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Research Article

In Vitro Skin Permeation and Bioassay of Chlorhexidine Phosphanilate, a New Antimicrobial Agent

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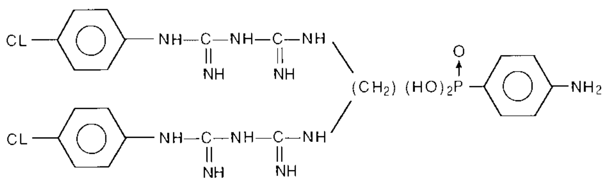
An *in vitro* technique was developed to study the permeation and antimicrobial activity of graded concentrations of a new antibacterial agent, chlorhexidine phosphanilate (CHP), in cream formulations using Franz diffusion cells. Formulations containing from 0.2 to 2% CHP were quantitatively applied to intact excised skin and to skin from which the stratum corneum and partial epidermis had been enzymatically removed. Receptor fluids from diffusion cells were sampled over time and assayed by HPLC methods for chlorhexidine and phosphanilic acid; 24- and 48-hr samples of the diffusate from studies with damaged skin were also bioassayed using clinical isolates of appropriate microbial species. Through intact skin almost no permeation of CHP was observed over 48 hr. The failure of CHP to penetrate intact human skin suggests that normal stratum corneum is the rate-limiting barrier to penetration by this antimicrobial agent. In damaged skin lacking stratum corneum barrier, the release of CHP from the formulation becomes the rate-determining step. Coincident with penetrating damaged skin, CHP dissociates, and the molar ratio of the chlorhexidine and phosphanilate moieties in the diffusate changes to favor phosphanilic acid. The extent of changes in the permeation rates of both moieties of CHP was directly related to the CHP concentration in cream. Both CHP moieties were found to reach equilibrium in the dermis within 24 hr after application. It was also observed that CHP creams down to 0.2% concentration yielded diffusates with activity exceeding the minimum inhibitory concentration of all test microorganisms within 24 hr.

KEY WORDS: *in vitro* skin permeation technique; chlorhexidine phosphanilate creams; permeability coefficients; *in vitro* microbial assay.

INTRODUCTION

Chlorhexidine phosphanilate (CHP) is currently in clinical development as a topical antimicrobial agent (see Scheme I). CHP was developed by the chemical modification of two known biologically active compounds, chlorhexidine (CH) and phosphanilic acid (P), to form a new salt substance at an average ratio of 1:1:83 which, when acted upon *in vivo* by nonenzymatic processes, will disassociate and liberate two antimicrobials, the parent compounds (CH and P). The molecule was designed to complement the potent, primarily Gram-positive activity of CH with P (1), a sulfonamide-like bacteriostatic agent with little Gram-positive activity but broad Gram-negative activity of the sulfonamides (2), particularly against *Pseudomonas* and the *Enterobacteriacae*. Phosphanilic acid has enhanced potency against virulent microbial strains including *Pseudomonas, Serratia, Proteus,* and *Providencia* compared to sulfonamides (2). CHP has shown activity against burn wound isolates resistant to sulfonamides, and it has been shown to be protective in infected burn mouse and rat models (3,4). Chlorhexidine has shown cytotoxic potential for epithelial cells (5,6). It is therefore valuable to study a range of concentrations to guide selection of the lowest effective concentration to balance biochemical toxicities with antimicrobial efficacy.



Scheme I. Chemical structure of chlorhexidine phosphanilate (1:1.8).

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The present study examines the release of CHP from cream vehicle, the penetration of CH and P of intact and damaged skin, and the delivery of antimicrobial activity to target tissues. A newly developed *in vitro* technique which evaluates skin permeation and the resulting antimicrobial activity of six concentrations of chlorhexidine phosphanilate after application to intact or damaged human skin is presented.

EXPERIMENTAL

Reagents and Materials. Chlorhexidine phosphanilate of assured purity for pharmaceutical use was prepared under Bristol-Myers control and formulated into an o/w cream, similar to USP hydrophilic ointment, at final analytically verified concentrations ranging from 0.2 to 2%. Permeation studies were done through intact and damaged skin by techniques described below. Sodium chloride was ACS grade, Fisher Scientific Corporation. All solvents used for analysis were HPLC grade, J. T. Baker Company. Bovine trypsin was obtained from Sigma Chemical Company. All chemicals were used as received.

Preparation of Excised Human Skin. Human skin samples were obtained from the abdomen or chest during autopsy. Skin specimens were immediately labeled, wrapped, and placed on ice. Only skin appearing normal on gross inspection was used. Historical evidence of chronic illness, skin disease, or skin injury would exclude use in the study. Within 2 hr of collection, the skin specimens were stored in a freezer at −30°C until needed. Before starting a skin permeation study, a sample was thawed at room temperature in normal saline. Skin was then frozen on the microtome with CO_2 and sectioned at a thickness of 150–240 μm. The prepared skin section was stored, labeled, in normal saline at 5°C until used in a permeation study. To mimic the compromised skin conditions where the barrier function of stratum corneum is either damaged or essentially absent, intact excised skin was damaged to simulate the wound in a deep cut or burn condition by controlled incubation of intact skin in a 0.2% trypsin solution in normal saline at 37°C for 0.5 hr to remove the stratum corneum from the epidermis. The prepared damaged skin was also stored in normal saline until used in the permeation study.

In Vitro *Human Skin Permeation Studies.* Nine-millimeter-ID silanized Franz diffusion cells filled with the necessary volume of normal saline as receptor fluid were adopted for the study. The designated specimen of prepared skin section was mounted on each cell, dermis side down. The membrane was secured with an O-ring and an open cap for the cell. A 50-μl sample of CHP cream was then dispensed from a microliter pipette and applied in an even layer onto the skin section. The receptor fluid was maintained at 37 ± 0.1°C throughout the study. Two hundred microliters of receptor fluid was collected at 1, 2, 3, 4, 7, 8, 12, 24, 32, and 48 hr for chemical assay. The samples collected at 24 and 48 hr were also used for the evaluation of antimicrobial activity by bioassay; volume was replenished with normal saline after these withdrawals. Two separate reversed-phase HPLC methods with UV detection were used to assay CH and P separately (7).

DATA ANALYSIS

The concentration–time course data collected from diffusion studies were statistically analyzed using Tukey's Studentized range test. The calculation of permeability constants was based upon Fick's law,

$$\frac{dQ}{dt} = \frac{DAK}{h}(C_o - C_i) \qquad (1)$$

where dQ/dt is the penetration rate, Q is the amount of drug penetrating the membrane, D is the mean diffusion coefficient of drug in the membrane, A is the area of the solution exposed to the absorbing membrane, K is the equilibrium distribution ratio or partition coefficient of the drug between the membrane (skin) and the cream in the donor site, h is the thickness of the membrane, the term $(C_o - C_i)$ is the difference between the drug concentration, C_o, in the donor (cream) and the drug concentration, C_i, in the receptor site. In a well-stirred system and sink condition, the term C_i can be ignored since it can reasonably be assumed that the drug's concentration in the receptor fluids after penetration is negligible compared to its concentration during the absorption at any time. Thus, Eq. (2) can be further simplified as

$$\frac{dQ}{dt} = \frac{DAK}{h}C_o \qquad (2)$$

Rearranging Eq. (2) and letting $k_p = (DK/h)$

$$k_p = \frac{(dQ/dt)}{A\,C_o} = \frac{DK}{h} \qquad (3)$$

where k_p is the mean permeability constant and dQ/dt is given by the slope of the steady-state plot of the amount of drug in the receptor side versus time. For diffusion of molecules through a homogeneous barrier, the lag time (time to reach the steady state, t_{lag}) can also be related to the thickness, h, in the following manner:

$$t_{lag} = \frac{h^2}{6D} \qquad (4)$$

t_{lag} was taken as the intercept on the X axis of the steady-state plot.

In Vitro *Microbial Assays.* Diffusion samples collected at 24 and 48 hr were bioassayed for antimicrobial activity by the cylinder-plate method described below. Five microorganisms isolated from burn or skin wound patients were selected for evaluation: *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes, Serratia liquifaciens,* and *Candida albicans.* The minimum inhibition concentration (MIC) values of CHP, CH, and P against these five microorganisms are listed in Table I.

Inoculum Preparation. Cultures were maintained on tryptic soy agar slants or frozen culture. The inoculum was incubated in BHI broth for 24 hr at 35°C. Working suspensions were prepared by diluting the 24-hr culture in 40 ml of sterile BHI broth. These suspensions were adjusted to 30% T (580 nm) with additional culture or broth using a Perkin-

Table I. Minimum Inhibition Concentration (MIC; µg/ml) of Chlorhexidine Phosphanilate (CHP), Chlorhexidine (CH), and Phosphanilic Acid (P) Against Five Tested Strains Collected from Clinical Skin Wound or Burn Wound Isolates

	Staphylococcus aureus	*Streptococcus pyogenes*[a]	*Pseudomonas aeruginosa*	*Serratia liquifaciens*	*Candida albicans*
CHP	1	2	8	8	16
CH	2	½	32	2–4	16[b]
P	>2048	>512	16	>512	>64[c]

[a] Collected from skin wound isolates.

[b] MIC value from 2% chlorhexidine digluconate cream.

[c] MIC value from 0.88% sodium phosphanilate cream.

Elmer LC 55 spectrophotometer. Fifteen milliliters of the working suspension was added to 500 ml of BHI agar for bioassay.

Standard Solution Preparation. Stock solutions were prepared by adding a sufficient amount of CHP to a 20-ml volumetric flask to attain a final activity of 1000 µg/ml. Two milliliters of DMSO was added to allow dissolution and the flask brought to volume Q.S. with phosphate buffer (pH 6.0). Working standard solution was prepared by pipetting 10 ml of the stock solution to a 100-ml volumetric flask brought to volume with phosphate buffer; this procedure made 100 µg/ml activity standard CHP.

Standard Curve Preparation. To ten 10-ml volumetric flasks 1.5 to 6.5 ml of the working standard solution was pipetted in 0.5-ml increments and the flasks brought to 10 ml with phosphate buffer (pH 6.0), yielding solutions of 15 to 65 µg/ml activity. Final dilutions were assayed to prepare the standard curve. The 35 µg/ml dilution (CHP) was used as the reference point for the standard curve.

Assay Procedure. Using a Draft dispenser, 15 ml of molten uninoculated BHI was pipetted into 100 × 15-mm petri dishes, then allowed to solidify to form a base layer, and 4.0 ml of inoculated BHI agar was pipetted on the surface. After plates cooled, sterile templates were placed onto the medium surface. The sample and the standard solutions (15 to 65 µg/ml) were assayed. Using a MLA Precision "D" pipette, 0.3-ml aliquots of the final dilution were transferred to three alternate template cups and 0.3-ml aliquots of the reference standard (35 µg/ml) to the other three cups. Plates were incubated 16–18 hr at 35°C. The diameter of each inhibition zone was measured with an antibiotic zone reader (mm).

RESULTS

Permeability of Human Skin to Chlorhexidine Phosphanilate (CHP) Cream Formulations

CHP was designed mainly to prevent the secondary infection caused by Gram-negative bacterial invasion which often occurred underneath the wound skin surface; it is believed that the concentrations of CH and P in the receptor phase would demonstrate the Gram-negative bacterial activity of P better. Therefore this study includes only the data collected from the receptor fluid.

In the study of intact human skin permeation, no chlorhexidine was found penetrating through the skin. The results indicate that the permeation of CHP through intact human skin is a slow process, with the stratum corneum being the rate-limiting barrier. Consequently, only the permeation data collected from damaged skin are presented.

Figures 1 and 2 illustrate the permeation profile of CH and P, respectively, through a section of 6.36 × 10^{-1} cm^2 damaged human skin for various concentrations of CHP

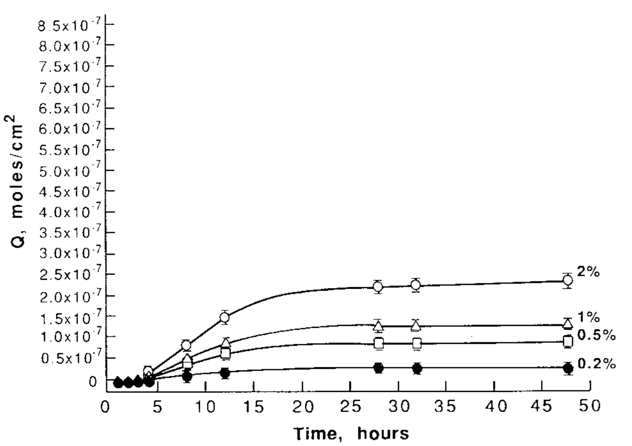


Fig. 1. The quantity of chlorhexidine penetrating 6.36 × 10^{-1} cm^2 of damaged human skin from 2, 1, 0.5, and 0.2% CHP cream formulations.

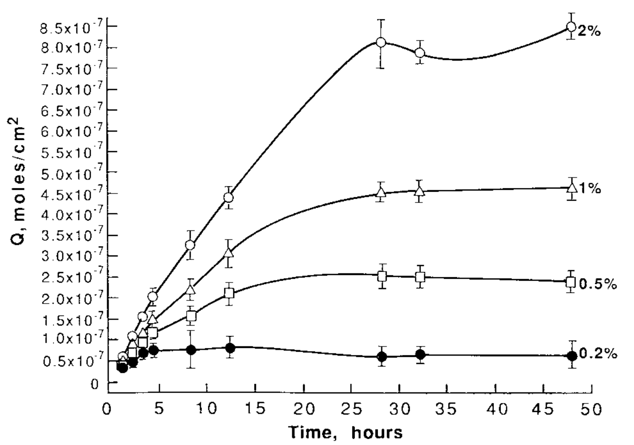


Fig. 2. The quantity of phosphanilic acid penetrating 6.36 × 10^{-1} cm^2 of damaged human skin from 2, 1, 0.5, and 0.2% CHP cream formulations.

Table II. Permeability of Damaged Human Skin to 2, 1, 0.5, and 0.2% CHP in o/w Cream Formulations (Mean Values of Three Runs)

Cream concentration (% CHP)	$10^2 \times h^a$ (cm)	$10^4 \times k_p{}^b$ (cm min^{-1})	$10^7 \times k_p h$ (cm^2 min^{-1})	t_{lag} (min)	$10^7 \times D^c$ (cm^2 min^{-1})	$10^8 \times Q_{24\ hr}{}^d$ (mol)
2%	1.93 (±0.34)					
	CH[e]	18.6 (±0.74)	358	168 (±6.72)	3.78	22.4
	P[f]	17.8 (±1.07)	343	1.8 (±0.04)	343	78.5
1%	2.10 (±0.59)					
	CH	21.3 (±0.64)	448	156 (±3.12)	4.78	12.8
	P	24.8 (±1.24)	520	1.2 (±0.04)	529	44.5
0.5%	1.70 (±0.50)					
	CH	29.3 (±0.59)	500	120 (2.4)	4.07	7.5
	P	31.2 (±1.25)	530	1.2 (±0.02)	530	24.5
0.2%	1.97 (±0.12)					
	CH	30.7 (±0.61)	605	81 (±4.15)	7.98	3.1
	P	18.0 (±0.54)	353	1.8 (±0.05)	353	6.3

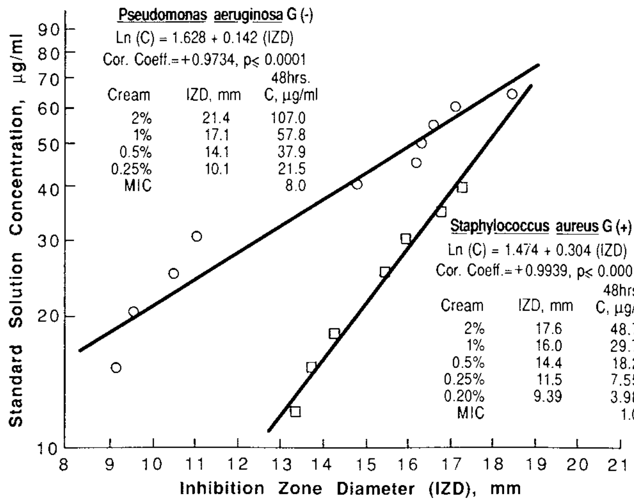
[a] h = skin thickness, and number in parentheses is the spread.
[b] k = permeability constant, and number in parentheses is the standard deviation.
[c] D = mean diffusion coefficient.
[d] $Q_{24\ hr}$ = quantity crossing 6.36×10^{-1} cm^2 of damaged skin in 24 hr.
[e] Chlorhexidine.
[f] Phosphanilic acid.

cream formulations (0.2–2.0%). The permeation of CH and P was measured by HPLC methods separately. Results are presented as the mean value of three runs. Means shown were generated from Tukey's Studentized range test. Since CHP is a salt formed by a dicationic base (CH) and a diprotic acid (P) which dissociates in the aqueous solution at various ratios depending on the pH of the solution. The evidence of the dissolution of CHP was demonstrated by the changing ratio of CH/P at different time points during the penetration (see Figs. 1 and 2) and also in the receptor fluid (see the last column in Tables III–VI). Should CHP remain undissociated during penetration, then the penetration profiles of CH and P would have been identical and their ratios would remain constant (CH:P = 1:1.8).

We therefore conclude from these chemical results that solubilized CHP dissociates into CH and P moieties on the skin surface. The individual component then diffuses through damaged skin membrane independently at different rates. The diffusion rates of CH and P through damaged skin are concentration dependent. The rate-limiting step in damaged skin is the release and dissolution rate of CHP from the cream formulations.

In Fig. 1, the permeation profile indicates that the steady-state penetration of CH through 170- to 200-μm damaged skin was achieved within 3 hr for all concentrations, and the penetration was maintained for at least 12–16 hr until equilibrium was reached.

On the other hand, the penetration profile of phospha-



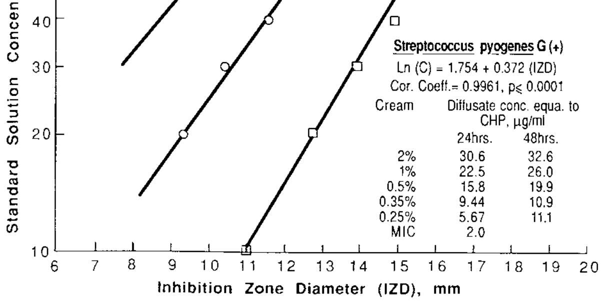
Pseudomonas aeruginosa G (-)
Ln (C) = 1.628 + 0.142 (IZD)
Cor. Coeff.=+0.9734, p≤ 0.0001

Cream	IZD, mm	48hrs. C, μg/ml
2%	21.4	107.0
1%	17.1	57.8
0.5%	14.1	37.9
0.25%	10.1	21.5
MIC		8.0

Staphylococcus aureus G (+)
Ln (C) = 1.474 + 0.304 (IZD)
Cor. Coeff.=+0.9939, p≤ 0.0001

Cream	IZD, mm	48hrs. C, μg/ml
2%	17.6	48.7
1%	16.0	29.7
0.5%	14.4	18.2
0.25%	11.5	7.55
0.20%	9.39	3.98
MIC		1.0

Fig. 3. *In vitro* microbial bioassay of the 48-hr CHP diffusates. ○, *Pseudomonas aeruginosa* G(−), MIC = 8 μg/ml. □, *Staphylococcus aureus* G(+), MIC = 1 μg/ml.



Candida albicans
Ln (C) = 1.500 + 0.253(IZD)
Cor. Coeff.= 0.9916, p≤ 0.0001

Cream	Diffusate conc. equa. to CHP, μg/ml 24hrs.	48hrs.
2%	41.3	57.7
1%	31.0	37.1
0.5%	26.3	27.0
0.35%	13.8	18.8
0.25%	17.1	17.1
MIC	2.0	

Serratia liquifaciens G (-)
Ln (C) = 0.102 + 0.310 (IZD)
Cor. Coeff.= 0.996, p≤ 0.0001

Cream	Diffusate conc. equa. to CHP, μg/ml 24hrs.	48hrs.
2%	33.6	35.5
1%	32.4	31.9
0.5%	27.2	30.2
0.35%	27.6	21.8
0.25%	22.8	24.9
MIC	8.0	

Streptococcus pyogenes G (+)
Ln (C) = 1.754 + 0.372 (IZD)
Cor. Coeff.= 0.9961, p≤ 0.0001

Cream	Diffusate conc. equa. to CHP, μg/ml 24hrs.	48hrs.
2%	30.6	32.6
1%	22.5	26.0
0.5%	15.8	19.9
0.35%	9.44	10.9
0.25%	5.67	11.1
MIC	2.0	

Fig. 4. *In vitro* microbial bioassay of the 24- and 48-hr diffusates. □, *Streptococcus pyogenes* G(+), MIC = 2.0 μg/ml. ○, *Serratia liquifaciens* G(−), MIC = 8.0 μg/ml. △, *Candida albicans*, MIC = 2.0 μg/ml.

Table III. *In Vitro* Bioassay of the CHP Standard Concentration Solution and the 48-hr Diffusant Obtained from the Skin Permeation Study (Microorganism, *Staphylococcus aureus* G(+); MIC = 1.0 μg/ml)

			48-hr diffusant				
			Bioassay		Chemical assay[d]		
CHP standard concentration solution (μg/ml)	Inhibition zone diameter (IZD) (mm)[a]	% CHP cream[b]	Inhibition zone diameter (mm)[a]	Conc. equiv. to CHP (μg/ml)[c]	CH (μg/ml)[e]	P (μg/ml)[f]	Molar ratio, CH/P
12	13.3 (0.73)	0.2	9.39 (0.32)	3.98	3.32	5.04	1/2.2
15	13.7 (0.45)	0.25	11.5 (0.32)	7.55	—[g]	—	—
18	14.2 (0.42)						
25	15.4 (0.45)	0.5	14.4 (0.40)	18.2	8.18	15.0	1/2.6
30	15.9 (0.37)	1	16.0 (0.27)	29.7	12.9	31.4	1/3.5
35	16.7 (0.82)	2	17.6 (0.27)	48.7	24.0	60.3	1/3.6
40	17.1 (0.37)						

Standard curve: $\ln(C) = -1.474 + 0.304$ (IZD)
Cor. coeff. = $+0.9939$, $P \leqslant 0.0001$

[a] Results are the mean values of nine determinations and the number in parentheses is the spread.
[b] Percentage of CHP cream used for the skin permeation study.
[c] Concentration equivalent to CHP in standard solution, obtained by the extrapolation of the inhibition zone diameter (IZD; mm) to the logarithmic value of CHP standard concentration from the standard curve: $\ln(C) = -1.474 + 0.304$ (IZD).
[d] Value of chemical assay by HPLC method.
[e] Chlorhexidine.
[f] Phosphanilic acid.
[g] Not determined.

Table IV. *In Vitro* Bioassay of the CHP Standard Concentration Solution and the 48-hr Diffusant Obtained from the Skin Permeation Study (Microorganism, *Pseudomonas aeruginosa* G(−); MIC = 8.0 μg/ml)

				48-hr diffusant				
concentration	CHP standard solution (μg/ml)	Inhibition zone diameter (IZD) (mm)[a]	% CHP cream[b]	Bioassay		Chemical assay[d]		
Inhibition zone diameter (mm)[a]		Conc. equiv. to CHP (μg/ml)[c]	CH (μg/ml)[e]	Inhibition zone diameter (mm)[a]	Conc. equiv. to CHP (μg/ml)[c]	CH (μg/ml)[e]	P (μg/ml)[f]	Molar ratio, CH/P
	15	9.17 (0.56)	0.2	—[g]	—	3.32	5.04	1/2.2
	20	9.73 (1.32)						
	25	10.7 (0.70)	0.25	10.1 (0.93)	21.5	—[g]	—	—
	30	11.0 (0.42)						
	40	14.7 (0.37)						
	45	16.1 (0.75)	0.5	14.1 (0.66)	31.9	8.18	15.0	1/2.6
	50	16.2 (0.56)						
	55	16.5 (2.40)	1	17.1 (0.96)	57.8	12.9	31.4	1/3.5
	60	17.0 (0.46)						
	65	18.3 (0.44)	2	21.4 (0.95)	107	24.0	60.3	1/3.6

Standard curve: $\ln(C) = 1.628 + 0.142$ (IZD)
Cor. coeff. = $+0.9734$, $P \leqslant 0.0001$

[a] Results are the mean values of six determinations and the number in parentheses is the spread.
[b] Percentage of CHP cream used for the skin permeation study.
[c] Concentration equivalent to CHP in standard solution, obtained by the extrapolation of the inhibition zone diameter (IZD; mm) to the logarithmic value of CHP standard concentration from the standard curve: $\ln(C) = -1.628 + 0.142$ (IZD).
[d] Value of chemical assay by HPLC method.
[e] Chlorhexidine.
[f] Phosphanilic acid.
[g] Not determined.

Table V. *In Vitro* Bioassay of the CHP Standard Concentration Solution and the 24-hr and 48-hr Diffusates Collected from the Skin Permeation Study (Microorganism, *Streptococcus pyogenes* G(+); MIC = 2 μg/ml)

CHP standard concentration solution (μg/ml)	Inhibition zone diameter (IZD) (mm)[a]	% CHP cream[b]	Bioassay: Inhibition zone diameter (IZD) (mm)[a] 24 hr	Bioassay: Inhibition zone diameter (IZD) (mm)[a] 48 hr	Bioassay: Conc. equiv. to CHP (μg/ml)[c] 24 hr	Bioassay: Conc. equiv. to CHP (μg/ml)[c] 48 hr	Chemical assay (μg/ml)[d]: CH[e] 24 hr	Chemical assay (μg/ml)[d]: CH[e] 48 hr	Chemical assay (μg/ml)[d]: P[f] 24 hr	Chemical assay (μg/ml)[d]: P[f] 48 hr	Molar ratio, CH/P 24 hr	Molar ratio, CH/P 48 hr
10	10.9 (0.24)	0.25	9.42 (0.66)	11.2 (0.85)	5.67	11.1	3.10	4.40	6.40	8.00	1/2.06	1/1.82
20	12.7 (0.70)											
		0.35	10.8 (1.70)	11.2 (1.16)	9.44	10.9	4.90	5.10	11.9	11.8	1/2.43	1/2.31
30	13.8 (1.11)											
		0.5	12.2 (1.60)	12.8 (0.56)	15.8	19.9	8.10	10.0	19.7	20.5	1/2.43	1/2.05
40	14.90 (0.84)											
50	15.1 (1.18)	1.0	13.3 (0.92)	13.5 (0.22)	22.5	26.0	12.2	13.2	28.6	31.0	1/2.34	1/2.35
		2.0	13.9 (0.39)	14.1 (0.41)	30.6	32.6	15.2	18.3	37.2	43.6	1/2.45	1/2.38

Standard curve; $\ln(C) = -1.754 + 0.372\ (\mathrm{IZD})$
Cor. coeff. = 0.9961, $P \leq 0.0001$

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[a] Results are the mean values of six determinations and the number in parentheses is the spread.

[b] Percentage of CHP cream used for the skin permeation study.

[c] Concentration equivalent to CHP in standard solution, obtained by the extrapolation of the inhibition zone diameter (IZD; mm) to the logarithmic value of CHP standard concentration from the standard curve: $\ln(C) = -1.754 + 0.372\ (\mathrm{IZD})$.

[d] Value of chemical assay by HPLC method.

[e] Chlorhexidine.

[f] Phosphanilic acid.

Table VI. *In Vitro* Bioassay of the CHP Standard Concentration Solution and the 24-hr and 48-hr Diffusates Collected from the Skin Permeation Study (Microorganism, *Serratia liquifaciens*; MIC = 8 μg/ml)

CHP standard concentration solution (μg/ml)	Inhibition zone diameter (IZD) (mm)[a]	% CHP cream[b]	Bioassay: Inhibition zone diameter (IZD) (mm)[a] 24 hr	Bioassay: Inhibition zone diameter (IZD) (mm)[a] 48 hr	Bioassay: Conc. equiv. to CHP (μg/ml) 24 hr	Bioassay: Conc. equiv. to CHP (μg/ml) 48 hr	Chemical assay (μg/ml)[d]: CH[e] 24 hr	Chemical assay (μg/ml)[d]: CH[e] 48 hr	Chemical assay (μg/ml)[d]: P[f] 24 hr	Chemical assay (μg/ml)[d]: P[f] 48 hr	Molar ratio, CH/P 24 hr	Molar ratio, CH/P 48 hr
20	9.40 (0.19)	0.25	9.77 (0.83)	10.1 (0.40)	22.8	24.9	3.10	6.40	6.40	8.00	1/2.06	1/1.82
30	10.5 (0.55)	0.35	10.4 (0.47)	9.63 (0.45)	27.6	21.8	4.90	5.10	11.9	11.8	1/2.43	1/2.31
40	11.6 (0.62)	0.50	10.3 (0.75)	10.7 (0.26)	27.2	30.2	8.10	10.0	19.7	20.5	1/2.43	1/2.05
50	12.5 (0.53)	1.0	10.9 (0.32)	10.9 (0.43)	32.4	31.9	12.2	13.2	28.6	31.0	1/2.34	1/1.35
60	12.8 (0.35)	2.0	11.0 (1.08)	11.2 (0.74)	33.6	35.5	15.2	18.3	37.2	43.6	1/2.45	1/2.38

Standard curve: $\ln(C) = 0.102 + 0.310(\mathrm{IZD})$
Cor. coeff. = 0.996, $P \leq 0.0001$

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[a] Results are the mean values of six determinations and the number in parentheses is the spread.

[b] Percentage of CHP cream used for the skin permeation study.

[c] Concentration equivalent to CHP in standard solution, obtained by the extrapolation of the inhibition zone diameter (IZD; mm) to the logarithmic value of CHP standard concentration from the standard curve: $\ln(C) = 0.102 + 0.310\ (\mathrm{IZD})$.

[d] Value of chemical assay by HPLC method.

[e] Chlorhexidine.

[f] Phosphanilic acid.

nilic acid in Fig. 2 shows that the steady-state penetration was achieved in less than 5 min and maintained for approximately 4–23 hr, depending upon its concentration. P appears to diffuse through damaged skin at a much faster rate than CH. Both molecules reached equilibrium within 24 hr after application.

Table II summarizes the diffusion parameters of CH and P. Assuming that damaged skin is a homogeneous barrier and that CHP diffuses by a passive transport mechanism, the permeability constants were calculated by Eq. (2) according to the permeation profile shown in Figs. 1 and 2. The permeability constants and the product of permeability constant and total skin thickness (k_p h) were approximately the same for each moiety. Theoretically, the lag time (t_{lag}) is a time-independent parameter for each concentration; however, it was found that there was a positive correlation, for CH only, between concentration and lag time ($r = 0.875$, $P \leq 0.001$). This can be attributed to the fact that there are different steady states at various concentration levels and different times are needed to reach the steady state. Since the apparent diffusion coefficients do not change significantly with concentration, it is also possible that different lag times may be due to concentration-dependent dissolution and thus a concentration-dependent dissociation. In the case of P, the diffusion rate is so fast that no difference in lag time can be found at various concentration levels.

There was a strong positive correlation between the concentration of CHP creams used on the skin surface and $Q_{24\ \mathrm{hr}}$ ($r = 0.9960$, $P \leq 0.001$, for CH and $r = 0.9938$, $P \leq 0.001$, for P), which revealed that the amount of CH and P that penetrated a section of 6.36×10^{-1} cm^2 damaged skin depended on the concentration and the dissolution rate of CHP in the cream. For diffusion of molecules through a homogeneous barrier, the values of the permeability constant (k_p) and t_{lag} can both be related to the skin thickness (h) by Eqs. (3) and (4). Values for these terms are included in Table II.

The diffusion rates were very different for CH and P since the $Q_{24\ \mathrm{hr}}$ of phosphanilic acid through the barrier with a thickness of 0.170–0.210 mm was 3.5-fold that of CH. In the case of CH, the mean value of the term $h^2/6\ t_{lag}$ through the thickness range 5.15×10^{-7} cm^2/min represents the apparent mean diffusion coefficient of CH in the damaged skin. The mean value of the term $k_p h$ [$= DK$; Eq. (3)] throughout the thickness range was 4.8×10^{-5} cm^2/min. Thus, on the basis that D was 5.15×10^{-7} cm^2/min, the apparent value of the partition coefficient (k) between the damaged skin and the cream was 93. In the case of P, the apparent mean diffusion coefficient was 4.58×10^{-5} cm^2/min and the mean value of the term $k_p h$ was 4.37×10^{-5} cm^2/min. This leads to the apparent value of k for P between the damaged skin and the cream of 0.95. Comparison of these calculated values of k (92 for CH and 0.95 for P) suggests that the dermis has a higher affinity for CH than for P.

In Vitro Microbial Assay of 24- and 48-hr CHP Diffusates

The 24- and 48-hr diffusates were selected for bioassay to assure that equilibrium had been reached and both moieties retained antimicrobial activity. The diffusates were an-

Table VII. *In Vitro* Bioassay of the CHP Standard Concentration Solution and the 24-hr and 48-hr Diffusates Collected from the Skin Permeation Study (Microorganism, *Candida albicans;* MIC = 2 μg/ml)

CHP standard concentration solution (μg/ml)	Inhibition zone diameter (IZD) (mm)[a]	% CHP cream[b]	Bioassay				Chemical assay (μg/ml)[d]				Molar ratio, CH/P	
			Inhibition zone diameter (IZD) (mm)[a]		Conc. equiv. to CHP (μg/ml)		CH[e]		P[f]			
			24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
50	9.66 (0.48)	0.25	5.39	5.39	17.1	17.1	3.10	6.40	6.40	8.00	1/2.06	1/1.82
60	10.1 (0.48)		(0.62)	(0.17)								
		0.35	4.56	5.76	13.8	18.8	4.90	5.10	11.9	11.8	1/2.43	1/2.31
70	10.9 (0.50)		(0.05)	(0.10)								
		0.50	7.06	7.16	26.3	27.0	8.10	10.0	19.7	20.5	1/2.43	1/2.05
80	11.5 (0.36)		(0.10)	(0.21)								
90	11.8 (0.23)	1.0	7.96	8.39	31.0	37.1	12.2	13.2	28.6	31.0	1/2.34	1/1.35
			(0.49)	(0.10)								
		2.0	8.81	10.1	41.3	57.7	15.2	18.3	37.2	43.6	1/2.45	1/2.38
			(0.28)	(0.42)								

Standard curve: $\ln(C) = -1.500 + 0.253\ (\mathrm{IZD})$
Cor. coeff. = 0.9961, $P \leq 0.0001$

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[a] Results are the mean values of six determinations and the number in parentheses is the spread.

[b] Percentage of CHP cream used for the skin permeation study.

[c] Concentration equivalent to CHP in standard solution, obtained by the extrapolation of the inhibition zone diameter (IZD; mm) to the logarithmic value of CHP standard concentration from the standard curve: $\ln(C) = -1.500 + 0.253\ (\mathrm{IZD})$.

[d] Value of chemical assay by HPLC method.

[e] Chlorhexidine.

[f] Phosphanilic acid.

alyzed chemically by HPLC methods for a quantitative evaluation of penetration and for the total antimicrobial activity of CHP on the basis of microbial growth inhibition assays. Figures 3 and 4 depict the standard curves for the microbial test strains (clinical isolates). Tables III and IV summarize the chemical and microbial assay results for the *P. aeruginosa* and *S. aureus* strains, respectively, with the 48-hr diffusates. Tables V, VI, and VII are the results for *S. pyogenes, S. liquifaciens,* and *C. albicans* strains, respectively, with the 24- and 48-hr diffusates. The molar ratio (CH:P) was separately determined by HPLC analysis for the same diffusates.

Since the chemical molar ratio changes during diffusion, the diffusates in the receptor fluid at equilibrium should not be treated as an actual CHP solution. However, it is difficult to interpret the standard MIC test for diffusate with a changing molar ratio. The concentrations of diffusates obtained from extrapolation of the standard curve equation (inhibition zone diameters vs CHP concentration) can only be considered as concentrations bioequivalent to CHP at the molar ratio of 1:1.83.

The bioequivalent concentrations obtained for *P. aeruginosa* were considerably higher than the chemical assay values. In previous *in vitro* studies, this *P. aeruginosa* strain was found to be more sensitive to P than to CH, which also agrees to the MIC results shown in Table I. The higher concentration of P in the diffusate, therefore, produced greater antimicrobial activity against the *P. aeruginosa* strain, resulting in increasing the bioequivalent concentrations in diffusates. In contrast, the *S. aureus, S. pyogenes, S. liquifaciens,* and *C. albicans* strains were sensitive only to the CH component. Therefore the bioequivalent concentrations obtained for these strains in the studies reported here were closer to the chemical results for CH. These results confirm the change in molar ratios of CHP favoring P after penetration. These data also indicate that both CHP moieties reach the steady state as well as the MIC of test strains within the first 24 hr after application, which is in agreement with the finding of the skin permeation study.

DISCUSSION AND CONCLUSIONS

A topical antimicrobial agent designed to control superfacial partial-thickness wound flora must diffuse into necrotic and ischemic zones in amounts adequate to inhibit microbial growth. Yet its access to the hyperaemic region and systemic circulation should, at the same time, be optimized to minimize local cellular, nonciceptive, and general systemic toxicities relative to the biochemical actions inherent in the active agent or its vehicle. Ideally, the delicate balance between the safety and the effectiveness of such an agent, factors such as patient and nurse acceptance must also be considered in its development.

This study demonstrates that CH and P are capable of penetrating damaged skin, yet at different rates. This fact agrees with preliminary findings obtained from burn patients treated with 2% CHP cream, showing a higher plasma concentration and urinary excretion of P than of CH (4).

The rate-determining step in damaged skin is the dissolution and release rate of CHP from its cream formulations. In contrast, the stratum corneum is the rate-limiting barrier in intact skin. Since the dissolution rate is the rate-limiting step, the transport of CHP through damaged skin is also found to be concentration dependent. The diffusion coefficient of P is similar to reported values for low to medium molecules in human dermis (9). But the *D* value of CH is much smaller than that of P. This may be due either to its large molecular size or to protein binding (10,11) during the diffusion process. We believe that the difference in diffusion rates between CH and P determines the change in the molar ratio in the receptor solution. Chemical assay of 48-hr diffusates indicates that the CH:P molar ratio in the receptor fluid changes from 1:1.83 (before penetration) to 1:2.2 (0.2% cream) or 1:3.6 (2% cream). P becomes the predominant species as the CHP concentration increases.

Because of the fast-penetrating nature of P, it is inferred that the broad-spectrum antimicrobial activity obtained with the combination of CH and P may be not only effective on the skin surface, but even more active in the deeper layers of skin, where Gram-negative bacterial invasion is of particular concern. These results support the original concept of complementing the predominately Gram-positive spectrum of CH with the significant Gram-negative activity of P. However, the data gathered from this *in vitro* model should be extrapolated only with caution to the *in vivo* situation since other as yet characterized factors may be operative, e.g., serum binding, tissue binding, and enzymatic degradation (10,11).

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