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Ex vivo absorption of promestriene from oil-in-water emulsion into infant foreskin

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ABSTRACT

Hypospadias is a birth defect in which the urinary tract opening is not at the tip of the penis. Hypospadias surgery is frequently complicated by healing deficiencies. Topical treatments with oestrogens were reported to improve healing. In the present study, ex vivo percutaneous absorption of promestriene, a synthetic oestrogen resulting of the double esterification of estradiol was conducted as a pre-requisite for further clinical trial in infants. Penetration of promestriene into infant foreskin treated with commercial oil in water emulsion ($10\,\mu g\,mg^{-1}$) for 24 h was characterized showing controlled release properties enabling epidermal concentration more than six times higher than dermal concentration ($4.13 \pm 2.46\,mg\,g^{-1}$ versus $0.62 \pm 0.84\,mg\,g^{-1}$, respectively). Furthermore, apparent promestriene fluxes into and through the skin (i.e., $1.5\,\mu g\,cm^{-2}\,h^{-1}$ and $< 0.89\,\mu g\,cm^{-2}\,h^{-1}$, respectively) were calculated from (i) drug amount retained into epidermis and dermis, or (ii) the limit of detection into the receptor fluid. In conclusion, less than 2% of initial dose were absorbed within 24 h which compared well with others steroids applied topically in colloidal systems.

1. Introduction

Hypospadias is a common development halt of the penis whose causes are currently under investigation. Endocrine and epigenetic steroid mediated aetiology involving androgens and oestrogens is suspected (Liu et al., 2007; Qiao et al., 2012, 2011; Vottero et al., 2011; Wang et al., 2007). Hypospadias is characterized by a hypoplasia of the tissues forming the ventral aspect of the penis and an abnormal ventral opening of the urethra (Ghirri et al., 2009; Kraft et al., 2010; Mouriquand et al., 2009). Since 2005, hypospadias are classified in the 46, XY disorders of sex development (Houk et al., 2006). Surgery of hypospadias is commonly performed in infants with a significant complication rate mostly related to healing deficiencies (fistula and dehiscence) which could reflect some congenital tissular deficiencies. Preparation of the penile tissues prior to surgery with hormonal treatments was therefore reported with the aim of boosting the tissular healing capacities (Borer and Retik, 1999).

The first line hormonal treatment used was testosterone which is well known to increase the size of the penis in infants. Besides, testosterone was reported to impact upon the proliferative phase of healing which involves immune processes such as re-epithelialization and angiogenesis (Engeland et al., 2009), whereas oestrogens may influence the various phases of cutaneous repair including inflammation, proliferation and remodelling (Calvin, 2000) and are known to accelerate cutaneous wound healing (Ashcroft et al., 1997) and keratinize human inner foreskin (Pask et al., 2008). Recently, several publications demonstrated the negative effects of androgens on the skin healing process whereas oestrogens were found to have more positive effects (Gilliver and Ashcroft, 2007; Gilliver et al., 2007a, 2006, 2007b, 2008, 2009, 2003; Mills et al., 2005). Similar conclusions were drawn from a series of hypospadiac patients submitted or not to

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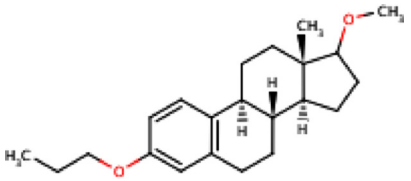
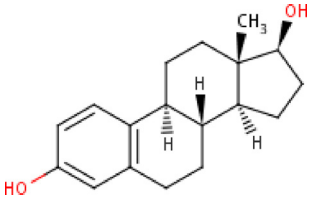
Table 1
Physico-chemical characteristics of promestriene and estradiol.

Steroids	Molecular weight (g mol^{-1})	Log P	Aqueous solubility (mg l^{-1})	Formula[a]
Promestriene	328.49[a]	5.61[b]	2.7.10^{-2}[c]	
Estradiol	272.39[a]	4.01[a]	3.6[a]	

[a] ChemIDplus advanced (http://chem.sis.nlm.nih.gov/chemidplus/).
[b] Advanced chemistry development/chemsketch® software.
[c] Virtual computational chemistry laboratory, ALOGPS 2.1 software.

pre-operative androgen stimulation (Gorduza et al., 2010). The next logical step is to evaluate the potential benefit of treating the penile tissues with pre-operative topical oestrogens without causing any systemic adverse effects. Promestriene (17β-methoxy-3-propoxyestra-1,3,5(10)-triene), a synthetic oestrogen resulting of the double esterification of estradiol, is topically used in the treatment of vaginal atrophy in postmenopausal women (Thomas et al., 1992) and shows interesting properties regarding cutaneous penetration (Table 1) (Del Pup, 2012; Del Pup et al., 2012; Lopez-Belmonte et al., 2012). Furthermore, comparative studies between promestriene and other oestrogenic compounds (i.e., estriol) in postmenopausal vaginal symptoms demonstrated effectiveness in reducing vaginal atrophy but discrepancies between studies concerning systemic exposure does not allow to conclude on harmlessness (Bruno et al., 2009; Lopez-Belmonte et al., 2012; Ouevedo et al., 2000). Therefore, in order to evaluate beneficial effects of promestriene in a clinical research project, a preliminary *ex vivo* study is mandatory to evaluate the penetration of promestriene into infant foreskin.

The aim of this *ex vivo* study was to evaluate penetration characteristics of promestriene into infant foreskin exposed to promestriene loaded commercial formulation (Colpotrophine®) for 24 h. Characterization of promestriene transport into infant foreskin was assessed from *ex vivo* penetration data enabling the evaluation of oestrogen cutaneous bioavailability.

2. Materials and methods

2.1. Promestriene loaded formulation

Promestriene loaded formulation (Colpotrophine®) was gratefully provided by Theramex® Laboratory–Merck Serono (Monaco). Colpotrophine® is an oil-in-water emulsion cream dosed at 10 μg mg^{-1} of promestriene mixed with different excipients (i.e., glycerol monostearate, macrogol 1000 cetostearate, decyloleate, triglycerides, glycerol, purified water, methyl- and propyl-*p*-hydroxybenzoate).

2.2. Reagents

Promestriene (purity: 100%) and 17β-3-dimethylestradiol (purity: 99.3%) provided by Theramex® Laboratory–Merck Serono (Monaco) were supplied by Monachem (Monaco). Methanol was purchased from VWR International S.A.S (Fontenay-sous-Bois, France). Fresh frozen human plasma was delivered by EFS Rhône-Alpes (Lyon, France).

2.3. Skin absorption studies

Infant foreskins were obtained during routine surgery of hypospadias or circumcision after inclusion in the Hypospades study protocole (A101311-16, ANSM, France) approved by the local centre of clinical investigation (Centre d'Investigation Clinique, Lyon, France). Foreskin samples were excised from infant foreskins, and then stored in aluminium foil at −20 °C before use. Freezing storage did not exceed two months. Skin specimens were cut into appropriate-sized pieces and mounted in carefully washed and disinfected static vertical Franz-type diffusion cells, with an effective surface area of 0.77 cm^2. The receptor compartments were filled, avoiding bubbles, by 9 mL of human plasma in which proteins have affinities with steroid hormones (Watanabe et al., 1991). Promestriene levels in human plasma were checked by high performance liquid chromatography to exclude interference with further dosage (cf. § 2.3). Therefore, the receptor compartments were immerged into a water-heated block maintained at 37 °C. The receptor fluids were stirred with a magnetic bar to provide homogenous circulation throughout the receptor compartment. Skin specimens were equilibrated with receptor fluid solution for 12 h before subsequent topical treatment. Eventually, the skin surface was exposed to 97.88 ± 13.14 mg (125 ± 17 mg cm^{-2}) of Colpotrophine® corresponding to 0.98 ± 0.13 mg (1.25 ± 0.17 mg cm^{-2}) of promestriene (n = 5 static vertical Franz-type diffusion cells mounted with 5 foreskin samples of different donors) delivered from pre-filled syringe. A large dose of test formulation was applied to skin surface instead of that recommended (1–5 mg cm^{-2}) in European procedure (OECD, 2004) to fit application procedure of the future clinical trial meant to facilitate further applications to infants by parents.

Receptor fluid solutions were collected (1 ml sample) at 1 h, 2 h, 4 h, 6 h, 8 h and 24 h and replaced by fresh medium to insure sink conditions. After 24 h, the skin surface of specimens was washed by 5 ml of demineralised water in order to remove unabsorbed formulation. Washing solutions were collected in tube containing 5 ml of 17β-3-dimethylestradiol (50 μg ml^{-1}) water-methanol solution (2.5:97.5, v/v). The skin surface areas were gently dried by using cotton swabs which were finally immerged in previous

Table 2
Determination of promestriene penetration into infant foreskin treated by ~100 mg of Colpotrophine® (promestriene concentration: 10 μg mg^{-1}) onto vertical static diffusion cells (skin surface area: 0.77 cm^2) for 24 h. Each data is the mean ± standard deviation of five experimental determinations.

Steroid	Dose applied (μg)	Dose absorbed (μg)		Concentration applied (mg g^{-1})	Tissue concentration (mg g^{-1})	
		Epidermis	Dermis		Epidermis	Dermis
Promestriene	979 ± 131	14 ± 4	14 ± 7	10	4.13 ± 2.46	0.62 ± 0.84

mixture of washing solution and water-methanol solution. The assessment of barrier function of foreskin was appreciated after absorption experiment by the measurements of transepidermal water loss (TEWL) and hydration recorded by SkinEvidence® Pro (La Licorne Laboratory, Grenoble, France) (Atrux-Tallau et al., 2010); both parameters fitted well with reference values found in literature (Sekkat et al., 2002). The epidermis was separated from the dermis (Atrux-Tallau et al., 2007) with a surgical sterile scalpel then weighted and finally homogenized in a vial filled with 1 ml 17β-3-dimethylestradiol (25 μg ml^{-1}) water-methanol solution (2.5:97.5, v/v) by using MiniBeadBeater® (Biospect Products, Bartlesville, Oklahoma, USA; 300 s, 2500 rpm). The tissue suspension was centrifuged for 10 min at 9000 rpm, and then the supernatants were filtered (Mura et al., 2007). The supernatants from receptor solutions and tissue suspensions as well as washing solutions were assayed for their promestriene and 17β-3-dimethylestradiol (internal standard) content by high performance liquid chromatography (cf. § 2.3).

2.4. Promestriene assay by high performance liquid chromatography (HPLC)

Standard solutions of promestriene (concentration range: 0–100 μg ml^{-1}) were prepared by dissolving drug in 17β-3-dimethylestradiol (25 μg ml^{-1}) water-methanol solution (2.5:97.5, v/v). Promestriene assays were carried out by reversed-phase adsorption chromatography using a Gemini 110 Å RP-18 (5 μm), 5 mm, (250 mm × 4.60 mm) HPLC cartridge mounted with a security guard cartridge (Phenomenex®, Le Pecq, France). The mobile phase was a mixture of demineralised water and methanol (2.5:97.5; v/v). The assays were carried out with a chromatograph HP 1200 Series (Hewlett Packard-France) equipped with a variable wavelength detector. Sample volume injected was 40 μl. The flow rate was 1.5 ml min^{-1}. Detection was performed at 278.5 nm. Retention times of promestriene and 17β-3-dimethylestradiol (internal standard) were 4.9 min and 3.9 min, respectively. For concentrations between 0 and 100 μg ml^{-1}, chromatograms were linear with a factor correlation of 0.999 ($P < 0.001$). The limit of detection and quantification of promestriene was about 1.83 μg ml^{-1} and 5.5 μg ml^{-1}, respectively.

2.5. Calculations

Permeation data analysis was carried out with Kaleidagraph® 3.6 software (Synergy software, Reading, PA, USA). Results were expressed as the mean ± standard deviation.

3. Results and discussion

3.1. Promestriene permeation through foreskin

In the present study, the percutaneous absorption of promestriene into and through foreskin was investigated *ex vivo* in order to appreciate its cutaneous bioavailability after 24 h skin exposure to commercial formulation dosed at 10 μg mg^{-1}. Promestriene assays were carried by HPLC for steroid contents in epidermis, dermis and receptor fluid solution. No promestriene content was detected, in the present study, into the receptor compartments (limit of detection 1.83 μg ml^{-1}) confirming minimal or poor permeation of promestriene as highly lipophilic steroid (Log *P* [octanol-water partition coefficient] ~ 6 calculated by using advanced chemistry development/chemsketch® software) into hydrophilic medium. Previous study using radiolabeled drug showed that less than 1% of initial dose (0.1–0.5 mg cm^{-2}) was absorbed within 6 h and subsequently excreted in human volunteers after topical application to forearms (Moore et al., 1983). Furthermore, the absorption of promestriene through vagina after unique 24 h application was found dramatically reduced as compared to estradiol, although promestriene traces were detected in blood of volunteers (0.2–0.5 ng ml^{-1}) (Thomas et al., 1992).

3.2. Promestriene penetration into foreskin

In the present study, promestriene penetration into epidermis and dermis was quantified after a unique application of commercial formulation dosed at 10 μg mg^{-1} for 24 h. Skin barrier function of foreskin samples estimated from TEWL (10 ± 3 g m^{-2} h^{-1}) and hydration (28 ± 6%) measurements was found effective confirming (i) the inocuity of treatment and (ii) the relevance of skin absorption procedures used in the present study. Table 2 reports foreskin absorption properties of promestriene into epidermis and dermis. The overall recovery of test substance was within the range of 85–115%. As expressed of absorbed dose, promestriene was found distributed equivalently between epidermis and dermis. However, taking account the weight of each tissue, the concentration of promestriene into the epidermis was found more than six-fold than that in the dermis. These results might be explained by (i) the efficiency of *stratum corneum* onto foreskin surface acting as barrier, (ii) the lipophilic structure of the epidermis constituting a reservoir for hydrophobic drugs and limiting further molecular penetration into the dermis. Therefore, after 24 h exposure, promestriene concentration in the epidermis and dermis counted for ~ 40 and 6 percent of initial vehicle concentration, respectively.

The apparent epidermis/vehicle, epidermis/dermis and dermis/receptor fluid partition coefficients (*Log K*) of promestriene were calculated from epidermal concentration determined after an equilibration period (i.e. 24 h skin exposure) following equations 1–3 respectively, in which concentration in vehicle was assumed to be constant over exposure time.

$$\mathrm{Log}\,K\,(e/v) = \mathrm{Log}\left[\frac{\mathrm{Concentration_{Epidermis}}}{\mathrm{Concentration_{Vehicle}}}\right] \quad (1)$$

$$\mathrm{Log}\,K\,(e/d) = \mathrm{Log}\left[\frac{\mathrm{Concentration_{Epidermis}}}{\mathrm{Concentration_{Dermis}}}\right] \quad (2)$$

$$\mathrm{Log}\,K\,(d/r) = \mathrm{Log}\left[\frac{\mathrm{Concentration_{Dermis}}}{\mathrm{Concentration_{Receptor\ fluid}}}\right] \quad (3)$$

Log *K*(*e*/*v*) (−0.40) was found uncorrelated with Log *P* (5.61). This discrepancy might be explained by the solubilization of promestriene in the oily dispersed phase of emulsion that would reduce subsequent steroid partitioning with *stratum corneum* intercellular lipids. Additionally, the average size of oily droplets

(~200 nm) would compromise further promestriene transport within intercellular lipids of the *stratum corneum*. Therefore, the high affinity of promestriene for the oily phase of the emulsified formulation provided release characteristics of the compound to the skin adapted to further secure clinical application to child.

Moreover, Log $K(e/d)$ (0.82) was found in favour of epidermis enabling to limit the penetration of promestriene to the dermis. Consequently, after 24 h, promestriene accumulation in the epidermis is more than 6 fold higher than that of dermis and its pharmacological activity is concentrated where the wound healing process occurs (i.e. in the upper layers of the skin). Notably, promestriene concentration was determined in whole epidermis instead of isolated *stratum corneum* which is recognized as a major barrier/reservoir for lipophilic compounds. Therefore, promestriene concentration in the upper parts of the epidermis (i.e., *stratum corneum*) might be underestimated.

Furthermore, taking into account the limit of detection of promestriene in the receptor fluid (i.e. 1.83 $\mu g\,ml^{-1}$), apparent fluxes of promestriene into and through the skin from oil-in-water emulsion formulation (1.52 $\mu g\,cm^{-2}\,h^{-1}$ and < 0.89 $\mu g\,cm^{-2}\,h^{-1}$, respectively) were calculated from skin surface, time exposure and (i) drug amount retained into epidermis and dermis, or (ii) maximal theoretical undetected amount of promestriene in the receptor fluid. Moreover, Log $K(d/r)$ (2.53) was found dramatically in favour of dermal retention. Consequently, less than 2% of initial dose might be absorbed through whole skin over 24 h skin exposure, confirming transcutaneous and transmucosal data reported elsewhere (Moore et al., 1983; Thomas et al., 1992; Wolff et al., 1982).

4. Conclusion

In the present report, an *ex vivo* study was designed for the characterization of promestriene absorption into and through foreskin treated by a commercial oil in water emulsion applied for 24 h. Permeation of promestriene through foreskin mounted onto vertical static diffusion cells was not detected. However, steroid penetration into both epidermis and dermis was quantified. Such permeability was found in accordance with previous studies dealing with skin absorption of steroids. Those results characterizing promestriene foreskin availability reply to pre-requisite mandatory for further clinical trial in infants.

Conflict of interest

The authors declare no conflict of interest

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