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In vitro percutaneous absorption of all-*trans* retinoic acid applied in free form or encapsulated in stratum corneum lipid liposomes

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Abstract

The objective of this study was to design an all-*trans* retinoic acid (RA) topical release system that modifies drug diffusion parameters in the vehicle and the skin in order to reduce systemic absorption and the side-effects associated with topical application of the drug to skin. Three cases of application of hydrogels containing RA either in free form or encapsulated in stratum corneum lipid liposomes (SCLLs) have been considered. For this purpose, we have evaluated the RA in formulations with combinations of Carbopol® Ultrez™ 10 (U10) and hyaluronic acid (HA) for percutaneous absorption. In vitro permeability experiments with [^{3}H]-*t*-RA were carried out using a Franz-type diffusion cell in abdominal rat skin samples. Accumulation of the drug in the surface and skin layers was evaluated by both the tape stripping method and a dissection technique, and subsequently, all the radiolabelled samples were analyzed by liquid scintillation counting. The results show that RA encapsulation not only prolongs drug release but also promotes drug retention by the viable skin. At the same time, interaction between RA and HA has an obstructive effect on diffusion, which contributes to the formation of a reservoir of the latter.

Keywords: Stratum corneum lipid liposomes (SCLLs); All-*trans* retinoic acid (RA); Skin permeation; Skin penetration; Drug reservoir

1. Introduction

Penetration of a topically formulated active compound into the skin involves two steps: the first is release of the drug from its vehicle and the second is the actual penetration of the released drug into the stratum corneum. Both processes depend on the physicochemical properties of the drug, its vehicle and the barrier. In this case, the stratum corneum constitutes the main barrier as well as the rate-limiting step for percutaneous absorption of the drug applied topically (Hosny et al., 1998).

Liposomes constitute an important potential as drug delivery systems, not only for intravenous delivery but also particularly for topical applications. In the latter case, beneficial effects on the skin itself could

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be demonstrated. In addition, liposomes have been shown to have certain advantages in comparison to other galenical formulations. Specifically, the use of liposomes has provided for a higher concentration of drugs in deeper layers of the skin (the epidermis and dermis) and a reduction in percutaneous absorption, which often causes unwanted side-effects (Kim et al., 1997; Kirjavainen et al., 1999a,b).

In recent years, there has been much interest in delivering drugs to and into the different layers of the skin using specific types of liposomes (Barry, 2002). Despite intensive research and the existence of numerous articles on the mechanism of liposome–skin interaction, investigation of the possible advantages of liposomes still constitutes an important objective of studies to improve dermatological formulations.

Many gelled water-soluble bases have recently been formulated to optimize topical drug delivery but no exhaustive rheological studies have been carried out. Nevertheless, knowledge of the rheological and mechanical properties is very important and may lead to the use of this type of parameters and empirical models to optimize topical non-polar drug delivery from dermatological formulations (Fresno et al., 2001).

Ultrez^{TM} 10, a new member of the Carbopols family, can be considered a hybrid between Carbopol® 940 and Carbopol® 934. It has better dispersion properties and a potentially wide range of applicability in the pharmaceutical and cosmetic fields. Hyaluronic acid (HA), a polymer used as another component of formulations, has recently been introduced as a vehicle for topical application of drugs to the skin. Due to its physico-chemical properties, hyaluronan performs numerous biological functions by exercising complex interactions with the matrix components and cells. At the same time, the discovery of certain interactions between HA and a specific group of proteins is evident that the former plays a part in the recognition and regulation of cellular activities. In addition, recent studies have propitiated the use of HA in the development of new pharmaceutical dosage delivery forms (Rivers and McLean, 1997; Brown et al., 1999).

The drug all-*trans* retinoic acid (RA) has demonstrated efficacy in keratinization disorders and in the treatment of other cutaneous lesions, when applied topically. Although the benefits of different therapies are often associated with several undesirable systemic side-effects, RA has a limited risk/benefit ratio, meaning that its retention by the skin – as opposed to systemic absorption – is desirable once it has been applied topically.

The aim of the present study was to evaluate the free RA and RA encapsulated in stratum corneum lipid liposomes (SCLLs) in formulations with combinations of Carbopol® Ultrez^{TM} 10 and hyaluronic acid (U10/HA) in hydroalcoholic gels. At the same time, we have studied [^{3}H]-*t*-RA permeation kinetics and its in vitro distribution in the different cutaneous strata of abdominal rat skin.

2. Materials and methods

2.1. Materials

The all-*trans* retinoic acid (tretinoin, Sigma Grade), 13-*cis* retinoic acid and retinol were furnished by Sigma Chem. Co. (St. Louis, USA). Ceramide type III (purity: approximately 99.0%), palmitic acid (hexadecanoic acid, Sigma Grade), cholesterol (Sigma Grade, purity > 99.0%) and cholesterol-6-sulphate (Sigma Grade) were provided by Sigma Chem. Co. Chloroform, de-ionized Milli-Q® grade water, glacial acetic acid, acetonitrile, ethanol, methanol and triethanolamine were supplied by Scharlau S.A. (Spain). All the solvents used in this study were high performance liquid chromatography (HPLC) grade. The TRIZMA® hydrochloride buffered solution [tris(hydroxymethyl)-aminemethane hydrochloride, purity 99.0%] (pH 7.4) was used as a dispersion medium was provided by Sigma Chem. Co. Carbopol® Ultrez^{TM} 10 from BF Goodrich Chemical Co. (Cleveland, USA) and cross-linked hyaluronic acid from Sigma Chem. Co.

The all-*trans* retinoic acid labelled 20-metil-^{3}H ([^{3}H]-*t*-RA) with a specific activity of 84.3 Ci/mmol (radioactive concentration: 1.0 mCi/ml ethanol) was supplied by American Radilabeled Chemicals Inc. (St. Louis, USA).

Disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, calcium chloride, magnesium chloride and potassium chloride were all used to elaborate a receptor fluid and were provided by Sigma Chem. Co. Formaldehyde from Scharlau S.A., glucose and bovine serum albumin (BSA) from Sigma

Chem. Co. were added to the receptor medium in order to serve as a preservative and to promote solubility.

We used scintillation liquid Optiphase 'HiSafe' 2 (Wallac Scintillation Products, Fisher Chemicals, England) for the sample analysis. The solubility of the skin samples was measured with Optisolv® (Wallac Scintillation Products, Fisher Chemicals, England) and tetrahydrofuran (HPLC grade, Scharlau, Spain).

2.2. Liposomes: preparation and characterization

Multilamellar large vesicles (MLV) of all-*trans* retinoic acid (RA) composed of similar stratum corneum lipids were obtained by the conventional solvent evaporation method or Bangham method (Bangham and Horne, 1964). Ceramide type III 40.0 mg, palmitic acid 25.0 mg, cholesterol 25.0 mg, cholesterol-6-sulphate 10.0 mg, and RA 5.0 mg were partially dissolved in 10 ml of chloroform–methanol (1:1, v/v) in a round-bottomed flask. In order to remove any undissolved lipids and to delay precipitation of the dissolved components during evaporation, the solution was filtered through a Millipore Millex-GN membrane with a pore size of 0.2 μm. The solvent was then removed by vacuum evaporation under a stream of nitrogen at 37.0 °C for 12 h using a rotary evaporator (Büchi R 114, Büchi, Switzerland) so that a thin lipid film could deposit itself on the flask wall (Coderch et al., 1999). At this point, the flask was heated at 60.0 °C and then 10 ml of TRIZMA® HCl tempered solution (pH 7.4) was added and the flask was hand-shaken vigorously for 20 min to rehydrate the lipid film and obtain a large multilamellar liposome dispersion (Kim et al., 1997). The suspension was centrifuged at 5000 rpm for 30 min in a Sorvall Centrifuge Mod. RC-5B and the pellet were resuspended in the TRIZMA® HCl buffered saline. This step was completed three times to remove any traces of non-encapsulated drug.

Finally, the resulting liposome suspension was treated in an ultrasonic Branson 3200 bath at 0 °C for 30 min until it became semi-transparent. All the suspensions were stored overnight at room temperature in order to reduce any preparation-induced membrane disturbances.

The SCLL characterization was determined using an high-performance liquid chromatographic quantification of the cholesterol and RA (HPLC, System Gold® Beckman Ltd., USA). Samples were chromatographed using a Genesis C18 – 4 μm bead size, 150 mm × 4.6 mm – octadecyl silica gel column.

The cholesterol quantification was carried out in isocratic conditions: mobile phase composition – 0.05% glacial acetic acid:methanol:acetonitrile – 5:70:25. The flow rate was 2 ml/min and cholesterol was detected at 205 nm. A stock solution of cholesterol (1 mg/ml) was prepared. The calibration curve was prepared with solutions of cholesterol at concentrations ranging from 250 to 1000 μg/ml. The curves were linear ($r = 0.997$). The validation results were established for three injections per concentration and a total of seven concentrations. The repeatability and the reproducibility of the method were expressed by the relative standard deviation at a concentration of 750 μg/ml cholesterol ($n = 8$); the values came to 0.25 and 0.45%, respectively. The detection limit was calculated to be 25 ng (Choudhari et al., 1996) and the relative retention time was found to be around 5 min.

For the RA quantification, we used a combination of two mobile phases: A 1.8% glacial acetic acid (pH 3.9) and B methanol. Minute 0–3 isocratic conditions, 85% of B; minute 3–8, gradient from 85 to 97% of B; minute 8–10, gradient from 97 to 85% of B. In this case, the flow rate was 1 ml/min and RA was detected at 345 nm (Barua and Olson, 1998; Sorg et al., 1999). A stock solution of all-*trans* retinoic (2.5 mg/ml) was prepared. The calibration curve was prepared with solutions of all-*trans* retinoic at concentrations ranging from 0.2 to 50 μg/ml. Again, the curves were linear ($r = 0.999$) and the validation results were established for three injections per concentration and a total of seven concentrations. The repeatability and the reproducibility of the method were expressed by the relative standard deviation at a concentration of 25 μg/ml all-*trans* retinoic ($n = 8$) with values of 0.22 and 0.39%, respectively. The detection limit was calculated to be 0.1 ng and the relative retention time was found to be 7.95 min.

The RA content in the vesicles was calculated according to the entrapment efficiency (EE) calculated using the following equation (Ahn et al., 1995):

$$\mathrm{EE}\,(\%) = 100 \times \frac{A}{B}$$

where A and B represent the drug content in the separated liposomal vesicular layer and the liposomal suspension before separation, respectively.

The liposome size analysis was determined by the dynamic light scattering method using a Microtrac Series 9200 particle analyzer with an Ultrafine Particle Analizer (UPA unit) to determine the particles between 0.005 and 3.0 μm (El Maghraby et al., 1999). The mean diameter (the *z*-average diameter, ZAve) is based on the intensity of scattered light and the width of the distribution (the polydispersity index) is a measure of particle uniformity. Quantitative information cannot be obtained from this technique. The *z*-average diameters and polydispersity index values of liposomes were calculated.

2.3. Vehicles

Free RA was incorporated in a hydrogel (F-I)—Carbopol® Ultrez™ 10, Ethanol and de-ionized Milli-Q® grade water. Also, free RA and RA encapsulated in liposomes containing stratum corneum lipid were incorporated in a binary hydrogel (F-II and F-III, respectively)—Carbopol® Ultrez™ 10, Cross-linked hyaluronic acid, Ethanol and de-ionized Milli-Q® grade water. Hyaluronic acid must be cross-linked or mixed with acrylic hydrogels to increase stability (Luo et al., 2000). The composition of the three formulations studied is summarized in Table 1.

Appropriate quantities of Carbopol® Ultrez™ 10 and hyaluronic acid (HA) (see Table 1) were added to a 10:90 (v/v) ethanol/de-ionized water mixture to obtain the three formulations F-I, F-II and F-III. These mixtures were agitated at 2000 rpm for 10 min with a Silverson L4R agitator equipped with an axial flow head in conjunction with a disintegrating head. The polymer dispersions were immediately neutralised with Triethanolamine 50% to pH 5.5 measured in situ with a digital Crison 2000 pH-meter. The RA dissolved in ethanol to a final concentration of 0.025% (w/w) for F-I and F-II was then incorporated. The RA-SCLL-hydrogel for F-III was formed under constant agitation at room temperature to ensure liposomal integrity and it was stored at $8 \pm 1\,^{\circ}C$ before further assays were carried out. Formulations were protected from the light by storing in dark-brown vials wrapped with aluminium foil to minimize the oxidation of the RA.

For radiolabelled determination, the tritiated RA ([^{3}H]-*t*-RA) was added to an ethanol solution of 25 μl of [^{3}H]-*t*-RA solution (concentration: 1.0 mCi/ml) per ml of formulation. The [^{3}H]-*t*-RA loaded SCLLs were obtained according to the procedure described above, and radiolabelled RA was added to the solution (Section 2.2). The specific end activity of the formulations was approximately 25 μCi/ml of gel. The homogeneity of the formulations was determined using liquid scintillating counting (Liquid Scintillation Counter 1414, Wallac, Finland) (Chung, 1999).

2.4. Skin preparation

The permeability of the RA was evaluated using hairless abdominal rat skin samples excised from animals aged about 3 months. This rat skin model is an intermediate stage for a future study using pig skin in vitro versus rat skin in vitro in order to establish the final human counterpart. Briefly, the animals were sacrificed by decapitation, skin from the abdominal area was removed carefully through surgical incision and any subcutaneous fat was removed completely. The skin surface (epidermal side) is cleaned with the aid of a cotton impregnated in 0.5 ml of a sodium lauryl sulphate (1%, w/w; 1 g sodium lauryl sulphate/99 g water) aqueous solution. The remaining fat in the dermal side is entirely removed by a cotton impregnated in ether. The skin is washed five times with bidistilled water and is dried very well with a clean and dry cotton. This rapid wash did not affect to the skin integrity. Integrity of the skin samples was examined by a physical method, transepidermal water loss (TEWL) for damage or diseased conditions. After 30 min for stabilization, the transepidermal water loss of the skin was measured (Tewameter TM 210, Courage-Khazaka, Monaderm,

Table 1
Composition of tritiated binary gels

Formula	Concentration (%, w/w)			[^{3}H]-*t*-RA (μCi/ml)	pH	Encap. SCLL
	RA	U10	HA			
F-I	0.025	0.50	–	25.0	5.5	No
F-II	0.025	0.50	0.05	25.0	5.5	No
F-III	0.025	0.50	0.05	25.0	5.5	Yes

Monaco). Before the measurement, the probe was positioned on the skin for 2 min to allow stabilization of its sensors. The TEWL was measured for 1 min (mean ± standard deviation (S.D.) $g\,h^{-1}\,cm^{-2}$). The skin in which the barrier was disrupted was not used in the study. We would like to point out that cells that gave a TEWL of more than $15\,g\,h^{-1}\,cm^{-2}$ were eliminated. Any samples which showed signs of a barrier disruption were eliminated the study (Modamio et al., 1998). Then, they were cut with a dermatome (model GA 630, Aesculap, Germany) into homogeneously thick layers. Again, TEWL for barrier assessment was performed after the dermatoming of the membrane. Only intact skin samples with an effective permeation area of 2.10 cm^2 were used.

2.5. In vitro permeability of all-trans retinoic acid

The skin samples were mounted in modified Franz diffusion cells with a surface of 2.10 cm^2 and a receptor volume of 10 ml so that the dermal side of the skin was exposed to the receptor fluid and the stratum corneum remained in contact with the donor compartment. The receptor fluid (pH 7.4) consisted of a phosphate buffered physiological albumin solution containing 0.1% (v/v) formaldehyde, 0.8% (w/v) sodium chloride, 0.02% (w/v) potassium chloride, 0.01% (w/v) calcium chloride, 0.01% magnesium chloride, 0.318% (w/v) disodium hydrogen phosphate, 0.02% (w/v) potassium dihydrogen phosphate and 1.0% (w/v) bovine serum albumin (BSA). Formaldehyde to that concentration of 0.1% (v/v) in the receptor medium neither volatilized nor was corrosive since the integrity of cutaneous barrier was verified in previous tests, maintaining in contact the receptor fluid during 48 h with the skin and analyzing its barrier integrity by TEWL. All-*trans* retinoic acid is practically insoluble in phosphate buffered solution, but with bovine serum albumin (BSA) and glucose is freely soluble. The solubility of RA in the receptor fluid was checked prior to beginning the experiments at 37 °C with glucose and BSA (3.3 mg/ml). RA under these conditions is readily soluble in the receptor fluid. Then, 100 μl of the formulation was placed with a curved spatula in the donor compartment enabling a gel film to cover the entire skin surface evenly.

The temperature was maintained at 37.0 ± 0.1 °C, and the receptor solution was continuously stirred at 400 rpm with a small Teflon-coated bar magnet placed inside the cell. Sampling was done at 2, 4, 6, 8, 10, 12, 24, 28, 32, 36 and 48 h. At each point, the receptor cell contents were completely replaced by 10 ml of new solution. The samples were diluted with 15 ml of Optiphase 'HiSafe' 2 scintillating fluid, and the total sample radioactivity was determined by liquid scintillating counting (Anissimov and Roberts, 1999; Modamio et al., 2000). Five replicates were used for the study.

2.6. In vitro distribution of all-trans [^{3}H] retinoic acid in the abdominal rat skin layers

After an exposure time of 2, 4 and 8 h, the receptor liquid and skin were removed. The skin sample surfaces were washed twice with 200 μl of 1% ammonium lauryl sulphate and then three times with 300 μl of Milli-Q® water to eliminate any remaining traces of drug. The solvents from the wash were collected separately and diluted with 3 ml of Optiphase 'HiSafe' 2 scintillating fluid to determine their levels of radioactivity. After they were rinsed, the skin samples were then dried with a cotton swab to eliminate any solvent and unabsorbed superficial formulation. At this point, the swab samples were transferred to vials and incubated for 12 h at 50 °C in 3 ml of scintillating liquid, and the radioactivity of all the swabs was determined by liquid scintillating counting (Hewitt et al., 1998).

Success of application in dermopharmacokinetic studies is based on the assumption that the stratum corneum (SC) concentration–time curves are directly related to the concentration–time curves of the drug in question in the epidermis and dermis (Shah et al., 1998). The tape stripping technique enabled quantification of the capture and elimination of the drug from the stratum corneum. The dried skin samples were mounted onto a mechanical support to separate the superficial layers of the stratum corneum according to the reported tape stripping method (Rougier and Lotte, 1993). For this purpose, we applied adhesive tape (Scotch™ Magic Tape 810, 3M, France) to the surface of each sample and then peeled it off with part of the stratum corneum adhered to it. This procedure was repeated 12 times. The strippings were dissolved in 5 ml of tetrahydrofuran, and afterwards the samples were diluted in 10 ml of 'HiSafe' 2 scintillating liquid to measure the radioactivity content.

After eliminating *stratum corneum* from skin samples by the tape-stripping procedure, the epidermis was separated from the dermis with dissection after being immersed in water at 60 °C for 45 s. Previous studies proved that in time and temperature selected conditions, water could not extract any quantity of RA—substance of extremely lipophylic nature (Ramírez et al., 2000a). The epidermis and dermis samples were digested separately in 2 ml of Optisolv® at 50 °C for 12 h. These solutions were then diluted in 10 ml of Optiphase 'HiSafe' 2 scintillating liquid and analyzed for the total radiolabel by liquid scintillation counting (Hewitt et al., 1998; Chung, 1999). Five replies were used for the study. Previous studies proved that in time and temperature selected conditions, stability of radiolabelled drug was maintained (Ramírez et al., 2000b).

2.7. Data treatment

The total quantity of drug (Q) that diffused to the receptor compartment in time (t) during the steady state was expressed by the following equation (Imanidis et al., 1998; Brown et al., 1999):

$$\frac{Q}{A} = KLC_0\left(\frac{Dt}{L^2} - \frac{1}{6}\right) \quad (1)$$

where A is the effective skin diffusion (cm^2), C_0 the initial concentration of RA in the gel ($\mu g/cm^3$), D the diffusion coefficient and corresponds to the drug diffusion capacity through the skin (cm^2/s), L the skin thickness and K is the partition coefficient of RA between the skin and the vehicle (Hosny et al., 1998). This equation is theoretical, and it is not used in calculation of results. This is, experimentally Q is calculated, and from Q the different parameters of permeation are obtained.

The flux at the steady state, J_s ($\mu g/(cm^2\,h)$), was calculated using the linear portion of the correlation between the accumulated quantity of RA that diffused through the skin by unit area and time, adjusted by minimal squares (Manitz et al., 1998):

$$J_s = \frac{C_0 KD}{L} = C_0 K_P \quad (2)$$

where K_P is the permeability coefficient (Shigeki et al., 1999).

The permeability coefficient, K_P (cm/h), of RA from the different formulations was obtained from the ratio between the flux of the drug and its initial concentration in the donor compartment, this is, from the formula J_s/C_0. The differences among the three formulations (F-I, F-II and F-III) were compared using a Kruskall–Wallis one-way analysis of variance (ANOVA), and afterwards each group was compared using the Mann–Whitney U-test (Sugibayashi et al., 1999).

3. Results and discussion

3.1. Liposomes: characterization

A relatively homogeneous population of small unilamellar vesicles was obtained using the Bangham method as mentioned above. The advantage of this method is the fact that it enables the efficient and reproducible formation of small liposomes, which have a high drug-loading capacity. The characteristics of the SCLLs loaded with RA are shown in Table 2.

The mean size of the liposomes was between 40 and 60 nm with a "relatively small" polydispersity index. The entrapment efficiency of encapsulation came to more than 95%. These parameters coincide with the behaviour of highly hydrophobic compounds (Montenegro et al., 1996).

3.2. In vitro permeability study of all-trans [^{3}H] retinoic acid in abdominal rat skin

The experimental radioactivity values (^{3}H DPM; disintegrations per minute) and the levels of concentration of the drug in the receptor compartment obtained as a function of time for the three formulations F-I, F-II and F-III were used to calculate the amount of drug that penetrated the skin. These figures were compared to the initial dose and expressed as a percentage of it

Table 2
Characteristics of the SCLLs loaded with RA

Parameter	Mean value
Particle size (nm, ±S.D.)	53.7 ± 11.6
Polydispersity index	0.3
Entrapment efficiency (%, ±S.D.)	95.5 ± 1.32

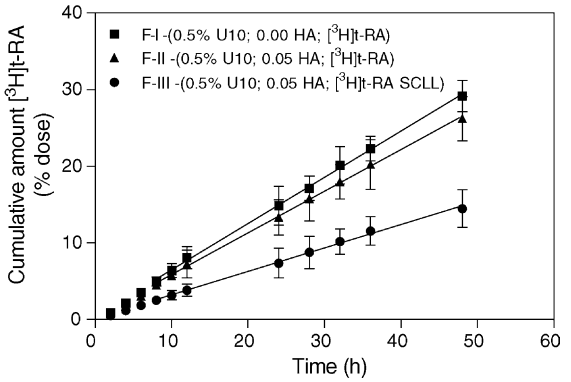

Fig. 1. Profiles of [^{3}H]-*t*-RA skin permeation from the formulation assays.

(% of dose; % of cumulative dose, Σ % of dose) and in micrograms of the drug (q, μg; cumulative amount, Σq, μg) detected in the receptor compartment at the different time intervals. In addition, the equations described in Section 2.7 (Eqs. (1) and (2)) were used to determine the values corresponding to the permeability parameters (J_S and K_P).

Fig. 1 shows the in vitro permeability kinetics of [^{3}H]-*t*-RA in the abdominal rat skin samples for the three tested formulations. The cumulative quantities of RA diffused through the samples from formulations F-I and F-II were significantly greater ($P < 0.005$) than the amounts of drug detected in the receptor compartment when F-III was used. Fig. 2 lists the mean values ($n = 5$, ±S.D.) in percentages of the applied [^{3}H]-*t*-RA dose that diffused through the skin in 48 h for each of

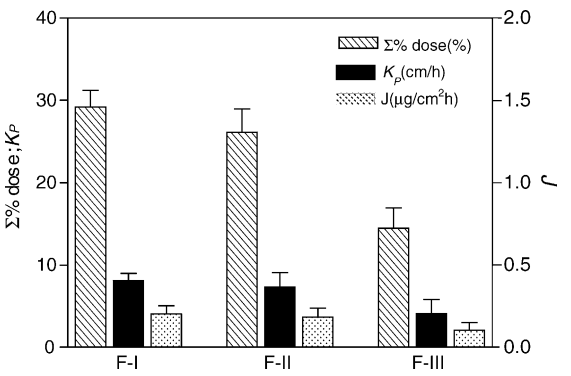

Fig. 2. Mean values ($n = 5$, ±S.D.) of cumulative dose percentage infiltrated at 48 h, flux in stationary state, and permeability coefficient of all-*trans* retinoic acid in F-I, F-II and F-III.

the formulations studied and for the respective in vitro permeability parameters of the drug in the rat skin [flux at steady state J_S ($\Sigma Q/A$ versus t; 6–48 h), μg/(cm^2 h); permeability coefficient K_P (cm/h)].

As can be observed in Fig. 2, the amounts of RA that diffused through the skin samples, the J_S and K_P values for the liposome formulation (F-III), were significantly lower ($P < 0.05$) that contained free RA (F-I and F-II). These results coincide with those of other authors who have reported that permeation of lipophilic compounds into the skin is lower than permeation of a drug applied in liposomal encapsulated form (Masini et al., 1993). At the same time, we have observed that the permeability of RA applied as the U10:HA binary formulation (F-II) was lower than when applied as the hydroalcoholic gel Carbopol® Ultrez™ 10 (F-I) and did result significantly ($P < 0.05$). This binary formulation can preserve RA just as the binding-type of controlled release of RA through chemical or ion bonds, due to the interaction between RA and HA (Levenberg and Langer, 2004; Hennink and van Nostrum, 2002).

At this point, certain concepts related to topical application of liposomes must be clarified in order to understand these results. Various studies have suggested that liposomes increase percutaneous penetration of compounds through interaction of vesicles with the stratum corneum and that this penetration is responsible for greater concentrations of the drug in question in the skin (Hofland et al., 1995; Kirjavainen et al., 1999a,b). Nevertheless, much debate concerning topical application of liposomes is whether they actually have the capacity to penetrate the stratum corneum or even the deeper skin layers in either intact or altered form. Mezei and Gulasekharam (1980) have suggested that after topical application of liposomes "the vesicle is capable of penetrating and traversing biological membranes, and possesses a determined degree of selectivity in the penetration and accumulation of targeted areas". This theory has been corroborated partially by Lasch et al. (1992), who found that intact liposomes reach the superficial layers of the stratum corneum but do not penetrate the deeper layers of the skin.

The mechanisms involved during interaction of vesicles with extracellular stratum corneum lipids are closely related to the lipid composition of the liposomes. In particular, PC liposomes favour permeation (El Maghraby et al., 1999) while SCLLs favour stratum

corneum retention (Coderch et al., 1999). The lipid composition can determine the character and intensity of interaction between the liposomes and stratum corneum and also influence significantly percutaneous absorption of the encapsulated drug (Coderch et al., 2000). This behaviour could be related to a high reservoir capacity of the stratum corneum when the drug is applied in a formulation that has a similar lipid composition to this skin layer and a high degree of ordenation in the superficial lipids, leading to an increase in the barrier function of topical application of SCLLs. In other studies (Zellmer et al., 1995; Manconi et al., 2002; Valenta and Janisch, 2003), liposomes have been reported to interact with intercellular lipids in deeper stratum corneum layers.

According to du Plessis et al. (1994), after topical application, lipid bilayers that make up the vesicles can mix with lipids from the stratum corneum to form a lipid deposit in this section of the skin. Specifically, application of SCLLs can induce greater storage of these vesicles in the horny layer, due fundamentally to affinity of the lipid composition. This behaviour can lead to increased amounts of RA deposited in the superficial strata of the horny layer if the drug is encapsulated in SCLLs (formulation F-III) before it is applied.

In addition, it can be asserted that the findings obtained for formulation F-III are the result of interaction among hyaluronic acid, RA encapsulated in SCLLs and the released RA, which reduces the degree of diffusion of the drug deposited in the superficial skin layers.

Masini et al. (1993) have compared the in vitro permeability of RA in rat skin as incorporated in gel form and in phosphatidylcholine–dipalmitoyl liposomes. They found that drug absorption was lower for the gel than for the liposomes, but the percentages of RA detected in the epidermis and dermis were greater with liposomes than with the gel. From these results, they concluded that encapsulation in liposomes affects distribution of RA in cutaneous structures.

The considerations and findings mentioned above (du Plessis et al., 1994; Cevc, 1996; Kirjavainen et al., 1999a,b) demonstrate that encapsulation in liposomes influences the distribution of lipid drugs throughout the different skin layers. Based on this fact, we can suggest that application of RA encapsulated in SCLLs leads to greater amounts of the drug deposited in the skin increasing the drug concentrations in the epidermis and dermis.

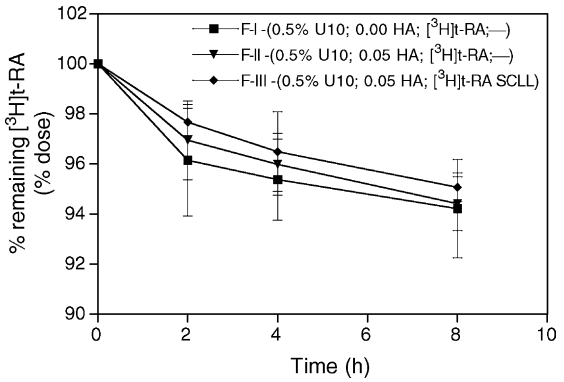

Fig. 3. Remaining amount of unabsorbed percentage [^{3}H]-*t*-RA on the skin surface as a function of time from the formulation assays.

The liposomal hydrogel might become dehydrated and form lipid structures, which resemble bilayers that form a strong link with the surface of the skin. The resulting bilayer structure might then act as a reservoir that slowly releases the drug. At the same time, interaction between HA, freed RA and encapsulated RA in the in vitro release assays of this study basically has an obstructer effect on diffusion and could also contribute to the formation of a drug reservoir with a limited diffusion capacity. In vitro formation of a reservoir from the liposomal RA vehiculized in HA/U10 binary gels was capable of reducing the local effects of the drug while maintaining clinical efficacy.

3.3. Study of the in vitro distribution of all-trans [^{3}H] retinoic acid in abdominal rat skin layers

The percentages of drug detected in the different layers of the skin (surface, stratum corneum, epidermis and dermis) were calculated using the radioactivity data (^{3}H DPM; disintegrations per minute) and the levels of drug concentration in the various cutaneous layers at different times (2, 4 and 8 h).

The mean values ($n = 5$, ±S.D.) of concentration of the unabsorbed all-*trans* (^{3}H) retinoic acid (% of the applied dose) that remained on the surface of the skin samples are found in Fig. 3. The difference between the amount of the remaining and the initially applied dose represented the quantity of the absorbed drug. The data obtained clearly demonstrate that the RA was absorbed in the case of all three formulations; that is, the drug was capable of penetrating the cutaneous

Table 3
Distribution (% dose; mean ± S.D.) of [^{3}H] RA in the different skin layers over time

Formulation	Time (h)	Cutaneous structure			
		Stratum corneum	Epidermis	Dermis	Recovery
F-I	2	1.481 ± 0.085	0.302 ± 0.050	0.196 ± 0.018	88.775 ± 1.663
	4	1.128 ± 0.113	0.205 ± 0.039	0.109 ± 0.022	81.999 ± 1.827
	8	0.834 ± 0.138	0.137 ± 0.011	0.093 ± 0.033	68.575 ± 1.375
F-II	2	1.343 ± 0.096	0.281 ± 0.082	0.151 ± 0.033	65.375 ± 8.807
	4	1.143 ± 0.240	0.204 ± 0.065	0.111 ± 0.043	94.164 ± 7.440
	8	0.952 ± 0.194	0.145 ± 0.064	0.123 ± 0.045	81.747 ± 2.004
F-III	2	1.257 ± 0.076	0.193 ± 0.022	0.155 ± 0.028	89.979 ± 4.916
	4	1.851 ± 0.086	0.234 ± 0.020	0.133 ± 0.036	87.397 ± 3.297
	8	2.057 ± 0.227	0.330 ± 0.122	0.115 ± 0.033	85.698 ± 2.167

structures regardless of whether it was in free form or it had been encapsulated in SCLLs. The most pronounced initial decrease within the first 2 h of application in the three cases (Fig. 3) possibly reflects absorption into the superficial layers of the stratum corneum (Kim et al., 1997). Percutaneous absorption was lower ($P < 0.05$) for formulation F-III (drug encapsulated in liposomes) than for formulations F-I and F-II (free drug). These results coincide with findings reported by other authors (Masini et al., 1993; Montenegro et al., 1996), who have indicated a decrease in percutaneous absorption of RA encapsulated in liposomes.

Table 3 shows the mean values ($n = 5$, ±S.D.) of RA-(^{3}H) distribution (% of the applied dose) in the different skin layers (stratum corneum, epidermis and dermis). The concentration–time profiles of [^{3}H]-*t*-RA in the stratum corneum and the viable tissues (epidermis plus dermis) are reflected in Figs. 4 and 5, respectively. The distribution data for the drug in the different skin layers suggest that the maximum concentrations of [^{3}H]-*t*-RA in the stratum corneum, the epidermis and the dermis were obtained at different time intervals for the three formulations. The drug concentrations for both the F-I and F-II formulations reached the highest values approximately 2 h after application, and subsequently, they decreased gradually in the three skin layers (Figs. 4 and 5). However, application of the liposome-containing HA/U10 binary gel (F-III) yielded a continuous increase in drug concentration in the stratum corneum and the viable skin tissues (Figs. 4 and 5).

As a result of this difference in the captation and elimination kinetics of [^{3}H]-*t*-RA, the drug concentra-

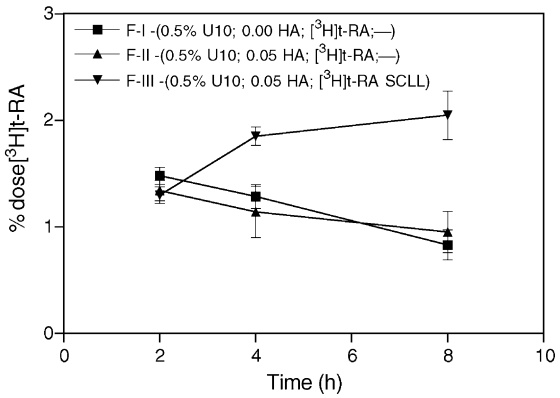

Fig. 4. All-*trans* [^{3}H] retinoic acid concentration–time profiles (% dose; mean ± S.D.-h) from the stratum corneum for the formulation assays.

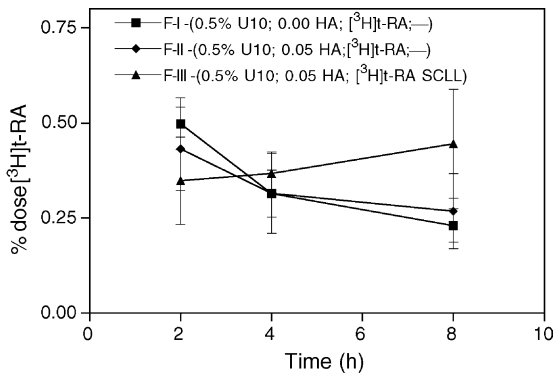

Fig. 5. All-*trans* [^{3}H] retinoic acid concentration–time profiles (% dose; mean ± S.D.-h) from the viable skin (epidermis plus dermis) for the formulation assays.

tion in the stratum corneum and the viable tissues for F-III reached approximately double the levels ($P < 0.05$) obtained for the F-I and F-II formulations at 8 h. The drug concentration in the viable skin is the balanced sum of percutaneous drug absorbed from the formulations and diffusion of the drug through the skin into the receptor compartment. Considering the fact that the level of drug absorption into the skin for the liposome-gel (F-III) was smaller than the amounts found for the non-liposomal F-I and F-II formulations (Fig. 4), the sustained or even increasing drug concentration for F-III could be attributed to retarded diffusion of the drug in the skin (Kim et al., 1997, 1998).

In addition, this may indicate that the phenomenon of higher, sustained skin concentration for [^{3}H]-*t*-RA was yielded in parallel with the decrease of total permeability of the drug in the skin (Fig. 4). Therefore, the higher, sustained drug concentration in the viable skin seems not to be due to "enhanced percutaneous absorption" but due to retarded diffusion of the absorbed drug in the skin (Roberts et al., 2002). The combination of both of these factors is desirable in order to treat skin pathology without significant systemic side-effects.

The findings of the distribution study confirm the hypothesis formulated in the discussion concerning the permeability results (Section 3.2), related to the formation of a drug reservoir – liposome/hyaluronic acid – which eventually retarded diffusion of [^{3}H]-*t*-RA and favoured a higher, sustained drug concentration at the site of action. Interaction between HA and liposomes could retard dehydration of the lipid structures by lowering partition of the drug between the liposomes and the stratum corneum (Kim et al., 1998). Moreover, RA is a highly lipophilic compound which penetrates into cutaneous structures associated with ceramides. The structural similarity of ceramides, a component of the skin and the components of the lipid bilayers of the stratum corneum might, however, explain the mechanism. Because the two are structurally similar, ceramides combine quickly and remain in the skin for a prolonged period of time, thus slowing down diffusion of the drug.

4. Conclusions

In this study, we have evaluated the hairless rat skin permeation behaviour of tritiated retinoic acid delivered in liposomal systems composed of stratum corneum lipids versus non-liposomal systems. From the results obtained here it can be concluded that:

The in vitro permeability of all-trans [3H] retinoic acid in abdominal rat skin was significantly lower in the case of the formulations that contained hyaluronic acid (F-II and F-III) and specifically in the case of the drug that was encapsulated in stratum corneum lipid liposomes (SCLLs) (F-III).

HA and encapsulation of the drug in SCLLs (F-III) modified the in vitro release and diffusion parameters of all-trans [3H] retinoic acid, and consequently, distribution of the drug in the different abdominal rat skin layers.

In vitro application of the liposomal gel formulation of RA (F-III) induced the formation of a drug reservoir that prolonged the action of the drug in the viable skin (epidermis plus dermis), improving its local effect in the skin.

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