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Accelerated photostability study of tretinoin and isotretinoin in liposome formulations

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Abstract

The photodegradation of retinoic acids, tretinoin and isotretinoin, in ethanol and liposomes was studied. The light irradiation was performed according to the conditions suggested by the ICH Guideline for photostability testing by using a Xenon lamp within a wavelength range of 300–800 nm. The photodegradation process was monitored by UV spectrophotometry. In ethanol solution, tretinoin and isotretinoin undergo complete isomerization just within a few seconds of light exposure to give 13-*cis* and 9-*cis* isomers, respectively. The 13-*cis* isomer from tretinoin undergoes in turn a slow isomerization to the same 9-*cis* isomer. Both retinoic acids incorporated in liposome complexes showed an increased stability in comparison to the ethanol solutions. In particular for tretinoin, a residual concentration of 60% was still present after a light irradiance of 3470 kJ/m^2, by means of a 250 W/m^2 light power for 240 min, versus a residual value of just 8% measured at the same time in ethanol solution. Moreover, the isomerization rate in liposomes resulted reduced for isotretinoin and practically irrelevant for tretinoin. The degradation rate was found to be dependent on the drug concentration. The better stability of the tretinoin in liposome complex was supposed to be related to its higher incorporation value due to the linear structure of the molecule.

Keywords: Tretinoin; 13-*cis* Retinoic acid; 9-*cis* Retinoic acid; Photoisomerization; Liposomes; Spectrophotometry

1. Introduction

Retinoids are involved in several biological processes, particularly vision, cell proliferation and differentiation. Particularly, three geometric isomers of the retinoic acid were demonstrated to be the main biologically-active forms in human tissues: all-*trans* retinoic acid or Tretinoin (RA), 13-*cis* retinoic acid or Isotretinoin (13RA) and 9-*cis* retinoic acid (9RA) or Alitretinoin. Particularly, 9RA is better than other isomers as both inducing morphological differentiation and inhibiting proliferation (Lovat et al., 1997).

In the pharmaceutical field, both RA and 13RA are widely used in the treatment of acne vulgaris

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and other disorders of keratinisation (Peck, 1984; Peinni and Vigolti, 1991; Layton and Cunlife, 1992). Nevertheless, researches with retinoids and clinical observations in humans sometimes give apparently contradictory results. On the one hand they stimulate the proliferation of normal epidermal cells, but on the other hand they may induce formation of psoriatic cells and carcinogenesis (Bollag, 1985). All retinoids are shown to be potential teratogens in humans and animals and, in particular for 13RA, teratogenicity is well documented (Hixson et al., 1979; Kochar et al., 1984).

With a feature common to all compounds of the retinoids class, retinoic acids undergo degradation when exposed to light (Lucero et al., 1994; Brisaert et al., 1995; Brisaert and Plaizier-Vercammen, 2000), so generating different isomers of which 9RA is the most important one (Curley and Fowble, 1988; Motto et al., 1989; Cahnmann, 1995). Retinoic acids effect in development and cells differentiation is mediated by nuclear retinoid receptors RARs (RA receptors) and RXRs (9-*cis* receptors). 9-*cis* retinoic acid seems particularly active, directly activating and repressing the transcription of target genes by binding to specific DNA sequences (Mangelsdorf et al., 1994). Therefore, the development of novel formulations characterized by high photo-protection towards the studied retinoic acids seems to be important, as well an efficient investigation about the photodegradation processes occurring in the retinoic acids in the studied formulations.

Drugs photostability represents a significant problem in pharmaceutical research, several articles and reviews dealing with different aspects of such studies (de Villiers et al., 1992; Beijersbergen van Henegouwen, 1997; Albini and Fasani, 1998; Tønnesen, 2001). Photostability testing for new drugs is included as integral part of stress testing in the ICH Guideline (ICH, 1996) and really is an essential activity of the pharmaceutical industry (Anderson et al., 1991; Merrifield et al., 1996). Formulation and manufacturing process seem to be of decisive importance for drug photostability (Aman and Thoma, 2002) and several approach systems have been proposed in order to enhance stability for a number of drugs (Tønnesen, 2001).

The inclusion of the drugs in the supramolecular systems represents an approach of growing interest to the problem of light-sensitive drugs (Jiménez et al., 1997; Csabai et al., 1998; Biloti et al., 1999; Mielcarek and Daczkowska, 1999; Ragno et al., 2003). Liposome matrix represents one of the most studied photo-protective carriers and successful results have been reported in many studies (Morgan et al., 1995; Bisby et al., 2000; Brisaert et al., 2001). The interest in the development of drug delivery systems based on liposomes is due also to their efficiency as vehicles for a wide number of hydro- and lipo-soluble agents. Really, liposomes are starting to be widely applied as an excellent novel formulation for drugs in topical treatment of diseases, especially in dermatology. Thus, they could have the potential to enhance the percutaneous penetration of the retinoic acids incorporated in them.

Photostability studies for RA formulations have been performed on lotion (Brisaert and Plaizier-Vercammen, 2000), pharmaceuticals (Wang, 2000) and cosmetics (Ragno et al., 1996; Wang, 2000). The inclusion of RA in liposomes was reported to protect the drug against photodegradation (Thoma and Joachan, 1992), as well as a deep characterization of RA-liposome (Brisaert et al., 2001) and niosome complexes (Manconi et al., 2002) has been described. No literature was found about the inclusion of 13RA in liposomes.

The present paper describes the increased photostability of tretinoin and isotretinoin when incorporated in liposomes. Drug-containing matrixes were subjected to stressed degradation studies, either in solution or in liposomes, by using a Xenon lamp as light source. Furthermore, the photo-isomerization of both drugs in liposomes and ethanol was investigated and the degradation kinetics curves were calculated. Analytical monitoring of the retinoic acids was performed by UV spectrophotometry and gaschromatography with mass detection.

2. Materials and methods

2.1. Apparatus

Absorption spectra were registered on the wavelength range of 190–500 nm in a 10 mm quartz cell on a Perkin-Elmer Lambda 40P Spectrophotometer at the following conditions: scan rate 1 nm s^{-1}; time response 1 s; spectral band 1 nm. The software UV Winlab 2.79.01 (Perkin-Elmer) was used for spectral acquisition and elaboration.

Photodegradation processes were performed in a light testing cabinet Suntest CPS+ (Heraeus, Milan, Italy), equipped with a Xenon lamp, according to the ICH Guideline for photostability testing (ICH, 1996). The apparatus was set up with an electronic device for both irradiation and temperature controlling inside the box. The system was able to closely simulate sunlight and appropriately select spectral regions by the interposition of filters. In the present study, samples were irradiated in a λ range between 300 and 800 nm, by means of a glass filter, according to the ID65 standard of ICH rules; irradiance power was set to 250 W/m^2 corresponding to a light dose of 21 $kJ\,min^{-1}\,m^{-2}$, at the constant temperature of 25 °C.

Gaschromatographic analysis was performed by using a gaschromatograph Agilent 6890N with a Mass Selective Detector Agilent 5973. The GC conditions were as follows: column, HP capillary (30 m × 0.25 mm I.D.), 250 nm film thickness; injection port temperature, 250 °C, carrier gas, helium; flow-rate, 0.3–0.6 mL/min; column temperature programmed from 60–280 °C at 13 °C/min, initial time 3 min, final time 54 min.

2.2. Laboratory precautions

To minimize drugs photodegradation, all handling of retinoic acids was carried out under red lamp (60 W) and whenever possible amberized glassware was used.

2.3. Chemicals

All-*trans* retinoic acid, 13-*cis* retinoic acid, 9-c*is* retinoic acid with a 98% purity grade, and Pipes [piperazine-*N*,*N*'-*bis*(2-ethanesulfonic acid) sodium salt] were purchased from Sigma-Aldrich (Germany). Turkey Egg Yolk Phospholipids (60% of phosphatidyl choline), (Fluka, Switzerland); Sephadex G-75 (Pharmacia, Sweden). All solvents and other reagents were of the highest purity that was commercially available.

2.4. Standard solutions of retinoic acids

Stock solutions in ethanol were prepared with a concentration of 1.40 $mg\,mL^{-1}$ for RA and 13RA and 0.70 $mg\,mL^{-1}$ for 9RA. Working standards, to obtain concentrations within a range 5.0–40.0 $\mu g\,mL^{-1}$ for RA and 13RA and 2.5–22.5 $\mu g\,mL^{-1}$ for 9RA, were prepared by appropriate dilution with ethanol of the stock solutions and used to set up the calibration curves.

2.5. Preparation of RA- and 13RA-liposomes

One gram of egg yolk phospholipids (60% of phosphatidyl choline) was added to 10 mL of water and unilamellar liposomes were prepared by using a Branson Sonifier B-15 in ice-bath and under a stream of nitrogen for 1–2 h (30 s sonication, 30 s intermission) until a clear solution was obtained. The entrapment of the retinoic acids was also carried out by sonication procedure. Thus, 6.20 mg of each retinoic acid, added to 1.5 mL of liposomes, were sonicated for 30 min under the same experimental conditions described above. As control, drug-free liposomes were prepared sonicating the liposomal suspension for an additional 30 min. To remove the unentrapped retinoic acids, the drug-liposomal suspension (1 mL) was chromatographed by gel-filtration through a column (Sephadex G-75, 0.75 cm × 30 cm) pre-equilibrated with a buffer of 50 mM NaCl, 10 mM Pipes and pH 7.0. The same NaCl/Pipes buffer was used as an eluent. The eluted liposomes (about 2 mL of slightly turbid suspension) were subjected to the controlled photodegradation process at increasing times. Analytical samples for UV analysis were prepared by diluting 100 μL liposomal suspension in 10 mL ethanol.

2.6. Characterization of the drug-liposome suspensions

A microscopical imagining analysis technique for the determination of RA- and 13RA-liposomes size distribution was applied. The particle size distribution was determined with a computer-controlled Olympus BH-2 microscope. In all determinations about 10,000 liposomes were checked, showing a spherical shape with 50–100 nm of inner diameter.

The drug inclusion efficiency in liposomes was determined spectrophotometrically. For this aim, the liposomal suspensions were 1:1 diluted with distilled water and ultracentrifugated at 150,000 × *g* (4 °C) for 90 min in a Centrikon T-1180 centrifuge (Kontron Instruments, Milano, Italy). The supernatant was removed and diluted 1:50 with ethanol for the determination of unentrapped drugs (UV absorbance at 349

and 346 nm for RA and 13RA, respectively). The liposomal pellet was suspended in 10 mL of 5% Triton X-100 and sonicated for 20 min; this solution was further 1:10 diluted with the same Triton X-100 solution for the determination of enclosed drugs. The recovery of entrapped drugs into liposomes was found to be $59.4 \pm 2.3\%$ and $26.7 \pm 3.1\%$ ($n = 3$) for RA and 13RA, respectively, of the amount initially added to the samples.

2.7. Photodegradation of retinoic acids in ethanol solution

An ethanol solution of 35.0 $\mu g\,mL^{-1}$ RA, in a 1 cm quartz cuvette perfectly stoppered, was irradiated with the Xenon lamp, recording UV spectra just after the preparation ($t = 0$) and at the following times: 0.5–10–30–60–80–120–140–170–200–240 min. The radiant power was adjusted to 250 W/m^2, which is the lower value in the instrumental scale, and the cabinet temperature to 25 °C. These gentle experimental conditions were set because of the high sensitivity of the drug to light, allowing so to obtain more accurate control of the photodegradation process.

Analogously, an ethanol solution of 30.2 $\mu g\,mL^{-1}$ 13RA was exposed to light under the same experimental conditions described above for RA, and recording the spectra at the same irradiation times.

2.8. Photodegradation of retinoic acids in liposomes

The liposomal suspension was prepared from egg yolk phospholipids (60% phosphatidyl choline), according to a described procedure (Genchi et al., 1999; Ragno et al., 2003). Retinoic acids inclusion in liposomes was promoted by prolonged sonication, in order to get homogeneous liposomal family with a small inner volume (∼50–100 nm diameter) and characterized by translucent aspect. The separation of the drug-enclosed vesicles from the unentrapped drug was performed by gel-filtration on Sephadex G-75. Spectrophotometric measurement was performed by appropriately diluting the liposomal complex with ethanol. The amount of drug entrapped into the complex was calculated to vary in a concentration range of 2.98–3.18 and 1.45–1.54 mg mL^{-1} for RA and 13RA, respectively.

3. Results

3.1. Photostability of retinoic acids in ethanol solution

The spectral curves recorded on the 35.0 $\mu g\,mL^{-1}$ RA ethanol solution at the various times of light exposure are shown in Fig. 1. The light exposure caused a sharp degradation occurring in the first seconds of irradiation with a contemporary shift of the maximum peak from 349 to 346 nm. A slower degradation was observed later on with the minimization of the 346 nm maximum peak with a 50% absorbance lowering, compared to the initial value after a light dose of about 800 kJ/m^2.

Fig. 2 shows the sequence of the obtained spectral curves recorded on the 13RA ethanol solution exposed to light. 13RA also undergoes a first-fast degradation in the early seconds of irradiance, with a simultaneous shift of the maximum peak from 346 to 340 nm. After further light exposure, this signal goes on shifting to lower wavelengths, with a minimization of its absorbance up to a 50% reduction, compared to the initial value after a light dose of about 650 kJ/m^2.

Such results point out the very high sensitivity to light of RA and 13RA, suggesting the importance of the development of new pharmaceutical formulations able to minimize photodegradation. Recent studies have demonstrated the positive action of the liposome matrix as carrier systems for a number of photosensitive drugs. The first goal of the present study was to investigate the photostabilization of the studied retinoic acids when enclosed in liposomes through data obtained under conditions of accelerated irradiation. Consequently, an interpretation of the photodegradation process of both drugs was tried as a complementary aim of the study.

3.2. Photostability of retinoic acids in liposomes

The photodegradation studies were carried out by spectrophotometric measurements performed on the drug-liposome complexes just before exposure and at increasing times (0.5–5–10–30–60–80–120–140–170–200–270–390–540 min) by diluting the samples with ethanol (0.1 to 10 mL) every time. Fig. 3 shows the spectral curves of the RA-liposome complex obtained after the sequential exposure times. As can be

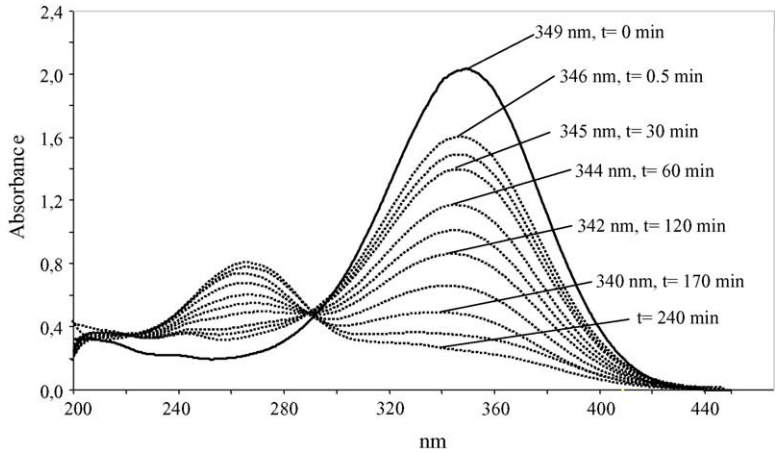

Fig. 1. UV spectra of a 35.0 μg mL^{-1} RA in ethanol solution after different times of light exposure.

seen, the drug entrapment into liposomes strongly reduced the photodegradation process if compared with that within the ethanol solution. Furthermore, no shift of the maximum peak at 349 nm was observed. This may be considered an interesting result, as demonstrates no direct isomerization of RA to 13- or 9-*cis* isomers.

On the other hand, the photostability of the 13RA-liposome complex improved when compared with that recorded in the ethanol solution, but in a lower measure when compared with the RA-liposome complex. Besides, the position of the maximum peak at 346 nm kept constant for just 10 min of light exposure, after that its shift to lower wavelengths could be observed.

Since several species of liposomes would show light sensitivity (Morgan et al., 1995; Bisby et al., 2000), a parallel analytical control on the matrix photostability was also performed. For this aim, three samples of liposomes were irradiated under the same experimental conditions adopted for the drugs–liposomes complex. No appreciable changes between the spectra recorded before and after light exposure were detected, clearly showing that any photodegradation occurring during experimental time was negligible.

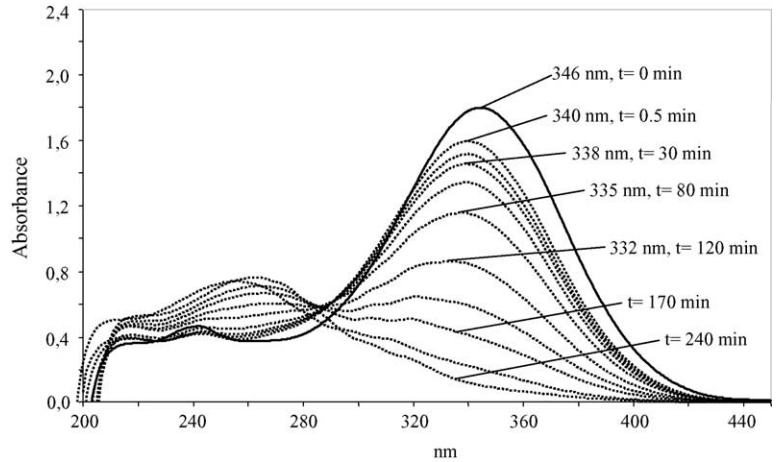

Fig. 2. UV spectra of a 30.2 μg mL^{-1} 13RA in ethanol solution after different times of light exposure.

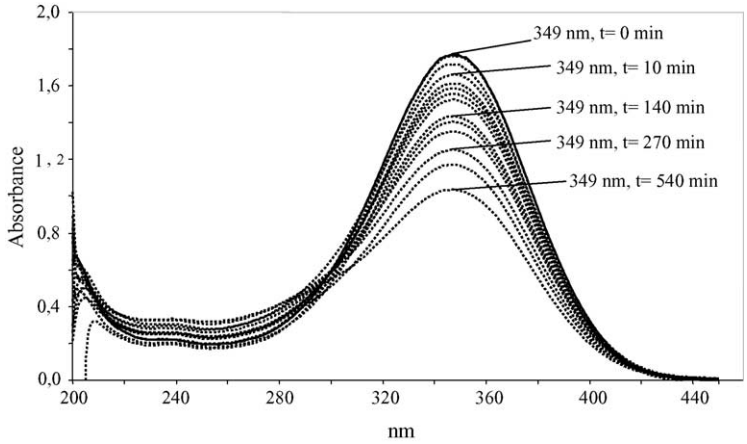

Fig. 3. UV spectra of a 2.40 μg mL^{-1} RA in liposomes after different times of light exposure.

To assure the complete release of the retinoic acids from liposomes, 100 μL of each drug-liposome complex were diluted to 20 mL with ethanol, and irradiated under the same conditions described above. The photodegradation kinetics data resulted practically overlapping those from ethanolic solutions degradation, therefore confirming a complete release of the drugs from liposome formulation when diluted with a large amount of solvent.

4. Discussion

4.1. UV spectra of retinoic acids

Fig. 4 shows the UV spectra of RA, 13RA and 9RA in ethanol solutions. As evident, all the products are characterized by the presence of one imposing-absorbance maximum at 349, 346 and 340 nm for RA, 13RA and 9RA, respectively. 9RA also presents high absorbance below 320 nm. Calibration graphs were obtained by applying least squares regression analysis to the absorbance amplitudes at the single-maximum peaks against the increasing concentrations of pure retinoic acids. The calibration curves parameters are summarized in Table 1.

4.2. Study of retinoic acids photodegradation process

The exposure to light of RA in ethanol solution caused a very rapid degradation of the compound with the exhibition of a spectral curve, practically superimposable with the 13-*cis* isomer spectrum, characterized by a shift of the maximum peak from 349 to 346 nm, and the appearance of a second small peak at 240 nm. This proved the complete isomerization of the drug just after a few seconds of exposure as reported by Cahnmann (1995). The further irradiation caused the degradation of the just formed 13RA to give spectral curves with a gradual shifting of the main peak up to 340 nm, which is the typical peak of the 9-*cis* isomer. Effectively, several authors report the formation of this isomer from tretinoin (Cahnmann, 1995; Simmons et al., 1997; Brisaert and Plaizier-Vercammen, 2000). A mixture of several isomers can be then supposed to form by ulterior irradiation (Bempong et al., 1995; Dimitrova et al., 1996). Analogously, when 13-*cis* isomer in ethanol solution was exposed to light, a quick and complete transformation of the spectral curve was observed with the shift of the main maximum peak from 346 to 340 nm, demonstrating in this case a rapid isomerization to the 9-*cis* isomer. As observed in RA degradation, further irradiation caused the minimization of this peak with its simultaneous shift to lower wavelengths, probably due to a complex mixture of several retinoic acid isomers.

The MS spectral analysis, performed on the samples before irradiation and just after few seconds of light exposure, confirmed the isomeric structure of the starting products and their photoproducts giving the identical mass spectrum with a molecular peak at 300.5 (*m*/*z*) and

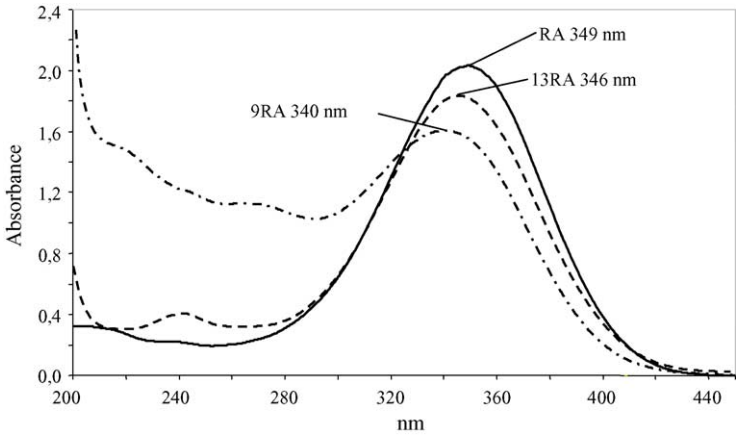

Fig. 4. Absorbance spectra in ethanol of 13.25 μg mL^{-1} RA, 13.02 μg mL^{-1} 13RA, 13.25 μg mL^{-1} 9RA.

Table 1
Calibration graphs for RA, 13RA and 9RA assay by spectrophotometry

Analite	Signal λ (nm)	Slope	Intercept	Correlation coefficient	Linearity range (μg mL^{-1})
RA	349	0.1493	0.0055	0.9990	5.0–40.0
13RA	346	0.1324	0.0012	0.9977	5.0–40.0
9RA	340	0.1228	0.0056	0.9985	2.5–22.5

a signal at 261 (*m*/*z*), due to the typical fragmentation of the acid residue.

4.3. Kinetic parameters determination

The two studied drugs and their main photoproduct 9RA present very similar spectral curves characterized by a single-maximum peak at three specific wavelengths, 349, 346 and 340 nm for RA, 13RA and 9RA, respectively, with a similar molar absorptivity. The photochemical reaction in ethanol solution was demonstrated to cause a very rapid isomerization of both RA and 13RA followed by a further slower degradation consisting in the minimization of the above mentioned absorbance peaks. Therefore, the absorbance values of these maxima were used to evaluate the kinetics of the photodegradation processes. Both RA and 13RA showed to follow a first order kinetics and a good linearity was obtained by plotting the logarithm of absorbances as a function of time, as in ethanol and in liposomes, in agreement with the following equation:

$$\log(\%A) = -kt + 2$$

where %*A* was percent residual absorbance, *k* was the photodegradation rate constant, *t* was the time (min), and 2 was the logarithm of initial percent absorbance (100%).

The degradation was evaluated on the basis of kinetic photodegradation constant *k* and half-life time ($t_{0.5}$), with respect to the initial percent absorbance. Retinoic acids degradation curves are plotted in Fig. 5, while Table 2 summarizes the mentioned degradation kinetic parameters based on data from three replicate analyses for each sample.

Table 2
Rate constants[a] of photodegradation for RA and 13RA in ethanol solution and liposomal complexes

Retinoic acid	Matrix	*k*	$t_{0.5}$	r^2
RA	Ethanol solution	4.23×10^{-3}	58.4	0.9962
	Liposomes	5.11×10^{-4}	560.4	0.9991
13RA	Ethanol solution	5.36×10^{-3}	47.7	0.9846
	Liposomes	3.10×10^{-3}	101.0	0.9910

[a] *t* is expressed in minutes.

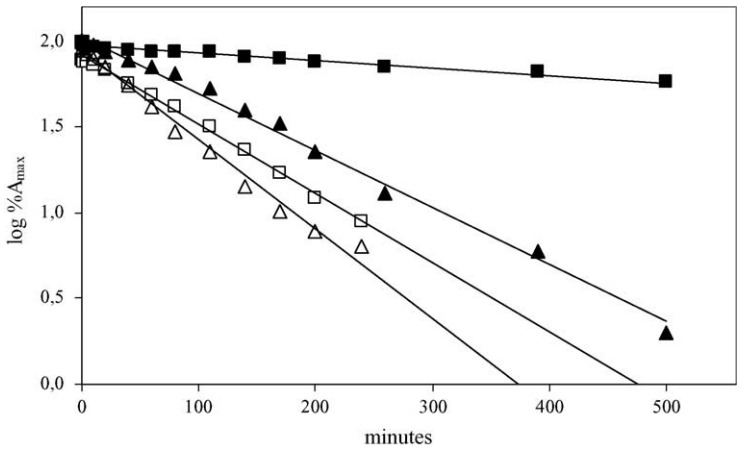

Fig. 5. Photodegradation plots in ethanol of RA (■) and 13RA (▲), and liposomes complexes containing RA (□) and 13RA (△). All values are means of three replicated experiments.

As it is evident, the RA inclusion in liposomes permitted a good photostability of the drug. Whereas in ethanol RA degraded almost completely after about 5200 kJ/m^2, corresponding to 360 min under a light power of 250 W/m^2, in the liposomal matrix still 65% of residual RA concentration could be measured. On the contrary, the inclusion of 13RA in liposomes did not give analogous satisfactory results, showing an increased stability if compared with the ethanol solution but a higher degradation rate than in the RA-liposomes complex.

Results obtained from this photostability study lead us to suppose that the better photoprotection of RA-liposome complex is the consequence of the higher inclusion value of this drug in liposome matrix, calculated as $59.4 \pm 2.3\%$, in comparison with the lower value of $26.7 \pm 3.1\%$ for 13RA-liposome complex. The relationships between the photodegradation rate versus the drug concentration has been well reported (Tan et al., 1993; Brisaert and Plaizer-Vercammen, 2000). The higher incorporation of RA is probably favoured by the linear structure of this molecule, whereas the presence of a *cis*-link in 13RA makes more difficult its inclusion.

5. Conclusions

Retinoic acids photodegradation represents a very complex subject involving several degradation processes, of which isomerization is the most important. Tretinoin and isotretinoin undergo in ethanol a stepwise photodegradation with a first very rapid isomerization to 13-*cis* and 9-*cis* isomers, respectively, both followed by a slower degradation to give several isomers. Inclusion of the drugs in liposome matrix was proved to show an improved stability to light. Furthermore, the fast isomerization realized in ethanol from both compounds is in liposomes avoided. Liposomes were demonstrated to decrease tretinoin half-life to about twelve times in comparison with an ethanol solution, under a light power of 250 W/m^2. The inclusion of isotretinoin in liposomes showed a worse performance, probably due to a low inclusion due to the non-linear molecular structure. The liposome systems described in the present paper should provide a valuable tool for the development of new pharmaceutical formulations of retinoic acids, capable of improving their photostability.

Acknowledgments

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