centrifuging, supernatant solution was decanted into another 250 mL centrifuge flask and the centrifuged solution was washed twice thoroughly with 50 mL of water, and the washed solutions were combined with the original extract and concentrated until the solution reaches 50 mL. The thiamine determinations were then made as same as described in the urine.

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2.7 Thiamine in Blood

The analysis of blood was carried out by Hussein, 2016 [14]. Drug-free blood samples were obtained from healthy volunteers. 100 mg of thiamine was added to each blood sample and 5 mL were drawn at different time points (1, 4, 8, 12, and 24) hours. The blood sample was allowed to clot, centrifuged and Serum was collected and stored. The volume of serum used for analysis was 0.5 mL.

2.8 Method development

We followed the method as per Kumar Reddy, 2019 [32].

2.8.1 Determination of solubility

Thiamine solubility was tested in different organic and aqueous solvents and solubility was found in water.

2.8.2 Selection of suitable reagent

Para dimethyl amino Benzaldehyde (PDAB) reagent was selected. The concentration and the volume of the reagent to be added were optimized by using design of experiment method.

2.8.3 Mechanism of colour production

Thiamine contains an Aromatic ring with a reactive primary amine. This reactive amine reacts with the carbonyl part of PDAB in the presence of an acidic medium with the elimination of water to form a yellow coloured Schiff base as shown in figure 1.

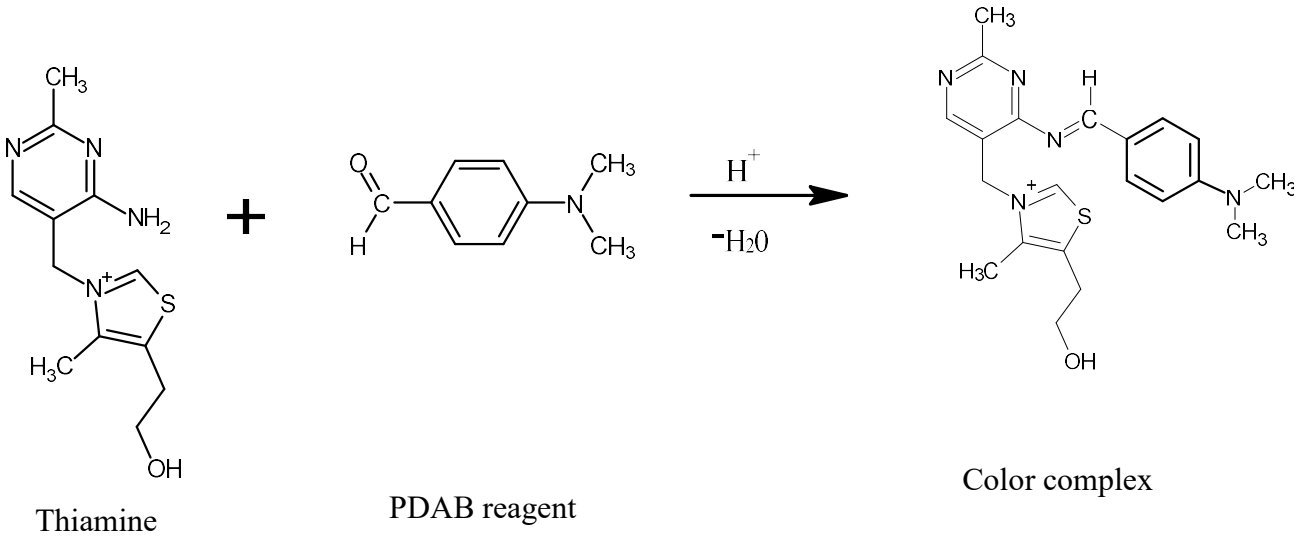

Figure 1. Formation of colour complex

3 Results and Discussions

3.1 Experimental design for optimization

The absorbance values (responses) obtained from the experiments proposed by CCD design were fitted to linear, 2FI and quadratic models to find the regression equations. The quadratic model was preferred because it consists of a low standard deviation (0.0954) and low *p*-value. The multi-regression analysis reveals the relationship between the absorbance and independent variables of experimental data. The predicted response i.e. absorbance can be calculated by the second-order polynomial expression in terms of significant actual factors as:

$$y = +0.6049+0.0450\ x_1+ 0.0340\ x_2 -0.0170\ x_3 -0.0175\ x_1x_2 -0.0100\ x_1x_3+0.0050\ x_2x_3- 0.0323\ x_1^2- 0.1473\ x_2^2-0.1023\ x_3^2$$

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The experimental domain of the selected variables is reported as represented in Table 1.

Table 1. Experimental design for optimization and response.

Run	Volume of PDAB (x_1)	Concentration of PDAB(x_2)	Volume of HCl (x_3)	Absorbance (y)
1	0.5	1	0.5	0.25
2	0.5	1	4.5	0.24
3	3.75	4	2.5	0.69
4	7	7	0.5	0.42
5	7	4	2.5	0.58
6	3.75	4	2.5	0.68
7	0.5	7	0.5	0.38
8	3.75	4	0.5	0.44
9	0.5	7	4.5	0.36
10	3.75	1	2.5	0.39
11	3.75	4	2.5	0.69
12	3.75	4	2.5	0.67
13	3.75	7	2.5	0.37
14	3.75	4	4.5	0.41
15	0.5	4	2.5	0.41
16	7	1	4.5	0.31
17	7	7	4.5	0.39
18	7	1	0.5	0.39

The response surface quadratic model was fitted and judged by analysis of variance (ANOVA) (Table 2). The F- and p-values (Prob > F) for the model were; 158.31 and <0.0412 respectively, suggesting that the quadratic model is highly significant.

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Table 2. Analysis of variance for the quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.2986	9	0.0332	3.64	0.0412	significant
x_1-volume of PDAB	0.0202	1	0.0202	2.22	0.1742	
x_2-Conc. of PDAB	0.0116	1	0.0116	1.27	0.2925	
x_3-Conc. of HCl	0.0029	1	0.0029	0.3174	0.5886	
x_1x_2	0.0025	1	0.0025	0.2691	0.6180	
x_1x_3	0.0008	1	0.0008	0.0879	0.7745	
$x_2\ x_3$	0.0002	1	0.0002	0.0220	0.8858	
x_1^2	0.0028	1	0.0028	0.3098	0.5930	
x_2^2	0.0588	1	0.0588	6.45	0.0347	
x_3^2	0.0283	1	0.0283	3.11	0.1157	
Residual	0.0728	8	0.0091			
Lack of Fit	0.0726	5	0.0145	158.31	0.0008	significant
Pure Error	0.0003	3	0.0001			
Cor Total	0.3715	17				

As per the value of coefficients from the polynomial equation; x_1 (volume of PDAB) x_2 (concentration of PDAB) and x_3 (Conc. of HCl) have a positive influence on the response i.e. absorbance; up to certain value and after it showed the abrupt decline, as shown in figure 2a-2c.

3.2 Optimization of reagent concentration, volume and HCl concentration

The effect of concentration of PDAB solution and HCl were studied on the related absorbance values. Different concentrations ranging from 0.5% to 7% and volumes of 0.5–7.0 mL of PDAB was examined. The investigations showed that 3.75 mL of 4% PDAB gave maximum absorbance hence selected as optimized volume, the strength of reagent respectively as shown in figure 2a. Different volumes of conc. HCl was studied and 2.5 mL of conc. HCl was selected for the volume of acid for analysis, as it gave the maximum absorbance among the volumes selected, as shown in figure 2b and 2c.

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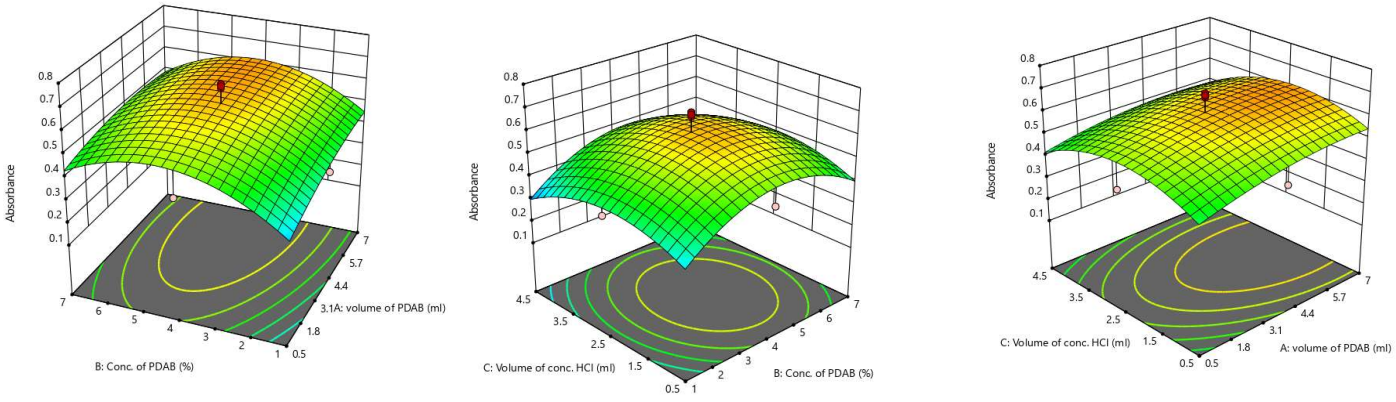

2a **2b** **2c**

Figure 2a, 2b, 2c. 3D plot for optimization of volume and concentration of PDAB and HCl

After optimizing PDAB and HCl separately, the optimized volumes of reagents are mixed with the drug to develop the colour. The reaction between Thiamine and PDAB was immediate and the colour was stable for 24 hours (The colour complex thus formed was stored up to24 hours and absorbance of the coloured complex was measured) and it was quite stable with precise measurements. The concentration for measuring the coloured complex was set at a higher level of calibration curve i.e. at 30 µg mL^{-1} to ensure the stability of the coloured complex, as shown in figure 3.

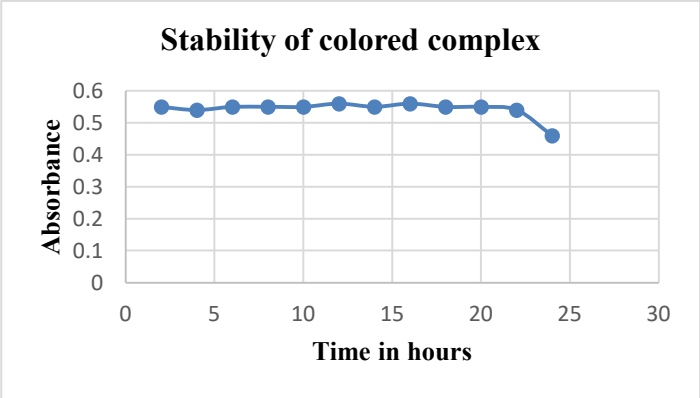

Figure 3. Stability of coloured complex of 30µg $mL^{-1}$with respect to hours

3.3 Validation of Analytical method for Thiamine

Validation of an analytical method processes to establish that the performance characteristics of the developed method meet the requirements of the intended analytical application. The Color complex of the drug with PDAB reagent and HCl was analyzed using a UV visible Spectrophotometry and the absorbance of all aliquots (converted to colour complex) was taken. The linearity complied with the regression plot in the concentration range of 5-30 µg mL^{-1} with a correlation coefficient (R^2) of 0.9981. The limit of detection (LOD) and limit of quantification (LOQ) was found to be 1.51 µg $mL^{-1}$and 4.57 µg mL^{-1}as shown in table 3. The data for intra-day and inter-day precision studies were obtained for three different concentrations 10, 20, and 30 µg mL^{-1} in linearity. The % RSD values for intra-day and inter-day precision were less than 2 and

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the result is shown in Table 3. The accuracy of the method was evaluated in triplicate at three concentration levels, i.e. 80%, 100%, and 120% of target test concentration (20 µg mL^{-1} of Thiamine) for biological samples and pharmaceutical formulations. The percentages of recoveries were calculated and a result is presented in Table 4.

Table 3. Linearity, LOD, LOQ and Precision

S. No.	Parameter	Values of Glycerol
1.	Concentration, µg mL^{-1}	5-30
2.	Regression equation	y = 0.018x + 0.187
3.	Correlation coefficient, (R^2)	0.998
4.	LOD, µgmL^{-1}	1.51
5.	LOQ, µgmL^{-1}	4.57
6.	Precision,(Intra and Interday)	<2

Table 4. Recovery of added Thiamine

S.No.	Name of Sample	Amount of drug (µg mL^{-1})	Recovery level (%)	Amount of drug added (µg mL^{-1})	Amount found (µg mL^{-1}) (Mean±SD)	% Recovery (n = 3)
1.	Urine	20	80	16	15.51± 0.59	96.97
			100	20	19.45± 0.44	97.28
			120	24	23.133± 0.7	96.38
2.	Faeces	20	80	16	15.67± 0.33	97.97
			100	20	19.38± 0.351	96.93
			120	24	23.08± 0.13	96.19
3.	Blood	20	80	16	15.20± 0.015	95.02
			100	20	19.3± 0.26	96.50
			120	24	22.97± 0.21	95.69
4.	Tablet-1	20	80	16	16.23± 0.088	101.43
			100	20	19.96± 0.107	99.8
			120	24	23.96± 0.049	99.86

n= number of replicates, SD= Standard deviation

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3.4 Application of the developed Method to Biological samples

Thiamine contents were analyzed in the urine, faeces of a healthy volunteer and marketed Thiamine tablet. During this research 100gm of faeces and 100mL of urine was used as the amount of sample for the study. Results reveal that the thiamine present in urine, faeces, blood and tablets complexes with PDAB resulting in 71.46 µg mL^{-1} thiamine in urine, 42.76 µg mL^{-1} in faeces and 60.66 µg mL^{-1} in the blood respectively. Thiamine content in Tablet-1 was found to be 99.63 µg mL^{-1} (Table 5). Due to simple operation, the cost-effective reagent used with good precision, the method was found to be reliable when comparing with the reference method [14] and can be used for routine analysis as shown in Table 6.

Table 5. Thiamine content of Biological and Pharmaceutical samples

SN	Samples	Amount analyzed	Time (hrs.) Conc. (µg mL^{-1}) (Mean ± SD) n=3				
			1	4	8	12	24
1	Urine	100mg	95.5±0.40	84.51±0.27	79.54±0.1	76.60±0.09	71.46±0.3
2	Faeces	100mg	81.66±0.20	75.63±0.30	60.53±0.4	50.06±0.15	42.76±0.15
3	Blood	100mg	79.73±0.11	68.43±0.20	65.7±0.17	62.7±0.79	60.66±0.25
4	Tablet-1	100mg	99.63±0.54	…..	….	….	…..

Table 6. Comparison of Thiamine content with the reference method

Sample	Proposed method	Reference method
Urine	71.46	47.98
Blood	60.66	52.89
Tablet	99.63	102.31

The experimental values of the proposed method and reference method were compared statistically by student t-test showing a significant difference (p=0.679, i.e. p>0.05).

The Committee on Food and Nutrition of the National Research Council [33] has stated that 1.8 mg is the optimal daily intake of thiamine for a moderately active man whose caloric intake is 3000 calories. Although the Fluorimetry method converting thiamine to thiochrome method is extremely sensitive, it has been found to lack specificity [34-36] because of the presence in normal urine, and in the urine of subjects taking certain drugs, of variable amounts of thiochrome and other fluorescent compounds for such the correction must be made. Approximately two-third of thiamine is converted to thiochrome [37]. Several colourimetric chemical methods are available for determining thiamine, based on the Ehrlich-Pauly reaction in which the vitamin is coupled with a diazotized amino compound. Prebluda and McCollum [38] have described a dye

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that was produced by the coupling of thiamine with diazotized p-aminoacetophenone [39] established a method for the determination of urinary thiamine which is based on the complex formed with diazotized ethyl p-aminobenzoate [30] have identified two interfering substances which make it more specific from earlier explained methods hence this method was used as a reference document to carryout extraction on biological samples. In the present research work, a simple spectrophotometric determination of Thiamine in Biological materials and Pharmaceutical formulations has been developed and validated. The main purpose of the present study is to establish a relatively simple, sensitive, validated and inexpensive UV spectrophotometric method for the determination of Thiamine using PDAB reagent in the presence of mineral acid like HCl. Since most of the previous methods comprised of tedious procedures such as heating utilize costly reagents which lead to cost-effective problems, it felt palpable stipulation in developing a method based on their activity between the drug and PDAB reagent in acidic medium without the application of high temperature and heat to stabilize the colour complex. The selected drug Thiamine was determined in biological samples and pharmaceutical formulations by a colour developing method using UV Spectrophotometry and this method were validated as per ICH guidelines [40, 41] . The linearity range for Thiamine was 5-30 µg mL^{-1}, with an R^2 value of 0.998. The % RSD for intraday and interday precision was <2%. The method has good recovery ranging from 95.02 to 101.43%.The developed method was compared with the PDAB proposed method with other aromatic amino group-containing drugs and we found it more reliable with all the parameters. While comparing with Hanamshetty, 2014 [26], where the colour was developed with the aid of heating at 100^0C for 10 min, whereas in this method the colour is developed without the introduction of heat, hence it is surely beneficial. Also Al-Ahmary, 2014 [12], reported a colorimetric assay of thiamine with good accuracy of 100.03% but has a limitation of heating to get a stable colour. The interference may cause a slight decrease in the recovery of biological samples even it has been followed an interference limiting articles whereas the accuracy of the formulated tablet sample was optimum. In contrast with the linearity of regression, while comparing with the earlier developed method of thiamine by M. A. Khan, 2009 [42], the drug linearity was 0.026-16.830 µg mL^{-1} with an R^2 value of 0.9964 and for Szpikowska-Sroka, 2013 [43], the drug linearity was 0.4-2.4 µg mL^{-1} whereas this developed method undergoes the extreme of linearity when compared with above articles.

4 Conclusions

A simple, rapid, cost effective, and sensitive UV-spectrophotometric method was established for the determination of thiamine in Biological and Pharmaceuticals formulation. The proposed procedures could be used in routine analysis of pharmaceutical products containing aromatic primary amines along with an estimation of biological samples like urine, blood, sweat, faeces and others to understand the level of primary amines within them.

Data Availability

All data used to support the findings of this study will be made available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

Authors are thankful to Mr. Shakti shrestha for his supportive guidance and Shree Medical and technical college for providing necessary facilities to carry out the research work.

Author Contributions

Hemraj Sharma, Hari Prasad Sapkota and Nim Bahadur Dangi designed and carryout the experiments; Sushant Aryal and Niranjan Koirala analyzed the data and helped to wrote the manuscript; Niranjan Koirala curated the data and advised the research group.

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