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Research paper

Skin protection against ultraviolet induced free radicals with ascorbyl palmitate in microemulsions

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Abstract

UV irradiation induces free radical formation in the skin. UV filters and antioxidants can be used for protection. In the present work, the amphiphilic antioxidant ascorbyl palmitate has been investigated and its effectiveness against free radical formation in porcine skin determined with electron paramagnetic resonance (EPR) spectroscopy with a spin trapping technique. 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) was used as spin trap. In this study, three different radicals were identified in UV irradiated porcine ear skin: two originated from sulphur centred radicals ($SO_3^{\cdot}$), while the third was the carbon-centred acyl (C═O$^{\cdot}$) radical. Ascorbyl palmitate applied on the skin decreased the level of formation of free radicals. Its effectiveness depended significantly on the carrier system – the type of microemulsion and its concentration, while the time of application had no influence on its effectiveness. Oil in water microemulsions delivered ascorbyl palmitate to the skin significantly better than water in oil microemulsions. In both types of microemulsions, the effectiveness increases at higher concentrations of ascorbyl palmitate.

Keywords: Skin; Free radicals; Ascorbyl palmitate; Microemulsions; Ultraviolet irradiation; Spin trapping

1. Introduction

Exposure of the body surface to ultraviolet (UV) irradiation produces free radicals in the skin, leading to premature aging and cancer. The skin possesses a wide range of coupled antioxidant defence systems to protect itself from damage by UV induced reactive oxygen species (ROS). However, the capacity of these systems is limited, and they can be overwhelmed by excessive exposure to irradiation. The endogenous defence systems include various low molecular weight antioxidants (ascorbate, glutathione, α-tocopherol, ubiquinol and β-carotene) and antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and thioredoxin reductase, etc.). After prolonged or excessive exposure to UV irradiation, these skin antioxidants are depleted [1], allowing free radicals to have damaging effects. Supporting the cutaneous antioxidant defence systems with exogenous antioxidants could thus prevent radical mediated damage in the skin. Different free radicals formed in UV irradiated skin include: superoxide ($O_2^{\cdot -}$), hydroxyl (HO$^{\cdot}$), peroxyl (HOO$^{\cdot}$), alkoxyl (RO$^{\cdot}$) and alkyl (R$^{\cdot}$), sulphur and some carbon centred radicals [2]. These are short-lived radicals, and can be measured by electron paramagnetic resonance (EPR) spectroscopy indirectly with a spin trapping method. The basic principle of this method is to introduce into the system a diamagnetic compound (spin trap), which reacts with the short-lived radical to form a more stable radical (spin adduct), which is detectable by EPR [3].

L-Ascorbic acid (vitamin C) is one of the important skin antioxidants. Therefore, it has also been used in cosmetic and dermatological products since it has many favourable effects on the skin. As a reducing agent, ascorbic acid can scavenge and destroy aggressive oxidising agents and radicals [4–6]. It also supports the action of α-tocopherol by its regeneration from α-tocopheryl radical. However, ascorbate can also act as pro-oxidant. This paradoxical behaviour is a consequence of its excellent reducing efficiency. The pro-oxidant activity is dependent on the availability of free metal ions. As a reducing agent, it is able

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to reduce metal ions such as Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{1+}, respectively, which can act in further reactions as oxidants. At very low concentration of metal ions and high concentrations of ascorbate, it acts as an antioxidant, but if the concentration of metals increases or ascorbate concentration decreases, then ascorbate could be dangerous since it could act as pro-oxidant [7].

The use of ascorbic acid is limited by its low chemical stability in heterogeneous systems. Ascorbyl palmitate, an amphiphilic ascorbic acid derivative, has better stability and ability to penetrate the skin than ascorbic acid [4,8]. So far only a few studies have been published on the antioxidative properties of ascorbyl palmitate applied on the skin. Its scavenging of free radicals has been reported only in in vitro model systems [9], and not in the skin.

The aim of our study was to evaluate the free radical scavenging effect of dermally applied ascorbyl palmitate on radical formation in UV irradiated porcine skin, together with the influence of carrier system and concentration of ascorbyl palmitate on its effectiveness. For this purpose, spin traps were used to capture the short-lived radicals that appear during UV irradiation of the skin. The decreased radical concentration after protection with ascorbyl palmitate formulations is a measure of its effectiveness. Microemulsions have been chosen as carrier systems for ascorbyl palmitate because of their thermodynamic stability and the simple technology of preparation. These systems have also high solubilising power, so that large amounts of poorly soluble compounds can be included [10]. After application to the skin, components of the microemulsions interact with the intercellular lipids of the skin, changing the intercellular space and facilitating drug transport [11,12].

2. Materials and methods

2.1. Materials

5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) was prepared according to the method described in Ref. [13]. Ascorbyl palmitate (AP) was provided by Fluka (Buchs, Switzerland) (Fig. 1). The microemulsions consisted of the caprylic/capric triglyceride, Mygliol 812® (Hüls, Marl, Germany), as oil phase; the PEG-8 caprylic/capric glycerides, Labrasol® (Gattefosse, Saint-Priest Cedex, France), as surfactant; the polyglyceryl-6-dioleate, Plurol oleique® (Gattefosse, Saint-Priest Cedex, France), as cosurfactant, and water as hydrophilic phase. This water was prepared by distillation of reverse osmosis water. Colloidal silica Aerosil 200® (Degussa AG, Rheinfelden, Germany) and Xanthan gum (Fluka, Buchs, Switzerland) were used as thickening agents.

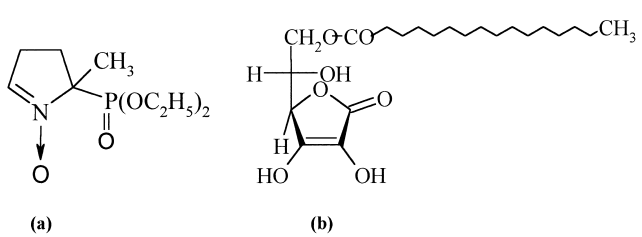

Fig. 1. Chemical structures of (a) DEPMPO and (b) ascorbyl palmitate.

Table 1
The composition of w/o and o/w microemulsions (w/w %)

Component	w/o	o/w
Mygliol 812®	23.8	7.4
Labrasol®	45.6	37.8
Plurol oleique®	11.4	9.5
Purified water	15.2	44.8
Aerosil 200®	4.0	–
Xanthan gum	–	0.5

2.2. Preparation of microemulsions with ascorbyl palmitate

AP was incorporated into the oil phase of microemulsions at concentrations of 0.5, 1.0, 2.5 and 5.0% w/w concentrations (0.012, 0.024, 0.060 and 0.120 mmol/g, respectively). It was dissolved in a mixture of surfactant, cosurfactant and oil, and water then added. Microemulsions were formed spontaneously after gentle hand mixing. The composition of the oil/water (o/w) and water/oil (w/o) microemulsion is shown in Table 1. Unfortunately, these microemulsions were quickly removed from the skin since they are insufficiently viscous. To increase their viscosity, thickening agents were added. Because the two types of microemulsion have different external phases, two thickening agents were used, Aerosil 200 in 4% w/w concentration for w/o and Xanthan gum in 0.5% w/w concentration for o/w microemulsions. The proportions of the microemulsion components were kept constant during the addition of active ingredient and thickening agent.

2.3. Application of ascorbyl palmitate microemulsions to the skin

Skin samples were cut from porcine ear obtained from a slaughter house and used not later than 24 h after killing of the animal. Before cutting, the ear sample was rinsed in water and dried. Different concentrations of AP in microemulsions were applied to full thickness skin before irradiation. For this purpose, an area of 5 cm^2 of skin was treated with 0.5 g of AP preparation using a plastic ring for different times. Excess preparation was then removed, the skin washed with water and dried with a cotton towel. In control experiment, microemulsions without active ingredient were used.

2.4. UV irradiation of skin

For EPR measurements, which can be performed only in the tissue cell (10 mm × 5 mm × 0.5 mm) skin slices with thickness of less than 200 μm can be measured. Therefore, from the full thickness untreated skin or skin treated with AP, only the surface layers; epidermis with dermis were taken. They were cut into 40 mm^2 pieces and placed on filter paper soaked with water to prevent drying. Five microliters of 0.4 M DEPMPO was added to the surface of the skin slice and irradiated with an UV lamp (Spectronics, New York, USA) at 365 nm (350 $\mu W/cm^2$) for 15, 30 or 45 min.

2.5. EPR measurement

The tissue slices were placed in the tissue cell and EPR spectra were measured at room temperature using a Bruker ESP 300 X-band spectrometer operated at 9.96 GHz with 100 kHz modulation frequency. The EPR spectrometer settings were: microwave power, 10 mW, modulation amplitude, 1.02 G, and sweep, 3320–3460 G. Because DEPMPO spin adducts disintegrate with time [14], spectra were measured as soon as possible but always at the same time after the end of irradiation. All EPR measurements were started 2 min after the end of irradiation. The amplitude of the first line of EPR spectra of DEPMPO spin adducts and its decrease with time was measured in untreated skin and in skin treated with AP. As the EPR spectra line shape does not change with time after irradiation, the amplitude of the first line can be used as a good estimate of the spectral intensity, which provides a measure of the quantity of radicals formed.

2.6. In vitro release of ascorbyl palmitate

The in vitro AP release experiments through cellulose acetate membrane were performed with a Franz diffusion cell (Hanson research, Chatsworth, USA) at 32°C (1.18 cm^2 surface area, 0.4 g vehicle on the donor side, 7 ml acceptor medium). The acceptor medium was 0.8% w/w solution of sodium lauryl sulphate. Samples were taken at intervals for 4 h following application of microemulsions with 2.5% w/w AP, and assayed by UV spectrometry at 246 nm (UV spectrophotometer 8453, Hewlet Packard, Waldbronn, Germany).

2.7. Statistical evaluation

Data are reported as arithmetic means ± standard error. Comparisons were performed by Student's *t*-test. Significance was tested at the 0.05 level of probability.

3. Results

3.1. Determination of free radicals in the skin after UV irradiation

Typical EPR spectra of DEPMPO adducts in skin obtained after different times of irradiation are shown in Fig. 2. The signal intensity of DEPMPO adducts, which is proportional to the number of captured radicals, increased with the increasing time of irradiation up to 30 min and decreased after that. No signals were detected before irradiation (0 min).

EPR spectra are superimposition of spectra of different radical adducts. By computer simulation of the experimental spectra using the Win Sim program (available at: http://epr.niehs.nih.gov/pest.html), we have found that at least three different types of radicals were formed and trapped in the irradiated skin. The calculated hyperfine splitting constants (*A*) of different DEPMPO radical adducts, which give the best fits to the experimental spectra are presented in Table 2. By comparing these values with the values from the literature [13–15], it can be concluded that species 1 and 2 originate from sulphur centred radicals ($SO_3^{\cdot}$), while the species 3 corresponds to carbon-centred acyl (C═O$^{\cdot}$) radical. From the simulation, it is evident that the proportion of species 3 relative to the total quantity of radicals increased with the time of irradiation. Sulphur-centred radicals probably arise from keratin, chondroitin sulphate, glutathione and other skin components with high sulphur content, while carbon-centred radicals originate from skin lipids.

3.2. Evaluation of the free radical scavenging effectiveness of dermally applied ascorbyl palmitate

The free radical scavenger effectiveness of AP was assessed by determining the amount of radicals formed in presence of AP with respect to the amount obtained in control, without AP. After 30 min of irradiation in control

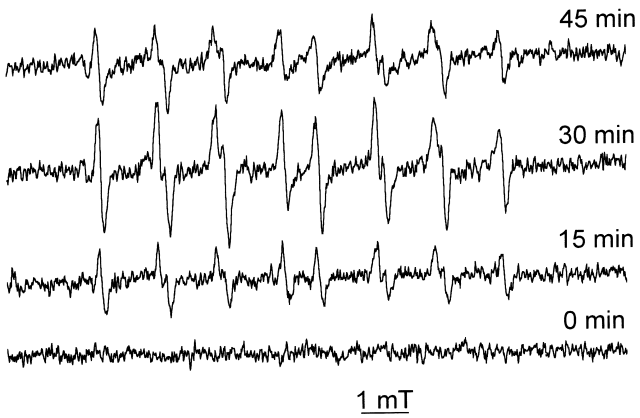

Fig. 2. EPR spectra of DEPMPO adducts in a porcine ear skin after 0, 15, 30 and 45 min of UV irradiation.

Table 2
Hyperfine splitting constants of the DEPMPO-adducts obtained after UV irradiation of in porcine ear skin determined by computer simulation of EPR spectra

Free radicals	A_N (G)	$A_{H\beta}$ (G)	$A_{H\gamma}$ (G)	A_P (G)
Species 1	13.59	14.66	0.37	49.26
Species 2	13.20	14.93	0.28	49.18
Species 3	14.33	17.40		51.46

experiment, the intensity of the EPR spectra was maximal and high enough for quantitative evaluation. After longer irradiation times (45 min), a decrease in the EPR spectra intensity was observed (Fig. 2). One of the reasons for the decrease could be the reduction of DEPMPO adducts to EPR non-visible hydroxylamine complexes by the reducing systems (tioredoxin reductase, tiol dependent processes, ascorbate etc. [16,17]) in the skin. It is possible that after longer irradiation reduction becomes faster as the free radical formation. At the same time, the skin starts to dry out, which could additionally change the EPR spectra intensity.

The difference between the untreated skin and skin treated with 5% w/w AP in thickened w/o microemulsion applied to the skin for 20 min, and irradiated for 30 min is shown in Fig. 3. Computer simulation of the EPR spectra of UV irradiated skin treated with AP showed that the same three types of radicals were trapped with DEPMPO as in untreated skin.

The concentration of radicals in skin treated with AP is lower than that in untreated skin. This can be a consequence of two reactions: (1) AP reacted with radicals formed after UV irradiation before they were trapped by DEPMPO; (2) AP as a strong reducing agent reduced DEPMPO adducts, making them invisible to EPR. These two reactions could occur simultaneously.

The amount of radicals captured by spin trap after application of AP (N_{AP}), which is proportional to the EPR spectra intensity I_{AP} in treated skin, can be described by the by following equation:

$$N_{AP} = N_0 - N_1 - N_2 \quad (1)$$

where N_0 is the amount of radicals captured in non-treated samples, N_1 the amount of radicals, which reacted with AP (were not captured by spin trap) and N_2 the amount of spin adducts, which were reduced by AP.

In order to evaluate the scavenging effectiveness of AP, we have to evaluate the contribution of spin adduct reduction by AP to the decrease of EPR spectra intensity. For this purpose, the rate of EPR spectra intensity decrease was measured in treated and in untreated samples (Fig. 3). If the reduction of the adducts by AP is dominant, the rate of intensity decrease in the skin treated with AP would be faster than in untreated skin, where the rate of decrease is a consequence of reduction of DEPMPO-adducts only by reducing agents in the skin. From Fig. 3, the zero order rate constants for the EPR spectra intensity decrease were calculated. It was 2.39 min^{-1} for untreated skin and 2.40 min^{-1} for skin treated with AP, which means that the possible reduction of DEPMPO adducts by AP is negligible and that the lower signal intensities in skin treated with AP than in untreated skin is the result of the scavenging activity of AP. For this reason, the term N_2 in Eq. (1) could be

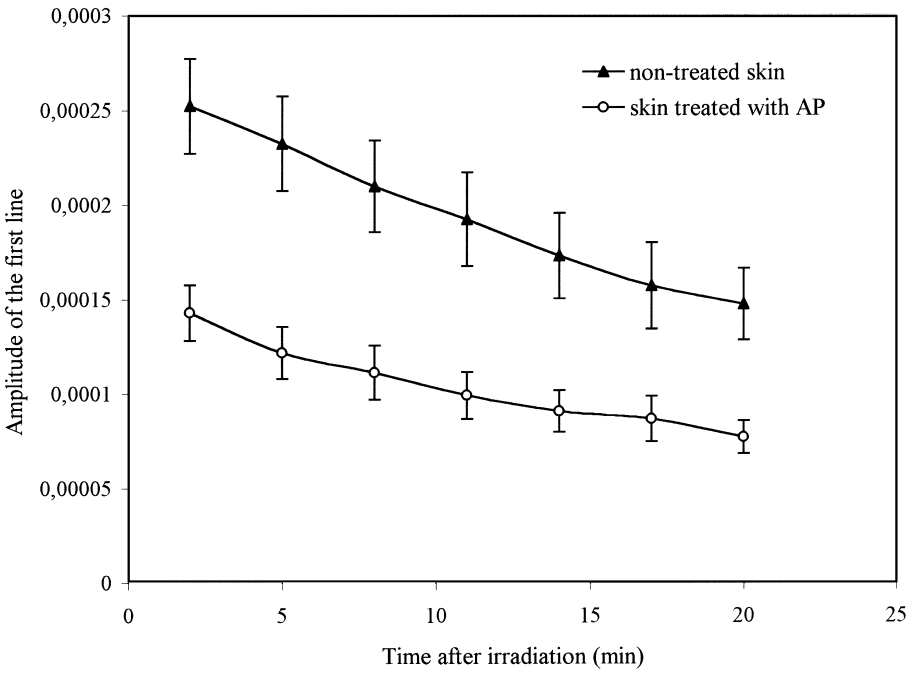

Fig. 3. DEPMPO adducts decrease in porcine ear skin with time after the end of irradiation (mean ± SE, $n = 3$).

neglected. The effectiveness (E) of dermally applied AP, which is defined as the relative ratio between the radicals scavenged by AP (N_1) and total amount of radicals captured in non-treated skin (N_0) was determined from the following equation:

$$E = N_1/N_0 = 1 - N_{AP}/N_0 = 1 - I_{AP}/I_0 \quad (2)$$

where I_{AP} and I_0 are amplitudes of the first line of the EPR spectra of DEPMPO adducts in skin treated with AP and untreated skin, respectively. $E = 1$ would mean that AP scavenged all formed radicals.

3.3. Influence of the time of application on the effectiveness of dermally applied ascorbyl palmitate

AP was applied to the skin at 5% w/w in thickened w/o microemulsion for 5, 10, 20, 30 and 40 min. The results are shown in Fig. 4, where the mean values of the measurements on skin samples obtained from four different animals are presented. The time of application did not significantly influence AP effectiveness. However, it can be observed that after longer application (especially 40 min), standard errors decreased. For further investigation, an application time of 40 min was chosen with the assumption that in this time steady state would be attained in all skin samples.

3.4. Influence of the carrier system and ascorbyl palmitate concentration on its effectiveness on the skin

Results of AP effectiveness against free radical formation in UV irradiated skin in different carrier systems and at different concentrations are presented in Table 3. AP in o/w microemulsions showed significantly greater effectiveness than in w/o microemulsions. At higher concentrations, the effect was more pronounced in both types of microemulsions.

It is important to note the effect observed at low concentration of AP (0.5%) in w/o thickened microemulsion. Out of five skin samples from different pigs, three showed higher free radical concentration in AP treated skin than in untreated skin from the same porcine ear skin samples (Fig. 5). The mean value is therefore negative with high standard error (-0.12 ± 0.17). This result could be related to the possible pro-oxidative action of AP that depends on many factors and not only on AP concentration. One of them is the level of catalytic metals in the skin. These levels are characteristic of individual skin and cannot be generalised. Pro-oxidative effects of ascorbic acid were also observed on lipid model systems by Trommer et al. [18]. They were concentration dependent and transition metal ions were required. Therefore, it is important to bear in mind when formulating AP or ascorbate in carrier systems that very low concentrations of active ingredients in some skins can act as pro-oxidant instead of as an antioxidant.

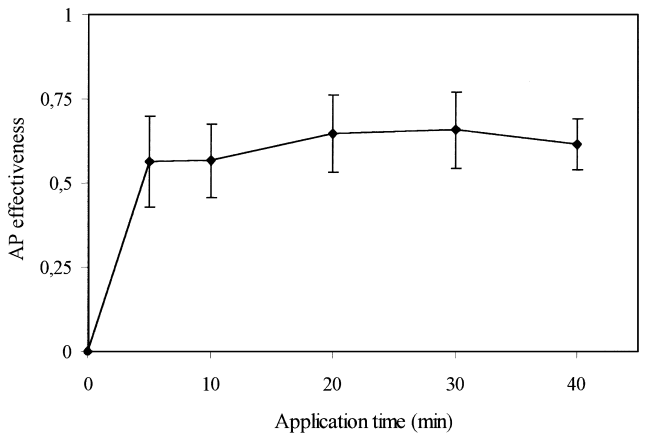

Fig. 4. The scavenging effectiveness of dermally applied 5.0% w/w AP in thickened w/o microemulsion as a function of application time (mean ± SE, $n = 4$).

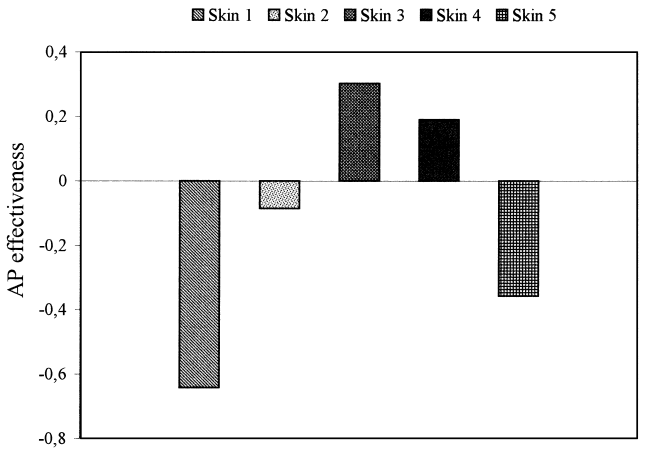

Fig. 5. The effectiveness of 0.5% w/w AP in w/o thickened microemulsion on five skin samples.

3.5. In vitro study of ascorbyl palmitate release from different carriers

The results of AP release from four different microemulsions (o/w, w/o in presence or absence of thickening agent) through cellulose acetate membrane are presented in Fig. 6. AP was released more slowly from both o/w systems than from w/o systems.

4. Discussion

Due to their chemical structure and amphiphilic character, the molecules of AP are orientated in the lipid bilayers with the palmitic residue in the lipophilic phase and the lactone ring in the lipid–water interphase [8,19]. Only the 3-hydroxy group of the lactone ring is responsible for the reaction with free radicals. Therefore, scavenging is possible particularly in hydrophilic compartments of the

Table 3
The influence of two carrier systems on the effectiveness of AP against free radical formation in UV irradiated skin at different concentrations of AP (mean ± SE, $n = 5$, skin of different pigs)

Samples	0.5% w/w	1.0% w/w	2.5% w/w	5.0% w/w
o/w thickened microemulsion	0.54 ± 0.05	0.46 ± 0.09	0.65 ± 0.06	0.68 ± 0.09
w/o thickened microemulsion	−0.12 ± 0.17	0.20 ± 0.09	0.40 ± 0.05	0.50 ± 0.08

skin. This presumption is in accordance with the already reported results on human erythrocytes, where it was found that AP helped to protect the membrane from oxidant damage originating from outside the cell indicating that AP was retained on the exterior of the cell surface [20]. With respect to these properties of AP, in our studies the hydrophilic spin trap was chosen to measure the scavenging effect of AP. Of the hydrophilic spin traps, 2-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone (POBN) and 5,5-dimethylpyrroline-1-oxide (DMPO) have already been used for spin trapping experiments in the skin [3]. In our experiments, DEPMPO was used as a spin trap, as it is reported as one of the most suitable spin traps for different types of radicals like superoxide and peroxyl radical, sulphur radical [14] etc, and also trapping of lipid derived radicals was reported [15]. Additionally, DEPMPO represents an improvement over the widely used DMPO in terms of the higher stability of the spin adducts [14].

After application on the skin, AP scavenged UV induced free radicals as evident from Fig. 3. The effectiveness of AP on the tested skin layers was found to depend on its concentration and the type of microemulsion. As expected, effectiveness was significantly higher at 2.5 and 5.0% (6.0 and 12.0 μmol AP/cm^2 skin) than at 0.5 and 1.0% concentrations of AP (1.2 and 2.4 μmol AP/cm^2 skin) in both types of microemulsions (Table 3). The difference between lower and higher concentrations was more pronounced in the case of w/o microemulsions. AP is highly soluble in the microemulsions used so a large amount of AP can be included in the formulation, which can result in a large concentration gradient from the vehicle to the skin. Therefore, increased drug delivery would be expected. However, no significant differences were found between 2.5 and 5.0% of AP. This indicates that saturation occurs in one of the many processes AP is undergoing before it finally becomes effective in the skin. AP has to be first released from the carrier system to the surface of the skin, the next step is penetration across the stratum corneum into deeper skin layers and finally AP should act as free radical scavenger. Saturation could occur at any of these steps.

The release of AP from the vehicle as well as penetration across the stratum corneum is influenced by interaction of the vehicle with the skin. Both processes are therefore strongly coupled with the applied carrier system [12,21]. To evaluate whether the diffusion of AP from the microemulsion or its penetration across the stratum corneum is more important for its effectiveness, in vitro AP release studies through an artificial membrane were performed. W/o

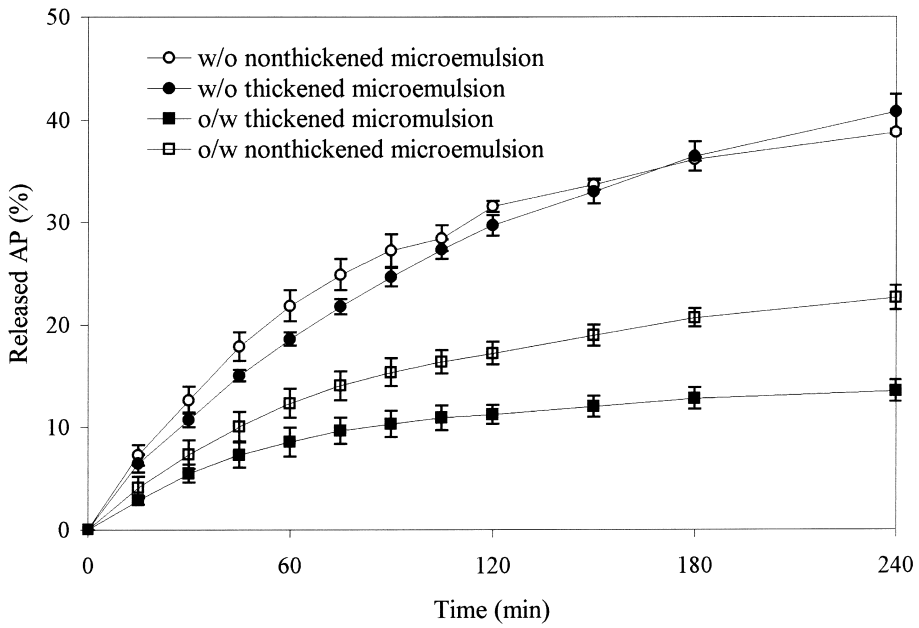

Fig. 6. The amount of AP released through cellulose acetate membrane from microemulsions with 2.5% w/w AP.

microemulsions were found to release more AP than o/w systems (Fig. 6), in keeping with the fact that AP was incorporated in the internal phase of o/w and in the external phase of w/o microemulsions. On the other hand, when we compared the effectiveness of AP applied in o/w and w/o thickened microemulsion, we found that o/w thickened microemulsions delivered AP to the skin significantly better than w/o thickened microemulsion (Table 3). The fact that o/w systems demonstrated better effectiveness on the skin than w/o systems is rather surprising because w/o microemulsions released higher amounts of AP. This indicates that the interaction of AP and components of microemulsion with the skin and its penetration through the stratum corneum is the most important mechanism in the whole process. One of the possible explanations is that AP, from the better releasing w/o system, which was applied to the full thickness skin is distributed to all skin layers but is delivered mostly into the deeper skin layers, cutis and subcutis, which are highly lipophilic and more amenable for AP. Cutis and subcutis were removed for EPR measurements while only epidermis with dermis can be measured as mentioned in Section 2. On the other hand, o/w systems, with more hydrophilic properties and with lower releasing capacity retained AP in stratum corneum and epidermis i.e. in the tested skin layers.

To obtain additional ex vivo data about the influence of the carrier system on AP delivery and effectiveness, permeation experiments with isolated stratum corneum, separated epidermis and full thickness porcine skin were performed. Preliminary results showed that the use of w/o thickened microemusions led to higher steady state fluxes and permeability coefficients of AP through stratum corneum and epidermis than the use of o/w thickened microemulsions. On the other hand, the stratum corneum and epidermis resistance for AP was higher when it was applied in o/w than in w/o thickened microemulsions indicating that w/o systems delivered AP also into deeper skin layers in comparison with o/w systems. This research is approaching the final stage and the results will be published in a separate publication.

It should be stressed that this would be a desired effect of AP formulation as UV irradiation causes damage above all in the skin epidermis and dermis. Therefore supporting of these skin layers with antioxidants is necessary. Another possible explanation for better effectiveness of AP in o/w systems is the hydration of the skin. It is generally accepted that most substances are absorbed through the stratum corneum more rapidly if this tissue is in a hydrated state rather than in its usual dry state. The permeating molecules are pushed across the barrier not only by the trans barrier concentration difference, but also by intrinsic osmotic pressure difference or some other permeant independent transbarrier gradient. Vehicles applied to the skin are capable of altering the hydration of the stratum corneum to some extent and water as an external phase of o/w vehicle increases hydration [22–24].

5. Conclusion

Dermally applied AP in microemulsions scavenged free radicals formed in UV irradiated porcine skin. These were sulphur centred radicals ($SO_3^{\cdot}$) and carbon-centred acyl ($C{=}O^{\cdot}$) radical. The effectiveness of AP was dependent on its concentration and the type of microemulsion. O/w microemulsions delivered AP to the skin significantly better than w/o microemulsions. It is also important to consider the influence of concentration on the effectiveness of AP because AP can act pro-oxidatively instead of antioxidatively if its concentration is too low.

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