ELSEVIER

International Journal of Pharmaceutics 117 (1995) 85–94

international journal of pharmaceutics

Novel liposome-based multicomponent systems for the protection of photolabile agents

Yannis L. Loukas [a], Pramukh Jayasekera [b], Gregory Gregoriadis [a,*]

[a] *Centre for Drug Delivery Research, School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK*
[b] *Chemical and Biological Defence Establishment, Porton Down, Salisbury SP4 0JQ, UK*

Received 1 September 1994; accepted 29 September 1994

Abstract

A photosensitive drug (riboflavin) was entrapped as such or in the form of β- or γ-cyclodextrin complexes into the aqueous phase of multilamellar dehydration-rehydration vesicles (DRV liposomes) made of equimolar egg phosphatidylcholine or dipalmitoylphosphatidylcholine and cholesterol. Riboflavin-containing DRV were prepared in the absence or presence of one or more of the lipid-soluble UV absorbers oil red O, oxybenzone and dioxybenzone (entrapped into the lipid phase) and the water-soluble sulisobenzone (entrapped in the aqueous phase of liposomes together with riboflavin). In some experiments, lipid-soluble absorbers were supplemented with the antioxidant β-carotene. Entrapment values for free (41–47%) and complexed (19–23%) riboflavin were estimated fluorimetrically with additional data from NMR studies confirming that the complexes were entrapped as intact entities. Entrapment values for each of the UV light lipid-soluble absorbers (79–98%) and β-carotene (78 and 88%) were estimated by the use of the second-order derivative of their UV spectra to circumvent interference from overlapping absorption spectra of the other agents, when present. A number of conditions of entrapment were found to reduce values, for instance co-entrapment of sulisobenzone together with the vitamin in the case of riboflavin and, for all other materials, the absence (or reduced content) of cholesterol in DRV or certain variations in their manufacture. Exposure of a variety of riboflavin-containing DRV preparations to UV light revealed optimal protection with a formulation containing the γ-cyclodextrin complex of the vitamin, all three lipid-soluble light absorbers and β-carotene, increasing the half-life of riboflavin 266-fold. Results suggest that liposome-based multicomponent systems could be developed for the protection of photolabile agents in therapeutics and other uses.

Keywords: Liposome; Sunscreen agent; Cyclodextrin; Antioxidant; Photodegradation

1. Introduction

Photosensitive drugs are known (Connors et al., 1979; Carstensen, 1990) to degrade on exposure to light and lose their activity. For example, the antibacterial activity of ciprofloxacin is reduced in the presence of ultraviolet light with associated photoreactions being hazardous to patients (Phillips, 1990). Such drugs can be protected from light during both storage and application (e.g., locally) by the use of appropriate light-

* Corresponding author.

SSDI 0378-5173(94)00320-3

absorbing agents present together with the drug in the same formulation (Thoma and Klimek, 1991). Light-absorbing materials protect drugs from light by absorbing, partially or wholly, visible and/or UV radiation and are classified as chemical absorbers or physical blockers (e.g., metal oxides). The former are generally aromatic compounds possessing a carbonyl group and function by transforming high energy UV radiation to energy of lesser magnitude (Shaath, 1990). Such materials can be formulated in combination with antioxidants (Krinsky and Deneke, 1982) which quench both singlet oxygen-driven photochemical and free radical reactions, for instance those involved in lipid peroxidation. Photoprotection can also be achieved to some extent by including photosensitive substances into the hydrophobic cavity of cyclodextrins (Andersen and Bundgaard, 1984). However, whilst such approaches to photostabilization have been adequate in some cases, there is a need for improving photostabilization further, particularly in therapeutics where a variety of photosensitive drugs are often administered in unstable forms.

We have recently proposed (Gregoriadis and Loukas, 1994; Loukas et al., 1994) that photostability of photosensitive agents may be greatly improved by combining these, as such or in the form of cyclodextrin inclusion complexes, with chemical light absorbers and antioxidants within the aqueous and/or lipid phase of liposomes. The proposed system is expected to provide several lines of defence against light for instance, through the lipid membranes of the vesicles, the presence of light absorbers and antioxidants embedded in the membranes or dissolved in the aqueous phase and, when appropriate, through the structure of the cyclodextrin cavity harbouring the photosensitive agent within the aqueous phase of liposomes. Here, we describe the preparation, quantitation and function of such liposome-based systems containing free or cyclodextrin-included riboflavin and incorporating one or more of the light absorbers oil red O, oxybenzone, dioxybenzone and the water-soluble sulisobenzone as well as the antioxidant β-carotene. Riboflavin was chosen as a model photosensitive agent because of its rapid degradation on exposure to UV light (Asker and Habib, 1990). Exposure of a number of liposome-based systems to UV light revealed that a formulation containing the γ-cyclodextrin inclusion complex of the vitamin and incorporating all of the lipid-soluble light absorbers together with β-carotene, provided maximum protection, increasing the half-life of riboflavin from 0.48 to 128 h.

2. Materials and methods

2.1. Materials

Riboflavin 5′-monosodium salt (dihydrogen phosphate) (R) was obtained from Aldrich Chemical Co. (Poole, Dorset, UK). β-Cyclodextrin (βCD) and γ-cyclodextrin (γCD) were purchased from Wacker Chemie (GmbH, Munich) and oil red O, oxybenzone, dioxybenzone, sulisobenzone, β-carotene, Triton X-100 and cholesterol from Sigma Chemical Co. (Poole, Dorset, UK). Egg phosphatidylcholine (PC) and 1-α-distearoylphosphatidylcholine (DSPC) were from Lipids Products (Nuthill, Surrey) and deuterium oxide (D_2O, 99.9% purity) from Fluka (Poole, Dorset, UK). All other reagents were of analytical grade. Double-distilled water was used throughout.

2.2. Instrumentation

Measurement of R in various preparations and the kinetics of its degradation was carried out in a Perkin Elmer LS-3 fluorescence spectrometer. Assay of components used for photoprotection was carried out in a Compuspec UV/visible spectrophotometer (Wallac) connected to a personal computer. ^{1}H-NMR spectra in D_2O were recorded in a Bruker AM 500 spectrometer connected to an Aspect 3000 computer. The chemical shifts were related to the residual solvent signal (hydrogen-deuterium oxide = 4.84 ppm at 293 K). Typical conditions were 16 K data points with zero filling, sweep width of 5 kHz giving a digital resolution of 0.61 Hz point^{-1}, pulse width 4 μs, acquisition time 1.64 s and number of scanning 128.

2.3. Preparation of R-cyclodextrin inclusion complexes

Inclusion complexes of R with βCD and γCD were prepared by the freeze-drying method. R (0.5 mmol) was dissolved in 10 ml water and the clear solution added dropwise into the cyclodextrin solution (0.5 mmol in 10 ml water) under stirring. The solution was allowed to stir in the dark at 20°C for 2 days. The final clear yellow solution was frozen at −40°C and freeze-dried overnight to yield an amorphous yellow powder.

2.4. Entrapment of agents into liposomes

Entrapment of R into liposomes was carried out by the dehydration-rehydration procedure (Kirby and Gregoriadis, 1984) with some modifications. In brief, small unilamellar vesicles (SUV) prepared (Kirby et al., 1980) at the appropriate temperatures from PC (0.04 mmol) or DSPC (0.04 mmol) and equimolar cholesterol were mixed with 0.004 mmol of R dissolved in water (10 ml final volume), rapidly frozen to −40°C and freeze-dried overnight. The dry powder was subjected to controlled (Kirby and Gregoriadis, 1984) rehydration at the same temperatures, initially with water and finally with 0.1 M sodium phosphate buffer supplemented with 0.9% NaCl, pH 7.4 (PBS) and centrifuged at 27300 × g for 20 min to separate the entrapped from non-entrapped R. The liposomal pellet containing multilamellar (Gregoriadis et al., 1993) dehydration-rehydration vesicles (DRV) was washed three times in PBS and resuspended in 4 ml PBS until further use. The same procedure was applied for the entrapment of the R-βCD (0.004 mmol R) or R-γCD (0.004 mmol R) complexes. With DRV preparations incorporating lipid-soluble light absorbers and β-carotene in addition to R or R-cyclodextrin complexes, oil red O, oxybenzone, dioxybenzone and β-carotene were dissolved in chloroform together with the lipids employed for the preparation of the SUV precursor vesicles. When the water-soluble sulisobenzone was used as a light absorber, this was dissolved together with free R in the aqueous solution to be subsequently mixed with SUV (see above). A comprehensive list of materials and amounts used is provided in Table 1.

2.5. Estimation of liposome-entrappped materials

Entrapment values for R, R-cyclodextrin complexes, light absorbers and β-carotene were estimated indirectly by measuring the concentrations

Table 1
Materials and amounts used for incorporation into DRV liposomes [a]

Liposomes	R	R-βCD	R-γCD	Oil red O	Oxybenzone	Dioxybenzone	Sulisobenzone	β-Carotene
DRV 1	0.004							
DRV 2	0.004			0.008				
DRV 3	0.004				0.008			
DRV 4	0.004					0.008		
DRV 5	0.004						0.004	
DRV 6	0.004				0.004	0.004		
DRV 7	0.004			0.004	0.002	0.002		
DRV 8	0.004			0.004	0.002	0.002	0.004	
DRV 9		0.004						
DRV 10			0.004					
DRV 11		0.004		0.008				
DRV 12			0.004	0.008				
DRV 13		0.004		0.004	0.002	0.002		
DRV 14			0.004	0.004	0.002	0.002		
DRV 15			0.004	0.004	0.0015	0.0015		0.0015

[a] All values are expressed in mmol. Riboflavin (R) (as such or complexed with βCD or γCD), oil red O, oxybenzone, dioxybenzone, sulisobenzone and β-carotene were entrapped in DRV liposomes made from 40 μmol PC and equimolar cholesterol. In some experiments preparation DRV 15 was made under a variety of conditions described in the legend to Table 2. For other details see section 2.

of materials in the pooled supernatants obtained following the isolation of the DRV liposomal pellets. Such materials were either non-entrapped in a free, water-soluble form (e.g., R, R-CD complexes or sulisobenzone) or, in the case of lipid-soluble materials, presumably associated with non-precipitable small liposomes or membrane fragments (see later). The combined supernatants (24 ml) were extracted twice with chloroform (8 ml) and the organic and aqueous phases obtained kept in the dark at 4°C until further use. Lipid-soluble light absorbers and β-carotene in the organic phase were determined by derivative UV spectroscopy (Mahrous et al., 1985), a useful technique for the analysis of multicomponent systems with extensive absorbance overlaps and spectra without a clear maximum (e.g., β-carotene spectrum). In the present work (see section 3), the use of the second-order derivative (D_2) of the spectra was found to provide both good resolution and high signal-to-noise (S/N) ratios. R, as such or as a cyclodextrin complex, in the aqueous phase (following extraction with chloroform) was measured fluorometrically at excitation and emission wavelengths 445 and 520 nm respectively. Similar aqueous phases containing the complexes were freeze-dried and the stoichiometry of the solid material obtained was determined by ^{1}H-NMR in D_2O. Digital integration of selected NMR signals from R and cyclodextrins provided direct access to the stoichiometry coefficient (Djedaini and Perly, 1991) which served to determine the R/cyclodextrin molar ratios of the non-entrapped complexes. Finally, assay of

Table 2
Entrapment of riboflavin and other agents into liposomes [a]

Liposomes	R	R-βCD	R-γCD	Oil red O	Oxybenzone	Dioxybenzone	Sulisobenzone	β-Carotene
DRV 1	47 [b]							
DRV 2	43			98				
DRV 3	41				96			
DRV 4	42					95		
DRV 5	25						20	
DRV 6	44				87	90		
DRV 7	44			89	85	89		
DRV 8	27			85	86	85	18	
DRV 9		23 [b](1.1) [c]						
DRV 10			20 [b](1.1) [c]					
DRV 11		21(1.17)		88				
DRV 12			19(1.1)	87				
DRV 13		21(1.05)		85	86	85		
DRV 14			20(1.1)	87	86	88		
DRV 15			19(1.1)	84	85	85		88
DRV 15 [d]			10(1.2)	48	52	50		50
DRV 15 [e]			16(1.2)	55	58	58		55
DRV 15 [f]			20(1.17)	80	79	80		78
DRV 15 [g]			8(1.14)	78	80	78		75
DRV 15 [h]			12(1.2)	79	81	81		74

[a] Riboflavin (R) (as such or included in βCD or γCD), oil red O, oxybenzone, dioxybenzone, sulisobenzone and β-carotene were entrapped in different combinations into the aqueous or lipid phase of DRV liposomes. Unless stated otherwise, DRV were made from 40 μmol PC and equimolar cholesterol. Numbers in parentheses denote R-cyclodextrin complex molar ratios. All entrapment values are expressed as % of the amounts used. For other details see Table 1 and section 2.

[b] Riboflavin values measured by fluorescence spectroscopy.

[c] R-cyclodextrin complex molar ratios determined by ^{1}H-NMR.

[d] Cholesterol-free DRV.

[e] PC/cholesterol molar ratio: 1:0.5.

[f] DRV prepared from 40 μmol DSPC and equimolar cholesterol.

[g] R-γCD added during rehydration.

[h] DRV prepared from unsonicated liposomes.

sulisobenzone present in the aqueous phase together with R, was carried spectrophotometrically at 316.8 nm. At this wavelength there is a minor interference by R which was estimated from its concentration and molar absorptivity ($\varepsilon_{316.8}$). This was subsequently substracted from the mixture's total absorbance at the same wavelength to give absorbance values for sulisobenzone only according to the law of additivity (Fell, 1986).

2.6. Exposure to UV radiation

Solutions or liposomal suspensions of R (3 ml) in cuvette cells ($1 \times 1 \times 4$ cm^3; 1 cm path length) were exposed sideways at 24°C ± 1 to UV light from a distance of 3 cm using a Black-Ray (365 nm) UVB lamp (San Gabriel, USA) with 6 W rating and 460 μW cm^{-2} dm^{-1} intensity. Samples in the cuvettes removed at time intervals were then appropriatelly diluted with distilled water and assayed for R fluorometrically as above. With liposome-entrapped R, samples were first solubilised with Triton X-100 (10% final concentration) at 70°C.

3. Results and discussion

3.1. Determination of entrapped materials in DRV liposomes and yield of entrapment

Entrapment values (Table 2) for materials in the DRV preparations (both aqueous and lipid liposomal phases) described in Table 1 were calculated according to the equation % entrapment $= [(A_0 - A)/A_0] \times 100$, where A_0 is the absorbance or fluorescence of the initial concentration of materials and A denotes the absorbance or fluorescence of non-entrapped materials in the organic and water phases (obtained on extraction of the combined supernatants with chloroform) after a dilution correction to achieve identical dilutions for both A_0 and A. Entrapment values for R (Table 2) in the absence or presence of one or more of the lipid-soluble materials incorporated into the lipid phase of liposomes were substantial (41–47% of the amounts used) and in agreement with those observed with other DRV-entrapped water-soluble solutes (Kirby and Gregoriadis, 1984; Gregoriadis et al., 1987, 1990). Moreover, values for R derived from fluorimetric measurements were similar to those (Table 3) obtained by measuring (entrapped) R in the liposomal pellets after their dissolution with Triton X-100 (10% final concentration), the standard deviation between the two sets of values being ±2%. When the water-soluble sulisobenzone was co-entrapped with R (DRV preparations 5 and 8; Table 1), however, values were nearly halved (25 and 27%; Table 2) and of a similarly low level to those for sulisobenzone (Table 2), presumably because of competition of the two solutes for entrapment in the aqueous phase of the vesicles.

Table 3
Direct estimation of entrapment values for riboflavin and oil red O [a]

Preparation	Entrapment (% of amount used)	
	Riboflavin	Oil red O
DRV 1	49	–
DRV 2	45	95
DRV 3	39	–
DRV 4	41	–
DRV 11	23	87
DRV 12	21	87

[a] Riboflavin and oil red O were measured in DRV liposomes after centrifugation to remove non-entrapped materials. For other details see Tables 1 and 2 and section 2.

Entrapment values for R used in the form of cyclodextrin complexes were, as with free R, estimated indirectly on the basis of fluorescence measurements in the water phase obtained on extraction of the superntants with chloroform: preliminary work had shown identical excitation and emission wavelengths for free and complexed R (results not shown). However, in order to confirm that such measurements did indeed reflect R entrapment in the cyclodextrin complex form, the R/βCD or R/γCD molar ratios were calculated. This was carried out by integrating the anomeric protons of the cyclodextrins with the phenyl protons of R according to: molar ratio = (7 or 8) × $I_R/2 \times I_{CD}$ where 7 or 8 are the numbers of anomeric protons (H1) in βCD and γCD, respectively, 2 represents the number of phenyl protons in R, I_R is the integration value of the phenyl

protons at 7.9 ppm and I_{CD} denotes the integration value for the anomeric protons at 5.2 ppm (Fig. 1). With all relevant preparations shown in Table 2, R/CD molar ratios were near unity (Table 2) indicating that R was entrapped as the intact complex. Table 2 also shows that, in contrast to values obtained with R as such, entrapment values for R in the form of cyclodextrin (βCD and γCD) complexes were modest (19–23% of the amount used for both PC and DSPC cholesterol-rich DRV) and similar to those measured directly in liposomal pellets (Table 3). Such values were in agreement with those previously obtained with other cyclodextrin-drug complexes entrapped in DRV liposomes (McCormack and Gregoriadis, 1994). As discussed elsewere (McCormack and Gregoriadis, 1994), relatively low entrapment values for cyclodextrin complexes could be attributed to the cryoprotective action of sugars (in this case cyclodextrins) on SUV leading to reduced DRV generation. Entrapment values (Table 2) for the cyclodextrin (γCD) complex of R were even lower (10%) when DRV made of PC were devoid of cholesterol, probably because of the reduced stability of such liposomes, leading to solute leakage (Gregoriadis, 1988). As expected (Kirby and Gregoriadis, 1984), similarly low entrapment values (8%) were also obtained when the complex was added during the step of rehydration (rather than mixed with SUV before de-

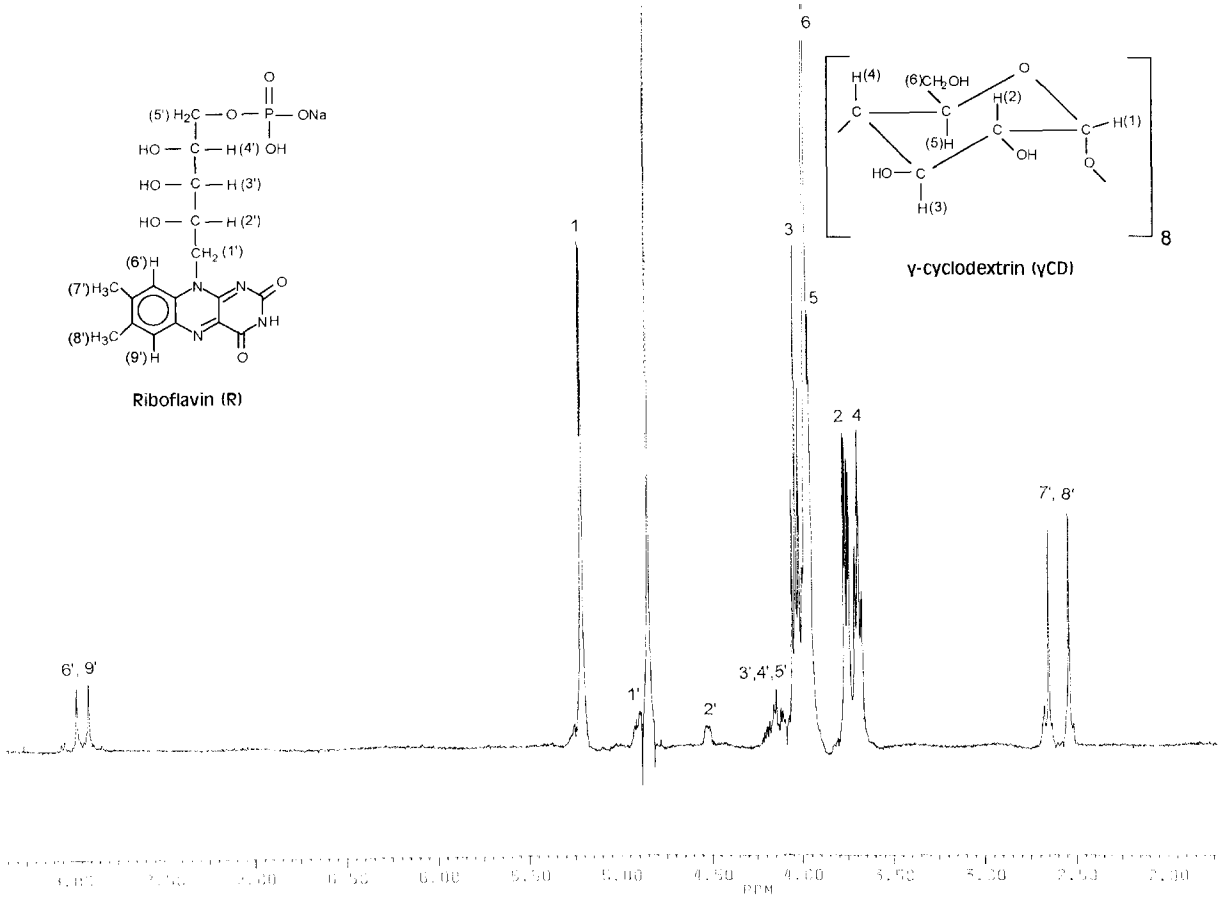

Fig. 1. 500 MHz ^{1}H-NMR spectrum of the R-γCD complex in D_2O. Numbers in parentheses in riboflavin and γCD structures show chemical shifts of their individual protons corresponding to numbered peaks in the spectrum.

hydration) or when MLV rather than SUV were the precursor vesicles for DRV in the procedure (12% of the amount used): both sets of conditions are thought (Kirby and Gregoriadis, 1984) to curtail the extent of intimate contact between solutes destined for entrapment and flattened bilayers and thus reduce entrapment during rehydration. Again, there was no interference with entrapment by the presence of lipid-soluble materials in the bilayers (e.g., compare preparations 9 and 10 with preparation 11–15; Table 2).

Incorporation of lipid-soluble absorbers and β-carotene into the liposomal membranes was monitored indirectly by spectrophotometric analysis of the organic (chloroform) phase obtained on extraction of the supernatants (see above). Because oil red O has an area (576–620 nm) in its absorption spectrum with which none of the other lipid-soluble materials (including β-carotene) interferes (Fig. 2a), its estimation in all relevant DRV preparations shown in Table 1 was carried out first. Since this absorption area does not exhibit a clear maximum, the second-order derivative (D_2) approach was applied to provide a clear maximum at 584.2 nm (Fig. 2b) with a calibration curve of oil red O based on the amplitude of $D_{2,584.2\ nm}$ maximum allowing for the selective determination of oil red O in the mixture. To that end, the vertical distance to the zero line (Fig. 2b) was measured according to the zero-crossing method (Talsky et al., 1978; Umapathi, 1994). Similarly, for the determination of β-carotene in the presence of other materials, the amplitude of the maximum of $D_{2,432.8\ nm}$ was measured as, at this wavelength, the amplitudes of the second-order derivative spectra, for the other materials are nearly zero. Estimation of β-carotene was therefore achieved by the use of a calibration curve which correlates the concentration of β-carotene with the amplitude of $D_{2,432.8\ nm}$.

As with oil red O and β-carotene, oxybenzone was determined by measuring the amplitude of its D_2 maximum at 332 nm. At this wavelength, the D_2 amplitude is due only to the oxybenzone, since the $D_{2,332\ nm}$ of oil red O, dioxybenzone and β-carotene are, on the basis of their spectra, nearly zero. Determination of dioxybenzone, on the other hand, was carried out by measuring the amplitude of the D_2 maximum at 358.4 nm. However, as at this wavelength, there is interference by the D_2 maxima of oxybenzone, oil red O and β-carotene specta, a mixture of known amounts of all four materials was prepared and analyzed quantitavely on the basis of standard solutions of individual materials. Recovery for all materials was calculated according to their second-order derivative spectra. Results in Table 2 indicate that incorporation of the lipid-soluble light absorbers (79–98%) and β-carotene (78 and 88% of the amounts used) into liposomes was generally high, regardless of the phospholipid (PC or DSPC) used. With one of the light absorbers (oil red O),

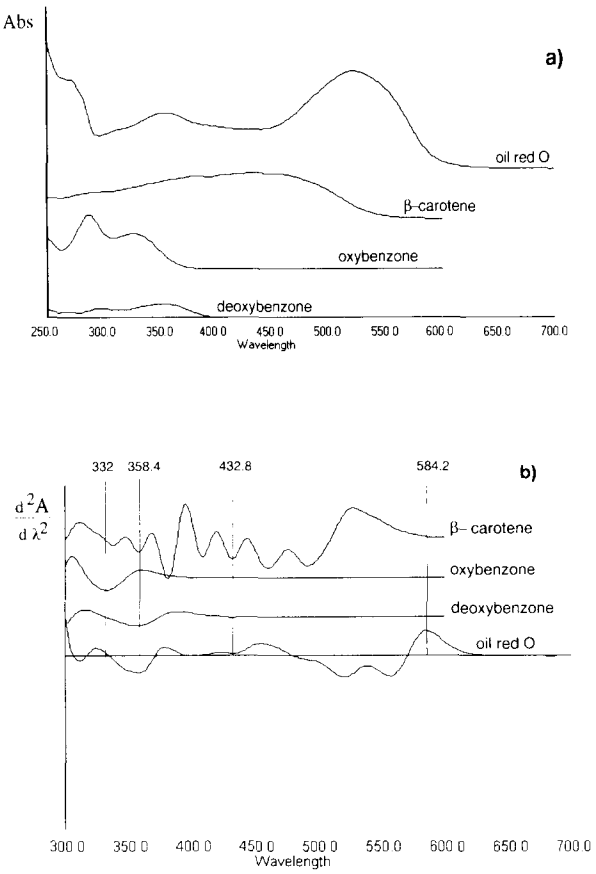

Fig. 2. Zero-order derivative UV spectra of light absorbers (a) and second-order derivative UV spectra (D_2) of light absorbers and wavelenghts used for the calculation of their concentrations (b). For other details see section 2.

such entrapment values were confirmed (see Table 3) by its direct measurement in the liposomal pellets after their dissolution with Triton X-100 as above (because of interference of Triton X-100 and high concentrations of R with the spectra, it was not possible to directly measure other lipid-soluble materials in the pellets). However, for DRV free of cholesterol or with a PC/cholesterol molar ratio of 1:0.5, incorporation values were lower (48–58%; Table 2). It thus appears that excess sterol (phospholipid/cholesterol molar ratio of 1:1) facilitates nearly complete accommodation of the lipid-soluble materials in the amounts used within the bilayers. As already mentioned, non-entrapped lipid-soluble materials recovered in the supernatants following centrifugation of the DRV were assumed to represent materials associated either with non-precipitable small vesicles or with bilayer fragments. This was supported by experiments where the DRV preparation procedure was carried out in the absence of liposomal lipids: as expected, no lipid-soluble materials were recovered in the supernatants obtained on centrifugation of the 'rehydrated' materials (results not shown).

3.2. *Photostabilization of liposome-entrapped riboflavin*

A number of riboflavin formulations described in Tables 1 and 2 were tested for their effectiveness in protecting the vitamin against UV light. Results in Table 4 indicate that entrapment of R into DRVs in the absence of other materials, modestly increases its half-life (in terms of protection from UV light) from 0.48 h (observed for free R) to 1.92 h. Half-life was, however, increased substantially to 31.2 h when oil red O was also present in the bilayers. In contrast, other light absorbers, for instance, oxybenzone, dioxybenzone and sulisobenzone incorporated individually in DRV did not improve the photostability of R significantly. On the other hand, there was a further increase in the half-life of R (to 36 h) when oil red O in the bilayers was supplemented with oxybenzone and dioxybenzone (results not shown).

Table 4
Photodegradation rate constants and half-lives of riboflavin exposed to UV light

Preparation	k ($\times 10^{-4}$) (min^{-1})	$t_{1/2}$ (h)	r
Free R	240	0.48	0.991
DRV-R	60	1.92	0.982
DRV-R [a]	3.7	31.22	0.986
DRV-RγCD	12	9.63	0.992
DRV-RγCD [a]	1.4	82.5	0.975
DRV-RγCD [a,b,c]	1.1	105	0.989
DRV-RγCD [a,b,c,d]	0.9	128.33	0.983

Free R or DRV liposomes composed of equimolar PC and cholesterol and containing R (DRV-R) or R-γCD (DRV-R-γCD) alone or together with oil red O (a), oxybenzone (b), dioxybenzone (c), and β-carotene (d) in combinations, were exposed to UV light. Photodegradation rate constants (k) were determined from R measurements in samples obtained at time intervals. Half-lives ($t_{1/2}$) in h were estimated according to the equation: $t_{1/2} = 0.693/k$, where k is the photodegradation rate constant in min. r, correlation coefficient. For other details see Table 1 and section 2.

Previous findings (Andersen and Bundgaard, 1984) that photosensitive agents included in cyclodextrins are protected to some extent from UV light, prompted us to investigate photoprotection of cyclodextrin-included R in conjuction with possible additional protection offered by entrapping the complex into liposomes. Preliminary work had established a half-life of 2.9 h for R included in γCD (results not shown). This was improved to 9.6 h when the RγCD complex was entrapped into DRV (Table 4). Interestingly, however, a much greater half-life (82.5 h) was achieved when oil red O was also present in the bilayers. As with DRV containing free R, stabilization was augmented further (to 105 h) in the presence of all lipid-soluble light absorbers (Table 4). Finally, incorporation of β-carotene in this formulation produced an even greater half-life (128 h; Table 4), presumably because of the above mentioned ability of antioxidants to quench oxygen driven photochemical reactions. Thus, by a combination of cyclodextrin inclusion, liposome entrapment and the use of light absorbers and an antioxidant in the vesicle structure, the half-life of riboflavin was increased 266.7-fold.

In conclusion, photolabile agents may be protected from UV light by incorporating these into a liposome-based multicomponent system. This functions through a series of barriers to light and the presence of an antioxidant, all assembled within the bilayer structure which, by itself, also appears to absorb light. Our data indicate that optimal protection of riboflavin (used as a model photolabile agent) is provided by liposomes containing the γ-cyclodextrin inclusion complex of the vitamin within their aqueous phase and the light absorbers oil red O, oxybenzone and dioxybenzone together with the antioxidant β-carotene in the lipid phase. Although each of the components, including γ-cyclodextrin, contributes to a lesser or greater extent to photoprotection, it is the combined presentation of all components that is most effective. It is likely that the present approach will apply to other photolabile agents as well, especially those of a molecular size that is compatible with cyclodextrin inclusion. However, larger molecules and particulates such as microorganisms (for which technology for entrapment into giant vesicles now exists; Antimisiaris et al., 1993) may also be realistically protected. To that end, the choice and number of light absorbers employed is expected to vary according to the structural characteristics of the photolabile agent which define the wavelength of light to which the agent is sensitive. Regardless of the potential complexity of combinations of the system's components, succesful application of the second-order derivative spectroscopy in the present work suggests that their analysis and quantitation might be feasible.

References

Andersen, F. and Bundgaard, H., The inclusion complexation of metronidazole benzoate with beta-cyclodextrin and its depression of anhydrate-hydrate transition in aqueous suspensions. *Int. J. Pharm.*, 19 (1984) 189–192.

Antimisiaris, S. Jayasekera, P. and Gregoriadis, G., Liposomes as vaccine carriers: Incorporation of soluble and particulate antigens in giant vesicles, *J. Immunol. Methods*, 166 (1993) 271–280.

Asker, A.F. and Habib, M.J., Effect of certain stabilizers on photobleaching of riboflavin solutions. *Drug Dev. Ind. Pharm.*, 16 (1990) 149–156.

Carstensen, J.T., *Drug Stability: Principles and Practices*, Dekker, New York, 1990, ch. 2.

Connors, K., Amidon, G. and Kennon, L., *Chemical Stability of Pharmaceuticals*, Wiley, New York, 1979, pp. 86–87.

Djedaini, F. and Perly, B., Nuclear magnetic resonance of cyclodextrins, derivatives and inclusion compounds. In Duchene, D. (Ed.), *New Trends in Cyclodextrins and Derivatives*, Edition de Sante, Paris, 1991, pp. 217–246.

Fell, A., Ultraviolet, visible, and fluorescence spectrophotometry. In Moffat, A. (Ed.), *Clarke's Isolation and Identification of Drugs*, The Pharmaceutical Press, London, 1986, pp. 221–236.

Gregoriadis, G., Fate of injected liposomes: Observations on entrapped solute retention, vesicle clearance and tissue distribution in vivo. *Liposomes as Drug Carriers: Recent Trends and Progress*, Wiley, Chicester, 1988, pp. 3–18.

Gregoriadis, G. and Loukas, Y., *British Patent Application 9320668.8*, 1994.

Gregoriadis, G., Da Silva, H. and Florence, A.T., A procedure for the efficient entrapment of drugs in dehydration-rehydration liposomes (DRV). *Int. J. Pharm.*, 65 (1990) 235–242.

Gregoriadis, G., Davies, A. and Davis, D., Liposomes as immunological adjuvants: Antigen incorporation studies. *Vaccine* 5 (1987) 143–149.

Gregoriadis, G., Garcon, N., Da Silva, H. and Steinberg, B., Coupling of ligands to liposomes independently of solute entrapment: Observation on the formed vesicles, *Biochim. Biophys. Acta*, 1147 (1993) 185–193.

Kirby, C. and Gregoriadis, G., Dehydration-rehydration vesicles: A simple method for high yield drug entrapment in liposomes. *Biotechnology*, 2 (1984) 979–984.

Kirby, C., Clarke, J. and Gregoriadis, G., Effect of cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. *Biochem. J.*, 186 (1980) 591–598.

Krinsky, N. and Deneke, S., Interaction of oxygen and oxyradicals with carotenoids. *J. Natl. Cancer Inst.*, 69 (1982) 205–210.

Loukas, Y., Jayasekera, P. and Gregoriadis, G., Co-entrapment of cyclodextrin inclusion complexes of riboflavin together with light absorbing agents in liposomes increases the photostability of the vitamin. *J. Pharm. Pharmacol.*, (1994) in press.

Mahrous, S.M., Abdel-khalek, M.M. and Abdel, M.E., Quantitation of indomethacin, naproxen and ibuprofen in pharmaceutical dosage forms by first and second derivative ultraviolet spectrometry. *J. Assoc. Off. Anal. Chem.*, 68 (1985) 535–539.

McCormack, B. and Gregoriadis, G., Drugs-in-cyclodextrins-in liposomes: A novel concept in drug delivery. *Int. J. Pharm.*, 112 (1994) 249–258.

Phillips, G., The loss of antibiotic activity of ciprofloxacin by photodegradation. *J. Antimicrob. Chemother.*, 26 (1990) 783–789.

Shaath, N.A., Evolution of modern sunscreen chemicals. In Lowe, N.J and Shaath, N.A. (Eds), *Sunscreens, Development, Evaluation and Regulatory Aspects*, Dekker, New York, 1990, pp. 3–37.

Talsky, G., Mayring, L. and Kreuner, H., High-resolution, higher-order UV/VIS derivative spectrophotometry. *Angew. Chem. Int. Ed.*, 17 (1978) 785–799.

Thoma, K. and Klimek, R., Photostabilization of drugs in dosage forms without protection from packaging materials. *Int. J. Pharm.*, 67 (1991) 169–175.

Umapathi, P., Determination of atenolol, nifedipine, aspirin and dipyridamole in tablet preparations by second-order derivative spectrometry. *Int. J. Pharm.*, 108 (1994) 11–19.