



Smart Spectrophotometric Methods for the Simultaneous Determination of Vitamin B1 and B2 Concentrations in Complex Mixtures

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ABSTRACT

Simple, selective and reproducible spectrophotometric methods have been developed for the determination of thiamin and riboflavin concentrations in bulk and their pharmaceutical dosage forms using pure formulations and pharmaceutical formulations of chromazurol S (CAS). This method is based on the ion-association between the drugs and the acid dye (CAS) in weak base media in the presence of Triton X-100 as a solubilizing agent. The absorption factor was calculated to estimate thiamin and riboflavin concentrations in mixtures without previous separation by applying the absorption factor method and absorbance subtraction method.

Key words: Thiamin, Riboflavin, Absorption factor, Spectrophotometry, Absorbance subtraction.

INTRODUCTION

Despite constituting only a very small fraction of the daily food intake, vitamins play a very important role in our health¹. Water-soluble vitamins are considered to be a relatively complete formulation and are among the pharmaceutical preparations that are most widely used as vitamins and mineral supplements.

Thiamin (vitamin B₁) contains two ring structures, 2-methyl-4-amino-5-hydroxymethyl pyrimidine (hydroxymethyl pyrimidine, HMP) and

4-methyl-5-β-hydroxyethylthiazole (hydroxyethyl thiazole, HET)². Thiamin has several known functions in the body that have the potential to affect brain activity and behavior. In its thiamin diphosphate (ThDP) form, thiamine serves as a cofactor for enzymes involved in energy metabolism and formation of essential body constituents³ as well as in the degradation of 3-methyl branched chain fatty acids and 2-hydroxy straight chain fatty acids^{4,5}.

Riboflavin (vitamin B₂) is a necessary nutrient. It is a precursor of flavin mononucleotide

and flavin adenine dinucleotide (FAD), which serve as coenzymes for numerous oxidases and dehydrogenases in eukaryotic cells⁶. Riboflavin is an essential vitamin that generally acts as a co-factor (flavin adenine mononucleotide and flavin adenine dinucleotide) in numerous enzymatic reactions in all forms of life. Additionally, it exhibits excellent photosensitizing properties, which have been used in photodynamic therapy for the treatment of various diseases. Various contemporary investigations strongly suggest that this vitamin has a tremendous potential for improving the chemotherapeutic potential of major anticancer drugs⁷⁻¹⁰. Many analytical techniques, such as chromatographic¹¹⁻¹³, spectrophotometric¹⁴⁻¹⁷, capillary electrophoresis¹⁸ and voltammetry^{19, 20} methods, have been developed and used for the quantitative determination of these vitamins in different samples.

Theory

Absorption factor method (AF)

The absorption factor method can be applied to analyze a mixture of two drugs, X and Y, with overlapping spectra. Y has interference at λ_{\max} of X (λ_x), but X does not show any absorption at another wavelength.

We can calculate the X absorption at λ_{\max} of x in a mixture (X+Y) using:

$$A_{X\lambda_x} = A_{\lambda_x}(X+Y) - \frac{A_{\text{of } Y_{\lambda_x}}}{A_{\text{of } Y_{\lambda_y}}} \times A_{\lambda_y}(X+Y)$$

$$\frac{A_{\text{of } Y_{\lambda_x}}}{A_{\text{of } Y_{\lambda_y}}} = \text{absorption factor}$$

The X concentrations can be estimated by using the corresponding regression equation and plotting the absorbance values of the zero order curves against the concentrations²¹.

Absorbance subtraction method (AS)

This method is based on the same principle as the absorption factor method. It was developed to analyze a mixture of two drugs, X and Y, with overlapping spectra intersecting at the isoabsorptive point; while Y extends over X, X does

not show any contribution at another wavelength (λ_2). In this method, the isoabsorptive point λ_{iso} can be used for separate quantitative estimations of each X and Y in the mixture (X+Y). This determination can be performed by using the mathematically calculated factor of one of these components with a simple manipulation step, obtaining the absorbance values corresponding to X and Y separately. The concentrations of each component can then be obtained via the isoabsorptive point regression equation without any need for a complementary method.

$$\text{The absorbance of Y in the mixture at } \lambda_{\text{iso}} = \frac{\text{abs}_1}{\text{abs}_2} \times \text{abs } \lambda_2(X+Y)$$

$$\text{The absorbance of X in the mixture at } \lambda_{\text{iso}} = \text{abs } \lambda_{\text{iso}}(X+Y) - \frac{\text{abs}_1}{\text{abs}_2} \times \text{abs } \lambda_2(X+Y)$$

Where $\text{abs } \lambda_{\text{iso}}$ and $\text{abs } \lambda_2$ are the absorbance values for Y at λ_{iso} and λ_2 , respectively; $\frac{\text{abs}_1}{\text{abs}_2}$ is the absorbance factor; and $\text{abs } \lambda_{\text{iso}}(X+Y)$ and $\text{abs } \lambda_2(X+Y)$ are the absorbance values of the mixture at these wavelengths, respectively²².

In this work, sensitive, selective and low-cost methods for the determination of thiamin and riboflavin present in many multivitamin formulations are developed. These methods can be applied simply in any pharmaceutical manufacturing process or drug quality control laboratory to determine the concentrations of each vitamin in the mixture without previous separation.

EXPERIMENTAL

The scanned spectra of riboflavin and thiamin, with concentrations of 2.94 to 15 $\mu\text{g/ml}$ and 4-20 $\mu\text{g/ml}$, respectively, were obtained in the 400-700 nm range. The thiamin and riboflavin absorbance values were measured at 450 nm and 410.5 nm, respectively. The absorption factor was the ratio of the absorbance at 428 nm and 495 nm. This was measured to determine the absorbance of thiamin and riboflavin in their mixtures without separation. The absorbance subtraction method was used to calculate the concentrations of each X and Y by using the isoabsorptive point unified regression equation obtained by plotting the absorbance values of the zero order spectra of either X or Y at the isoabsorptive point (λ_{iso}) against the corresponding concentrations X or Y, respectively²⁸.

MATERIALS AND METHODS

All of the absorption spectra were obtained using a Shimadzu UV-1601(UV- visible) spectrophotometer, with a scanning rate of 200 nm/min and a bandwidth of 2.0 nm, equipped with 10-mm matched quartz cells.

Thiamin and riboflavin were obtained from BDH. The stock solution of vitamins was prepared in double distilled water and stored in a suitable dark bottle covered with aluminum foil. The solutions were stored in a cool environment and were replaced every week. Working solutions of the vitamins were prepared daily by diluting the stock solution in double distilled water and were then stored in dark conditions. Methavet- Dinurine from Mobedco (vitamin B₁ 8 mg, vitamin B₂ 9 mg) and B complex from Cenovis (15 mg vitamin B₁ and vitamin B₂) were used as the pharmaceutical preparations. Ammonium and ammonium chloride buffer (7.6-7.8).

One percent nonionic surfactants in double distilled water (Triton X-100 and Tween 80) were obtained from BDH.

Chromazurol S (CAS), 1×10^{-3} , was obtained from Fluka and prepared by dissolving an accurately weighed amount of the pure solid in double distilled water.

General Procedure

Spectrophotometric method

Transfer an appropriate amount of thiamin and riboflavin in a 10-ml calibrated flask, add 1 ml of buffer solution (pH 7.6-7.8), then add 1.0 ml of Triton X-100 and 1.5 ml of CAS. Dilute to the mark with water. After 10 minutes, measure the absorbance at 450 nm for thiamin and at 410.5 nm for riboflavin.

The absorbance values were measured at 410.5 and 575.5 nm for use in the absorption factor method. The absorbance of riboflavin at 410.5 nm, after the subtraction of thiamin interference, was calculated by applying the absorption factor equation. For use with the absorbance subtraction method, the absorbance was measured at $\lambda_{\text{iso}}=505$ nm and $\lambda_2=584$ nm.

For pharmaceutical preparations

Twenty tablets of each product (Cenovis B Complex and Methavet – Dinurine) were powdered. An amount of the powder was weighed and dissolved in water. The volume was placed into a 10-ml measuring flask.

RESULTS AND DISCUSSION

The zero order absorbance spectra of riboflavin and thiamin complexes (Fig1) show a severe overlap. The main task of this work is to establish novel, simple, sensitive and accurate analytical methods for the simultaneous determination of the riboflavin and thiamin concentrations in their bulk powders and in the pharmaceutical dosage form with satisfactory precision and accuracy.

Absorption spectra

Several parameters, such as acidity, effect of temperature, effect of time and type of surfactants, were optimized to achieve high sensitivity and stability as well as reproducible results.

Reaction conditions

Effect of the sequence of addition

For both thiamin and riboflavin, it was found that the addition sequence of the drug, buffer solution, surfactant and dye was optimal for giving high and stable absorbance.

Effect of temperature

The effect of temperature was studied by measuring the absorbance values at different temperatures. The optimal reaction temperature was found to be 23-25°C.

Effect of time

The optimal reaction time was greater than 10 minutes. We found that high and stable absorbance was obtained after 10 minutes of reaction.

Effect of pH

It is well known that riboflavin and thiamin are not stable in alkaline conditions[23], but their complexes with CAS are more stable in weak base media. It was noticed that the maximum color intensity and highest absorbance values were

Table 1: Analytical parameters for the determination of pure thiamin and riboflavin concentrations using CAS dye

Parameters	Vitamin B	
	Thiamin	Riboflavin
λ_{\max}	445 nm	428 nm
Beer's law ($\mu\text{g/ml}$)	4-20	2.94- 15.9
Molar absorptivity ($\text{Lmol}^{-1}\text{cm}^{-1}$)	1.24×10^4	1.602×10^4
Sandell sensitivity (μgml^{-1})	2.39×10^{-2}	1.66×10^{-2}
Limit of detection (LOD) (μgml^{-1})	1.3	1.32
Limit of quantification (LOQ) (μgml^{-1})	4.0	4.01
Slope (a) ($\text{ml}\mu\text{g}^{-1}\text{ml}^{-1}$)	0.0053	0.0048
Intercept (b)	0.0001	-0.07
Correlation coefficient (r)	0.9998	0.9999
RSD%	0.21	0.02
RE%	0.0014	-0.002

Table 2: Determination of the thiamin and riboflavin concentrations in laboratory prepared mixtures and in tablets by the absorption factor method

Drugs	Thiamin	Riboflavin
Laboratory prepared method (n=5)*		
Linearity	3.5-19.5	2.5-15
% Recovery \pm RSD%	100 ± 0.31	100.02 ± 0.4
Inter-day**		
% Recovery \pm RSD%	100.19 ± 1.24	100.37 ± 1.01
Intra-day**		
% Recovery \pm RSD%	99.47 ± 1.17	100.23 ± 0.99
Methavet-Dinurine (8mg B ₁ and 9mg B ₂ /tablet)	100.28 ± 0.34	99.36 ± 0.29
B complex (15mg/tablet), Cenovis	100.45 ± 0.829	100.9 ± 0.66

*5 sets each of 3 replicates.

**Average value of four determinations

Table 3 Statistical analysis of the proposed method and the reported method [25]

Parameters	Thiamin		Riboflavin	
	Proposed method	Reported method [25]	Proposed method	Reported method [25]
Mean \pm SD*	99.87 ± 1.11	99.75 ± 0.66	100.08 ± 0.81	99.99 ± 0.76
n	5	5	5	5
Variance	1.232	0.444	0.658	0.542
t-Value**	1.89		0.464	
F-Value**	2.775		1.214	

*Mean of six independent analysis.

**The theoretical values of t and F are 2.306 and 6.39, respectively, for four degree of freedom and 95% confidence limit.

observed in the buffer of 7.6 to 7.8 for thiamin and riboflavin.

Effect of surfactants

In the absence of the solubilization agent, the ion-association complex would gradually separate out as a precipitate because of its hydrophobicity, which would affect the spectrophotometric measurement¹⁷. The effects of the type and amount of surfactants on the formation and stability of the associated ions were studied by adding different types of surfactants (ionic, nonionic and cationic). It was found that 1% of nonionic surfactant (Triton X-100) gave a stable and clear color.

Stoichiometric relationship

Job's method of continuous variation was employed. The molar ratio of (dye:drug) in the complexes was 1:1.

The zero order absorbance spectra of thiamin and riboflavin (Fig. 1) shows an overlap that would lead to difficulties for the determination of the thiamin and riboflavin concentrations by the direct method.

The aim of this work is to develop a simple and sensitive method for the simultaneous determination of the thiamin and riboflavin concentrations at their pharmaceutical dosage.

Validation of the proposed method

Standard calibration curves with a reagent were obtained under the optimal conditions using the relationship between the absorbance and the

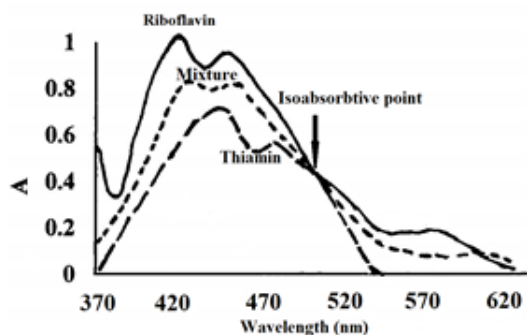


Fig. 1: Zero-order absorbance spectra of the riboflavin complex (—) and thiamin complex (---) and their complexes' mixture (- -)

concentrations. The calibration graphs were described by the regression equation. The statistical parameters, correlation coefficient, intercept and slope are listed in Table 1.

Sensitivity

The molar absorptivity and the relative standard deviation values were calculated and are presented in Table 1. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the formulas^{24, 25}:

$$\text{LOD} = 3.3 \frac{S}{K}$$

$$\text{LOQ} = 10 \frac{S}{K}$$

where S is the standard deviation for the data obtained in five trials and K is the slope of the calibration curve.

Accuracy and Precision

To evaluate the precision of the suggested methods, the percentage relative deviation (RSD%) was calculated, and the percentage relative error (RE%) was estimated to evaluate the accuracy of the proposed method.

The precision was evaluated by preparing solutions of three different concentrations of thiamin and riboflavin and analyzing them in six different trials.

The percentage relative error was calculated by using the following equation:

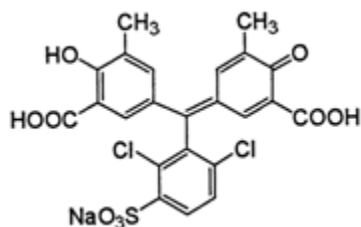
$$\text{RE}\% = \frac{\text{found} - \text{taken}}{\text{added}} \times 100$$

The obtained RSD% and RE% values indicate that the recommended method is highly accurate and reproducible.

The precision and accuracy values were determined within the same day to evaluate repeatability (Intra-day) and over five days to evaluate intermediate precision (Inter-day) and are presented in Table 2.

The percentage recovery values for each drug were calculated and are listed in Table 2. The experimental results are presented in Tables 1 and 2. The obtained recoveries were good. The recovery values are close to 100%, which means that the suggested methods are highly accurate.

CAS has the following structure:

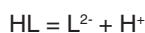


Chromazurol S

Riboflavin and thiamin form ion-association complexes with this dye. The composition of this complex was established by Job's method. The results showed that drug:CAS ratio was 1:1 for both riboflavin and thiamin.

The main species of CAS was L^{2-} in weak base media.

CAS has the following balance:



Therefore, the combination of riboflavin and thiamin with L^{2-} formed ion-association complexes between CAS and the vitamins with λ_{max} at 410.5 nm for VB_1 and at 450 nm for VB_2 [17][27]. VB_1 or $VB_2 + L^{2-} = [VB_1$ or $VB_2] [L^{2-}]$

The maximum negative charge density of L^{2-} is found at the dissociated phenolic hydroxyl, while the charge density of $-SO_3^-$ is lower; therefore, riboflavin and thiamin should react with the dye at the phenolic hydroxyl rather than at the $-SO_3^-$ group. The ion-association complexes were formed due to weak bonding forces, such as electrostatic attraction and hydrophobic interactions¹⁷.

AF method

The λ_{max} values for riboflavin and thiamin complexes were 410.5 nm and 450 nm,

respectively. Because their λ_{max} differs by more than 20 nm, the absorption factor method was applied for the estimation of their concentrations in the formulation. Quantitative determination of the thiamin concentrations was carried out by subtracting the interference of riboflavin using the experimentally calculated absorption factor.

The value of the absorption factor was found to be 3.24.

Absorption of thiamin at 410.5 nm =

$$= A_{\text{mix}410.5\text{nm}} - \frac{A_{(\text{riboflavin at } 410.5)}}{A_{(\text{riboflavin in } 575.5)}} \times A_{\text{mix}575.5\text{nm}}$$

AS method

For the determination of the concentration of riboflavin and thiamin in their mixtures, we measured the absorbance values corresponding to VB_1 or VB_2 separately at an isoabsorptive point of 505 nm; the absorbance values for thiamin and riboflavin can then be calculated using the absorbance factor.

$$\text{Absorbance of thiamin in the mixture at } \lambda_{iso} = \frac{A_{\text{thiamin at } \lambda_{iso}}}{A_{\text{thiamin at } \lambda_2}} \times A_{\lambda_2}(X+Y) =$$

$$\text{Absorbance of X in the mixture at } \lambda_{iso} = A_{\lambda_{iso}}(X+Y) - \frac{A_{\text{thiamin at } \lambda_{iso}}}{A_{\text{thiamin at } \lambda_2}} \times A_{\lambda_2}(X+Y)$$

Analysis of pharmaceutical formulations

The proposed method was applied to determine the thiamin and riboflavin concentrations in their combined pharmaceutical formulations, and the results were compared with those obtained using a previously reported method [25]. The obtained results are presented in Table 3 and show that our methods are suitable for the routine determination of the thiamin and riboflavin concentrations in their combined formulation.

CONCLUSION

In this work, smart, novel and simple spectrophotometric methods were applied for the simultaneous determination of the concentrations of two components in their mixture. The application of these techniques without any preliminary separation step to two commercial pharmaceutical preparations of vitamin B_1 and B_2 (tablets) was highly successful.

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