International Journal of Pharmaceutics 423 (2012) 535–542

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Effect of γ-cyclodextrin on the in vitro skin permeation of a steroidal drug from nanoemulsions: Impact of experimental setup

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ARTICLE INFO

Article history:
Received 2 November 2011
Received in revised form
22 November 2011
Accepted 23 November 2011
Available online 1 December 2011

Keywords:
γ-Cyclodextrin
Skin penetration
Skin permeation
Fludrocortisone acetate
Permeation enhancement
Franz-type diffusion cell

ABSTRACT

Numerous reports on the enhancement effect of cyclodextrins (CDs) on the skin permeation of dermally applied drugs exist, the majority of which is based on in vitro diffusion cell studies. The specific experimental setup of such studies may skew the obtained results, which is rarely discussed in the context of CD studies. Thus, the aim of this work was to conduct a systematic in vitro investigation of the permeation enhancement potential of γ-CD on a steroidal drug from a nanoemulsion. The role of critical diffusion cell parameters such as the dose of application, occlusive conditions, the nature of the receptor medium and the skin thickness were investigated. The results showed that significantly enhanced skin permeation rates of fludrocortisone acetate were indeed caused by 1% (w/w) of γ-CD at both finite and infinite dose conditions. At 0.5% (w/w) of γ-CD, significant enhancement was only achieved at infinite dose application. Additional in vitro tape stripping experiments confirmed these tendencies, but the observed effects did not reach statistical significance. It may be concluded that the full permeation enhancement potential of the CD as observed in the franz-cell setup can only be realised at infinite dose conditions while preserving the formulation structure.

1. Introduction

Cyclodextrins (CDs) are commonly used pharmaceutical excipients which are well known for their ability to complex lipophilic compounds. Both the natural cyclic oligosaccharides α-, β- and γ-CD as well as their derivatives can be employed for the solubilisation or stabilisation of drugs. In dermal drug delivery, CDs are highly useful for the stabilisation of sensitive compounds which are prone to chemical degradation (Lopez et al., 2000; Scalia et al., 2007). In addition, they may serve to stabilise formulations themselves or to influence the skin penetration of active substances by either promoting or decreasing drug penetration (Klang et al., 2010; Loftsson et al., 1991; Montassier et al., 1998; Rajewski and Stella, 1996; Trichard et al., 2007). This effect depends on the affinity of the respective drug to the lipophilic cavity of the employed CD and its solubility within the vehicle. Since CDs can interact with various lipophilic molecule structures, their use in topical formulations has to be carefully evaluated for each new product. Such preliminary investigations frequently consist of in vitro skin permeation experiments using diffusion chambers and excised human skin or suitable substitutes. Numerous reports dealing with the effect of incorporated CDs on the skin permeation of model drugs from different formulations can be found in the literature (Loftsson and Olafsson, 1998; Loftsson and Masson, 2001; Loftsson and Bodor, 2006; Matsuda and Arima, 1999). However, the effect of the experimental setup on the outcome of skin permeation studies involving CDs has not been discussed or systematically investigated so far. Important parameters in this context are the amount of applied dose, the nature of the receptor medium and the effect of occlusion conditions. Since CDs may contribute to enhanced skin permeation rates of lipophilic drugs by simply increasing their solubility in the donor medium, such an effect might be severely overestimated during in vitro diffusion cell studies where an infinite dose of sample is provided in the donor chamber. Under such conditions, the CD might indeed facilitate the diffusion of the drug within the surplus formulation and lead to an increased concentration of drug at the skin surface which is available for permeation. However, such an effect might not be reached under in vivo conditions where a finite dose of a semi-solid sample is rubbed into the skin.

Therefore, the aim of this work was to systematically investigate the permeation enhancement effect of γ-CD on a steroidal model drug and to elucidate the influence of different in vitro setups on the results. To this end, skin permeation experiments were conducted using established nanoemulsion formulations containing the lipophilic drug fludrocortisone acetate (Klang et al., 2011a). For these fluid submicron-sized O/W emulsions, remarkably enhanced skin permeation rates of steroidal drugs had been observed after

Abbreviations: CD, cyclodextrin; DLS, dynamic light scattering; PDI, polydispersity index; ZP, zeta potential; BSA, bovine serum albumin; PEG 400, polyethylene glycol 400; TEWL, transepidermal water loss.

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doi:10.1016/j.ijpharm.2011.11.037

incorporation of γ-CD using franz-type diffusion cells (Klang et al., 2010, 2011a). The validity of these results should be tested in comprehensive in vitro skin permeation studies to address the questions discussed above. It should be clarified whether the observed enhancement effect could be reproduced under different experimental setups. To this end, extensive franz-type diffusion cell studies using porcine abdominal skin were performed with corresponding formulations containing fludrocortisone acetate and different amounts of γ-CD. The comparison of these different formulations containing 0, 0.5 or 1% (w/w) of γ-CD served to elucidate the importance of the amount of incorporated γ-CD for the permeation enhancement effect. Experimental diffusion cell parameters such as the dose of application, occlusion conditions, pre-treatment of the skin, skin thickness and the nature of the receptor medium were systematically modified and optimised for the envisioned studies.

In addition, comparative tape stripping experiments were performed to provide a different in vitro setup using a finite dose application while avoiding the maceration effects of a receptor fluid. Thus, a more realistic estimation of the actual enhancement potential of CDs in topically applied formulations should be gained and the observed trends were compared to those of the diffusion cell experiments.

2. Materials and methods

2.1. Materials

Egg lecithin Lipoid E-80 was kindly donated by Lipoid GmbH (Ludwigshafen, Germany). PCL-liquid (cetearyl ethylhexanoate, isopropyl myristate) was purchased from Dr. Temt Laboratories (Vienna, Austria). Potassium sorbate was obtained from Herba Chemosan Apotheker-AG (Vienna, Austria). Fludrocortisone acetate (CAS: 514-36-3) and bovine serum albumin (CAS: 9048-46-8) were purchased from Sigma Aldrich (St. Louis, USA). Polyethylene glycol 400 (CAS: 25322-68-3) was obtained from Gatt-Koller GmbH, Absam, Austria. Cyclodextrin γ (Cavamax® W8 Pharma) was obtained from Wacker Chemie AG (Munich, Germany). All further chemicals used were of analytical reagent grade and used without further purification. Standard Corneofix® adhesive films with a square area of 4.0 cm^2 were obtained from Courage + Khazaka GmbH (Cologne, Germany).

2.2. Formulations

The model nanoemulsions were prepared as previously described (Klang et al., 2011a). Briefly, the aqueous phase, consisting of freshly distilled water and potassium sorbate, was stirred at 50 °C. Lecithin E-80 was dissolved in PCL-liquid and the lipophilic drug fludrocortisone acetate was incorporated into the resulting oil phase. Additional γ-CD was dissolved in the aqueous phase. The two phases were mixed and pre-homogenised with an ultra-turrax (Omni 500, 4 min, 2500 rpm). Afterwards, the mixture was stirred and heated to 50 °C before further homogenisation with a high-pressure homogeniser (EmulsiFlex C3, Avestin) for 20 homogenisation cycles at 750 bars. The composition of the resulting formulations and the corresponding abbreviations are given in Table 1.

Table 1
Composition of the model nanoemulsion NE with fludrocortisone acetate. For the additional nanoemulsions γ-0.5% NE and γ-1% NE, additional γ-CD was incorporated at either 0.5 or 1.0% (w/w). Abbreviations of the three formulations are detailed below.

Excipients	Composition (%, w/w)
PCL liquid	20.0
Lecithin E-80	2.5
Potassium sorbate	0.1
Fludrocortison acetate	1.0
Distilled water	ad 100

Abbreviations: NE = basic nanoemulsion without additional γ-CD; γ-0.5% NE = nanoemulsion with 0.5% (w/w) of γ-CD; γ-1% NE = nanoemulsion with 1% (w/w) of γ-CD.

2.3. Emulsion characterisation

2.3.1. Particle size, polydispersity index and particle surface charge

The systems were analysed for their particle size and particle size distribution by dynamic light scattering (DLS, photon correlation spectroscopy) using a Zetasizer Nano ZS (Malvern, UK) at 25 °C. Samples were diluted with freshly distilled water 1:100 (v/v) to diminish opalescence. The measured parameters were the hydrodynamic diameter expressed as *z*-average value, i.e. the intensity weighted mean diameter of the bulk population of oil droplets, as well as the polydispersity index (PDI). The latter represents the particle size distribution within the formulations; values below 0.2 indicate a narrow size distribution and thus good long-term stability (Mueller, 1996a). All samples were analysed in triplicate ($n = 3$) and each individual result was automatically calculated as the average of 3 measurements with 20 sub-measurements each.

In addition, the particle surface charge or zeta potential (ZP) of the nanoemulsions was determined by laser Doppler electrophoresis, again using the Zetasizer Nano ZS (Malvern, UK) at 25 °C. The samples were diluted with distilled water (1:100 v/v) containing sodium chloride (0.01 mmol) to ensure constant conductivity below 0.05 mS/cm and thus reproducible measurement conditions (Klang et al., 2011a). The ZP was determined in triplicate for all nanoemulsions ($n = 3$); again each individual result was calculated as the average of 3 measurements with 20 sub-runs each. Overall, the obtained data should ensure the use of representative and intact formulations for all further studies.

2.3.2. Drug content and pH

The drug content of all nanoemulsions was analysed immediately after preparation to ensure appropriate drug incorporation. Briefly, 10 mg of each system were dissolved in 1 ml of methanol, centrifuged for 6 min at 12,000 rpm (Hermle Z323K, MIDSCI, USA) and analysed by HPLC. Samples were taken at least in triplicate ($n \geq 3$). In addition, the pH of the formulations was determined using a pH meter (Orion 420A, Bartelt, Austria) at ambient temperature (25 °C, $n = 3$).

2.4. Preparation of porcine abdominal skin and porcine ear skin

For the diffusion cell studies, porcine abdominal skin was freed from hair and dermatomed (GB 228R, Aesculap, Germany) to a thickness of 1.2 mm. Since full-thickness skin represents an unphysiologically strong barrier for the diffusion of lipophilic drugs (Bosman et al., 1998; Lehman et al., 2011), additional experiments were performed using porcine abdominal skin that was dermatomed to a thickness of 0.5 mm. The dermatomed skin was stored in aluminium foil and polyethylene bags at −20 °C and was thawed prior to the experiments. Under such conditions the skin is stable in regard to SC properties for diffusion studies for up to 6 months (Leveque et al., 2004; Wagner et al., 2000).

2.5. Solubility of the model drug and choice of the receptor medium

The investigated receptor media were pure aqueous phosphate buffer (Pharmacopoea Europea, pH 7.4, 0.012 M) as well as buffer

containing 1.4% or 5% (w/v) of bovine serum albumin (BSA) or 20% (v/v) of either propylene glycol, polyethylene glycol 400 (PEG 400) or ethanol.

The saturation solubility of the fludrocortisone acetate in the designated receptor fluids was determined by HPLC analysis. To this end, an excess of fludrocortisone acetate was dissolved in 1 ml of the respective medium at 25 °C. The dispersion was then shaken for 24 h at room temperature, centrifuged to separate undissolved drug and was filtered (Minisart RC4 0.45 μm, Sartorius stedim Biotech GmbH, Göttingen, Germany) and analysed by HPLC. At least 4 individual experiments were performed for each medium ($n \geq 4$).

2.6. Skin permeation experiments using franz-type diffusion cells

In vitro skin permeation studies were performed using Franz-type diffusion cells (Permegear, USA) (Franz, 1975). Porcine abdominal skin was chosen as model membrane because of its similarity to human skin regarding morphology and permeability (Michniak-Kohn et al., 2005). Appropriately cut skin pieces were clamped between the donor and the receptor chamber of the diffusion cells having a permeation area of 0.95 cm^2. The receptor compartment was filled with 2 ml of the respective receptor medium. Pure phosphate buffer (pH 7.4, 0.012 M) was employed as standard receptor medium. Comparative studies were performed with buffer media containing 1.4% (w/v) of BSA or 20% (v/v) of either propylene glycol, PEG 400 or ethanol to evaluate the benefit of an adapted receptor medium. The diffusion cells were kept at the average skin surface temperature of 32 ± 0.5 °C and were continuously stirred with magnetic bars for 24 h. The accurately weighted formulation was placed on the excised skin in the donor chamber. Samples of 200 μl were removed at defined time intervals for HPLC analysis and were replaced by fresh receptor medium. Permeation profiles of fludrocortisone acetate were constructed by plotting the time (hours) against the cumulative amount of the drug (μg/cm^2) as determined in the receptor solution. The steady state flux (J, μg cm^{-2} h^{-1}) was calculated by linear regression after the respective lag-times.

Different sets of experiments were performed. The influence of the amount of applied formulation was tested. To this end, respectively 5, 50 and 500 mg/cm^2 of each nanoemulsion, corresponding to a finite, semi-infinite and infinite dose application (Henning et al., 2009), were applied onto the skin ($n \geq 8$, respectively). Information about the influence of the incorporated γ-CD at the different amounts of formulation could likewise be obtained in these experiments. Additional experiments served to investigate whether pre-treatment with the CD would affect the skin permeation of the drug from the applied standard nanoemulsion as well. To this end, the skin was pre-macerated within the franz-cell setup using 50 μl of either 1% (w/w) aqueous buffer solution of γ-CD, pure aqueous buffer solution or no additional solute for 16 h. These experiments were carried at all three amounts of applied formulation.

During all experiments, the donor compartment as well as the sampling arm was occluded to prevent evaporation. For selected comparative studies, additional non-occluded conditions with an open donor compartment were employed.

Further experiments to optimise the setup for the standard nanoemulsion involved variation of the model skin thickness as well as the nature of the receptor medium. These experiments served to identify the most suitable working protocol for general studies of nanoemulsions containing highly lipophilic drugs.

2.7. Skin penetration experiments via in vitro tape stripping

In vitro tape stripping was conducted to elucidate whether the enhancement effect of CD incorporation would be reproducible under a different experimental setup. Thus, the penetration behaviour of fludrocortisone acetate into the stratum corneum of porcine ear skin was investigated. Penetration profiles were constructed from the results of at least eight individual tape stripping experiments for each formulation ($n \geq 8$). Fresh porcine ears were kindly donated by the Clinic for Swine, University of Veterinary Medicine, Vienna. The ears were stored at −20 °C, thawed prior to the experiments, cleaned with cold water and blotted dry. The skin was freed from hair with scissors and the skin barrier function of representative areas was confirmed by determination of the transepidermal water loss (TEWL) using the closed-chamber device AquaFlux® (Biox Ltd., London, UK) (Klang et al., 2011b). The formulations were then applied to a marked area at a concentration of 6 mg/cm^2 with a saturated vinyl glove finger for 30 s.

After 1 h of penetration time, Corneofix® tapes were employed to remove the superficial stratum corneum layers of the porcine ear skin (Klang et al., 2011b,c). The outline of the first adhesive film was indicated with a permanent marker. Pressure was applied for 3 s with a rolling movement of the vinyl glove-covered thumb at a constant pressure of 49 N (5 kg). The tape was subsequently removed in a single rapid movement. This procedure was repeated with 25 tapes per experiment. The amount of adherent corneocytes was determined using the infrared densitometer SquameScan™ 850A (Heiland electronic GmbH, Wetzlar, Germany) (Voegeli et al., 2007). The optical pseudo-absorption of the adhesive films at 850 nm (A, in %) was employed to quantify the amount of stratum corneum proteins by employing the equation $m = A/0.41$ (μg/cm^2) (Klang et al., 2011b). The mean cumulative amount of the stratum corneum proteins removed with the tapes was employed to establish the penetration depth of fludrocortisone acetate in relation to the complete horny layer thickness. The latter was determined by continuous stripping of the complete stratum corneum in four of the experiments ($n = 4$) until the detection limit of the IR-densitometer was reached.

2.8. HPLC analysis

The samples were analysed for their drug content by HPLC (Series ISS-200, Perkin Elmer, USA), consisting of an auto sampler, a lc pump and an UV-diode array detector (235 C) using a Nucleosil 100-5 C18 column (250 mm × 4 mm, Macherey-Nagel, USA) plus pre-column (CC8/4, 40 mm × 4 mm). The oven temperature was set at 50 °C and the injection volume was 20 μl for all samples. Data analysis was performed using the TotalChrom Navigator 6.2.0 software. The quantification of fludrocortisone acetate was performed as previously reported (Klang et al., 2011a) at a detection wavelength of 240 nm using acetonitrile/water (40/60, v/v) as the mobile phase. The retention time was around 11 min at a flow rate of 0.8 ml/min. Standard solutions of fludrocortisone acetate in methanol were prepared and a calibration curve was calculated by plotting the analysed drug concentrations against the obtained peak area values. The concentration range of the standard solutions was between 0.018 μg/ml and 35.88 μg/ml with a coefficient of determination of $R^2 = 1$. However, the limit of reasonable quantification was set at 0.06 μg/ml since values below this concentration showed limited reproducibility.

2.9. Statistical data analysis

All results are expressed as means of at least three experiments ± SD. Statistical data analyses were performed using the GraphPadPrism3 program. Student's t-test or ANOVA with $P < 0.05$ as respective minimum level of significance were employed for the analysis of parametric data. Non-parametric data were analysed using the Mann–Whitney test or the Kruskal–Wallis-test with $P < 0.05$ as minimum level of significance, respectively. For the

Table 2
Physicochemical properties of nanoemulsions with fludrocortisone acetate (1%, w/w). All given values are means of at least three experiments ± SD ($n \geq 3$). The parameters shown in Table 2a are the mean hydrodynamic diameter or mean droplet size in nm, the PDI, the ZP values in mV as well as the conductivity of the diluted samples. The parameters given in Table 2b are the mean drug content in % of the originally incorporated drug amount as well as the pH of the investigated formulations.

(2a)

Formulation	Size (d, nm)	PDI	ZP (mV)	Cond (mS/cm)
NE	178.40 ± 0.66	0.040 ± 0.029	−31.27 ± 1.55	0.024 ± 0.006
γ-0.5% NE	171.03 ± 0.32	0.098 ± 0.042	−33.17 ± 0.75	0.021 ± 0.001
γ-1% NE	169.73 ± 2.35	0.033 ± 0.049	−31.73 ± 1.52	0.019 ± 0.001

(2b)

Formulation	Drug content (%)	pH
NE	96.17 ± 05.64	6.84 ± 0.02
γ-0.5% NE	91.93 ± 01.73	6.74 ± 0.01
γ-1% NE	93.13 ± 02.11	6.78 ± 0.02

diffusion cell studies, both cumulative amounts permeated and drug fluxes were statistically evaluated in parallel. The results were consistent in all cases.

3. Results and discussion

3.1. Nanoemulsion characterisation

The physicochemical properties of the produced nanoemulsions are given in Table 2. The mean hydrodynamic diameter of the droplets showed only a slight change upon incorporation of the CD (Table 2a). The PDI values after CD incorporation showed slightly increased standard deviations, but largely unaltered absolute values below 0.1, which represents a narrow droplet size distribution (Mueller and Schuhmann, 1996b). The ZP values, which characterise the surface charge of the emulsion droplets in solution, ranged slightly above −30 mV for all formulations, which indicates sufficient electrochemical stability. The low conductivity confirmed the validity of the ZP measurements (Mueller, 1996a). Overall, these results are in good agreement with previous studies (Klang et al., 2011a) and the produced formulations were therefore employed for the subsequent experiments on porcine skin.

As shown in Table 2b, both the mean drug content and the pH values of all formulations were in a satisfying range and confirmed the representative nature of the formulations for the envisioned studies.

3.2. In vitro skin permeation: franz-type diffusion cells

A standard nanoemulsion containing a steroidal model drug was chosen for comprehensive diffusion cell studies in order to systematically identify the most suitable experimental setup to obtain reliable permeation data for this and similar systems. In addition, these basic data served as a basis for comparison with formulations containing additional γ-CD.

3.2.1. Effect of the applied dose: finite vs. infinite dose

The effect of the applied dose on the skin permeation of fludrocortisone acetate into the aqueous buffer solution was investigated. In context of this finite/infinite dose comparison, two additional formulations with different amounts of γ-CD were evaluated in the same manner. The results of these experiments are given in Table 3. Quite expectedly, the skin permeation of fludrocortisone acetate from the standard nanoemulsion could be ranked in the order 5 mg/cm^2 < 50 mg/cm^2 < 500 mg/cm^2. Both the cumulative drug amount after 24 h and the mean drug flux were roughly ten-fold increased for the semi-infinite and infinite dose conditions ($P < 0.05$, respectively). No significant difference, however, was observed between the 50 and 500 mg/cm^2 application ($P > 0.05$). This suggests that infinite dose conditions were already reached at 50 mg/cm^2 although different reports exist (Lehman et al., 2011).

The ten-fold increase in applied dose from 5 to 50 mg/cm^2 was nicely reflected in the respective permeated drug amounts and fluxes. Comparatively large standard deviations were observed in all experiments since the overall permeated drug amounts were very low and biological material was involved as a model membrane.

In case of both systems containing additional γ-CD, the ten-fold increase in applied dose from 5 to 50 and from 50 to 500 mg/cm^2 was clearly reflected in the permeated drug amounts and fluxes ($P < 0.05$, respectively). Apparently, the addition of the CD magnified the increase in permeation with the increasing doses.

The enhancement effects caused by the incorporated γ-CD were clearly related to the amount of CD as well as the experimental setup. When comparing the formulation containing 0.5% (w/w) of γ-CD to the standard nanoemulsion, the permeation behaviour was quite similar at applied doses of 5 and 50 mg/cm^2 ($P > 0.05$, respectively). A significant enhancement effect was only observed at infinite dose conditions (500 mg/cm^2, $P < 0.05$). In case of 1.0% (w/w) of γ-CD, a significant enhancement effect was observed at all applied doses of 5, 50 and 500 mg/cm^2 when compared to the standard nanoemulsion ($P < 0.05$, respectively). This indicates that an enhancement effect can be achieved at both finite and infinite dose conditions if the amount of incorporated CD within the formulation is sufficiently high.

To exclude any direct effect of the CD on the skin, additional experiments were performed using the standard nanoemulsion. The skin was pre-impregnated within the franz-cell setup with 50 μl of either pure aqueous buffer, buffer containing 1% (w/w) of

Table 3
Skin permeation data of fludrocortisone acetate from standard nanoemulsions (NE) and corresponding formulations with 0.5% or 1.0% of additional γ-CD (γ-0.5% NE and γ-1% NE). Given values are means ± SD of at least 8 experiments ($n \geq 8$). Finite, semi-infinite and infinite dose conditions were tested with applied amounts of 5, 50 or 500 mg/cm^2 of formulation. For each setup, statistically significant differences with $P < 0.05$ against the standard nanoemulsion (NE) are marked with a '*'.

Formulation	Applied amount (mg)	Cumulative amount after 24 h ± SD (μg cm^{-2})	Mean drug flux ± SD (J, μg cm^{-2} h^{-1})
NE control	5	0.12 ± 0.11	0.008 ± 0.007
γ-0.5% NE	5	0.11 ± 0.09	0.005 ± 0.004
γ-1% NE	5	0.45 ± 0.23 *	0.067 ± 0.047 *
NE control	50	1.31 ± 1.11	0.08 ± 0.07
γ-0.5% NE	50	1.83 ± 0.63	0.08 ± 0.03
γ-1% NE	50	4.68 ± 2.44 *	0.90 ± 0.64 *
NE control	500	1.36 ± 1.10	0.09 ± 0.07
γ-0.5% NE	500	12.99 ± 2.53 *	0.63 ± 0.11 *
γ-1% NE	500	54.23 ± 15.65 *	2.48 ± 0.68 *

dissolved γ-CD or no additional solute. The skin permeation of fludrocortisone acetate in this setup was highly comparable to that observed in the conventional studies with untreated model skin ($n \geq 4$, $P > 0.05$ at all applied doses, data not shown). It may thus be assumed that the CD does not have a direct effect on the skin barrier function, which is in accordante with previous theories (Klang et al., 2011a) and studies where no permeation enhancement was observed after pre-treatment of skin with CDs in vitro (Williams et al., 1998). It likewise confirms that maceration effects, which are known to skew the results of diffusion cell studies after extended experiment times of over 24 h, are mainly a problem when investigating hydrophilic drugs (Otberg et al., 2008).

In conclusion, the assumed mechanism behind the enhanced permeation of fludrocortisone acetate is not related to any direct effects on skin, but most likely to solubility effects within the vehicle. In literature, the most frequently suggested mechanism of action relies on the better dispersion of the drug molecules within the vehicle through complexation with the CD and thus better solubilisation of the drug (Loftsson and Masson, 2001; Loftsson et al., 2007). As a consequence, more drug is available at the skin surface to enter the SC through diffusion. The CD molecules themselves are too large to penetrate into the skin themselves. As previously discussed (Klang et al., 2011a), it remains to be clarified whether this mechanism can be held responsible for the observed strong effects if only small molar amounts of CD are present within the vehicle. CDs may also affect the interfacial films of the emulsion droplets (Klang et al., 2011a). Thus, the modified emulsion structure could also contribute to the altered drug release from the droplets. Since we are dealing with dynamic systems, a combination of both mechanisms is well possible.

In summary, the amount of applied dose was confirmed to be a crucial parameter in the diffusion cell studies of the fluid nanoemulsions. For experiments with semi-solid systems, finite dose conditions are usually recommended although certain applications for infinite doses exist as well (Gillet et al., 2011). For theoretical evaluations, infinite dose conditions offer the advantage of standardised kinetic conditions as the influence of the dose on skin permeation is minimised (Wagner et al., 2002). Thus, the permeation of different drugs might be compared more directly. In our case, we primarily benefited from more accurate drug quantification by HPLC grace to the increased amounts of drug detected in the receptor medium. Likewise, application of the formulation onto the diffusion cells was facilitated due to the larger amount of sample. Thus, errors due to inaccurate weighing are minimised (Wagner et al., 2002). Since the permeation data obtained for the 50 mg/cm^2 dose of the standard nanoemulsion were a very accurate upscale from the finite dose of 5 mg/cm^2, the former dose was chosen for further studies with this system to investigate the effects of other methodological parameters.

3.2.2. Occluded vs. non-occluded conditions

For comparative reasons, additional studies were performed using diffusion cells with non-occluded upper chambers. The skin permeation of fludrocortisone acetate from the standard nanoemulsion was investigated at all three amounts of applied dose (5, 50 and 500 mg/cm^2, Fig. 1). Large standard deviations were observed under non-occluded conditions at all amounts of applied dose. At occluded conditions, this was primarily the case for finite dose application. Apart from this slight trend, no statistically significant differences were observed between the experiments at occluded or non-occluded conditions regarding cumulative amounts drug amounts or drug fluxes at all amounts of applied dose ($P > 0.05$, respectively).

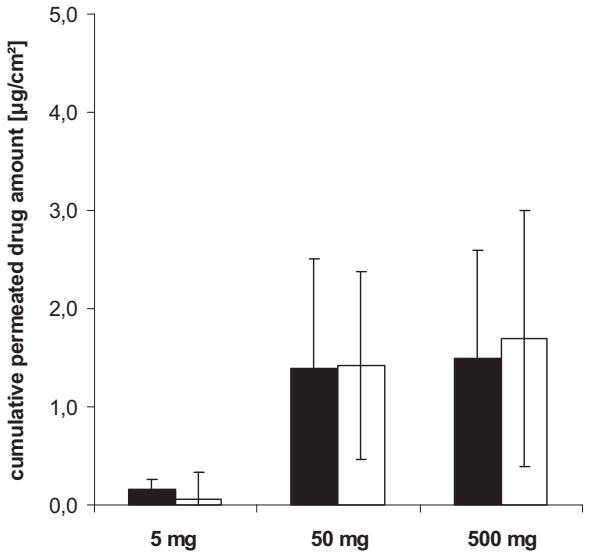

Fig. 1. Cumulative permeated amounts of fludrocortisone acetate after 24 h from standard nanoemulsions (NE) at occluded (black bars) or non-occluded conditions (white bars). Finite (5 mg/cm^2), semi-infinite (50 mg/cm^2) and infinite dose (500 mg/cm^2) application of the formulation were employed. Given values are means ± SD of at least 8 experiments ($n \geq 8$).

3.2.3. Effect of the skin thickness

Since the thickness of the employed model skin plays a major role in diffusion cell experiments, two differently dermatomed skin samples of 1.2 or 0.5 mm thickness were employed for comparative studies. Again, the experiments were performed using the model nanoemulsion at all amounts of applied dose (5, 50 and 500 mg/cm^2, Fig. 2). Quite expectedly, significantly higher cumulative drug amounts and drug fluxes were observed at a skin thickness of 0.5 mm for all applied amounts of standard nanoemulsion ($P < 0.05$, respectively). The use of split-thickness or strongly dermatomed skin is indeed recommended for absorption studies of poorly water soluble drugs (Lehman et al., 2011). However, the drug fluxes for the thin skin samples were rather irregular and

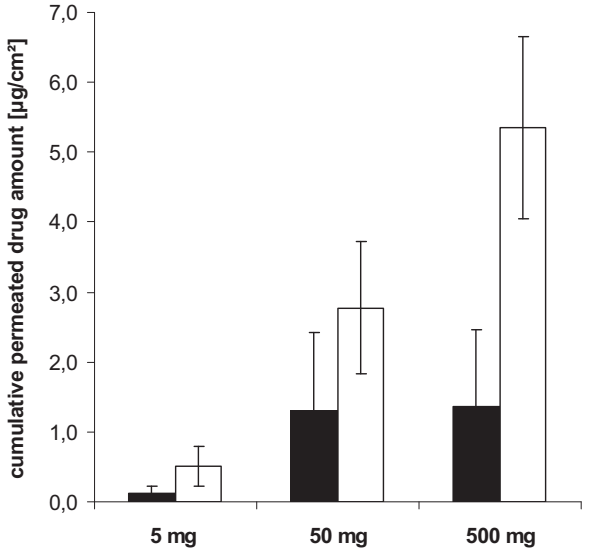

Fig. 2. Cumulative permeated amounts of fludrocortisone acetate from standard nanoemulsions (NE) after 24 h through porcine abdominal skin at a skin thickness of either 1.2 mm (black bars) or 0.5 mm (white bars). Finite (5 mg/cm^2), semi-infinite (50 mg/cm^2) and infinite dose (500 mg/cm^2) application of the formulation were employed. Given values are means ± SD of at least 5 experiments ($n \geq 5$).

Table 4
Solubility of fludrocortisone acetate in different receptor media. All values were determined at least in quadruplicate ($n \geq 4$).

Receptor fluid	Drug solubility (μg/ml) ± SD
Phosphate buffer pH 7.4	15.04 ± 2.43
Phosphate buffer/BSA (1.4%, w/v)	34.69 ± 0.19
Phosphate buffer/BSA (5%, w/v)	63.91 ± 0.16
Phosphate buffer/propylene glycol (80/20, v/v)	57.81 ± 0.66
Phosphate buffer/PEG 400 (80/20, v/v)	72.34 ± 7.65
Phosphate buffer/ethanol (80/20, v/v)	105.09 ± 1.38

hardly showed a consistent increase over time. This might be due to the fact that producing dermatomed skin samples of such small thickness becomes increasingly difficult and already small aberrations in the skin may lead to variable results. Thus, a compromise regarding skin thickness should be found to produce adequately homogeneous skin samples without creating an unphysiologically strong barrier for lipophilic drugs.

3.2.4. Effect of the acceptor medium

The solubility data obtained for the different receptor media are given in Table 4. As expected, the solubility of fludrocortisone acetate was markedly increased in the presence of the different additives. The strongest increase in solubility was caused by the presence of 20% (v/v) of ethanol, followed by the respective amounts of PEG 400, propylene glycol and finally BSA at 1.4% (w/v). The buffer containing 5% (w/v) of BSA was excluded from further studies due to its high foaming tendency, which rendered this medium impractical for the envisioned studies.

Pure phosphate buffer still represents the receptor fluid of choice if undesired interactions with constituents of the skin or the employed formulation are to be avoided. This was particularly important in our case where the specific effect of the CD should be investigated separately and undisturbed by any additional factors. Thus, the phosphate buffer was employed for all basic studies. The different receptor media were compared in additional studies using the standard nanoemulsion at 50 mg/cm^2 application (Table 5). In this setup, semi-infinite dose application was employed to emphasise potential differences in drug permeation. A comparison of the different receptor media revealed that both cumulative drug amounts and drug fluxes were highly comparable for the control buffer and the buffer media containing either BSA, propylene glycol or PEG 400 ($P > 0.05$ in all cases). Merely in case of the ethanol-containing buffer a significantly increased drug permeation was noticeable when compared to the control buffer ($P < 0.05$). This indicates that in case of this steroidal model drug, the increased solubility in the different receptor media caused by BSA, propylene glycol or PEG 400 was apparently not sufficient to promote its skin permeation. Comparative studies employing an application dose of 500 mg/cm^2 revealed the same tendency, at the same time confirming that no significant difference between the two amounts of applied dose are to be expected irrespective of the employed receptor medium ($n \geq 5$, $P > 0.05$ in all cases, data not shown). The remarkably enhanced skin permeation in context with ethanol that was observed for both 50 and 500 mg/cm^2 application is in accordance with the solubility data given in Table 4. It may however be assumed that the impact of ethanol on the skin itself may have contributed to this pronounced effect.

Table 5
Skin permeation of fludrocortisone acetate from standard nanoemulsions (NE) at semi-infinite dose conditions using 50 mg/cm^2 of applied formulation and different acceptor media. Given values are means ± SD of at least 8 experiments ($n \geq 8$).

Investigated receptor medium	Applied dose (mg)	Cumulative amount after 24 h ± SD (μg cm^{-2})	Mean drug flux ± SD (J, μg cm^{-2} h^{-1})
Buffer	50	0.80 ± 0.88	0.29 ± 0.21
Buffer with BSA	50	0.19 ± 0.13	0.17 ± 0.13
Buffer with PG	50	0.57 ± 0.62	0.20 ± 0.17
Bufffer with PEG	50	0.47 ± 0.48	0.21 ± 0.13
Buffer with EtOH	50	5.46 ± 3.43	2.09 ± 0.98

As commonly known, current recommendations such as the OECD guidelines rely on the use of isotonic buffer media for skin permeation studies with franz-type diffusion cells. The receptor fluid should provide adequate solubility of the permeant so as not to hinder its absorption, but at the same time be innocuous to the skin (Gillet et al., 2011). In case of lipophilic drugs, the solubility requirements are usually not met by standard buffer media such as phosphate buffered saline. Thus, additives which increase the solubility of the permeant may be incorporated in the receptor medium, such as surfactants, propylene glycol, bovine serum albumin or even ethanol. However, these additives may affect the skin samples and can alter the apparent skin diffusion rates of applied substances (Gillet et al., 2011; Lehman et al., 2011). This is frequently observed for both propylene glycol and ethanol, which may act as permeation enhancers (Megrab et al., 1995a, b). They supposedly act via increasing drug solubility in the stratum corneum, but may also directly affect stratum corneum components. However, solvent concentrations of over 60% may lead to dehydration of the skin, thus decreasing drug flux. Likewise, a solvent gradient across the membrane may influence the results due to osmotic or solvent drag effects, i.e. the co-transport of water and solvent through the membrane and back-diffusion effects (Megrab et al., 1995a; Sznitowska, 1996). For these reasons, we chose to limit the amounts of additives in the receptor medium to 20% (v/v). If the rate of absorption of a given compound is quite low even isotonic buffer can serve as an adequate receptor for some compounds that are otherwise considered water insoluble (Lehman et al., 2011). In context with the presented experiments, this might explain why the aqueous buffer medium performed equally well when compared to other media containing BSA, propylene glycol or PEG 400.

3.3. In vitro skin penetration: tape stripping

Additional skin penetration studies without the use of buffer media were conducted to exclude any maceration or skin hydration effects on the outcome. Since it is well-known that a strong inter-individual variability in SC thickness exists (Dickel et al., 2010; Jacobi et al., 2007; Kalia et al., 2000; Schwindt et al., 1998), at least 8 individual experiments were performed for each formulation ($n \geq 8$). The experiments served to determine the skin penetration potential of the standard nanoemulsion itself. Furthermore, the effect of additionally incorporated γ-CD should be evaluated in this in vitro setup as well. Fig. 3 shows the skin penetration profile (Lademann et al., 2009) of fludrocortisone acetate from the standard nanoemulsion. The penetration depth of the three formulations was in the order of γ-1% NE > γ-0.5% NE > NE (Fig. 4). However, these differences did not reach statistical significance ($P > 0.05$ in all cases). In case of the nanoemulsion containing 1% of γ-CD, up to 87% of the total SC thickness were reached; the latter was found to be around 8.06 ± 1.22 μm ($n = 4$) for the employed porcine ears. The summarised penetrated drug amounts were in the range of 20–30 μg/cm^2 in all cases.

In additional studies, corresponding formulations with a different oil phase consisting of the commonly used squalene were produced and investigated in identical tape stripping experiments with fludrocortisone acetate (data not shown). Again, a slightly deeper skin penetration of the formulations containing γ-CD was observed. The differences did not reach statistical significance in this case, either, but the same trends were observed.

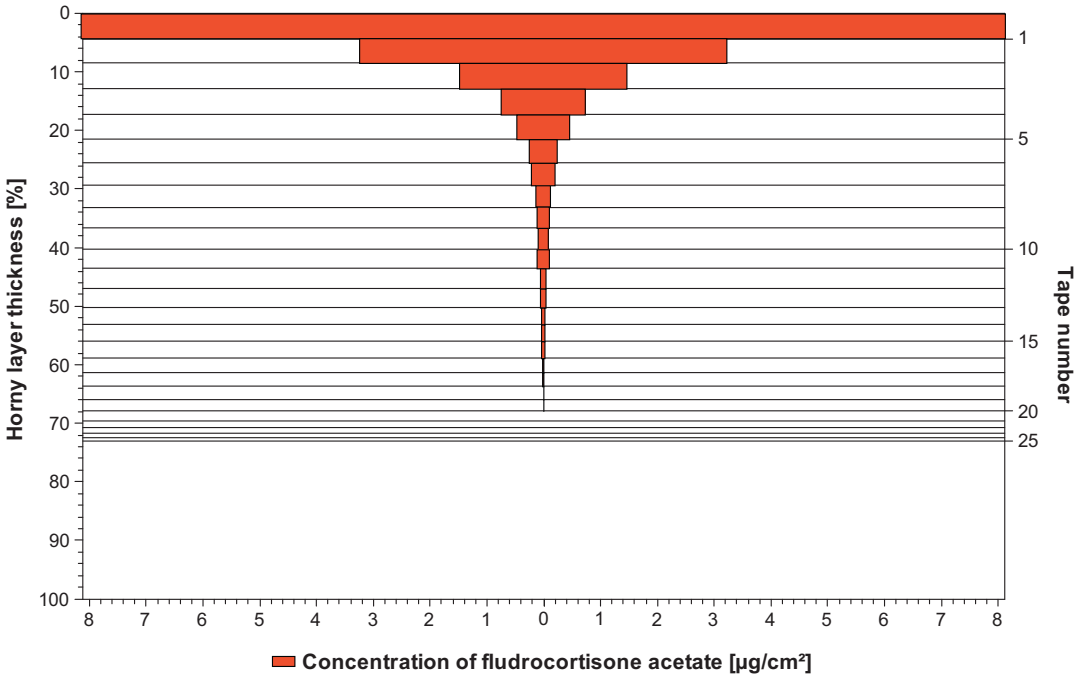

Fig. 3. Skin penetration profile of fludrocortisone acetate from a standard nanoemulsion (NE). The depicted values are means of eight individual experiments ($n = 8$). The distances between the horizontal lines correspond to the respective amounts of removed SC while the coloured areas represent the corresponding amounts of recovered drug on the respective tape strips. The upper line of the profiles corresponds to the skin surface, the lowest line to the boundary of living cells below the SC.

When comparing these findings to the results of the diffusion cell experiments, it may be assumed that the non-destructive manner of formulation application in the franz-cell setup might be associated with the permeation enhancement. In diffusion cell setups, the formulation is not massaged into the skin as during tape stripping and the emulsion structure remains intact. This preservation of the internal formulation structure might be the underlying cause of the disproportionately strong permeation enhancement effect through γ-CD especially at infinite dose conditions. Thus, the discussed solubility effects or changes in formulation structure caused by the incorporation of the CD may take hold.

It may be advisable to conduct additional tape stripping experiments when investigating the permeation enhancement potential of CDs in diffusion cell studies. With a conventional finite-dose in vivo application, the strong enhancement potential of the CD as observed in the franz-cell setup will not be realised to its full extent. The development of infinite-dose vehicles for in vivo application, such as matrix patches, might serve to overcome this limitation.

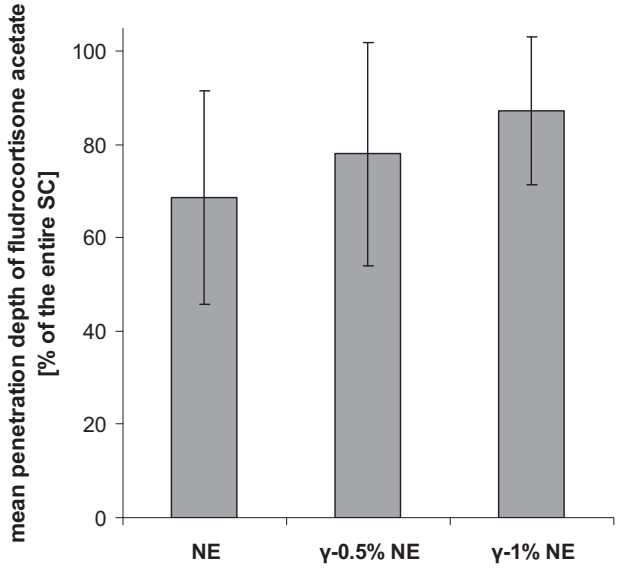

Fig. 4. Skin penetration depth observed after in vitro tape stripping with a standard nanoemulsion containing fludrocortisone acetate (NE) as well as corresponding nanoemulsions containing additional amounts of 0.5 or 1% of γ-CD. The presented values are means ± SD of at least 8 individual experiments ($n \geq 8$).

3.4. Optimisation of the franz-cell technique for nanoemulsion studies with steroidal drugs: conclusive remarks

Overall, we found that the classical franz-cell method consisting of using a skin thickness of 1.2 mm, infinite dose application of the formulation and pure phosphate buffer as acceptor medium led to the most reproducible results and is likewise the most practical working procedure in regard to skin preparation, sampling accuracy and quantification limits. In the case of formulations containing CDs, additional in vitro methods such as tape stripping experiments are recommended to detect potential overestimations caused by the conditions of the franz-cell setup.

4. Conclusion

Remarkably strong permeation enhancement effects for the steroidal model drug were obtained with the franz-cell setup at sufficiently high amounts of γ-CD, especially at infinite dose conditions. These effects were obviously related to the peculiarities of the experimental setup, where large amounts of formulation are applied without massaging them into the skin. The potential of this enhancement strategy cannot be realised to its full extent upon mechanical application of a finite dose on skin, as was shown in tape stripping experiments in vitro. As a next step, in vivo experiments would be of interest. Future research should focus on the development of novel dermal delivery systems such as matrix patches

enforced by CDs where infinite dose application may be employed while maintaining the formulations' original structure.

Conflicts of interest

No conflicts of interest occurred in the context of this study for any of the authors.

Acknowledgements

The authors would like to thank Wolfgang Sipos and the Veterinary University of Vienna for providing fresh porcine ears. The financial support of the research platform "Characterisation of Drug Delivery Systems on Skin and Investigation of Involved Mechanisms" is gratefully acknowledged.

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