Regional differences in the in vitro penetration of hydrocortisone through equine skin

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Little is known about the transdermal penetration of hydrocortisone in the horse and, although commercial formulations containing hydrocortisone are registered for topical use in the horse, there have been no studies investigating the movement of this glucocorticoid through different regions of equine skin. Skin was harvested from the thorax, groin and leg (dorsal metacarpal) regions of five Thoroughbred geldings and frozen (−20 °C) until required. Defrosted skin was placed in Franz-type diffusion cells and the amount of radiolabelled (3H) hydrocortisone, in a saturated solution of unlabelled hydrocortisone in 50% ethanol (w/w), which penetrated through and remained within skin samples was measured over 24 h. Significantly higher (P < 0.001) maximum flux (Jmax; mol/cm²/h) was measured when hydrocortisone was applied to skin from the leg, compared to thorax and groin, although significantly less hydrocortisone (P < 0.001) was retained within skin from the leg at 24 h. Topical application of hydrocortisone in a vehicle containing ethanol would penetrate faster through leg skin from the lower leg when compared with the thorax or groin, which depending on cutaneous blood flow, may result in higher systemic drug concentrations or greater efficiency in treating local inflamed tissue.

(Paper received 4 August 2005; accepted for publication 21 October 2005)

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INTRODUCTION

Glucocorticoids are a potent class of anti-inflammatory drugs that affect virtually every cell type and system in mammals (Adams, 2001; Schacke et al., 2002). Administration of exogenous glucocorticoids in horses, while therapeutically beneficial, has been implicated in a range of adverse effects, including early induction of parturition (Jelfcott & Rossdale, 1977), laminitis (Johnson et al., 2002) and suppression of the hypothalamus-hypophysis-pituitary axis (Schacke et al., 2002).

Topical administration of glucocorticoids is frequently used in inflammatory skin disease to achieve relatively high local drug concentration (Magnusson et al., 2001; Roberts et al., 2002), yet minimize the systemic drug absorption and, therefore, the likelihood of adverse effects (Schacke et al., 2002). Furthermore, low or negligible systemic glucocorticoid concentrations following topical application will minimize the risk of infringing the Rules of Racing if inflammatory skin diseases require treatment close to the race day.

Little is known about transdermal penetration of glucocorticoids in the horse. One of the more popular active ingredients of topical anti-inflammatory creams is hydrocortisone, which is available in a range (0.5–1.0%) of concentrations (Australian Prescription Products Guide, 2002). Active drug is normally formulated into a vehicle comprising non-active constituents, such as ethanol (EtOH) or propylene glycol, which may enhance transdermal drug movement. However, many of the commercially available topical formulations of hydrocortisone were developed following studies for human use, with little or no studies investigating the specific penetration of hydrocortisone through equine skin. Since extrapolation of transdermal penetration of drugs between species is highly unreliable (Walters & Roberts, 2002), there is no scientific evidence to demonstrate the permeability of hydrocortisone into or through equine skin.

In this study, we used a saturated solution of hydrocortisone in an aqueous ethanol vehicle, a common vehicle in topical formulations, to determine penetration of hydrocortisone into and through equine skin. The effect of anatomical site on hydrocortisone penetration was also compared by using skin collected from three different regions: thorax, groin and leg (dorsal metacarpus).
MATERIALS AND METHODS

Horse skin

Horse skin was harvested from five Thoroughbred geldings, 4–8 years old and weighing 405 ± 14 kg, which had been presented to the University Of Queensland Veterinary School for killing. The horses were killed by an i.v. injection of sodium pentobarbital (Lethabarb; Virbac, Peakhurst, NSW, Australia) and the hair over the skin harvest sites was removed by electric clippers. The skin over the thorax (central thorax, approximately mid-way between costochondral junction and vertebrae, groin and leg (dorsal metacarpal) regions was dissected away, with care to trim off subcutaneous fat, and frozen at −20 °C and used within 48 h (Mills et al., 2004). Sections from each region were prepared for microscopic examination and stained with haematoxylin and eosin to compare gross ultrastructural differences (Fig. 1). This protocol was approved by the Animal Ethics Committee of the University of Queensland (approval number: SVS/087/04/RIRDC).

Chemicals

Radiolabelled [³H]-hydrocortisone (specific activity 25 mCi/ mmol) was purchased from American Radiolabelled Chemicals Inc (St Louis, MO, USA). Scintillation fluid (Emulsifier Safe, OCS) was purchased from Packard (Meriden, CT, USA). All other chemicals, including hydrocortisone and albumin (bovine-fraction V) were purchased from Sigma (St Louis, MO, USA).

In vitro skin penetration

Skin was allowed to thaw at room temperature and cut into circular sections (approximately 2 cm diameter) and mounted in Franz-type diffusion cells with the stratum corneum side being uppermost. A measured volume (approximately 3.5 mL) of phosphate-buffered saline (PBS, pH 7.4) containing 4% bovine serum albumin as a receptor fluid was added to the lower reservoir with a magnetic flea for stirring. One milliliter of PBS was added to the donor reservoir and the skin cell was placed in a water bath containing a magnetic stirring plate and allowed to equilibrate at 35 °C for 60 min. The temperature of the skin surface in the diffusion cell was approximately 32 °C. The PBS was removed from the donor reservoir and 1 mL of donor solution (EtOH 50% in PBS w/w) saturated with hydrocortisone and containing a tracer concentration of radiolabelled hydrocortisone (0.5–1.0 μCi), was added (t = 0). A glass cover slip was used to occlude the donor chamber and prevent evaporative loss. A 200 µL sample was collected from the receptor fluid via a side-port of each diffusion cell and immediately replaced with fresh solution at 2, 4, 8, 14, 20 and 24 h. The 200 µL sample was placed in scintillation vials with 2.0 mL of scintillation fluid and the radioactivity in each vial was measured using preset channels of a TriCarb 2700TR Liquid Scintillation Analyzer (Packard). An aliquot (20 µL) was collected from the donor reservoir of each diffusion cell at t = 24 h to determine the depletion of radiolabelled drug, compared with the initial donor solution (donor recovery). At the completion of each study, skin samples were removed from the diffusion cell, rinsed in distilled water, placed in preweighed scintillation vials and accurately weighed (Sartorius CP Analytical Balance, Goettingen, Germany). Two milliliters of tissue solubilizer was added before incubation at 60 °C (140 °F) for 48 h. Two milliliters of scintillation fluid was then added to each sample and radioactivity was
assessed as described above. The reported values represent a mean of the five horses [each time point and site represent the mean of three replicates (three different skin samples) for each horse] for each data point.

**Analysis of saturated solubility concentration of hydrocortisone**

Saturated solutions of hydrocortisone in the donor phase were prepared by dissolving excess hydrocortisone in 50% EtOH in PBS (w/w) and then roller-mixing the solutions at 30 °C for 24 h. The resulting suspension was centrifuged (300 × g for 10 min) and the supernatant was then diluted to four different concentrations (1:10; 1:100; 1:500 and 1:1000) with the donor phase. These were analysed for hydrocortisone concentration using a high-pressure liquid chromatography system (HPLC) consisting of a Shimadzu 10A pumping system, a Shimadzu Sil 9A auto-injector (Sil 9A) and a Shimadzu 10AXL UV/Vis detector at 254 nm, using Shimadzu VP Chromatography software (Rydalmere, NSW, Australia). A Phenomenex Luna C18 5 μ 150 × 4.6 mm steel column was used. The mobile phase was 40% acetonitrile in water, filtered and degassed through a 0.45 μm filter, pumped at a flow rate of 1.0 mL/min. Inter- and intrarun precision (CV%) were 4.2% and 2.7% respectively.

**Data analysis**

Permeability coefficients (\(k_p\); cm/h), relating solute flux to the concentration gradient across the membrane (i.e. how fast a drug molecule travels through skin), were calculated from the pseudo-steady-state portion of the receptor compartment concentration versus time profile, according to the following formula (Flynn et al., 1981):

\[
k_p = V_R \left( \frac{dC}{dt} \frac{A}{\Delta C} \right)
\]

where \(V_R\) is the receiver volume, \(dC/dt\) is the steady-state rate of change in the receptor concentration (counts per minute), \(A\) is the exposed cross-sectional area of the membrane and \(\Delta C\) is the concentration (counts per minute of radioactivity) differential between compartments. Maximum flux \(J_{max};\) mol/cm²/h was predicted from \(k_p\) multiplied by the solubility in the donor phase. In aqueous solutions, \(J_{max}\) estimates the maximum amount of drug that can travel across a defined area of skin per unit time from the product of skin area, \(k_p\) and aqueous solubility (Hagraft, 1996). The apparent diffusion coefficient \(D_R\), describing the rate of drug movement through skin, was calculated according to the formula (Rosado et al., 2003):

\[
D_R = J_R/K_R
\]

where \(K_R = (\text{dpm in membrane at end of experiment})/(\text{dpm in donor})\), \(J_R = (\text{cumulative dpm in receptor vs. time gradient})/(\text{dpm in donor})\).

Differences in \(k_p, J_{max}\) and skin retention for each site (mean of five horses per site) from each horse were compared using one-way ANOVA over application site using Microsoft Office Excel 2003 with Tukey’s post hoc test for pairwise comparisons.

**RESULTS**

Histological examination of the skin revealed a similar thickness in the stratum corneum and dermis, but visibly less numbers of hair follicles from skin harvested from the leg region. The solubility of hydrocortisone in 50% EtOH in PBS w/w was 8.68 ± 0.49 g/L. Hydrocortisone recovery from donor solution applied to the thorax, groin and leg were 113.1 ± 4.6%, 110.2 ± 5.1% and 107.6 ± 3.8% respectively. Values for \(k_p\) and \(D_R\) were lower in the thorax (5.88 ± 0.56 × 10⁻⁵ cm/h and 2.86) and groin (5.33 ± 1.56 × 10⁻⁵ cm/h and 3.97) regions when compared with the leg (10.01 ± 0.89 × 10⁻⁵ cm/h and 11.02; \(P < 0.001\)). Similarly, \(J_{max}\) was significantly higher \((P < 0.001)\) when hydrocortisone in 50% EtOH was applied to the leg, compared with thorax and groin (Fig. 2). In contrast, significantly less hydrocortisone \((P < 0.001)\) was retained within skin from the leg when compared with thorax and groin regions after 24 h of application (Fig. 3).

**DISCUSSION**

We have measured, for the first time, the penetration of hydrocortisone through different regions of equine skin. This model of *in vitro* drug penetration permits us to study transdermal drug movement over 24 h, where we were able to observe a significantly higher \((P < 0.001)\) permeability rate \((k_p)\), maximum achievable total hydrocortisone penetration \((J_{max})\) and estimated diffusivity \((D_R)\) within the skin of the leg (dorsal metacarpal region) compared with other sites. Previous studies (Mills et al., 2005) comparing the application of different vehicle systems containing hydrocortisone on dog skin showed that the maximum flux of hydrocortisone was significantly higher for all sites (neck, thorax and groin) when dissolved in a vehicle containing 50% EtOH, compared with PBS alone or 50% propylene glycol with differences more prominent in skin from...
the neck region ($P < 0.0001$). The findings in the horse suggest that hydrocortisone applied topically would penetrate through skin of the lower leg at almost twice the rate as through upper body regions using the vehicle and application conditions used in the current study.

Several factors may account for regional differences in transdermal drug penetration, particularly appendageal density (Tur et al., 1991; Hueber et al., 1992, 1994) and the thickness of the major barrier to drug and water movement through skin, the stratum corneum (Magnusson et al., 2001; Roberts et al., 2002). It has been suggested that certain drugs may penetrate via appendageal openings (Hueber et al., 1992, 1994), although in the current study, there was no significant difference between hydrocortisone penetration through skin from the thorax (quite hairy) compared with the groin (only some fine hair visible). These results are consistent with studies performed using dog skin where hydrocortisone penetration through thorax and groin skin was similar, yet significantly higher values were observed in the neck (Mills et al., 2005). Previous studies have shown a similar thickness of stratum corneum and blood flow in the thorax and groin regions of the horse (Monteiro-Riviere et al., 1990; Manning et al., 1991), but thickness of the stratum corneum overlying the leg has not been reported. In addition, the number of layers of the stratum corneum may influence solute permeation due to possible lateral bilayer diffusion (Johnson et al., 1997). Preliminary histological examination in the current study suggests that the stratum corneum is of a similar thickness in the three regions, although there appears to be substantially lower numbers of hair follicles visible in skin from the leg. Further studies are required to specifically measure appendageal density from different regions of the horse and to compare the biochemical composition of the stratum corneum, particularly the type and amount of lipid components.

One factor that may affect the composition of the stratum corneum, including the lipid components, is the constituents of the vehicle in any topical formulation (Barry, 2001). Mixtures of ethanol and water have been shown to extract lipids from the stratum corneum and alter the partitioning of drugs into the stratum corneum (Levang et al., 1999; Van der Merwe & Riviere, 2005). Ethanol–water systems can therefore be used to enhance the flux of solutes, including nitroglycerin (Berner et al., 1989), salicylate (Kurihara-Bergstrom et al., 1990) and naloxone (Panchagnula et al., 2001). There appears to be an optimal ethanol volume fraction of 0.6–0.7 to enhance solute flux (Berner et al., 1989; Kurihara-Bergstrom et al., 1990) with the ethanol–water (PBS) vehicle system used in the current study within this fraction, which would therefore be expected to enhance the penetration of hydrocortisone through equine skin.

The permeability of the stratum corneum will also be affected by hydration. Keratinocytes swell as they absorb water into the intracellular keratin matrix, disrupting the organized layers of the stratum corneum. Stratum corneum permeability has been shown to increase rapidly with water uptake then reach a steady-state of diffusion (Roberts & Walker, 1993), which contributes to the effectiveness of transdermal drug delivery from skin patches (Riviere & Papich, 2001). In the current study, it was assumed that since the thickness of the stratum corneum was similar at each site, the effect of hydration caused by application of the solvent system in the receptor chamber of the diffusion cell would also be similar and not contribute to regional differences of drug penetration.

A difference of the in vitro model of drug penetration compared with in vivo models in the horse is that, drug movement is independent of blood flow. This is important because variation in blood flow can significantly affect transdermal drug movement (Monteiro-Riviere et al., 1993; Qiao et al., 1993). The in vitro skin sections do not have functional intact microcapillaries whereas an in vivo study may provide greater regional differences in transdermal drug penetration due to blood flow differences. Furthermore, hydrocortisone will induce vasoconstriction of local vasculature, a feature used to determine efficacy of hydrocortisone formulations in vivo (Barry, 1976; Caron et al., 1990). However, laser-Doppler velocimetry studies have shown that blood flow through skin over the leg (dorsal metacarpal region: $10.16 \pm 2.92 \text{ mL/min/100 g of tissue}$) was slightly higher than the groin (ventral abdomen: $8.91 \pm 1.46 \text{ mL/min/100 g of tissue}$) and thorax (at humeroscapular junction: $6.76 \pm 1.49 \text{ mL/min/100 g of tissue}$) (Manning et al., 1991). A visible inspection of the histological section also suggested similar dermal thickness, although specific differences in the dermal thickness and composition were not considered in this study. It would appear likely that greater penetration of hydrocortisone in 50% EtOH, combined with a higher blood flow, may result in higher systemic drug concentrations if this formulation was applied in vivo to the leg of a horse, compared with thoracic and groin regions.

A potential criticism of this model is the use of skin that has been frozen and then defrosted as required. Grafted equine skin has been shown to retain viability when refrigerated for up to 3 weeks (Schumacher et al., 1987), but it is generally acknowledged that frozen skin will have diminished metabolic and biochemical function (Riviere & Papich, 2001; Roberts et al.,
2002). However, freezing is unlikely to affect the structure of the stratum corneum (Harrison et al., 1990). In vitro use offers similar barriers to topically applied compounds and diffusion cells are suitable to estimate the penetration of topically applied xenobiotics. Individual variation is also reduced as different regions on the same animal can be compared simultaneously.

In addition to the regional differences between leg and upper body, the current study has also demonstrated the necessity to investigate transdermal drug penetration in the species of interest. Differences in skin thickness, composition and number of appendages (hair follicles and glands) make extrapolation between species impractical (Magnusson et al., 2002; Walters & Roberts, 2002). A comparison of a similar study in the Greyhound dog (Mills et al., 2005) reveals a higher (five-fold for the groin region) maximum flux of hydrocortisone in 50% EtOH in the horse. Blood flow through the groin region is similar for the dog and horse, although a thicker stratum corneum (12.20 ± 2.12 vs. 8.19 ± 1.38 μm) in the dog has been reported (Monteiro-Riviere et al., 1990). Ultrastructural differences in skin from the dog and horse have also been reported (Monteiro-Riviere, 1991). For example, the relative proportion of individual lipids within the epidermis of pigs was constant across several body regions, while greater total lipids were found on the back region (Monteiro-Riviere et al., 2001). Significantly lower skin residues after application of hydrocortisone in 50% ethanol to the leg may reflect variability in dermal and epidermal constituents, compared with upper body regions. Further studies are required with commercial formulations (0.5 and 1.0% hydrocortisone creams) to confirm this regional variation.

In conclusion, regional differences in the penetration of hydrocortisone through equine skin have been found, with significantly greater maximum flux through skin of the dorsal metacarpal region, compared with thorax and groin. This finding may have implications for commercial hydrocortisone preparations that may be applied topically to the horse.

ACKNOWLEDGMENTS

This study was made possible by the generous support of the Rural Industries Research Development Corporation (RIRDC).

REFERENCES


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