

ENZYMATIC AND SPECTROSCOPIC DETERMINATION OF RIBOFLAVIN USING AMYLASE PHOSPHATE

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Received: 23 Jan 2012, Revised and Accepted: 29 Feb 2012

ABSTRACT

Sophisticated analytical methods viz. Spectroscopic methods (A and B) which being employed for analysis, are relatively expensive and hence need for simple analytical methods arises. Such methods has been developed and applied for routine determination of riboflavin in pharmaceutical formulations and bulk dosage forms. These methods were based on the formation of colored species via reaction of the enzyme amylase phosphate with Ferric chloride, n-methyl benzothiazolone hydra zone (MBTH), Potassium ferrocyanide in the presence of HCl and finally with the drug to be estimated namely riboflavin to produce light green and blue colored chromogen (λ_{max} at 430 and 700nm). Statistical analysis of these methods exhibited a molar absorptivity value $10^4 M^{-1} CM^{-1}$ and Sandal's Sensitivity of 0.0165 and 0.01574, Relative standard deviation (RSD)% of these methods was found as 1.51 and 1.85 indicating that these methods are reproducible, based on the principle of absorption visible spectrophotometer for the determination of riboflavin in pharmaceutical formulations and bulk dosage forms.

Keywords: Riboflavin, Amylase phosphate, Spectroscopy, Molar absorptivity, Beer's Law.

INTRODUCTION

Riboflavin

Riboflavin[7,8-Dimethyl-70-[(2s,3s,4r)-2,3,4,5-tetrahydroxy-pentyl] Benzo[g]pteridine-2,4-dione (chemical name), is also known as "Vitamin B2" (required for a wide variety of cellular processes), is an easily absorbed micronutrient with a key role in maintaining health in humans and animals, and in energy metabolism, and also for the metabolism of fats, ketone bodies, carbohydrates, and proteins. It is the central component of the cofactors FAD and FMN, and is therefore required by all flavono proteins. It is also used as an orange-red food colour additive, designated in Europe as the E number E101. Milk, cheese, leafy green vegetables, liver, kidneys, legumes etc., are good sources of vitamin B2, but exposure to light destroys riboflavin. The name "riboflavin" comes from "ribose" (the sugar whose reduced form, Ribitol, forms part of its structure) and "Flavin", the ring-moiety which imparts the yellow colour to the oxidized molecule. Vitamin B2 was necessary for preventing pellagra. Riboflavin is also needed to help the body change vitamin B6 and folate into forms it can use. It is also important for body growth and red blood cell production.

The effectiveness ratings for Riboflavin (vitamin B2) are as follows:

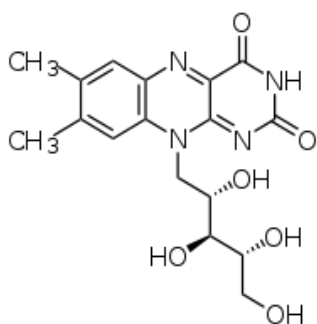


Fig. 1: Structure of Riboflavin

Effective for

- Preventing and treating riboflavin deficiency and conditions related to riboflavin deficiency.

Possibly effective for

- Preventing migraine headaches. Taking high-dose riboflavin (400 mg/day) seems to significantly reduce the number of

migraine headache attacks. However, taking riboflavin does not appear to reduce the amount of pain or the amount of time a migraine headache lasts.

- Preventing cataracts, an eye disorder.

Other names for riboflavin were

B complex Vitamin, Flavin, Lactoflavin, Riboflavin, Riboflavin, Vitamin B-2, Vitamin G, Vitamin B2, Vitamin B2.

Soybean

Soybeans (Glycine max) are the cheapest form of dietary protein is classed as a "Legume" and is native to South East Asia. It is the main ingredient in Tofu, Soy-flour and Soy-milk (important protein sources) especially for vegans and vegetarians which are rich in Vitamins and Minerals. These are necessary for manufacturing and production of Soaps, Plastics, Cosmetics, Crayons and even bio-diesel and also in Muscle repair and creation as well as for Skin, Hair and Nail growth and repair, it also reduces the risk of colon and breast cancer. Soy protein products can be good substitutes for animal products because, unlike some other beans, soy offers a 'complete' protein. Soy protein products can replace animal-based foods—which also have complete proteins but tend to contain more fat, especially saturated fat—without requiring major adjustments elsewhere in the diet. The beans contain significant amounts of phytic acid, alpha-linolenic acid, and the is flavones genistein and daidzein. Soybeans also contain the is flavones genistein and daidzein, types of phytoestrogen, that are considered by some dietitians and physicians to be useful in the prevention of cancer and by others to be carcinogenic!

MATERIALS AND METHODS

Instrumentation

After due calibration of the instrument, spectral and absorbance measurements were made using UV-Visible spectrophotometer model SL-159, Mumbai, India. All the chemicals used were of Analytical grade. All the solutions were freshly prepared using millipore double distilled water. Freshly prepared solutions were used for analysis. In the propose methods aqueous solutions of MBTH(0.2%), Ferric chloride(0.3%w/v) and HCl(0.5N) were used for method A and Potassium Ferrocyanide(0.1%), ferric chloride (0.5%w/v) and HCl (1N) ere used for method B.

Standard and Sample solution of Riboflavin

About 100mg of Riboflavin was accurately weighed on a digital single pan balance and dissolved in a volumetric flask containing

100ml of water to prepare a standard solution with a concentration equal to 1mg/ml and further dilutions are made with the same solvent for this method.

Isolation of the Enzyme

Weigh 60gms of Soyabeans and transfer it into motor & pestle for crush and grinding process. Add 40ml of sodium PBS (sodium Phosphate Buffer Saline) to it and transfer it into blue capped test tube. Enzyme extract over 1hour time period at room temperature. Filter the extract and collect in a new test tube and centrifuge at (12000rpm/20min). Next transfer the supernatant into another blue capped test tube and stored at 4°C.

Preparation of Reagents

All the chemicals used were of analytical grade. All solutions were freshly prepared with distilled water and always used for analysis. Following aqueous solutions were used:

Method A

MBTH (0.2% W/V)
FeCl₃ (0.7% W/V)
HCl (0.5N)

Method B

Potassium Ferro cyanide (0.01M)
FeCl₃ (0.005M)
HCl (0.1N)

Assay procedure

Method A

Into a series of 25 ml calibrated tubes; enzyme solution (0.5ml W/V), FeCl₃, MBTH, HCl (1ml) were taken. These tubes were incubated for 10 minutes for color development. Aliquots of 0.4-2.0 ml of standard tannic acid solution were transferred to the incubate solution and the absorbance of green colored chromogen were measured at 430nm against the reagent blank.

Method B

Into a series of 25 ml calibrated tubes; 0.3ml of drug (Riboflavin), FeCl₃, Potassium ferrocyanide (1ml) each were taken. These tubes

were kept in a hot water bath for 15 minutes at 100°C and then cooled to room temperature. To develop initial color HCl (1ml) was added to each tube. Aliquots of 0.4-2.0 ml of standard Riboflavin solution were transferred to the resulting solution and the absorbance of Blue colored complex were measured at 700nm against reagent blank.

RESULTS AND DISCUSSIONS

The results of analysis for method A and B were validated through systematic statistical analysis and results are tabulated. The statistical analysis values are reported in Table -1 and assay and recovery results for these methods are tabulated in Table-2.

Method A

The proposed method is based on the mechanism of oxidation followed by complex formation, where in the initial reaction the anti-oxidant undergoes oxidation in the presence of ferric chloride and then the oxidized anti-oxidant reacts with MBTH to form a green colored complex which exhibits maximum absorption at wavelength of 430 nm.

Method B

The results obtained in this method were due to redox reaction followed by complex formation between the drug and ferric chloride and potassium ferrocyanide to form a bluish green colored solution that exhibited maximum absorption at 700nm against the corresponding reagent blank.

For these methods optical characteristics such as absorption maxima, Beer's law limits, molar absorptivity regression analysis using the method of least squares, slopes (a), intercept (b) and correlation coefficients (r) obtained from different concentrations are summarized in Table-1. The precision and accuracy were found by analyzing five replicate samples containing known amounts of the drug and the results summarized in Table-1. The accuracy of these methods in the case of formulations was thoroughly studied by recovery experiments and the results were presented in Table-2. Additional checks on the accuracy of these methods were analyzed by adding known amounts of pure drug to pre-analyzed formulations.

Table 1: Optical characteristics, precision and accuracy of Riboflavin

Parameters	Developed Method A	Developed Method B
Name of the method	MBTH, ferric chloride	Potassium ferrocyanide, Ferric chloride
λ max(nm)	430nm	700nm
Beer's law limit(μ g/ml)	0.4-2.0	0.4-2.0
Sandell's Sensitivity (μ g/cm ² /0.001 abs. Unit)	0.0165	0.01574
Molar absorptivity	2.2707 \times 10 ⁴	2.389 \times 10 ⁴
Correlation coefficient(r)	9998.49	9998.15
Regression Equation(Y)*		
Slope(b)	0.299	0.4374
Intercept(a)	0.00439	0.00339
Standard deviation	11X10 ⁻³	132X10 ⁻⁴
%RSD**	1.51	1.85
% range of error(confidence Limits):		
0.05 significance levels	1.262	1.546
0.01 significance levels	1.868	2.239

Y= a+bx, where 'Y' is the absorbance and x is the concentration of Riboflavin in μ g/ml.

Table 2: Estimation of Riboflavin in pharmaceutical formulations

Formulations	Labeled amount mg/vial	Amount found		% Recovery by Developed method	
		Method-A	Method-B	Method-A	Method-B
Sample -1	50 μ g	48	48	96.00	96.00
Sample- 2	70 μ g	64	66	94.2	81.42

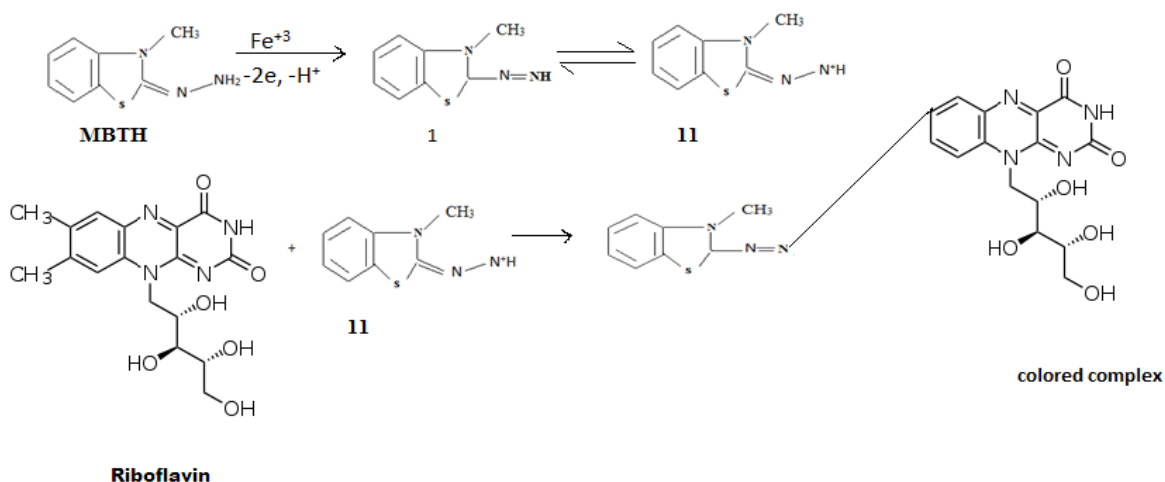


Fig. 2: Mechanism of reaction between riboflavin and MBTH

DISCUSSIONS

The developed method is based on lyses mechanism of the enzyme amylase phosphate. For carrying out this mechanism, after considering a wide range of electron donors, it was identified that MBTH and Potassium Ferro cyanide, were more effective in the formation of coupling species as compared earlier methods. The proposed drug riboflavin reaction with coupling agent MBTH and Potassium Ferro cyanide results in the primary color in the presence HCl. The active functional groups present in the drug react with the coupling species, resulting in the formation of a light green and blue colored chromogen and its absorbance was measured at the wavelength of 430nm and 700nm against the corresponding reagent blank.

CONCLUSIONS

Performance recovery experiments and percent recovery values obtained in this work indicated the absence of interferences from commonly encountered pharmaceutical additives and excipients. Though in earlier reported methods of analysis for riboflavin, the methods including the application of enzyme amylase phosphate were not found, and hence for now this could be for now considered as a protocol for the estimation of riboflavin using amylase phosphate. The developed method is simple and sensitive with reasonable precision and accuracy and can be used as a standard method for the routine determination of Riboflavin in quality control analysis.

ACKNOWLEDGEMENTS

The authors are grateful to the management of Koneru Lakshmaiah University, Vaddeswaram, Guntur Dist. for their continuous support and encouragement and for providing the necessary infrastructure facilities for executing this work.

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