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1 **Penetration of chlorhexidine into human skin**

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3 **Short running title:** Skin penetration of chlorhexidine

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16 **Key words**

17 Skin permeation, antiseptic, HPLC, Franz diffusion cell, chlorhexidine

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18 **Abstract**

19

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

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38 **Introduction**

39

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

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61 The aim of this current study was to use the Franz-cell skin model (6) to

62 determine the penetration profile for CHG through excised human skin, and to evaluate

63 the skin permeation of 2% (w/v) aqueous CHG into the skin using this model.

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64 **Materials and methods**

65

66 **Materials**

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

71

72 **Skin samples**

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

78

79 **Quantification of CHG**

80 High-performance liquid chromatography (HPLC) was used to measure the amount of
81 CHG in the skin samples obtained during the permeation studies. The analyses were
82 performed using an Agilent 1200 series HPLC system (Agilent Technologies, UK). The
83 samples were run at a flow rate of 1.2 mL/ min at room temperature through a reverse
84 phase chromatography column (CPS-2 Hypersil 5µm column dimension 150 x 4.6mm
85 (Thermo Electron Corporation, UK)), with ultraviolet detection at 254 nm. The isocratic
86 mobile phase consisted of methanol: water mixture (75:25) with 0.005 M sodium heptane

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87 sulphonate and 0.1% (v/v) diethylamine adjusted to pH 4 with glacial acetic acid. The
88 HPLC method was validated by repeating a series of standardised CHG concentrations
89 five times and plotting a graph of peak area versus CHG concentration. The level of
90 detection (LOD) and level of quantification (LOQ) were calculated from the standard
91 curve according to the following equation:
92
93 LOD= 3 x standard deviation (SD) / slope, and the LOQ= 10x SD/ slope.
94
95 **Skin permeation studies**
96 Skin permeation studies were performed with vertical Franz diffusion cells (figure 1).
97 The receptor compartment was filled with 29 mL of PBS and maintained at 37˚ C by a
98 circulating water jacket and agitated by stirring with a magnetic bar. Skin samples were
99 thawed in PBS at room temperature, dried with an absorbent towel and mounted onto
100 Franz diffusion cells with the stratum corneum (SC) uppermost facing the donor
101 compartment. The surface area exposed to the test compound was 3.14 cm^2 (2 cm in
102 diameter). All entrapped air was removed between the skin and receptor fluid and the
103 skin was left to equilibrate for 30 min to reach the skin surface temperature of 32˚C.
104 Twenty percent (w/v) aqueous CHG was diluted with distilled water and 0.1% (v/v)
105 Tween 80 to obtain the final test solution 2% (w/v) CHG. One millilitre of test solution
106 was spread over the skin surface in the donor compartment and the compartment was
107 sealed with a moisture resistant film (Parafilm M®, Alcan packaging, USA) to prevent
108 evaporation. One millilitre of receptor fluid was removed every 30 min for 2 h, every
109 hour between 2 to 6 h and at 8 h, 12 h and 24 h. Fluid removed from the receptor

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110 compartment was immediately replaced with an equal volume of fresh PBS solution. All
111 samples were filtered through a 0.45 µm nylon filter (Kinesis, UK) and analysed by
112 HPLC. The assay was performed in triplicate and on two different donor skin samples.
113

114 **CHG penetration profile studies**

115 Excised full thickness human skin samples were mounted onto the Franz diffusion cells
116 as described above, and exposed to 2% (w/v) CHG for 2 min, 30 min and 24 h. Following
117 exposure, the skin samples were removed, washed with PBS and dried with an absorbent
118 towel. The skin samples were immediately sprayed with a cryospray (Bright Instruments)
119 and frozen at -20˚C. Punch biopsies (7 mm in diameter) were cut from each frozen
120 sample in triplicate and placed onto a cork disc in embedding compound (Bright
121 Instruments, Cambs, UK), The frozen samples were sectioned horizontally with a
122 microtome (Bright Instruments) into 20 µm sections (from the surface to a depth of 600
123 µm) and 30µm sections (from depths of 600 to 1500 µm). Each section was placed into
124 an Eppendorf tube and the total weight of each skin sample determined. Chlorhexidine
125 was extracted from the skin by placing 1 mL of HPLC mobile phase solution into each
126 tube followed by incubation of the sealed tubes at 60˚C for 1 h. Following this, the
127 samples were analysed by HPLC and the concentration of CHG (µg/ mg of skin)
128 determined. Control skin (skin without treatment) was analysed parallel to the test
129 samples. Effective elution and recovery of CHG from the skin by this method was
130 confirmed prior to the experiment by injecting a standardised quantity of CHG (128 µg)
131 into ten skin samples, extracting the CHG and determining the recovered amount (94.4 ±
132 1.82 %, data not shown).

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133 **Results**

134

135 **HPLC validation**

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

141

142 **Skin permeation studies**

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

145

146 **CHG retention studies**

147 After 2 min, 30 min and 24 h, concentrations of chlorhexidine within the skin were
148 highest in the surface 100 µm sections, and reduced below depths of 300 µm (Figures 2
149 and 3). The concentration of CHG within the top 100 µm sections of skin were 0.157 (±
150 0.047) µg/ mg tissue and 0.077 (± 0.015) µg/ mg tissue after 2 min and 30 min exposure
151 to 2% (w/v) CHG respectively (figure 2). The concentration of CHG within deeper layers
152 (below 300 µm) fell to less than 0.002 µg/ mg tissue following both 2 min and 30 min
153 exposure. The difference between the amount of chlorhexidine within the top layers
154 between 2 min and 30 min exposure was not significant ($p > 0.05$) (student t-test,
155 INSTAT2, Graphpad, San Diego, CA, USA). The concentration of CHG was

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156 significantly higher within all skin sections following 24 h exposure to CHG compared to

157 the shorter exposure times. The concentration of CHG was 7.88 (± 1.37) µg/ mg tissue

158 within the upper 100 µm sections, and less than 1 µg/ mg of tissue at depths of 300 µm

159 and below.

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160 **Discussion**

161

162 This study demonstrates that 2 % (w/v) chlorhexidine, the antiseptic agent recommended
163 within EPIC and CDC guidelines for skin antisepsis prior to central venous catheter
164 (CVC) insertion, poorly permeates into deeper layers of the skin after 2 min and 30 min
165 exposure to the antiseptic. The concentration of CHG within the upper 100 μm sections
166 of skin was 0.157 (± 0.047) μg/ mg tissue and 0.077 (± 0.015) μg/ mg tissue after 2 min
167 and 30 min respectively. If 1 g of tissue is estimated to equal 1 ml, these levels are higher
168 than the concentrations required to kill many common skin microorganisms such as
169 *S.epidermidis* in *in vitro* conditions (10). Below 300 μm, the CHG concentration
170 remained less than 0.002 μg/ mg tissue, which may not be effective at eradicating
171 microorganisms on skin (17), especially microorganisms residing deep in the hair
172 follicles. Furthermore, chlorhexidine activity is reduced in the presence of organic
173 compounds, such as fatty acids and at lower pH (16) and therefore may reduce the
174 efficacy of skin antisepsis with CHG. An exposure time of 2 minutes was used to reflect
175 the clinical conditions used prior to surgery (5). Although the 2 minute study appears to
176 show a higher amount of bound chlorhexidine than the 30 minute study, there is
177 variability in concentrations measured in the top layers, as is expected at the shorter
178 exposure period (24), and the difference between 2 min and 30 min exposure is not
179 significant ($p > 0.05$). It is likely that a steady state has not been reached yet at 2 min. A
180 similar phenomenon was reported by Wagner *et al*. (23). Skin was also exposed to 2%
181 (w/v) CHG for 24 h and the concentration of CHG in the deeper sections, i.e., beyond
182 300 μm, was less than 1 μg/ mg tissue. These levels of CHG are more than the minimum

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183 bactericidal concentrations for many skin commensals (10), however this level of CHG
184 was only obtained after a prolonged contact time of the skin with CHG. In this study no
185 detectable levels of CHG was recovered from the receptor compartment suggesting that
186 aqueous CHG does not permeate through the full thickness of excised skin and is retained
187 within the tissue. These results support previous research on another CHG-based
188 compound, chlorhexidine phosphanilate, which was also shown not to permeate through
189 full thickness skin samples (25).

190 In this study, a model for studying the delivery of CHG into excised full thickness
191 human skin was evaluated. Skin permeation studies are commonly performed *in vitro*
192 with vertical or horizontal diffusion cells using skin or artificial membranes. This study
193 was performed using vertical diffusion cells (Franz type diffusion cells), to evaluate the
194 delivery of CHG through excised full thickness human skin. Such conditions mimic the
195 *in vivo* environment by maintaining the physiological receptor fluid at body temperature
196 and the skin surface temperature of 32˚C (6,23). Skin permeation studies generally
197 evaluate drug delivery through the skin by measuring drug diffusion into the receptor
198 fluid through the SC or epidermis, which are the main barriers for skin permeation.
199 However, the use of stripped skin layers, such as isolated SC or epidermal layers, for
200 drug permeation studies may influence the results with possible retention of the drug in
201 the dermal layers of the skin will not be considered. Full thickness skin was used in this
202 study to determine the location of CHG throughout the skins, rather than studying flux of
203 the drug through the barrier layers. Following exposure to CHG, the full thickness human
204 skin was sectioned to a depth of 1500 µm by sequential sectioning with a microtome
205 producing a total of 60 sections per skin sample. Skin sectioning has been used in many

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206 previous studies (21), however the SC is often removed by tape stripping prior to
207 sectioning of the skin. In this study the full thickness skin samples were sectioned
208 throughout the sample without prior removal of the surface layers. This study
209 demonstrates that the CHG permeation through the full thickness skin was not linear,
210 which was expected due to the variation in structure at various layers. The top 100 μm
211 layer of the skin, which contains SC (average 10- 20 μm thick) and other epidermal
212 layers (50 – 100 μm thick), contained the highest amount of CHG following exposure to
213 2% (w/v) CHG over all time points studied. Previous research has shown that the main
214 permeation barrier for skin absorption is the SC (3,11,25), which is thought to be due to
215 its high lipid matrix and packed layers of keratinised epithelial cells. Furthermore, this
216 study found that below 300 μm, at the dermal layer, the level of CHG remained
217 constantly low. Depending on the body site, dermis contains hair follicles and other skin
218 appendages, including sebaceous glands and sudoriferous glands (sweat producing
219 glands), which are of interest in skin antisepsis as they may be niches for microbial
220 colonisation of the skin following skin antisepsis (7,8). It is generally recognised that skin
221 antisepsis does not sterilize the skin; our study confirms this and demonstrates that it may
222 be due to poor permeation of chlorhexidine into the deeper layers of the skin.

223 In conclusion, this study showed poor permeation of chlorhexidine through
224 excised full thickness human skin after 2 min and 30 min exposure to aqueous 2 % (w/v)
225 CHG. The level of CHG were highest within the top 100 μm sections of skin, and
226 remained consistently low within the deeper layers. Furthermore, the model presented in
227 this study is a valuable tool in determining a permeation profile for chlorhexidine through
228 human skin *in vitro*. This study lays the foundation for further research within this area

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229 with a view to potentially adopting alternative strategies for enhanced skin antisepsis in

230 clinical practice.

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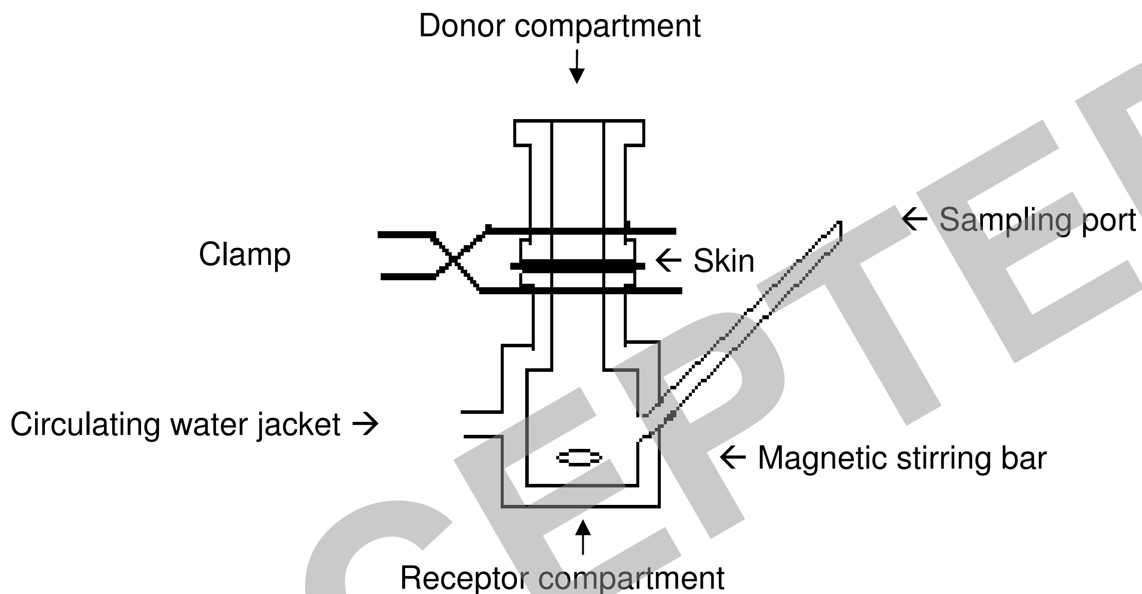

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339 **Figure 1.** Diagram of Franz diffusion cell. Receptor compartment was filled with PBS,

340 which was kept at 37˚C by circulating water jacket. The skin was mounted between the

341 receptor and donor compartment and clamped. The test drug was aliquoted onto the

342 donor compartment. The drug diffused through the skin was sampled by drawing up

343 receptor fluid via sampling port.

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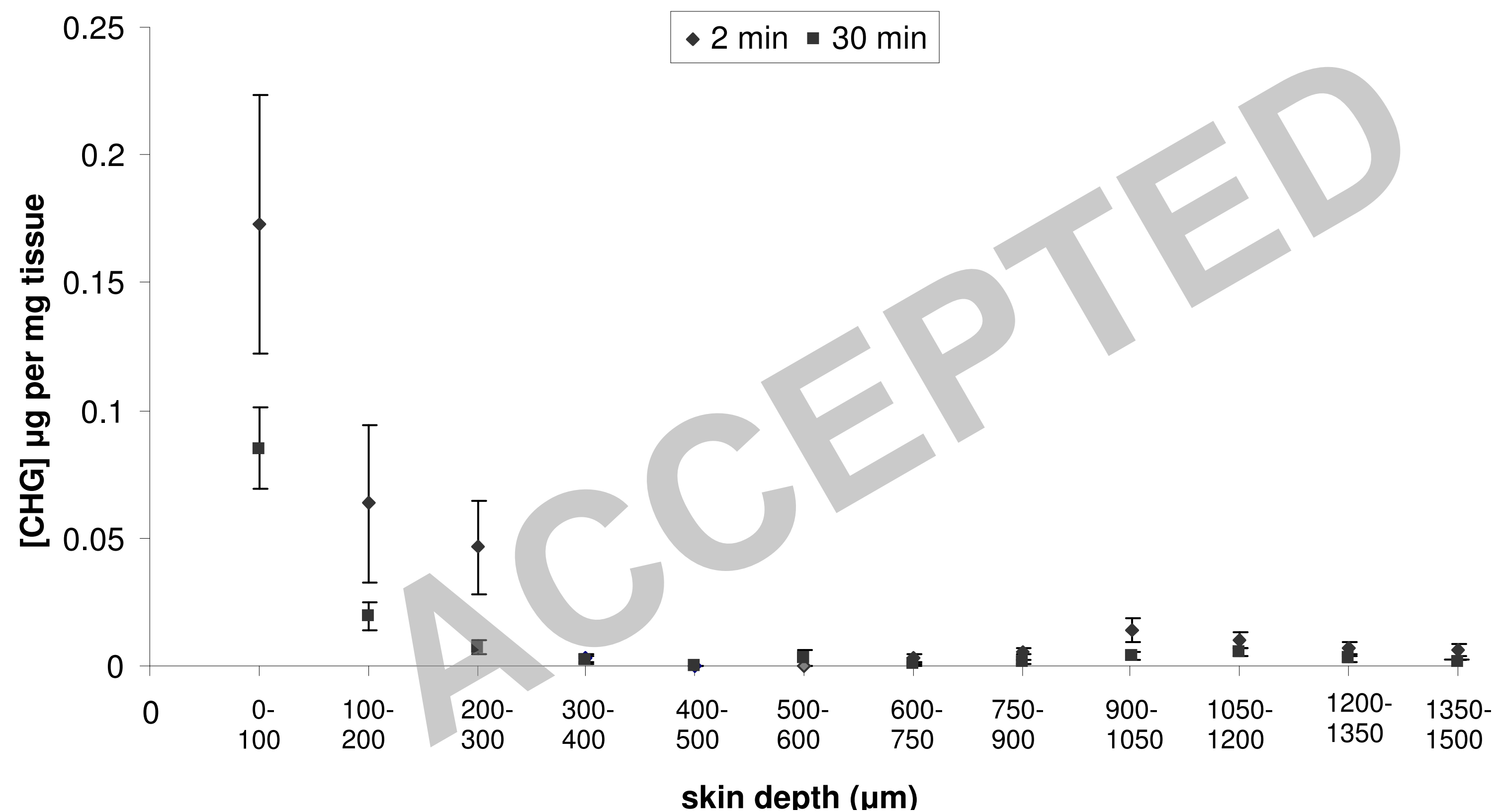

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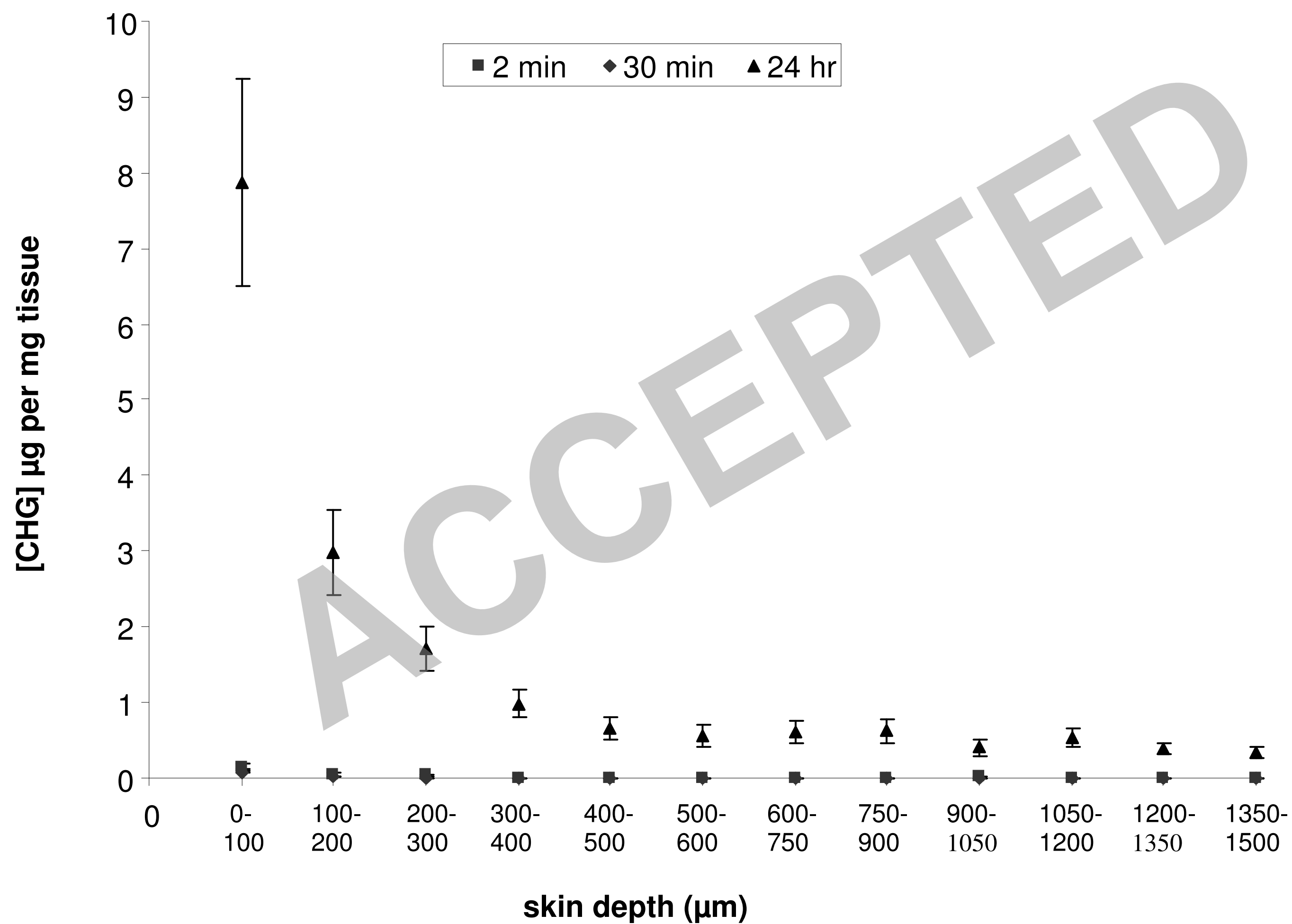
345 **Figure 2.** Penetration profile showing the location of chlorhexidine (µg/mg tissue) in excised human skin after 2 min and 30 min

346 exposure to aqueous 2% (w/v) chlorhexidine digluconate (mean ± s.e., n=15).



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347

348 **Figure 3.** Penetration profile showing the location of chlorhexidine (µg/ mg tissue) in excised human skin after 2 min and 30 min

349 (n=15) and 24 h (n=30) exposure to aqueous 2% (w/v) chlorhexidine digluconate (mean ± s.e).