

Research Article

Stability-Indicating Photochemical Method for the Assay of Thiamine by Spectrophotometry

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A stability-indicating photochemical method has been developed for the assay of thiamine (TH) salts in aqueous solution and in fresh and aged vitamin preparations. It is based on the photooxidation of TH by UV irradiation to form thiochrome (TC) in alkaline solution. The TC : TH ratio under controlled conditions of light intensity, temperature, pH, exposure time, and irradiation distance is constant and can be used to determine the concentration of UV irradiated TH solutions. TC, on extraction with isobutanol from the photodegraded solution of TH, has been determined by the UV spectrophotometric method at 370 nm. It exhibits a high intensity of absorption in the UV region that can be used for the assay of even low concentrations of TH. Under optimum conditions, Beer's law is obeyed in the concentration range of 0.20–2.00 mg/100 ml ($R^2 = 0.9998$). The limit of detection (LOD) and limit of quantification (LOQ) are 0.0076 and 0.0231 mg/100 ml, respectively. The method has been validated and applied to aqueous solutions and vitamin preparations. The results have statistically been compared with the United States Pharmacopeia liquid chromatography method. It has been found that there is no significant difference between the two methods at 95% confidence level.

1. Introduction

Thiamine (vitamin B₁) (TH) is a water-soluble vitamin and is used in the prevention and cure of the disease beriberi [1]. It is extensively used as a component of single vitamin B complex and multivitamin preparations [2–4]. Several analytical methods, including UV and visible spectrophotometric and multivariate analysis [5–7], fluorimetric [8–21], high performance liquid chromatography (HPLC) [22–35], UHPLC/MS-MS [36], turbidimetric and nephelometric [37], amperometric [38], and voltammetric methods [39–45], have been used for the assay of thiamine and its salts in pharmaceutical preparations, food materials and biological fluids. Some of these methods have previously been reviewed [46–49]. A flow injection analysis- (FIA-) spectrophotometric method for the determination of TH after UV irradiation, based on difference

spectrophotometry, has been reported [50]. The official methods for the assay of thiamine salts are based on non-aqueous titration [2] and liquid chromatography [3]. Thiamine undergoes chemical [1, 8, 9, 11, 14, 16, 17, 19, 51–54] and photochemical oxidation [55, 56] to form thiochrome (TC) which exhibits a blue fluorescence (440 nm). This has been made as the basis of fluorimetric determination of TH [8, 9, 11, 14, 16, 17, 19, 54–60]. The photochemical dehydrogenation of TH (1) to TC (3) probably proceeds through an intermediate (2) [52] as shown in Figure 1. The formation of TC is pH-dependent, and its yield is maximum at pH 10 [52]. TC exhibits strong absorption in aqueous solution in the UV region [61].

The objective of this study is to develop and validate a simple, economical, and stability-indicating photochemical method for the assay of TH on the basis of the formation

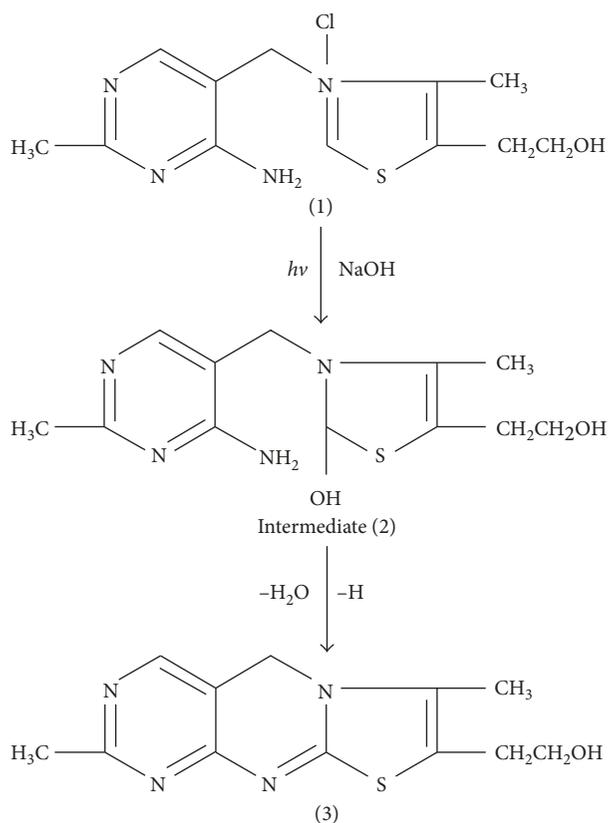


FIGURE 1: Photochemical dehydrogenation of thiamine (1) to thiochrome (3).

of TC, its spectrophotometric determination, and to establish conditions for its quantitative relationship with TH. The method would be applied to the assay of TH in pure solutions and in vitamin preparations.

2. Experimental

2.1. Materials. Thiamine hydrochloride (TH) and thiochrome (TC) were purchased from Sigma-Aldrich (USA). All reagents and solvents were of the purest form available from Merck (Germany). The buffer systems used were $Na_2B_4O_7$ -NaOH (pH 9–10) and Na_2HPO_4 -NaOH (pH 11); the ionic strength was maintained at 0.002 M.

2.2. Precautions. The experimental work was carried out in a dark chamber under subdued light. The TH solutions were protected from light to avoid the effect of any photochemical change before UV irradiation.

2.3. Photooxidation of TH. Aqueous solutions containing 4 mg/100 ml of TH were prepared at pH 9–11 using appropriate buffers with the addition of 2 ml of a 30% H_2O_2 solution. A 50 ml quantity of the solution spread in a Petri dish was placed in a thermostat bath at $25 \pm 1^\circ C$ and irradiated using a Philips 36 W TUV tube (100% emission at 254 nm) for 30 min. The tube was fixed horizontally at a distance of 25 cm from the centre of the Petri dish in

a radiation chamber. Samples were withdrawn at appropriate intervals and subjected to chromatographic examination and spectrophotometric assay.

2.4. Thin-Layer Chromatography (TLC). The formation of TC in UV irradiated solutions of TH was confirmed by TLC using 250 μm silica gel GF₂₅₄ plates and solvent system: (a) pyridine-isobutanol-water (66:17:17, v/v) and (b) 1-butanol-acetic acid-water (40:10:50, v/v, organic layer) [62].

The compounds were detected on comparison of their R_f values and fluorescence colour under 365 nm (TC, blue) and fluorescence quenching under 254 nm excitation (TH, dark), with those of the reference standards.

2.5. pH Measurements. The pH of TH solutions was measured on an Elmetron LCD display pH meter (Model CP 501, sensitivity ± 0.01 pH units, Poland) using a combination pH electrode. The calibration of the electrode was carried out using commercially available buffer tablets of pH 7 and 9 (Merck).

2.6. Spectral Measurements. The spectral measurements on TH and TC solutions were performed on a Thermo Fisher Scientific UV-Vis spectrophotometer (Evolution 201, USA) using quartz cells of 10 mm path length.

2.7. Light Intensity Measurement. The intensity of the Philips 36 W TUV tube was determined by potassium ferrioxalate actinometry [63], and a value of $5.50 \pm 0.11 \times 10^{18}$ quanta s^{-1} was obtained.

2.8. Spectrophotometric Assay of TH

2.8.1. Extraction of TC. A 5 ml aliquot of the photooxidized solution of TH was placed in a 100 ml separating funnel, 2 ml of dehydrated ethanol was added, and the solution extracted with 10 ml of isobutanol to isolate TC. The isobutanol layer was dried over Na_2SO_4 and used for the determination of the concentration of TC in the solution at 370 nm using $20,400 M^{-1} \cdot cm^{-1}$ as the value of molar absorptivity [61] or 654 as the value of A (1% 1 cm) at that wavelength. The same procedure was used for the determination of the concentration of TC in diluted solutions of photooxidized vitamin preparations.

2.8.2. Determination of TC:TH Ratios. The TC:TH ratios are determined by the formation of a certain amount of TC on the photooxidation of a fixed amount of TH under controlled experimental conditions (i.e., concentration of TH, concentration of oxidizing agent, pH of the solution, irradiation source, and irradiation time). These values are determined from the concentrations of TC and TH and have been used for the assay of TH in different samples.

2.8.3. Assay of TH. The spectrophotometric assay of TH in pure solutions and in vitamin preparations has been carried

TABLE 1: R_f values of thiamine HCl (TH) and thiochrome (TC).

Compound	Solvent system	
	<i>a</i>	<i>b</i>
TH	0.03	0.27
TC	0.97	0.52

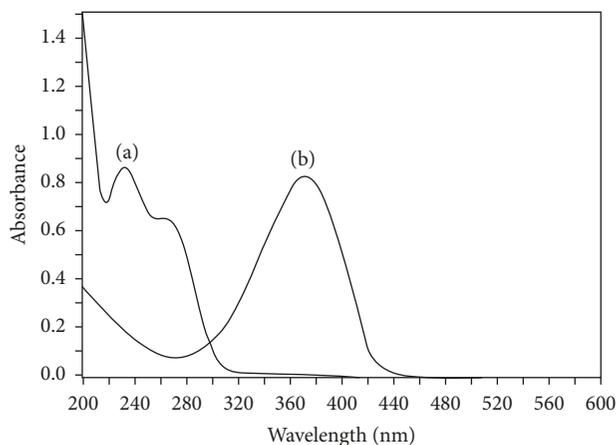


FIGURE 2: Absorption spectra of (a) thiamine HCl and (b) thiochrome, at pH 7.0.

out on the basis of the TC:TH ratios determined under controlled conditions as described in the above section. For example, if the TC:TH ratio obtained on the irradiation of a vitamin preparation containing 30 mg TH per 100 ml is 0.405:1, the corresponding TH concentration can be calculated by the following formula:

$$\begin{aligned} \text{TH} &= \frac{\text{amount of TC formed}}{\text{TC : TH ratio}} \\ &\times \text{dilution factor of vitamin preparation,} \\ &= \frac{1.600}{0.405} \times 7.5, \\ &= \frac{29.7 \text{ mg}}{100 \text{ ml}} \end{aligned} \quad (1)$$

3. Results and Discussion

3.1. Identification of Photoproduct of TH. The photooxidation of TH in alkaline solution leads to the formation of TC. The identification of TC in UV irradiated solutions of TH at pH 9–11 has been carried out by TLC using the solvent systems (a) and (b) (Section 2.4). TC was detected by comparison of its characteristic fluorescence (blue) and R_f values with those of the authentic compounds (Table 1). TC was only detected in TH solutions irradiated at pH 9–11 since it is known to be formed by photooxidation in alkaline solution [52]. Judging from the intensity of the fluorescence of TC in the pH range studied, the maximum yield was found for the reactions carried out at pH 10.

TABLE 2: Analytical parameters for the assay of thiochrome (TC).

λ_{max}	370 nm
Linearity ^a	
Concentration range	0.20–2.00 mg%
Correlation coefficient	0.9999
Slope	654
SE of slope	0.0048
Intercept	0.0031
SE of intercept	0.00325
SD of intercept	0.0103
Recovery range (%)	99.3–100.7
Accuracy (%) ^b \pm SD	99.71 \pm 0.50
Precision (% RSD) ^c	0.50
LOD ^d (mg)	0.052
LOQ ^e (mg)	0.157

^aLinearity, $n = 5$. ^bAccuracy, $n = 6$ at 100% concentration. ^cPrecision, $n = 6$ at 100% concentration. ^dLimit of detection (LOD) = $3.3 \times \sigma/S$, where σ is the standard deviation and S is the slope of the standard curve. ^eLimit of quantitation (LOQ) = $10 \times \sigma/S$, where σ is the standard deviation and S is the slope of the standard curve.

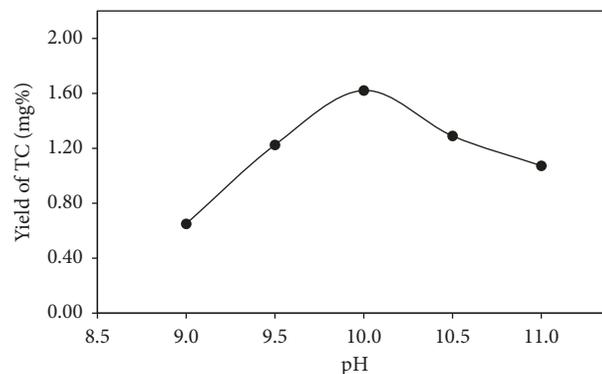


FIGURE 3: Yield of TC as a function of reaction pH.

3.2. Spectral Characteristics of TH and TC. TH exhibits absorption maximum at pH 7 at 246 nm with a molar absorptivity value of $15,200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and an A (1% 1 cm) value of 450 [64]. TC shows an absorption maximum at 370 nm with a molar absorptivity of $20,400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [61] and an A (1% 1 cm) value of 654 (Figure 2). In the present study, a molar absorptivity value of $19,800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and an A (1%, 1 cm) value of 635 for TC at 370 nm have been obtained (Table 2). In view of the very high value of the molar absorptivity of TC at 370 nm, it can be conveniently used for the assay of TH in vitamin preparations on its photooxidation to TC under controlled conditions as described in Section 3.5. The absorption maximum of TC at 370 nm provides maximum specificity and sensitivity for absorbance measurements in the method. The validation data for the test method is reported in Table 2.

3.3. Yield of TC. The photooxidation reaction of TH has been carried out at pH 9–11. It has been observed that the yield of TC on UV irradiation for a fixed period of time is maximum at pH 10 compared to that of pH 9 and 11 (Figure 3). Therefore, pH 10 was considered suitable for the photooxidation reaction of TH under the conditions used in this

TABLE 3: TC:TH ratios for UV irradiated TH solutions (4 mg %) at pH 9.0–11.0.

pH	TH concentration (mg %)	Time (min)	$A_{370\text{nm}}$	TC concentration (mg %) ^a	TC:TH ratio
9.0	4.0	0	0	0	—
		15	0.221	0.334	0.084:1
		30	0.425	0.650	0.162:1
10.0	4.0	0	0	0	—
		15	0.512	0.782	0.196:1
		30	1.06	1.621	0.405:1 (optimum value)
11.0	4.0	0	0	0	—
		15	0.411	0.628	0.157:1
		30	0.702	1.073	0.268:1

^aThe concentration of TC in photooxidized solution of TH has been calculated from the increase in absorbance at 370 nm of the extracted solution at 30 min irradiation using 654 as the value of A (1% 1 cm) [61].

TABLE 4: Assay of TH in vitamin preparations ($25 \pm 1^\circ\text{C}$).

Method	Labeled ^a (mg/100 ml)	Vitamin preparations stored in original package (pH 3-4)					
		Found ^b (mg/100 ml)	Fresh		Aged (6 months)		
			Recovery ^c (%)	RSD (%)	Found (mg/100 ml)	Recovery (%)	RSD (%)
<i>USP LC method</i>							
Sample 1	30	30.2	100.7	1.7	28.8	96.0	1.6
Sample 2	50	49.8	99.6	1.9	46.5	93.0	1.8
Sample 3	83	83.6	100.7	1.6	78.5	94.6	1.7
<i>Proposed method</i>							
Sample 1	30	30.0	100.0	1.2	27.5	91.7	1.6
Sample 2	50	48.2	98.6	1.4	45.9	91.8	1.5
Sample 3	83	82.5	99.4	1.7	73.6	88.6	1.8

^aThe labeled amount of thiamin is the amount (mg/100 ml) stated on the label of the vitamin preparations. ^b $n = 3$. ^cPercent recovery of TH in vitamin preparation = ((amount found in mg/100 ml)/(labeled amount in mg/100 ml)) \times 100.

study (Table 3). It has earlier been reported that the formation of TC depends on the pH of the medium, and its yield is maximum at pH 10 [52]. Above pH 10, TH is ionized to the negatively charged thiol form (pK_a 11.6) which undergoes hydrolytic degradation to thiazole and pyrimidine moieties [53]. Therefore, there is a decrease in the formation of TC with an increase in pH above 10.

3.4. Application of the Assay Method. The photochemical method has been applied to the assay of TH in fresh and aged pharmaceutical vitamin preparations, and the results are compared with the United States Pharmacopeia liquid chromatography method (Table 4). At 95% confidence level, it has been found that the calculated t value for each concentration is less than the tabulated t value, indicating no significant difference between the two methods. The accuracy and precision of the method has been determined, and its sensitivity has been calculated (Table 2). The method is stability-indicating for the assay of TH in vitamin preparations on the basis of the amount of TC photochemically formed under controlled conditions and its quantitative relationship with TH, that is, TC:TH ratio. A similar photochemical method for the assay of riboflavin (RF) on the basis of the formation of its photoproduct, lumichrome (LC) and LC:RF ratio, has been reported [65].

3.5. Experimental Conditions

3.5.1. Radiation Source. A 36 W TUV tube (100% emission at 254 nm) has been found to be suitable for the photooxidation of TH. The absorption maximum of TH (246 nm) [64] overlaps the emission band of the TUV tube that provides maximum radiation energy for the photochemical reaction and the formation of TC.

3.5.2. Radiation Source-Vessel Distance. The distance between the radiation source and the reaction vessel must be constant for a fixed number of quanta to be absorbed to perform a uniform photochemical reaction. A distance of 25 cm between the radiation source and the reaction vessel has been found to be appropriate for this work.

3.5.3. Temperature. The control of temperature is essential for photochemical studies to avoid any thermal effect on the rate of reaction. This has been achieved by irradiation of the solutions in a thermostat bath maintained at a constant temperature, that is, $25 \pm 1^\circ\text{C}$ during the reaction.

3.5.4. Irradiation Time. The irradiation of the solutions for a fixed period of time is necessary to achieve uniform results

in the formation of TC. In the present study, a time period of 30 min has been found to be sufficient to avoid any photodegradation of TC [52].

3.5.5. Oxidizing Agent. The oxidation of TH in alkaline solutions occurs in the presence of an oxidizing agent, that is, potassium ferricyanide [66] or hydrogen peroxide [67]. In the present study, the photooxidation of TH has been carried out in the presence of hydrogen peroxide to avoid any interference of the oxidizing agent in the absorbance of TC.

4. Conclusion

A simple photochemical method has been developed and validated for the assay of TH in aqueous solution and pharmaceutical vitamin preparations. It is based on the photooxidation of TH to TC, determination of their quantitative relationship (TC: TH ratio) under controlled conditions and on this basis the assay of TH in different pharmaceutical samples. TC has a high value of A (1%, 1 cm) and can be conveniently determined by spectrophotometry at 370 nm. The method is comparable to the United States Pharmacopeia method.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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