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Photolysis of riboflavin in aqueous solution: a kinetic study

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Abstract

The kinetics of photolysis of aqueous riboflavin solutions on UV and visible irradiation has been studied in the pH range 1–12 using a specific multicomponent spectrophotometric method for the simultaneous determination of riboflavin and its major photoproducts (formylmethylflavin, lumichrome and lumiflavin). The apparent first-order rate constants for the photodegradation reactions in the pH range have been determined. The log *k*–pH profiles indicate that riboflavin has maximum photostability around pH 5–6, at which the rate of oxidation–reduction of the molecule is lowest. The cationic and anionic forms of riboflavin are non-fluorescent and less susceptible to photolysis than the non-ionised molecule as indicated by the relatively slow rates below pH 3.0 and above pH 10.0. The rate of photolysis is increased up to 80-fold at pH 10.0, compared to that at pH 5.0, due to increase in redox potentials with an increase in pH and consequently the ease with which the molecule is oxidised. The increase in rate at pH 3.0, compared to that at pH 5.0, appears to be due to the involvement of the excited singlet state as well as the triplet state in riboflavin degradation. The apparent first-order rate constants for the photolysis of riboflavin at pH 5.0–10.0 with UV and visible radiation are 0.185×10^{-2} to 13.182×10^{-2} min^{-1} and 0.098×10^{-2} to 7.762×10^{-2} min^{-1}, respectively.

Keywords: Riboflavin; Photolysis; Photoproducts; Kinetics; Rate–pH profile

1. Introduction

Aqueous solutions of riboflavin are sensitive to light (DeRitter, 1982; British Pharmacopoeia, 1998; Martindale, 1999) and are degraded through a variety of reactions (Penzer and Radda, 1967; Hemmerich, 1976; Ahmad and Tollin, 1981a; Ahmad et al., 1981, 2004; Heelis, 1982, 1991; Stevens et al., 1997), resulting in a number of products under aerobic and anaerobic conditions (Smith and Metzler, 1963; Treadwell et al., 1968; Cairns and Metzler, 1971; Cerman and Hais, 1972; Schuman Jorns et al., 1975; Ahmad and Rapson, 1990). Several studies have been conducted on the photodegradation of riboflavin in pharmaceutical preparations (Yamaji et al., 1981; Yamaoka et al., 1982, 1995; Bhatia et al., 1983; Buxton et al., 1983; Chen et al., 1983; Allwood, 1984; Allwood and Kearney, 1998; Smith et al., 1988; Martens, 1989; Zhan and Yin, 1992; Garcia and Silva, 1997; Silva et al., 1998; Edwards et al., 1999; Park et al., 1999) and attempts have been made to improve the photostability of the vitamin by various methods (Shin et al., 1970; Kostenbauder et al., 1971; Saleh, 1974; Casini et al., 1981; Asker and Habib, 1990; Habib and Asker, 1991; Loukas et al., 1995, 1996). Riboflavin plays an important role in photosensitised degradation of a wide range of substrates (Silva, 1992; Silva and

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Godoy, 1994; Silva et al., 1991, 1993, 1994, 1995, 1999, 2002; Lu et al., 2000, 2004; Rochette et al., 2000; Edwards and Silva, 2001; Haggi et al., 2002; Min and Boff, 2002; Glover et al., 2003; Montana et al., 2003; Viteri et al., 2003; Huvaere et al., 2004).

The photolysis of riboflavin in aqueous solution occurs through 7,8-dimethyl-10-(formylmethyl) isoalloxazine (formylmethylflavin) as an intermediate which is hydrolysed to lumichrome and lumiflavin as the major photoproducts (Smith and Metzler, 1963; McBride and Metzler, 1967; Ahmad and Rapson, 1990; Ahmad et al., 1980; Heelis et al., 1980). The kinetics of photolysis of riboflavin or formylmethylflavin or hydrolysis of formylmethylflavin has been studied by following the loss of absorbance at 445 nm, without any consideration of interference from photoproducts at this wavelength (Halwer, 1951; Smith and Metzler, 1963; Holmstrom, 1964; Radda and Calvin, 1964; Song et al., 1965; McBride and Metzler, 1967; McBride and Moore, 1967; Sato et al., 1982, 1983, 1984). Thus the kinetic information obtained from these studies may not be reliable.

Riboflavin is a highly photosensitive compound and is extensively used as a component of liquid vitamin preparations and parenteral nutrition solutions. A knowledge of its photochemical behaviour in aqueous solution over an appropriate pH range has important pharmaceutical implications and is needed to predict the shelf-life. The choice of the optimum pH is crucial for liquid preparations. In the present work aerobic photolysis of riboflavin has been studied over a wide range of pH (1–12) by UV and visible radiations using a specific multicomponent spectrophotometric method (Ahmad and Rapson, 1990) and the kinetics of the reaction has been evaluated to determine the effect of pH on the rate of photolysis and the range of optimum stability. Similar studies have been conducted on the photolysis of cyanocobalamin (Ahmad et al., 1992, 2003) and folic acid (Akhtar et al., 1999, 2000).

2. Materials and methods

Riboflavin (RF), lumiflavin (LF) and lumichrome (LC) were obtained from Sigma Chemical Co. Formylmethylflavin (FMF) and carboxymethylflavin (CMF) were synthesized by the methods of Fall and Petering (1956) and Fukumachi and Sakurai (1954), respectively. 1,2-Dihydro-6,7-dimethyl-2-keto-1-D-ribityl-quinoxaline-3-carboxylic acid (β-ketoacid) and 6,7-dimethyl-4-D-ribityl-2,3-dioxo-1,2,3,4-tetrahydroquinoxaline (flavo-violet) were prepared according to the methods of Surrey and Nachod (1951) and Ina (1959), respectively. All reagents and solvents were of the purest form available from BDH/Merck. The following buffer systems were used throughout. KCl–HCl, pH 1.0–2.0; citric acid–Na_2HPO_4, pH 2.5–8.0; $Na_2B_4O_7$–HCl, pH 8.5–9.0; $Na_2B_4O_7$–NaOH, pH 9.5–10.5; Na_2HPO_4–NaOH, pH 11.0–12.0; the ionic strength was 0.005 M in each case.

2.1. Precautions

All experimental procedures were carried out in a dark chamber under subdued light. Riboflavin solutions were protected from light before irradiation. Freshly prepared solutions were used for each experiment to avoid any chemical or photochemical effects.

2.2. Photolysis

2.2.1. UV lamp

A 10^{-4} M aqueous solution of riboflavin (500 ml) at the appropriate pH was placed in a 1-l Pyrex flask and irradiated with 125 W medium pressure mercury vapour lamp with emission at 313 and 366 nm (Applied Photophysics Ltd., UK). The solution was continuously bubbled with oxygen during irradiation. The lamp was located in a double walled tube with provision for circulation of water and fitted at the centre of the reaction flask. The temperature of the solution during irradiation was maintained at 25 ± 1 °C by circulation of water from a thermostat cooling unit (Frigomix 1496, B. Braun). Samples were withdrawn at appropriate intervals for thin-layer chromatography and spectrophotometric assay.

2.2.2. Visible lamp

A 10^{-4} M aqueous solution of riboflavin (100 ml) at the appropriate pH was prepared in a 100 ml volumetric flask (Pyrex) and placed in a water bath maintained at 25 ± 1 °C. The solution was irradiated with Philips HPLN 125 W high pressure mercury vapour fluorescent lamp (emission at 405 and 435 nm) fixed horizontally at a distance of 30 cm from the centre

of the flask. The solution was continuously stirred by bubbling a stream of oxygen into the flask.

2.3. Thin-layer chromatography

Thin-layer chromatography (TLC) of the photolysed solutions of riboflavin was carried out on 250-μm cellulose plates (Whatman CC 41) using the solvent systems: (A) 1-butanol–acetic acid–water (40:10:50, v/v, organic phase); and (B) 1-butanol–1-propanol–acetic acid–water (50:30:2:18, v/v) (Ahmad et al., 1980). The flavins were detected by their characteristic fluorescence emission under UV (365 nm) excitation.

2.4. Spectral measurements

All spectral measurements on riboflavin and its photolysed solutions were carried out on a Shimadzu UV-240 recording spectrophotometer using quartz cells of 10-mm pathlength.

2.5. Light intensity measurements

The intensities of the 125 W medium pressure mercury vapour lamp and HPLN high pressure mercury vapour fluorescent lamp were determined by potassium ferrioxalate actinometry (Hatchard and Parker, 1956) as $2.19 \pm 0.12 \times 10^{18}$ quanta s^{-1} and $1.14 \pm 0.10 \times 10^{17}$ quanta s^{-1}, respectively.

2.6. Assay method

The assay of riboflavin and its photoproducts in degraded solutions was carried out by a previously reported multicomponent spectrophotometric method (Ahmad and Rapson, 1990).

3. Results and discussion

3.1. Photoproducts of riboflavin

The photolysed solutions of riboflavin were subjected to TLC using solvent systems A and B to identify the products formed on UV and visible irradiation. The following products were detected on comparison of their R_f values and fluorescence emission with those of the authentic compounds.

pH 1–6: FMF, LC (major), CMF (minor)	
pH 7–9: FMF, LC, LF (major), CMF (minor)	
pH 10–12: FMF, LC, LF (major), CMF, β-keto acid, flavo-violet (minor)	
Fluorescence emission:	RF, FMF, LF, CMF—yellow green LC, β-keto acid—blue Flavo-violet—violet

The main photoproducts of RF at pH 1–12 are FMF, LC and LF, which are obtained by the oxidation of ribityl side-chain. A minor photoproduct, CMF, is obtained at pH 7–12. In addition to these, two minor isoalloxazine ring cleavage products, i.e., β-keto acid and flavo-violet are obtained by alkaline hydrolysis of RF at pH 10–12. All these products have been previously reported (Surrey and Nachod, 1951; Ina, 1959; Guttman, 1962; Treadwell et al., 1968; Ahmad and Rapson, 1990). The photoproducts of riboflavin formed on UV or visible irradiation appeared to be the same except that the reaction was faster in solutions exposed to UV radiation as indicated by the spot intensities of various compounds.

3.2. Assay of photoproducts

A specific multicomponent spectrophotometric method (Ahmad and Rapson, 1990) has been used to assay riboflavin and its main photoproducts, FMF, LC and LF. The results of the assay of four compounds in a photolysed solution (pH 7.0) are reported in Table 1. The assay method gives uniformly increasing values of FMF, LC and LF, with an almost constant molar balance, with time. The values of the molar balance are in good agreement with the initial concentration of RF. The slightly higher molar balance may result from the presence of some minor products absorbing in the region of analytical wavelengths. The analytical data obtained for riboflavin photolysis by this method are accurate and free of any interference from its photoproducts and are thus reliable for kinetic studies

Table 1
Photolysis of 10^{-4} M riboflavin solution at pH 7.0 (citro-phosphate buffer) concentrations of riboflavin and photoproducts

Time (min)	RF (M × 10^5)	FMF (M × 10^5)	LC (M × 10^5)	LF (M × 10^5)	Total (M × 10^5)
0	10.00	–	–	–	10.00
2.5	9.82	–	0.17	0.15	10.14
5	9.73	–	0.21	0.21	10.15
7.5	9.54	0.03	0.28	0.21	10.06
10	9.51	0.04	0.33	0.19	10.07
15	9.47	0.04	0.46	0.19	10.16
20	9.06	0.11	0.55	0.19	9.91
25	9.09	0.17	0.59	0.24	10.09
30	8.77	0.26	0.82	0.18	10.03
40	8.39	0.38	1.18	0.21	10.16
50	7.82	0.44	1.49	0.23	10.00
60	7.54	0.52	1.89	0.26	10.23
70	7.07	0.62	2.29	0.24	10.25
80	6.91	0.65	2.36	0.32	10.24

125-W medium pressure mercury vapour lamp.

compared to those obtained by some workers by direct absorbance measurement at 445 nm (see Section 1).

3.3. Effect of pH

Riboflavin is sensitive to pH (pK_{a1} 1.7, pK_{a2} 10.2; Budavari, 1989) and undergoes a number of acid–base equilibria to produce cationic, neutral and anionic species at various pH values (Hemmerich et al., 1965). The rate of photolysis of riboflavin depends upon the state of ionisation of the molecule and its susceptibility to excitation and subsequent degradation on exposure to light. The photophysical and photochemical processes involved and the factors affecting the rate of degradation of drugs have been reviewed in detail (Lachman et al., 1986; Laidler, 1987; Florence and Attwood, 1988; Tonnesen, 1991; Martin, 1993; Moore, 1996). In the present work aqueous solutions of riboflavin were exposed to UV and visible radiations and the analytical data were subjected to kinetic treatment. The apparent first-order rate constants for the photolysis reactions at pH 1–12 were determined and are reported in Table 2.

In order to evaluate the effect of pH on the rate of photolysis of riboflavin, log k–pH profiles for the reactions carried out using UV (313 and 366 nm) and visible (405 and 435 nm) radiations were constructed. Both profiles are similar in shape indicating that the wavelengths of irradiation used have no significant effect on the nature of the reaction, however, the rates of photolysis are higher with UV radiation, compared to that of visible radiation, due to the energy of the radiation involved. Several authors have dealt with the interpretation of rate–pH profiles of drugs on the basis of their shapes and the processes involved (Garrett, 1967; Parrott, 1970; Rawlins, 1977; DeRitter, 1982; Connors et al., 1986; Lachman et al., 1986; Florence and Attwood, 1988; Carstensen, 1990; Fung, 1990; Martin, 1993), this would now be discussed with reference to the photolysis of riboflavin.

Riboflavin is amphoteric in nature (isoelectric point, pH 6.0; Budavari, 1989) and its rate of photolysis, in general, increases with an increase in pH. The log k–pH profile of riboflavin (Fig. 1) may be considered as a bell-shaped curve indicating the presence of two ionisable groups affecting rate with a $pH_{max} = 1/2(pK_{a1} + pK_{a2}) = 5.9$. It is evident from the log k–pH profile that the rate is slowest in the pH range of 5–6, increases about two-fold at lower pH values to a maximum around pH 3.0 and then falls a little at pH 2.0 due to ionisation of the molecule (pK_{a1} 1.7). Above pH 5.0 there is a tremendous increase in the rate reaching about 80-fold at pH 10.0. The data in acid region appear to be somewhat in agreement with the observations of Cairns and Metzler (1971) who reported a 2.5-fold increase in the rate of anaerobic photolysis at pH 3.0 (maximum in acid region) and 30-fold increase at pH 8.0 (maximum in alkaline region), relative to the rate at pH 5.0, on the basis of the time required to achieve 20% bleaching by absorption

Table 2
Apparent first-order rate constants for the photolysis of riboflavin at pH 1.0–12.0

pH	$k \times 10^2$ (min^{-1})[a]	Correlation coefficient	$k \times 10^2$ (min^{-1})[b]	Correlation coefficient
1.0	0.204	0.998	0.098	0.999
2.0	0.317	0.999	0.123	0.998
3.0	0.415	0.998	0.158	0.999
3.5	0.331	0.999	0.142	0.998
4.0	0.236	0.998	0.137	0.999
5.0	0.185	0.998	0.098	0.999
6.0	0.314	0.999	0.120	0.999
7.0	0.426	0.997	0.414	0.999
7.5	0.816	0.999	0.740	0.998
8.0	2.610	0.999	1.489	0.999
8.5	4.560	0.999	3.013	0.999
9.0	10.008	0.998	5.688	0.999
9.5	11.121	0.999	7.116	0.998
10.0	13.182	0.999	7.762	0.999
11.0	9.885	0.999	6.442	0.999
12.0	6.170	0.999	3.589	0.999

[a] 125-W medium pressure mercury vapour lamp (UV).
[b] 125-W high pressure mercury vapour fluorescent lamp (visible).

measurement at 445 nm. However, the photoproducts of RF (i.e., FMF and LF) would also be bleached under the conditions employed and, therefore, the assay values for RF may not be accurate for a comparative study. Unlike the anaerobic photolysis of RF in alkaline solution (Cairns and Metzler, 1971), the rate of aerobic photolysis continues to increase and reaches to a maximum value at pH 10.0, with subsequent decline due to anion formation (pK_{a2} 10.2) (Table 2). The ionised species of riboflavin appears to be less susceptible to photodegradation than the non-ionised species. It is important to recognize that the aerobic photolysis of riboflavin is irreversible (unlike that under anaerobic conditions) and that a greater degree of degradation is observed when aerobic photobleaching is carried out to the same degree as that with anaerobic photobleaching (Treadwell et al., 1968).

An important factor in the photolysis of riboflavin is the consideration of its redox behaviour. The redox potentials of riboflavin are pH dependent (Clark, 1960; Wells, 1988; Martin, 1993; Mayhew, 1999; Yamashita et al., 2002) and have a profound influence on the rate of photolysis involving intramolecular photoreduction and subsequent oxidation of the ribityl side-chain (Heelis, 1982). They are lowest in the pH range 5–6 (E° pH 5.0 = −0.117 V; Wells, 1988; Martin, 1993) resulting in the lowest rate of oxidation–reduction and hence the greatest stability of the molecule around pH 5.0, the region most suitable for maintaining the pH of vitamin preparations. The rate is increased with an increase in pH and redox potentials (E° pH 7.0 = −0.207 V; Wells, 1988; Mayhew, 1999) up to pH 10.0 as shown in Fig. 1. The occurrence of higher redox potentials at higher pH values causes a greater tendency to oxidation as indicated by a tremendously high value of rate constant for photodegradation at pH 10.0 (∼80-fold), compared to that at pH 5.0. The effect of solvent on the redox reactions of flavins have been reported by Song (1971) and Ahmad and Tollin (1981b).

In the acid region the nearly two-fold increase in the rate at pH 3.0, compared to that at pH 5.0, may be due to the photodegradation of RF to LC (main photoproduct) through the excited singlet state (directly) as well as the triplet state (through FMF as intermediate), as suggested by Song and Metzler (1967) and Cairns and Metzler (1971). The possibility of the involvement of two pathways leading to greater degradation of RF in this region is substantiated by the fact that almost equal rate constants are obtained at pH 1.0 and 5.0, indicating that the formation of LC is independent of FMF which would be protonated to the extent of 99.7% at pH 1.0 and 3.0% at pH 5.0 (pK_a 3.5; Suelter and Metzler, 1960) and is thus unable to yield LC in the lower pH range due to resistance of the protonated form to photoreduction and subsequent

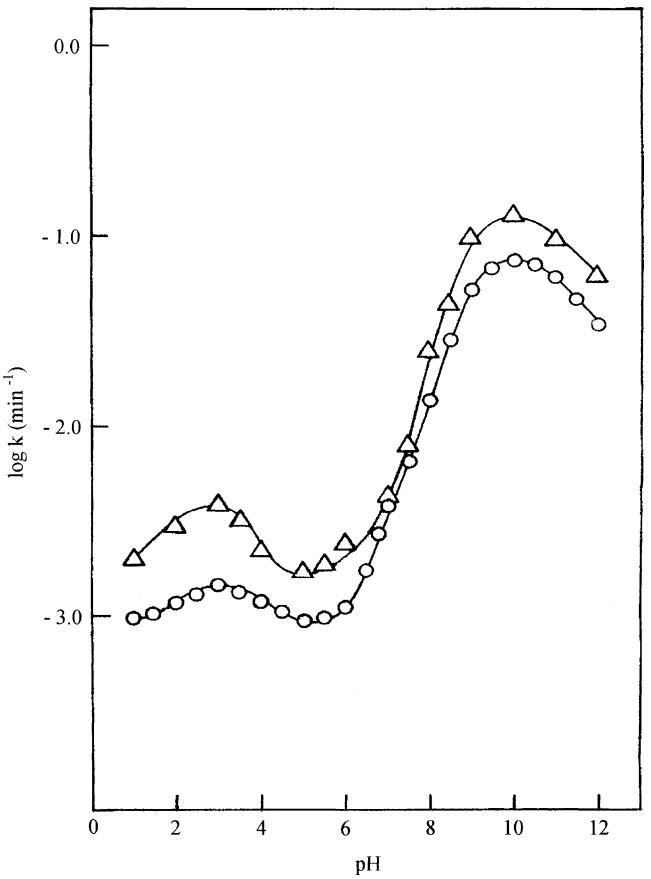

Fig. 1. log *k*–pH profiles for the photolysis of riboflavin in aqueous solution using UV light (△) and visible light (○).

degradation. Thus an increase in the rate at pH 3.0 would appear largely due to the degradation of RF to LC through the excited singlet state and to a smaller extent through the triplet state.

Some further explanation may be offered to the log *k*–pH profile for the photolysis of riboflavin. The slight decrease in the rate below pH 3.0 could be due to similar decrease in the fluorescence of riboflavin (Weber, 1950) resulting partly from the formation of a non-fluorescent cation (pK_a 1.7) but more from the quenching of the singlet state (^{1}Fl*). The gradual slow down in the rate in alkaline medium above pH 9.0 appears to be due to the formation of the anion (also non-fluorescent) by deprotonation of N-3 (pK_a 10.2). The rates of photolysis at various pH values may be influenced by the reactivity of the triplet state (^{3}Fl*) which is more basic (pK_a 4–5, Schreiner et al., 1975) than the ground state flavin (Fl_{ox}). In order to explain the increased reactivity of ^{3}Fl* at higher pH, Cairns and Metzler (1971) suggested the existence of a bent triplet and a planar excited singlet state of riboflavin, differing in the centre for hydrogen abstraction and protonation, to be responsible for the dependence of the rate of photolysis on pH.

The present study has shown that riboflavin has the lowest rate of photodegradation at pH 5–6, the range most suitable for maintaining the pH of vitamin preparations. However, the possibility of mutual interaction between riboflavin and other vitamins and any adverse effects of pH on other vitamins in this range has to be considered and appropriate measures to be taken to avoid the loss of the vitamins. The various aspects of photostability of drugs and drug formulations have been dealt in detail by Tonnesen (1996) and modern

approaches to photostability testing of drugs and standardization of experimental conditions have been discussed by Anderson et al. (1991), Tonnesen (1991), Tonnsen and Moore (1993), and Tonnesen and Karlsen (1995, 1997).

4. Conclusion

The photolysis behaviour of riboflavin in the pH range 1–12, on UV and visible irradiation, may be explained on the basis of the shape of log *k*–pH profile involving the existence of different species of the molecule (i.e., ionised/non-ionised) in this pH range and their redox potentials and fluorescence characteristics. The non-ionised species of riboflavin is more susceptible to photolysis than the ionised species. The increase in rate between pH 5.0 and 10.0 is largely due to the existence of the molecule in the non-ionised state and a gradual change, with pH, in redox potentials. The relatively low rates of photolysis below pH 2.0 and above pH 10.0 are due to the cation and anion formation, respectively, both of which are non-fluorescent and due to acid–base quenching of the excited singlet state. Riboflavin solutions are most stable to UV and visible radiations at pH 5–6, the range suitable for maintaining the pH of vitamin preparations provided vitamins other than riboflavin are not adversely affected.

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