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Doxorubicin skin penetration from monoolein-containing propylene glycol formulations

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Abstract

Topical chemotherapy with the antineoplastic doxorubicin (DXR) could be an alternative to treat skin cancer, however its poor skin penetration often limits the efficacy of topical formulations. The aim of this work was to study the effect of monoolein (MO), a penetration enhancer, on the *in vitro* skin permeation and retention of DXR. DXR was incorporated in a propylene glycol preparation containing 0–20% of MO. DXR release rate and topical delivery were evaluated *in vitro* using acetate cellulose membrane and porcine skin, respectively, mounted in a Franz diffusion cell. At 5%, MO did not significantly change DXR release rate, but MO concentrations larger than 10% decreased almost twice its release. *In vitro* skin penetration studies showed that the presence of MO in the propylene glycol formulations markedly increased DXR presence in the stratum corneum (SC). At 5%, MO significantly increased the amount of DXR in the SC already in the first hours, attained a maximum in 6 h. Comparing propylene glycol formulations containing more than 10% MO with that containing 5%, the former took the double of the time (12 h) to reach the same amount of DXR in the skin, result that is in agreement with *in vitro* release studies. Interesting, despite the fact that MO significantly increased the amount of DXR in the SC, drug transdermal delivery did not change. These findings suggest a cutaneous delivery of DXR that is an important condition for topical treatment of skin tumors. Further *in vivo* experiments can show DXR delivery to deeper skin layers.
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Keywords: Topical delivery; Monoolein; Doxorubicin; Skin penetration; Topical tumor therapy

1. Introduction

Doxorubicin hydrochloride (DXR) is an anthracyclin antibiotic widely used in antitumor chemotherapy especially for the treatment of solid tumors (Heywang et al., 1998; Gewirtz, 1999). However, DXR, in common with many other chemotherapeutic agents, has toxic side effects such as myelosupression, mucositis and cardiac toxicity (Chabner and Longo, 2001). Topical chemotherapy for the treatment of cutaneous malignancies, as basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), could be an alternative to reduce drug systemic toxicity. Also, smaller residual tumors could be completely removed by less extensive surgery (Guthrie and Porubsky, 1982; Wieman et al., 1983; Guthrie et al., 1985; Luxenberg and Guthrei, 1986; Chang et al., 1993). Partially effective DXR local application in selected cases of advanced and recurrent breast cancer (Shimizu et al., 1986) and carcinosarcoma (Moore et al., 1985) has been reported. However, formulations used in these cases of DXR topical administration were simple ointments. Theoretically, to improve tumor DXR bioavailability in topical treatments, drug skin penetration should be increased either by varying vehicle composition in order to increase drug solubility in the skin, or by altering skin permeability to the drug using, for example, chemical penetration enhancers (Pereira et al., 2002; Guy and Hadgraft, 2003). Glyceryl monooleate (Monoolein, MO), a fusogenic and polar lipid, has been proposed as a enhancer as it causes temporary and reversible disruption of the ordered lamellar structure of the bilayers in the stratum corneum, leading to increased fluidization of the intercellular lipid medium (Maggio and Lucy,

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1976; Giannakou et al., 1995; Ogiso et al., 1995; Lopes et al., 2005).

In the present study, the influence of MO on the percutaneous absorption of DXR and its retention in the skin was investigated *in vitro*. Drug release from different formulations was also studied to better understand skin permeation experiments.

2. Materials and methods

2.1. Chemicals

Myverol 18–99 K, as a source of monoolein, was kindly offered by Quest International (Campinas, Brazil) and was used as received. Doxorubicin hydrochloride (DXR) was purchased from Eurofarma (São Paulo, Brazil), methanol and tetrahidrofuran from Ominsolve (Merck, Darmstadt, Germany), propylene glycol and $ZnSO_4$ from Synth (Diadema, Brazil). All other chemicals were analytical grade. Deionized water (Milli-Q Millipore Simplicity 185, Bedford, MA) was used to prepare all solutions.

2.2. Skin

Experiments were performed in full thickness pig ear skins. Tissue was obtained less than 2 h after slaughter of the animal (Frigorífico Pontal Ltda, Brazil) and used at once or stored frozen for a maximum of 7 days before use.

2.3. Analytical chemistry

The amounts of DXR, which had diffused into or through the skin, were determined by a fluorimetric high-performance liquid chromatography (HPLC) method (Álvarez-Cédron et al., 1999). Before injection into the chromatograph, samples were treated as follows (Álvarez-Cédron et al., 1999): 200 μL of a 50:50 (v/v) mixture of methanol – 40% $ZnSO_4$ in water, were added to 150 μL of sample (receptor solution or homogenized skin preparation). After 1 min of vigorous vortex-mixing (Phoenix, model AP56), the fluid was centrifuged using a LC-1Excelsa Baby centrifuge (model II 206-R) at 1500 × *g* for 10 min. Hundred microliters of the supernatant were automatically injected (Shimatzu Instruments, Japan, SIL10AD) onto a Lichrosphere 100 RP-18 column (125 mm × 4 mm, 5 μm, Darmstadt, Germany, Merck), connected to a HPLC chromatograph (Shimatzu Instruments, Japan, LC 10-AD). Elution was performed at 35 °C with a mobile phase consisting of a mixture of methanol, 0.01 M phosphate buffer pH 3 and tetrahidrofuran (50:50:0.5, v/v), at a flow rate of 1.5 mL/min. The eluates fluorescence intensities were monitored (Shimatzu fluorescent detector RF-10AXL) at 480 nm/555 nm (excitation/emission). The retention time for DXR was 5.2 min. The calibration curve was linear ($r = 0.999$) for DXR over the concentration range of 10–320 ng/mL. Intra- and inter-days precision and accuracy of the method showed a % CV and a relative error (% *E*) not greater than 5% and 4%, respectively. The recovery percentages obtained were not less than 98.5%.

2.4. In vitro release studies

DXR release rates from formulations containing different MO concentrations (DXR at 0.1% in propylene glycol (PG), 5% monoolein (MO) in PG, 10% MO in PG and 20% MO in PG) were measured through a 23 μm cellulose membrane (MW 12,000–14,000, Fisher Scientific, USA) in a Franz-type diffusion cell with a diffusional area of 1.2 cm^2. 1.0 mL of the formulation was placed on the membrane surface in the donor compartment while the receptor contained 4.7 mL of pH 7.4 phosphate buffer. The receptor solution stirred at 300 rpm and kept at 37 °C was perfused continuously at 2 mL/h, and samples were collected automatically every hour, up to 12 h. At the end of the experiment, the amount of drug that permeated across the membrane was analyzed as described above.

2.5. In vitro permeation studies

Full thickness skin was mounted in a Franz-type diffusion cell, with the dermal side facing downward into the receptor medium, 4.7 mL of isotonic phosphate buffer, pH 7.4. To achieve higher reproducibility, the skin samples were allowed to prehydrate with receptor fluid for 1 h before the formulation was applied. The donor compartment was then filled with 1.0 mL of solutions containing DXR at 0.1% in (i) PG, or (ii) 5% MO in PG, or (iii) 10% MO in PG or (iv) 20% MO in PG. The total available diffusion area of the cell was 1.2 cm^2. The system was maintained at 37 °C and the receptor medium stirred at 300 rpm.

At the end of the experiment (after 3, 6 or 12 h), the amount of drug that permeated across the skin was analyzed by HPLC as described before. The amount of DXR retained in the stratum corneum (SC) was also evaluated. For that the skin was removed from the diffusion cell, cleaned with cotton soaked in methanol, and pinned to a piece of Parafilm™ with the SC face up. The skin area that had been exposed to the formulation (1.2 cm^2), was then tape-stripped 15 times (Lopez et al., 2003) using Scotch Book Tape no. 845 (3 M, St. Paul, MN). The tape strips were subsequently immersed in a vial containing 5 mL methanol/water (1:1) for permeant extraction during 24 h; extract aliquots were then subjected to protein precipitation and analyzed by HPLC. Removal of the SC was almost complete after 15 tape-strippings as indicated by the glistening of the exposed (viable epidermal) surface (Lopez et al., 2003).

2.6. Transmission electron microscopy

Skin ultra structure was analyzed by transmission electron microscopy (TEM) after the following treatments: (A) 12 h in contact with physiological phosphate buffer and (B) 12 h in contact with DXR solution. Skin samples were cut into appropriate-sized cubes and immediately fixed at 4 °C in 3% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4) for 3 h and postfixed in 1% osmium tetroxide in 0.2 M phosphate buffer, pH 7.4, at 4 °C. The specimens were gradually dehydrated in acetone, stained with uranyl acetate and lead citrate and then embedded in eponaraldite resin. Ultrathin sections were cut by an ultramicrotome (Leica, Ultracut, UCT) and examined in a Philips EM 208

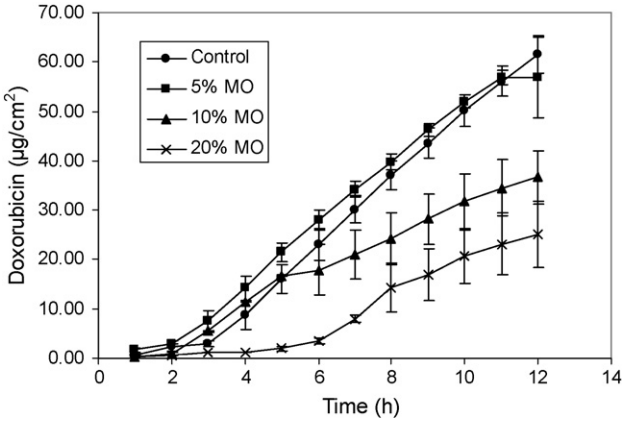

Fig. 1. DXR release profiles from formulations containing different concentrations of monoolein. Data shown are the mean ± S.D. of four replicates.

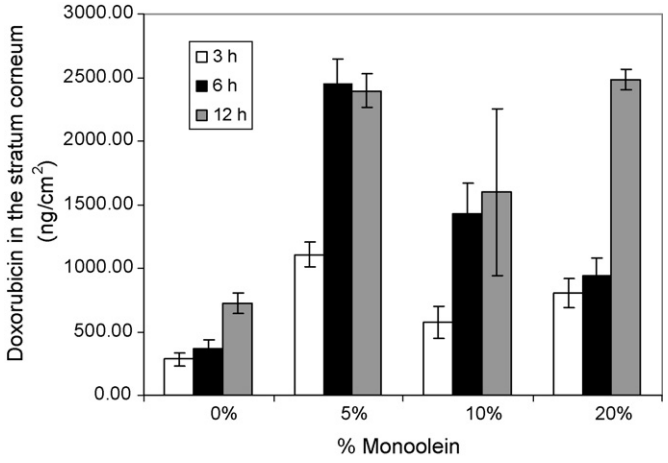

Fig. 2. Uptake of DXR into the stratum corneum as a function of time and monoolein concentration. Data shown are the mean ± S.D. of between three and five replicates.

transmission electron microscope and photographed in a Kodak electron microscopy film 4489.

2.7. Statistics

All results were expressed as the mean ± standard deviation. Statistical comparisons were performed using the Mann–Whitney test by GraphPad Prism software. A 0.05 level of probability ($p < 0.05$) was taken as the level of significance.

3. Results and discussion

Monoolein assumes a variety of structures in aqueous media, forming different mesophases, by self-association depending on water content, temperature and monoolein concentration (Engström, 1990; Esposito et al., 2003). Monoolein-containing propylene glycol formulations in the concentrations used in this report were characterized by polarized light microscopy as isotropic liquids over a temperature range of 25–40 °C (Lopes et al., 2005). Fig. 1 shows the DXR release profile from the formulations studied. A linear relationship was obtained for each vehicle ($r > 0.99$) when the amount of drug released was plotted against the time square root, indicating that the Higuchi model could describe the release. The rapid release rate of DXR from propylene glycol, calculated from the slope of that line was 35 μg/cm^2 h$^{1/2}$. Addition of 5% of monoolein did not significantly change the DXR release rate (Table 1). However, the release of DXR decreased significantly when monoolein concentrations larger than 10% were added to propylene glycol, rendering release rates of 19.36 ± 0.81 μg/cm^2 h$^{1/2}$ and 18.72 ± 1.97 μg/cm^2 h$^{1/2}$ in the presence of 10% and 20% of the lipid, respectively. Similar results have been reported for piroxicam (Hsu et al., 1994) and tenoxicam (Larrucea et al., 2001) in the presence of high concentrations of fatty acids, especially oleic acid that is structurally similar to monoolein. The lower release was attributed to an increase in vehicle lipophilicity or viscosity. As can be seen in Table 1, for formulations containing 20% of monoolein, a steady state process was attained approximately 4 h after release started. This relatively long lag time can also influence the drug skin penetration profile.

Table 1
DXR release rate from control and formulations containing different concentrations of monoolein and corresponding lag times (t_L)

Formulation	K (μg/cm^2 h$^{1/2}$)*	t_L (h)
PROP[a]	35.07 ± 2.11	3.0
PROP + 5% MO[b]	30.52 ± 2.28	2.3
PROP + 10% MO	19.36 ± 0.81	2.4
PROP + 20% MO	18.72 ± 1.97	4.4

[a] Propylene glycol.
[b] Monoolein.
* Means ± S.D. of the results of four experiments are shown.

Fig. 2 shows the *in vitro* skin retention of DXR in the stratum corneum (SC) as a function of time and monoolein concentration. The amount of drug in the stratum corneum was 2–6.5 times higher than controls (propylene glycol only) for formulations containing monoolein at all times tested. 5% monoolein significantly increased the amount of DXR already in the first 3 h of the experiment, attained a maximum at 6 h, which seems to be the drug penetration steady state, and almost the same amount was found into the skin after 12 h. As can be noticed in Fig. 2, propylene glycol formulations containing 10% and 20% of monoolein increased drug skin retention more slowly than the 5% formulation probably because the drug release rates from formulations containing high concentrations of lipids are approximately two times lower (Table 1).

Cumulative amounts of drug in the receptor solutions after 12 h of permeation are represented in Fig. 3. As can be noticed, despite the fact that monoolein increased significantly the amount of DXR in the skin, its presence did not change drug concentration in the receptor phase in comparison to the control at the same time. According to the literature, monoolein is capable of interacting with phospholipids bilayers (Maggio and Lucy, 1976; Ogiso et al., 1995; Pereira et al., 2002), thus showing a penetration enhancing activity in the same manner as oleic acid because of its unsaturated alkyl chain (C18). The long chain with a *cis*-configuration is expected to disturb intercellular lipid packing (Williams and Barry, 2004) increasing skin penetration of

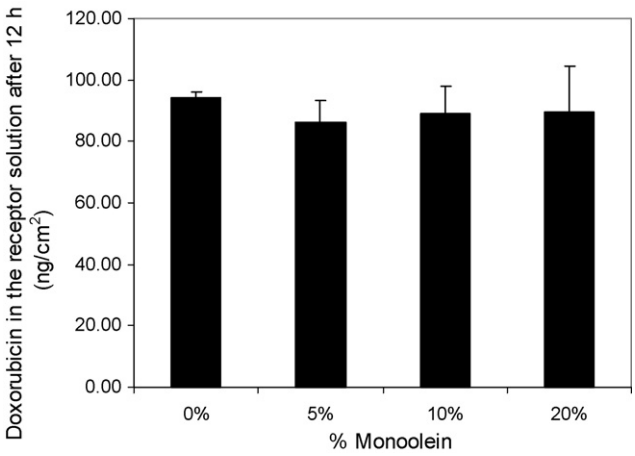

Fig. 3. Cumulative amount of DXR in the receptor solution in *in vitro* permeation studies after 12 h. Data shown are the mean ± S.D. of three to five replicates.

both lipophilic and hydrophilic permeants (Bucks and Maibach, 1999). However, some authors have previously observed that the type of functional group present in the enhancer agent, may have an influence on the extent of penetration enhancer. For example, the replacement of the carboxyl group by either a hydroxyl group (e.g. oleyl alcohol) or a methyl ester group, as monolein, brought about a decrease in the enhancing effect (Yamada and Uda, 1987; Ganem-Quintanar et al., 1998).

The observation that monoolein may increase topical delivery without promoting transdermal delivery was recently reported for cyclosporine A dissolved in formulations containing 20% or more of monoolein in propylene glycol (Lopes et al., 2005). An explanation for our results may lie more on the physicochemical properties of DXR than in the monoolein actions on the skin. It is already known that monoolein partitions well into the skin (Cornwell and Barry, 1994). Therefore, it can facilitate DXR penetration into SC, as can be seen in Fig. 2, but the drug diffusion from this skin layer is probably dependent, among other factors, on its molecular weight, partition coefficient and solubility. Furthermore, it seems that DXR can interact with anionic lipids (Goormaghtigh et al., 1980; Speelmans et al., 1994; Gaber et al., 1998; Heywang et al., 1998): its antracycline structure consists of an aglycon, adriamycinone, combined with an amino sugar, daunosamine. The pK_a of the amino function on the sugar is 8.6. As formulations developed in this work have a pH of ~5.5, DXR is positively charged. It is well documented that DXR in solutions in this same pH interacts widely with some membrane models such as lipid monolayers, lipid bilayers and liposomes (Goldman et al., 1978; de Wolf et al., 1990; Gaber et al., 1998). The affinity of DXR to lipid membranes which bear no net charge is moderate. An electrostact interaction is observed between the ammonium group of DXR and the phosphate group of neutral phospholipids (Gaber et al., 1998). In contrast, DXR interacts strongly with positively or negatively charged liposomes. Studies in model membranes suggest that DXR exhibits a specific affinity to membrane lipid domains (Duarte-Karim et al., 1976; Vilallonga and Phillips, 1978; Murphree et al., 1982), and modifies the lipid thermotropic properties (Goldman et al., 1978; Tritton et al., 1978). To relate these findings to the present work, it is important to clarify that it is well described by the literature that human skin as well as the porcine skin model used here present an isoelectric point (pI) in the range of 4.0–4.5 (Merino et al., 1999; Marro et al., 2001). That is, at pH values above this region, the skin becomes negatively charged (Burnette and Marrero, 1986; Pikal, 1992; Delgado-Charro and Guy, 1994; Lopez et al., 2001). It is possible that DXR interacts with negative lipids inside the stratum corneum, making it difficult to diffuse to the receptor solution. It is clear, however, that monoolein improves DXR penetration in the SC.

The presence of DXR in the "viable epidermis" (epidermis without SC plus dermis) was not determined in our *in vitro* experiments because, as can be observed in Fig. 4, these layers of the skin are not at all viable. Photomicrographs of the skin after 12 h of treatment with DXR showed picnotic nucleus, cytoplasmatic vacuolization and cytoplasm leaking in almost all layers of the epidermis (Fig. 4A). These alterations were also noticed in the skin treated only with phosphate buffer (Fig. 4B). For a

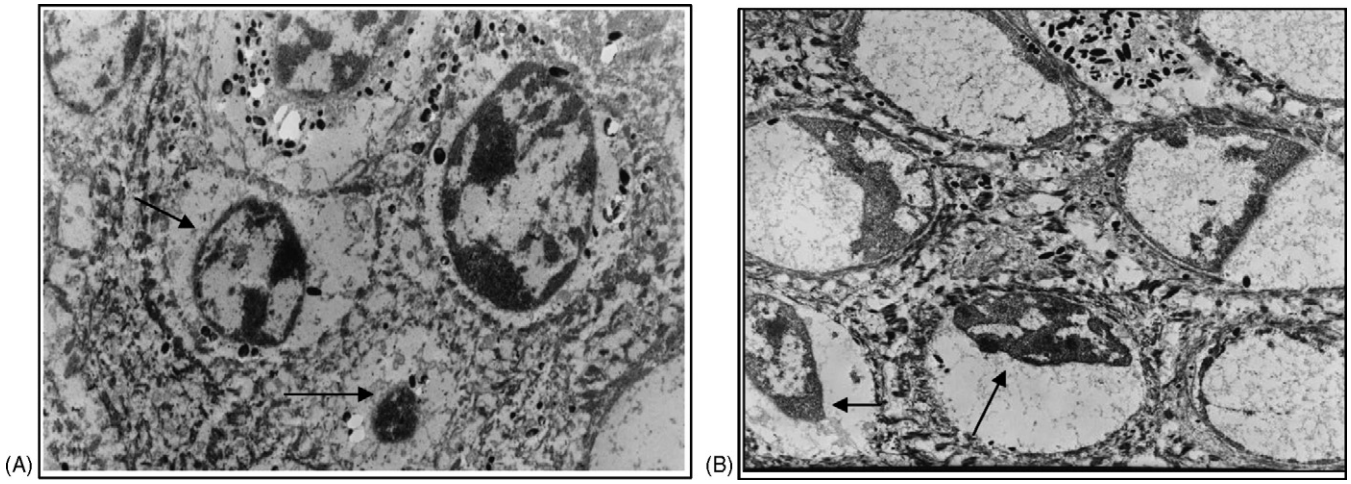

Fig. 4. Transmission electron micrographs of pig ear skins after treatment for 12 h with (A) phosphate saline buffer and (B) 0.1% DXR aqueous solution. Basal cells, 5000×; (→) picnotic nucleus.

lipophilic drug, that diffuses through these layers as a limited step, it is possible that drug concentration may offer some penetration insights. However, for a hydrophilic drug, as DXR-HCl, the quantification of the drug in a completely disorganized layer is not interesting. Therefore, in this case only *in vivo* experiments can show the delivery of the drug to the "viable epidermis".

In conclusion, DXR penetration in the SC was significantly improved by the presence of monoolein in propylene glycol formulations in a time- and concentration-dependent manner without apparent effect on its transdermal delivery. These formulations, with low concentrations of monoolein (5%), may be a simple approach to obtain higher amounts of DXR in the skin associated to minimal systemic side effects.

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