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Methods of Analysis of Riboflavin (Vitamin B_2): A Review

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ABSTRACT

Riboflavin (vitamin B2) is a component of vitamin B complex and multivitamin preparations. It exhibits several physical properties, such as light absorption, florescence emission and electroreduction. These properties have been used to develop spectrometric, fluorimetirc, electrochemical, chromatographic and electrophoretic methods for the assay of riboflavin in pharmaceutical preparations and clinical samples. In addition to these enzymatic and microbiological methods have also been used for the assay of the vitamin. The fluorimetric methods are much more sensitive than the spectrometric methods. Chromatographic methods have the advantage of separation and simultaneous determination of riboflavin and other components of vitamin preparations. All the above mentioned methods have been reviewed.

Keywords: Riboflavin, Spectrometry, Fluorimetry, Chromatography, Electrophoresis

INTRODUCTION

Riboflavin (vitamin B2) is a yellow-green fluorescent water-soluble vitamin and is responsible for imparting yellow colouration to B-vitamin preparations. The structure of this vitamin consists of a heterocyclic isoalloxazine ring attached to ribitol, a sugar alcohol. Major sources of riboflavin include almonds, milk, liver, legumes, mushrooms and vegetables. It is not prepared by mammals but plants and microorganisms can synthesize it. The physiochemical properties of riboflavin [1-5] are reported in Table 1. Riboflavin is stable to heat in acidic solutions but decomposes in alkaline solutions by the cleavage of isoalloxazine ring. The aqueous solutions of riboflavin are unstable to light and degrade to give formylmethylflavin, lumichrome and lumiflavin as the main photoproducts. Formylmethylflavin is the intermediate degradation product in these reactions and it is hydrolysed to give lumiflacin and lumichrome. Lumichrome is detected in neutral and acidic conditions while lumiflavin also appears in basic conditions [6]. Ascorbic acid protects riboflavin from degradation. The kinetics of riboflavin photolysis has been studied in detail [7-11]. Riboflavin is the prosthetic group in the two enzymes FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide) that are involved in many of the oxidation-reduction reactions in biological system. Certain hormones (e.g. thyroid), nutritional factors and some drugs (e.g. chlorpromazine) can help conversion of the vitamin to these enzymes [12].

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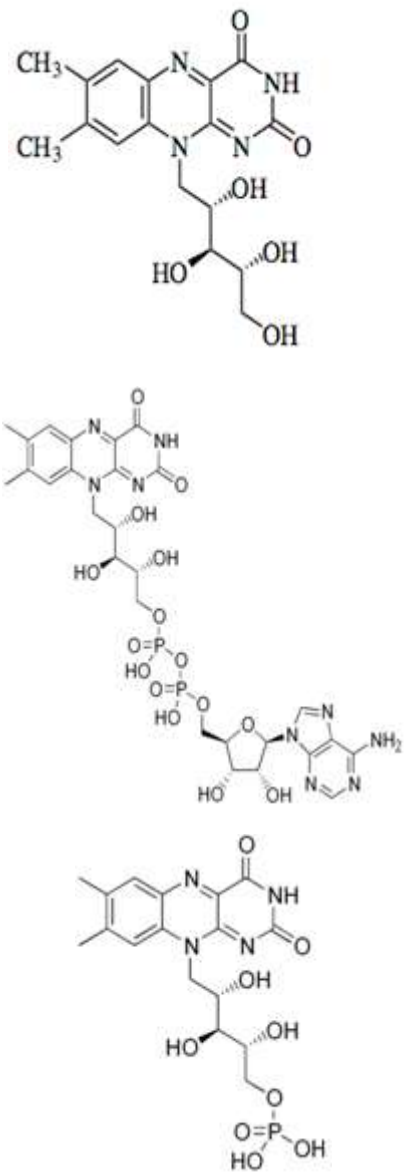

Figure 1: Chemical structures of (a) riboflavin; (b) FMN and (c) FAD

The RDA recommendations of riboflavin are 1.3 mg/day and 1.1 mg/day for an adult male and female respectively. The intake increases during pregnancy and lactation to 1.4 mg and 1.6 mg, respectively. The requirement may also increase after injury, burn and during oral broad spectrum antibiotic therapy. For children the recommendation is 0.6-0.9 mg/day and for infants its 0.3-0.4 mg/day [13]

Analytical Methods

The earlier analytical methods for the determination of riboflavin [14-21] and the pharmacopoeial method [5] have been summarized by several workers. Most of the methods of analysis of riboflavin developed since 2000 are presented in the following sections.

Spectrometric methods

UV spectrophotometry

A spectrophotometric method has been proposed for the analysis of riboflavin and other water-soluble vitamins in pharmaceutical preparations using a multicommuted flow system. The concentrations between 5.00-50.00 mg/ml of riboflavin give linear response at the wavelength of 290 nm used for assay [22].

Riboflavin along with vitamin B1 and B6 in pharmaceutical preparations has been assayed spectrometrically using partial least-square modeling and least-square support vector machines. The root mean square for riboflavin was found to be 0.3755 with PLS and 0.0318 with LS-SVM [23]. Spectrophotometric assay of riboflavin and its photoproducts (formylmethylflavin, lumichrome and lumiflavin) have been reported. The photoproducts are separated by the extracting with chloroform. The method has been found to be specific, rapid and convinent for riboflavin and formylmethylfalvin [24].

The flex tolerance simplex method (FTSM) has been applied for the spectrometric determination of riboflavin, thiamine, niacin and pyridoxine. The recoveries of riboflavin have been found to be 100.1 ± 0.8 % [25].

A new method has been developed for the determination of riboflavin along with other water soluble vitamins by partial least-squares regression method. The limit of detection has been found to be 0.09 µg/ml of riboflavin. The method can resolve complex mixture of compounds in strong over lapping signals [26].

Soft independent modeling of class analogy and PLS regression methods have been used for the

identification and quantitation of thiamine, riboflavin, nicotinamide and pyridoxine by UV-visible spectrometry without separation or pre concentration steps in the procedure [27].

Simultaneous determination of naphazoline hydrochloride (NAP), chloropheniramine maleate (CLO) and methylene blue (MB) has been carried out by spectrometric methods. NAP and MB have been determined using second derivative spectrometry using the peak amplitudes at 299 nm and 377 nm for NAP and MB, respectively. CLO has been determined using the peak amplitude at 276.6 nm. In a chemometric method, the technique of principal component regression (PCR) and partial least squares (PLS) has been used for the assay of NAP, CLO and MB. These methods have been applied for the analysis of these compounds in pharmaceutical formulations and the results compare with these reported methods [28].

A new method has been developed for the determination of riboflavin along with other water-soluble vitamins by partial least-squares regression method. The limit of detection has been found to be 0.09 µg/ml for riboflavin. The method an resolve complex mixture of compounds in strong overlapping signals [29].

A group of workers have conducted two spectrophotometric methods i.e. derivative and multivariate method for the determination of riboflavin in multivitamin preparations. The method has shown good accuracy and precision and linear calibration curves were obtained. The concentration of the solutions was in the range of 2.5-90 µg/ml and was measured in the range of 200-500 nm [30]. Multicomponent spectrometric methods have been developed for the simultaneous assay of drugs and their degradation products. These methods include the assay of cyanocobalamin and its photoproduct, hydroxocobolamin at 550 and 525 nm [31, 32]. Cyanocobalamin, hydroxocobalamin and riboflavin [33]. Ascorbic acid, cyanocobalamin and hydroxocobalamin [34], 7,8-dimethyl-10 (formyl methyl) isoalloxazine and its hydrolytic products, lumichrome and lumiflavin [35]. Riboflavin and photoreduction products, formylmethyl flavin, lumichrome and lumiflavin [36-38], riboflavin and its photoaddition product cyclodehydroriboflavin [29, 30]. These methods has been used to study the kinetics of chemical and photodegradation reactions of these vitamin.

Spectrofluorimetry

A fluorimetric method has been developed for the determination of riboflavin and pyridoxine in acetate buffer pH 6 in the presences of cyanocobalamine. The method has been found to be sensitive and has shown good precision with no interference from the pharmaceutical additives. [39]. A new rapid synchronous spectrofluorimetric method for the simultaneous determination of riboflavin and folic acid in nutritional beverages has been formed to give satisfactory results. The calibration curves are linear in the range of 1-250 µg/l and the detection limits is 0.014 µg/l [40]. Riboflavin along with thiamine and pyridoxine in a pharmaceutical preparation has been assayed spectrofluorimetrically. The method has proved to be stability-indicating with the sensitivity ranging between 0.4-2.0 µg/ml [41]. A previous synchronous fluorimetric method has been used for the determination of riboflavin along with thiamine and pyridoxine in pharmaceutical products. The method is sensitive and has good repeatability. The detection limits were found to be 9µg/lit for riboflavin [42]. Riboflavin along with thiamine and pyridoxine has been determined fluorimetrically by appling parallel factor analysis (PARAFAC) to the resolution of overlapped spectra of mixtures of the three vitamins. The delta wavelenght and the excitation wavelength has been in the range of 20-120 nm and 200-500 nm respectively [43]. A multivariate method for the simultaneous determination of riboflavin, caffeine and caramel has been reported. The synchronous fluorescence spectra has been recorded from 200-500 nm with the difference of 90 nm [44]. A direct fluorimetric determination of riboflavin in powders has been reported [45].

Mass spectrometry

With the help of laser depletion mass spectrometry has been used for the assay of certain vitamins. The molecular fragmentation pattern of the vitamins were helpful in the analysis of the vitamins including vitamin A, D3, C, B1, B2 and B6 [46]. A method has been developed for the analysis of water-soluble vitamins in multi-vitamin, multi-mineral dietry supplements using multiple reaction mode LC/UV/MS-MRM. The extraction was carried out with a 10 mM phosphate buffer at pH 2.5. The technique does not need any treatment before proceeding [47].

A simple and reliable method has been reported for the determination of riboflavin along with vitamin B3, B6, caffeine and taurine in energy drinks by planar chromatography electro-spray ionization mass spectrometry. The fluorescence measurement of riboflavin was performed at 366/>400 nm. All the calibrations were linear and the repeatabilities were found to be between 0.8 and 1.5% [48].

Electrochemical Methods

Riboflavin and its photoproducts, formylmethylflavin and lumichrome has also been determined by polarographic methods using half-wave potentials of -0.47V, -0.45V and -0.58V, respectively [49, 50].

Chromatographic Methods

High-performance liquid chromatography (HPLC)
The determination of riboflavin along with other B-vitamins has been carried out by RP-HPLC method. The mean recoveries are between 95.2-103.9% while the RSD for riboflavin is 0.7% [51]. The water-soluble vitamins including riboflavin, thiamine, pyridoxine and pantothenic acid in multivitamin mixtures have been separated by HPLC technique using FTIR detector [52]. A RP-LC method has been used for the determination of riboflavin in baby foods using an amide-based stationery phase. The method has been successfully applied to the separation and determination of other vitamins including B1, B3, B6, B9 and B12 [53]. Several other HPLC methods for the simultaneous determination of riboflavin and other vitamins in food [54-58]; multivitamin blends [59], pharmaceuticals [60, 61], milk [62] and biological fluids [63] have been reported.

Electrokinetic chromatography / Capillary electrophoresis

Riboflavin along with 2-aminoethansulfonic acid, nicotinamide, pyridoxine, caffeine and thiamine in a vitamin enriched drink has been separated by micellar electrokinetic chromatography and detected using a diode-array detector at 210 nm. The % recoveries and % RSD has been found to be between 99.0-1.1.2 and 0.4-2.5 [64]. A fast, accurate, simple and inexpensive method has been developed for the simultaneous determination of six water-soluble vitamins including riboflavin using capillary electrophoresis operated in micellar mode. The % RSD have been found to be 1.26-3.35 (inter-day) and 1.08-3.68 (intra-day) [65]. A method has been developed that can be applied for the determination of riboflavin and other water-soluble vitamins in pharmaceuticals. On separation by micellar electrokinetic chromatography. The method has shown good recoveries and RSD values in the range of 99.3-101.8% and 0.1-2.5% respectively [66]. A simple and novel method for the analysis and separation of vitamin B2 has been reported using CEC technique with monolithic column. The method can be used for the separation and assay of B-vitamins from human urine [67]. Six water-soluble vitamins including riboflavin in a pharmaceutical formulation have been determined by CZE method. Some of the excipients get adsorbed to the capillary surface causing decrease of electroosmotic flaow and increase of analyte migration time. However the results have shown the method to be good with respect to linearity, precision and accuracy [68].

Enzymatic Assay Method

An enzyme-linked ligand-sorbent assay (ELLSA) of vitamin B2 has been reported. The conjugates obtained by coupling 3-carboxymethylriboflavin and bovine serum albumin were adsorbed on the multi-well microtitre plates. The detection limit of the

method has been found to be 0.8 pmol. The method can be used for the determination of riboflavin in human plasma and urine [69].

Microbiological Methods

A microbiological method has been reported which can be used for the assay of riboflavin, thiamine, pyridoxine, cyanocobalamine, calcium pantothenate, nicotinic acid, pantothenol and folic acid. The turbidity produced has been measured at the rate of 300/hr [70].

Table 1: Physicochemical properties of Riboflavin

Empirical formula	$C_{17}H_{20}N_4O_6$
Molar mass	376.4
Crystalline form	fine needles
Melting point	278 to 282 ^oC
$[a]_D^{25}$	-112 to -122^o
pH of saturated solution	~6
pKa	1.9, 10.2 (20^o)
Redox potential , pH 7.0	-0.208 V
Solubility, mg /100 ml	
Water	3.3-6.06
Absolute ethanol	0.45
Acetone, chloroform,	insoluble
ether, benzene	
Absorption maxima	223, 267, 373, 444 nm
Fluorescence emission	520 nm
Principle infrared peaks	1544, 1575, 1641,
(KBr disk)	1715, 1235, 1070 cm^{-1}

Table 2: Analytical parameters for HPLC methods of riboflavin assay.

Material	Technique	Column	Mobile phase	Flow rate ml/min^{-1}	Detection nm	Conc. range µg/ml	References
Riboflavin, B_1 and B_6	HPLC	Inertsil ODS-3	0.05 mol/l KH2PO4 (pH 6) – methanol	-	265	-	[71]
B_2 and B_1	HPLC with fluorescence detection	RP 18 column	72% 0.005 M NH4OAc and 28% methanol	1.450	220, 288	-	[72]
Riboflavin and thiamine	LC	C 18	72% 0.005 M NH4OAc (pH 5.0) and 28% methanol	-	370-435	-	[73]
Riboflavin, B_1, B_3, B_6 and sorbic acid	HPLC	RP C18	0.1% methanolic hexane sulphonic acid Na salt and 0.01 M phosphate buffer containing 0.1% hexane sulphonic acid Na salt pH 2.7	-	220 and 288	-	[74]

Riboflavin and multivitamin and mineral tablets	RP-HPLC	Hypersil C(18)	Methanol-aqueous 0.5% acetic acid solution (18:82, v/v; containing 2.5 mM Na hexanesulfonate, pH 2.8)	1.2	280	5-90	[75]
Riboflavin and multivitamin mixture	HPLC	Zorbax SB- Aq 1C 18	0.0125 M hexane -1-sulfonic acid Na salt in 0.1 % o-phosphoric acid pH 2.4-2.5 and acetonitrile	1	210, 230, 254	-	[76]
Riboflavin and multi vitamin mixture	HPLC	Bondapak C 18	Methanol-water (27:73, v/v), pH 3 using glacial acetic acid	0.35	250-295	20-90	[77]
Riboflavin and other water-soluble vitamins	RPLC	RP C18	Mixture of 15 mM ammonium formate buffer and 0.1% triethylamine (pH 4), formic acid, acetonitrile	-	275, 360	-	[78]
Riboflavin and thiamine in mushrooms	RP-LC	Spherisorb ODS column	Methanol/water	-	422, 515	-	[79]
Riboflavin and other water-soluble vitamins in infants formula	HPLC	C 18	Methanol: water (15:85), 5 mM octanesulfonic acid with 0.5% triethylamine at pH 3.6	1	-	-	[80]
B- complex vitamins	Capillary zone electrophoresis (CZE)	Silica capillary	20 mM tetra borate buffer, pH 10	-	214	-	[81]

Riboflavin in multivitamin preparation	RP-HPLC	Nova-pack C 18	Methanol-ammonium acetate	2.0	285	-	[82]
Riboflavin in water-soluble vitamin tablets	RP-HPLC	ODS	1% HCOOH in water	0.25	Diode assay	-	[83]
Riboflavin in multivitamin-mineral preparations	Ion pair HPLC	C 18	Methanol: water (15:85)	2.0	280	-	[84]
B_2, B_1, B_3, B_6, caffeine and sodium benzoate in pharmaceutical formulation	LC	C 18	Acetonitrile: 0.01M potassium dihydrogen phosphate: triethylamine (8:91.5:0.5, v/v/v)		254		[85]

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