Penetration of chlorhexidine into human skin

Short running title: Skin penetration of chlorhexidine

T. J. Karpanen*, T. Worthington¹, B. R. Conway¹, A. C. Hilton¹, T. S. J. Elliott² and P. A. Lambert¹.

1. Life& Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, UK
2. Selly Oak Hospital, University Hospital Birmingham NHS Foundation Trust, Raddlebarn Road, Selly Oak, Birmingham B29 6JD.

* Corresponding author. Mailing address: Life and Health Sciences, Aston University, Aston Triangle, B4 7ET, UK. Tel. (44) 121 204 3951, fax. (44) 121 204 4187. E-mail: karpantj@aston.ac.uk.

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Abstract

This study evaluated a model of skin permeation to determine the depth of delivery of chlorhexidine into full thickness excised human skin following topical application of 2% (w/v) aqueous chlorhexidine digluconate. Skin permeation studies were performed on full-thickness human skin using Franz diffusion cells and exposed to chlorhexidine for 2 min, 30 min and 24 h. The concentration of chlorhexidine extracted from skin sections was determined to a depth of 1500 µm following serial sectioning of the skin using a microtome and analysis by high performance liquid chromatography. Poor penetration of chlorhexidine into skin following 2 min and 30 min exposure to chlorhexidine was observed (0.157 ± 0.047 and 0.077 ± 0.015 µg/ mg tissue within the top 100 µm) and levels of chlorhexidine were minimal at deeper skin depths (less than 0.002 µg/ mg tissue below 300 µm). After 24 h exposure, there was more chlorhexidine within the upper 100 µm sections (7.88 ±1.37 µg/ mg tissue), however the levels remained low (less than 1 µg/ mg tissue) at depths below 300 µm. There was no detectable penetration through the full thickness skin. The model presented in this study can be used to assess the permeation of antiseptic agents through various layers of skin in vitro. Aqueous chlorhexidine demonstrated poor permeation into the deeper layers of the skin, which may restrict the efficacy of skin antisepsis with this agent. This study lays the foundation for further research in adopting alternative strategies for enhanced skin antisepsis in clinical practice.
Introduction

Effective skin antisepsis is essential in preventing infections associated with invasive procedures, such as intravascular catheter insertion or surgery. A range of skin antiseptic agents are available in the clinical setting, such as povidone-iodine and chlorhexidine compounds at various concentrations with alcoholic or aqueous solutions. However, 2% (w/v) chlorhexidine solution is the recommended agent to be used prior to invasive procedures by the EPIC (Evidence-based practice in infection control) and CDC (Centres for disease control and prevention) guidelines (18,19). Two percent chlorhexidine digluconate (CHG) has been shown to significantly reduce intravascular catheter related infections (14), yet 2% (w/v) CHG in 70% (v/v) isopropyl alcohol (IPA) demonstrates superior activity compared to aqueous CHG solution in preoperative skin preparation (9) and in vitro carrier tests (1). However, there is little known about the kinetics of chlorhexidine skin permeation from either of these solutions (11,25). Microorganisms colonising the skin not only reside on the skin surface but are also found to inhabit hair follicles and lower skin depths (8). Many antimicrobial agents exhibit restricted permeation of the skin (8) and fail to reach the deeper layers, including the hair follicles, which harbour coagulase negative staphylococci (2,7,8,13,15) and propionibacteria (13). Commensal microorganisms may therefore persist at the site of incision following skin antisepsis (4,22) and such resident organisms may cause infection when the protective skin barrier is breached during surgical procedures (12,20,26). Therefore, effective and rapid permeation of the applied antiseptic agent into the deeper layers of the skin is essential in preventing infections associated with invasive procedures.
The aim of this current study was to use the Franz-cell skin model (6) to
determine the penetration profile for CHG through excised human skin, and to evaluate
the skin permeation of 2% (w/v) aqueous CHG into the skin using this model.
Materials and methods

Materials

Chlorhexidine digluconate (CHG), diethylamine (HPLC grade), dimethyl sulphoxide (DMSO), phosphate buffered saline (PBS), sodium heptane sulphonate (HPLC grade) and Tween 80 were purchased from Sigma-Aldrich (Dorset, UK). Acetic acid and methanol (both HPLC grade) were purchased from Fisher Scientific (Leicestershire, UK).

Skin samples

Full thickness human skin samples were obtained from patients undergoing breast reduction surgery (The Stephen Kirby Skin Bank, Queen Mary’s Hospital, London, UK) and full ethical committee approval was obtained prior to this study (REC 2002/169). The full thickness human skin was frozen on the day of excision and stored at -70°C until required.

Quantification of CHG

High-performance liquid chromatography (HPLC) was used to measure the amount of CHG in the skin samples obtained during the permeation studies. The analyses were performed using an Agilent 1200 series HPLC system (Agilent Technologies, UK). The samples were run at a flow rate of 1.2 mL/min at room temperature through a reverse phase chromatography column (CPS-2 Hypersil 5µm column dimension 150 x 4.6mm (Thermo Electron Corporation, UK)), with ultraviolet detection at 254 nm. The isocratic mobile phase consisted of methanol: water mixture (75:25) with 0.005 M sodium heptane.
sulphonate and 0.1% (v/v) diethylamine adjusted to pH 4 with glacial acetic acid. The HPLC method was validated by repeating a series of standardised CHG concentrations five times and plotting a graph of peak area versus CHG concentration. The level of detection (LOD) and level of quantification (LOQ) were calculated from the standard curve according to the following equation:

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\text{LOD} = 3 \times \text{standard deviation (SD) } / \text{slope}, \text{ and the LOQ} = 10 \times \text{SD} / \text{slope.}
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**Skin permeation studies**

Skin permeation studies were performed with vertical Franz diffusion cells (figure 1). The receptor compartment was filled with 29 mL of PBS and maintained at 37˚C by a circulating water jacket and agitated by stirring with a magnetic bar. Skin samples were thawed in PBS at room temperature, dried with an absorbent towel and mounted onto Franz diffusion cells with the stratum corneum (SC) uppermost facing the donor compartment. The surface area exposed to the test compound was 3.14 cm² (2 cm in diameter). All entrapped air was removed between the skin and receptor fluid and the skin was left to equilibrate for 30 min to reach the skin surface temperature of 32˚C.

Twenty percent (w/v) aqueous CHG was diluted with distilled water and 0.1% (v/v) Tween 80 to obtain the final test solution 2% (w/v) CHG. One millilitre of test solution was spread over the skin surface in the donor compartment and the compartment was sealed with a moisture resistant film (Parafilm M®, Alcan packaging, USA) to prevent evaporation. One millilitre of receptor fluid was removed every 30 min for 2 h, every hour between 2 to 6 h and at 8 h, 12 h and 24 h. Fluid removed from the receptor
compartment was immediately replaced with an equal volume of fresh PBS solution. All
samples were filtered through a 0.45 µm nylon filter (Kinesis, UK) and analysed by
HPLC. The assay was performed in triplicate and on two different donor skin samples.

**CHG penetration profile studies**

Excised full thickness human skin samples were mounted onto the Franz diffusion cells
as described above, and exposed to 2% (w/v) CHG for 2 min, 30 min and 24 h. Following
exposure, the skin samples were removed, washed with PBS and dried with an absorbent
towel. The skin samples were immediately sprayed with a cryospray (Bright Instruments)
and frozen at -20°C. Punch biopsies (7 mm in diameter) were cut from each frozen
sample in triplicate and placed onto a cork disc in embedding compound (Bright
Instruments, Cambs, UK). The frozen samples were sectioned horizontally with a
microtome (Bright Instruments) into 20 µm sections (from the surface to a depth of 600
µm) and 30µm sections (from depths of 600 to 1500 µm). Each section was placed into
an Eppendorf tube and the total weight of each skin sample determined. Chlorhexidine
was extracted from the skin by placing 1 mL of HPLC mobile phase solution into each
tube followed by incubation of the sealed tubes at 60°C for 1 h. Following this, the
samples were analysed by HPLC and the concentration of CHG (µg/ mg of skin)
determined. Control skin (skin without treatment) was analysed parallel to the test
samples. Effective elution and recovery of CHG from the skin by this method was
confirmed prior to the experiment by injecting a standardised quantity of CHG (128 µg)
to ten skin samples, extracting the CHG and determining the recovered amount (94.4 ±
1.82 %, data not shown).
Results

HPLC validation

The mean retention time for CHG was 3.6 min. There were no intervening peaks from endogenous contaminating compounds within skin samples. The HPLC method gave a linear response ($R^2 = 0.999$) over the concentration range of 0.0039 µg/mL to 128 µg/mL. The level of detection and LOQ were calculated at 0.016 µg/mL and 0.052 µg/mL respectively.

Skin permeation studies

No CHG was detected in the receptor compartment during the 24 h exposure of excised full thickness human skin to 2% (w/v) aqueous CHG.

CHG retention studies

After 2 min, 30 min and 24 h, concentrations of chlorhexidine within the skin were highest in the surface 100 µm sections, and reduced below depths of 300 µm (Figures 2 and 3). The concentration of CHG within the top 100 µm sections of skin were 0.157 (± 0.047) µg/ mg tissue and 0.077 (± 0.015) µg/ mg tissue after 2 min and 30 min exposure to 2% (w/v) CHG respectively (figure 2). The concentration of CHG within deeper layers (below 300 µm) fell to less than 0.002 µg/ mg tissue following both 2 min and 30 min exposure. The difference between the amount of chlorhexidine within the top layers between 2 min and 30 min exposure was not significant (p> 0.05) (student t-test, INSTAT2, Graphpad, San Diego, CA, USA). The concentration of CHG was
significantly higher within all skin sections following 24 h exposure to CHG compared to the shorter exposure times. The concentration of CHG was 7.88 (± 1.37) µg/ mg tissue within the upper 100 µm sections, and less than 1 µg/ mg of tissue at depths of 300 µm and below.
Discussion

This study demonstrates that 2% (w/v) chlorhexidine, the antiseptic agent recommended within EPIC and CDC guidelines for skin antisepsis prior to central venous catheter (CVC) insertion, poorly permeates into deeper layers of the skin after 2 min and 30 min exposure to the antiseptic. The concentration of CHG within the upper 100 µm sections of skin was 0.157 (± 0.047) µg/ mg tissue and 0.077 (± 0.015) µg/ mg tissue after 2 min and 30 min respectively. If 1 g of tissue is estimated to equal 1 ml, these levels are higher than the concentrations required to kill many common skin microorganisms such as *S. epidermidis* in *in vitro* conditions (10). Below 300 µm, the CHG concentration remained less than 0.002 µg/ mg tissue, which may not be effective at eradicating microorganisms on skin (17), especially microorganisms residing deep in the hair follicles. Furthermore, chlorhexidine activity is reduced in the presence of organic compounds, such as fatty acids and at lower pH (16) and therefore may reduce the efficacy of skin antisepsis with CHG. An exposure time of 2 minutes was used to reflect the clinical conditions used prior to surgery (5). Although the 2 minute study appears to show a higher amount of bound chlorhexidine than the 30 minute study, there is variability in concentrations measured in the top layers, as is expected at the shorter exposure period (24), and the difference between 2 min and 30 min exposure is not significant (p> 0.05). It is likely that a steady state has not been reached yet at 2 min. A similar phenomenon was reported by Wagner *et al.* (23). Skin was also exposed to 2% (w/v) CHG for 24 h and the concentration of CHG in the deeper sections, i.e., beyond 300 µm, was less than 1 µg/ mg tissue. These levels of CHG are more than the minimum
bactericidal concentrations for many skin commensals (10), however this level of CHG was only obtained after a prolonged contact time of the skin with CHG. In this study no detectable levels of CHG was recovered from the receptor compartment suggesting that aqueous CHG does not permeate through the full thickness of excised skin and is retained within the tissue. These results support previous research on another CHG-based compound, chlorhexidine phosphanilate, which was also shown not to permeate through full thickness skin samples (25).

In this study, a model for studying the delivery of CHG into excised full thickness human skin was evaluated. Skin permeation studies are commonly performed in vitro with vertical or horizontal diffusion cells using skin or artificial membranes. This study was performed using vertical diffusion cells (Franz type diffusion cells), to evaluate the delivery of CHG through excised full thickness human skin. Such conditions mimic the in vivo environment by maintaining the physiological receptor fluid at body temperature and the skin surface temperature of 32°C (6,23). Skin permeation studies generally evaluate drug delivery through the skin by measuring drug diffusion into the receptor fluid through the SC or epidermis, which are the main barriers for skin permeation. However, the use of stripped skin layers, such as isolated SC or epidermal layers, for drug permeation studies may influence the results with possible retention of the drug in the dermal layers of the skin will not be considered. Full thickness skin was used in this study to determine the location of CHG throughout the skins, rather than studying flux of the drug through the barrier layers. Following exposure to CHG, the full thickness human skin was sectioned to a depth of 1500 µm by sequential sectioning with a microtome producing a total of 60 sections per skin sample. Skin sectioning has been used in many
previous studies (21), however the SC is often removed by tape stripping prior to sectioning of the skin. In this study the full thickness skin samples were sectioned throughout the sample without prior removal of the surface layers. This study demonstrates that the CHG permeation through the full thickness skin was not linear, which was expected due to the variation in structure at various layers. The top 100 µm layer of the skin, which contains SC (average 10- 20 µm thick) and other epidermal layers (50 – 100 µm thick), contained the highest amount of CHG following exposure to 2% (w/v) CHG over all time points studied. Previous research has shown that the main permeation barrier for skin absorption is the SC (3,11,25), which is thought to be due to its high lipid matrix and packed layers of keratinised epithelial cells. Furthermore, this study found that below 300 µm, at the dermal layer, the level of CHG remained constantly low. Depending on the body site, dermis contains hair follicles and other skin appendages, including sebaceous glands and sudoriferous glands (sweat producing glands), which are of interest in skin antisepsis as they may be niches for microbial colonisation of the skin following skin antisepsis (7,8). It is generally recognised that skin antisepsis does not sterilize the skin; our study confirms this and demonstrates that it may be due to poor permeation of chlorhexidine into the deeper layers of the skin.

In conclusion, this study showed poor permeation of chlorhexidine through excised full thickness human skin after 2 min and 30 min exposure to aqueous 2 % (w/v) CHG. The level of CHG were highest within the top 100 µm sections of skin, and remained consistently low within the deeper layers. Furthermore, the model presented in this study is a valuable tool in determining a permeation profile for chlorhexidine through human skin in vitro. This study lays the foundation for further research within this area.
with a view to potentially adopting alternative strategies for enhanced skin antisepsis in clinical practice.
Acknowledgments

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References


Figure 1. Diagram of Franz diffusion cell. Receptor compartment was filled with PBS, which was kept at 37°C by circulating water jacket. The skin was mounted between the receptor and donor compartment and clamped. The test drug was aliquoted onto the donor compartment. The drug diffused through the skin was sampled by drawing up receptor fluid via sampling port.
Figure 2. Penetration profile showing the location of chlorhexidine (µg/mg tissue) in excised human skin after 2 min and 30 min exposure to aqueous 2% (w/v) chlorhexidine digluconate (mean ± s.e., n=15).
Figure 3. Penetration profile showing the location of chlorhexidine (µg/ mg tissue) in excised human skin after 2 min and 30 min (n=15) and 24 h (n=30) exposure to aqueous 2% (w/v) chlorhexidine digluconate (mean ± s.e).