Journal of Photochemistry & Photobiology, B: Biology 153 (2015) 358–366

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jpb

Formulation and stabilization of riboflavin in liposomal preparations

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ARTICLE INFO

Article history:
Received 16 August 2015
Received in revised form 5 October 2015
Accepted 19 October 2015
Available online 22 October 2015

Keywords:
Liposomal formulation
Riboflavin
Kinetics
Photolysis
Stabilization
Fluorescence quenching

ABSTRACT

A study of the formulation of liposomal preparations of riboflavin (RF) with compositional variations in the content of phosphatidylcholine (PC) and their entrapment efficiency (26–42%) have been conducted. Light transmission characteristics of the liposomal preparations have been determined to evaluate their effect on the amount of light passing through the system to initiate a photochemical reaction. Dynamic light scattering (DLS) and atomic force microscopy (AFM) have been used to study the physical characteristics of liposomes. The liposomal preparations of RF have been subjected to photolysis using visible light and the apparent first- order rate constant, k_{obs}, for the degradation of RF have been determined. The values of k_{obs} ($1.73–2.29 \times 10^{-3}$ min^{-1}) have been found to decrease linearly with an increase in PC concentration in the range of 12.15 to 14.85 mM. Thus, an increase in PC concentration of liposomes leads to an increase in the stability of RF. RF and its main photoproduct, lumichrome (LC), formed in liposomes have been assayed by a two-component spectrometric method at 356 and 445 nm using an irrelevant absorption correction to compensate for the interference of liposomal components. The fluorescence measurements of RF in liposomes indicate excited singlet state quenching and the formation of a charge-transfer complex between RF and PC. It results in electron transfer from PC to RF to cause photoreduction and stabilization of RF.

1. Introduction

Liposomes are artificial microscopic and submicroscopic phospholipid vesicles of spherical shape with a bilayered membrane structure. They have been used to target a drug to a specific site of action in the body to overcome stability and toxicity problems. The photodegradation and stabilization of photolabile drugs in liposomal formulations have been reviewed by several researchers [1–6]. The drugs used in these studies include riboflavin [7–11], doxorubicin [12] Vitamin A palmitate [13], fluoroquinolones [14,15], rose bangal [16], amlodipine [17], tretinoin [18], 2,7-dichlorodihydrofluorescein [19], *o*-palmitoyl amylopectin [20], anthralin [21] and zinc phthalocyanine and chloroaluminum phthalocyanine [22]. The chemical degradation and stabilization of phenolic, acidic and miscellaneous drugs [23], anesthetics [24], indomethacin [25,26] and natural compounds [27] in liposomes have also been studied. The enhancement of the encapsulation efficiency of liposomes may improve the stability of photolabile drugs [9,18,24,26,28,29].

Riboflavin (RF; i.e. vitamin B_2) (Fig. 1) is a highly photosensitive compound [30] and its photolysis in aqueous and organic solvents leading to a number of products by different pathways has been studied [6,31–34]. Attempts have been made to achieve photoprotection of RF by the use of stabilizers [35], borate [36] and citrate buffers [37], complexation with chemical agents [6,38–43] and cyclodextrins [8,9,44,45], light absorbers [8,9] and liposomes [7–11,46]. The stability of liposomes has been enhanced by the addition of α-tocopherol to retard the oxidation of bilayers of phospholipid component [47,48]. One of the problems in the study of photolysis of RF in liposomal preparations is the accurate assay of RF since its photoproducts (formylmethylflavin, FMF; lumichrome, LC; and lumiflavin, LF) [34,49,50] as well as the component of liposomes interfere with the assay of RF. Most of the workers have used fluorimetric methods [8–11] for the assay of RF in liposomes without any consideration of the interference of its intermediate photoproduct, FMF, and its hydrolytic product, LF (λ_{fluor} of both compounds 528 nm) [51] in the region of RF fluorescence emission (λ_{fluor} 520 nm) [8,9]. Therefore, the assay data of RF obtained by fluorimetric method may not be reliable. Multicomponent spectrometric methods have been developed for the assay of RF in the presence of its photoproducts [52] and to follow the kinetics of its photodegradation reactions [39,52,53]. These methods involve the extraction of LC and LF from photolysed solutions by chloroform which in the case of liposomes forms an emulsion with liposomal components and, therefore, cannot be used for the assay of RF in these preparations.

In the present study the assay of RF and its main photoproduct (i.e. LC) formed in liposomes at pH 7.4 has been carried out by a two-component spectrometric method using an irrelevant absorption correction to compensate for the interference of liposomal

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http://dx.doi.org/10.1016/j.jphotobiol.2015.10.017
1011-1344/

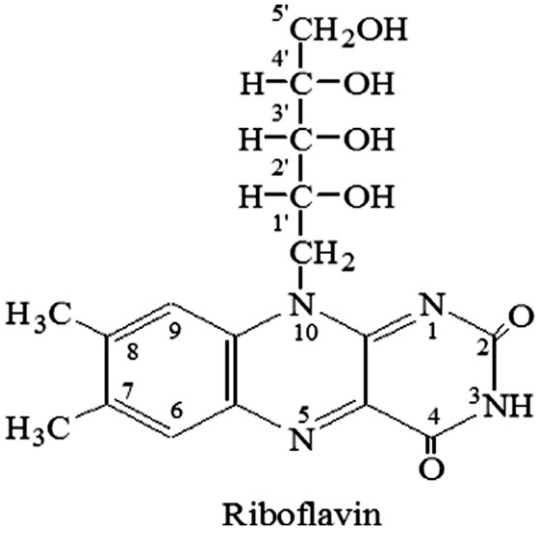

Fig. 1. Chemical structure of riboflavin (RF).

components at the analytical wavelengths [53,54]. Such interference has been observed on photodegradation of tretinoin in liposomal formulations [18]. In a two-component spectrometric assay [55], absorbance measurements are made at two suitably selected wavelengths and the concentrations are evaluated by solving simultaneous Eqs. (A.1) and (B.1).

$$A_1 = {}_{11} \cdot {}_1C + {}_{21} \cdot {}_2C \tag{A.1}$$

$$A_2 = {}_{12} \cdot {}_1C + {}_{22} \cdot {}_2C \tag{B.1}$$

where A_1 and A_2 are the absorbances at wavelength λ_1 and λ_2, respectively. ${}_{11}$, ${}_1\epsilon_2$, ${}_2\epsilon_1$ and ${}_2\epsilon_2$ are the molar absorptivity-cell path length products for component 1 at λ_1 and λ_2, and component 2 at λ_1 and λ_2 and ${}_1C$ and ${}_2C$ are the concentrations of component 1 and component 2, respectively.

The solution of Eqs. (A.1) and (B.1) for ${}_1C$ and ${}_2C$ is:

$${}_1C = ({}_{22} \cdot A_1 - {}_{21} \cdot A_2)/({}_{11} \cdot {}_{22} - {}_{21} \cdot {}_{12}) \tag{A.2}$$

$${}_2C = ({}_{11} \cdot A_2 - {}_{12} \cdot A_1)/({}_{11} \cdot {}_{22} - {}_{21} \cdot {}_{12}) \tag{B.2}$$

If the background or irrelevant absorption varies linearity with wavelength, a multipoint correction can be applied. In this case it is assumed that the irrelevant absorption (${}_1e$) obeys the following relation (Eq. (3)).

$${}_1e_{\mathbf{i}} = m \cdot \lambda_{\mathbf{i}} + c \tag{3}$$

where m and c are constants for a preparation containing the two components and the interfering substances.

Thus, the total absorbance $A_{\mathbf{i}}$ at $\lambda_{\mathbf{i}}$ is expressed by Eq. (4).

$$A_{\mathbf{i}} = {}_1C \cdot k_{\mathbf{i}} \cdot e + m \cdot \lambda_{\mathbf{i}} + c \tag{4}$$

Using this equation it is intended to find out the most probable concentration, ${}_1C$, of the component 1 from a series of n absorbance measurements, $A_{\mathbf{i}}$, at the wavelengths i = 1 to n.

The unknowns are ${}_1C$, m and c; the knowns are $\lambda_{\mathbf{i}}$ (the wavelength of ith measurement), $A_{\mathbf{i}}$ (absorbance measurement at $\lambda_{\mathbf{i}}$), the e and k_i are the values for absorptivity (usually at λ_{max}), and the factor appropriate to the wavelength λ_i.

The following matrix equation (Eq. (5)) can be used for the four appropriately chosen wavelengths which are required for a two-component assay, with a linear irrelevant absorption correction.

$$\begin{pmatrix} A_1 \\ A_2 \\ A_3 \\ A_4 \end{pmatrix} = \begin{pmatrix} {}_1\epsilon_1 & {}_2\epsilon_1 & \lambda_1 & 1 \\ {}_1\epsilon_2 & {}_2\epsilon_2 & \lambda_2 & 1 \\ {}_1\epsilon_3 & {}_2\epsilon_3 & \lambda_3 & 1 \\ {}_1\epsilon_4 & {}_2\epsilon_4 & \lambda_4 & 1 \end{pmatrix} \begin{pmatrix} {}_1a \\ {}_2a \\ m \\ c \end{pmatrix} \tag{5}$$

where, A_1, A_2, A_3, A_4 are the absorbance at the wavelengths λ_1, λ_2, λ_3, λ_4, ϵ is the molar absorptivity- cell path length product at these wavelengths; a is the concentration and m and c are constants. To obtain ${}_1a$ and ${}_2a$ solution of only two equations is required.

The present study involves the formulation and entrapment of RF in various liposomal preparations, determination of entrapment efficiency, and evaluation of the kinetics of its photolysis reactions on variations in liposomal composition to achieve optimum stability of the photolabile drug. This would involve the use of a specific spectrometric method with irrelevant absorption correction for the assay of RF in liposomal preparations and other techniques such as dynamic light scattering (DLS) and atomic force microscopy (AFM) to determine physical characteristics as well as spectrofluorimetry to study the interaction between RF and phospholipid component of liposomes to understand the mode of stabilization of RF.

2. Materials and Methods

Riboflavin (RF), lumichrome (LC), lumiflavin (LF) and cholesterol (CH) were purchased from Sigma chemicals Co., St. Louis, MD, USA. Phosphatidylcholine (PC) was from Avanti Polar Lipids, Alabaster, AL, USA. Formylmethylflavin (FMF) was prepared according to the method of Fall and Petering [56]. All solvents and reagents were of analytical grade or of purest form available from Merck & Co. Whitehouse Station, NJ, USA. Freshly boiled distilled water was used for the preparation of buffers and liposomal preparations. The following buffer system was used Na_2HPO_4–KH_2PO_4 (0.005 M), pH 7.4.

2.1. Preparation of Liposomes

The liposomes were prepared by the reverse evaporation method described by Szoka and Papahadjopoulos [57] with slight modification in the composition. PC (12.15–14.85 mM) and CH (13.50 mM) were weighed and dissolved in 2:3 ratios of chloroform and methanol in a 25 ml volumetric flask. Nitrogen gas was flushed in the solution to remove the oxygen to avoid its effect on the oxidation of lipids. It was then placed in a 100 ml round bottom flask and the solvent was removed under reduced pressure using a rotary evaporator. Vacuum was created and the flask was kept overnight to remove any traces of organic solvent. Then the thin film formed was hydrated by using a solution of RF (1×10^{-4} M) in 0.005 M phosphate buffer saline (PBS), pH 7.4. The flask was rotated at a temperature ~40 °C at 100–120 rpm. The resultant RF solution was converted into an opalescent viscous liposomal preparation. The liposomes were removed by centrifugation at a speed of 14,000 rpm at 4 °C. The pellets of liposomes then settled down. The supernatant was removed by a micropipette and the remaining pellets were washed three times with 0.005 M PBS, pH 7.4. The size of liposomes was reduced by passing through an extruder fitted with a polycarbonate filter. The encapsulated liposomal preparation was stored at 4 °C for further use. The physical characteristics of the liposomes were studied by DLS and AFM.

2.2. pH measurements

The pH of the solutions was measured with an Elmetron LCD display pH meter (Model-CP 501 sensitivity ±0.01 pH unit, Poland) using a