Full Length Research Paper

In-vitro Bactericidal Kinetics of Chlorhexidine Gluconate Disinfectant/ Antiseptic Formulations Containing Different Additives

Idowu P. A[1*] and Idowu O. S[2]

[1]*Department of Pharmaceutical Microbiology,and* [2]*Department of Pharmaceutical Chemistry Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria.*

ABSTRACT

Chlorhexidine gluconate(CHG) is a popular disinfectant/antiseptic which is often formulated with additives. We investigated the effect of additives type on the in vitro bactericidal kinetics of CHG in three commercially available formulations: Hibiscrub®, Savlon® and Purit® commonly used as household and hospital disinfectants/antiseptics. The bactericidal kinetics of the products was determined by time-survival curve method using Pseudomonas aeruginosa NCTC 6750 and Staphylococcus aureus NCTC 6571 as model organisms. Best-fit rate constant and half-life was computed by exponential decay curve-fitting. Half-life was; 76.5, 65.5, 66 and 74 (min) for Control solution, Hibiscrub®, Savlon® and Purit® respectively, against Ps. aeruginosa. The corresponding values obtained against Staph. aureus are 51.0, 61.7, 29.3, and 49.0 (min) (95% CI). Presence of alcohol (e.g. Hibiscrub® and Savlon®) caused an insignificant increase in the rate of killing of Ps. aeruginosa relative to preparations that are devoid of alcohol. (p>0.05, 1-way ANOVA). Toward Staphylococcus aureus, the combined effect of cetrimide and alcohol (e.g. Savlon®) is higher than any enhancement due to combination of alcohol and surfactant (e.g. Hibiscrub). Savlon® show a significantly higher bactericidal effect of all the preparations (P<0.0001, 1-way ANOVA). The choice of additives in the formulation of chlorhexidine antiseptic solutions significantly alters the kinetics and overall bactericidal effect of CHG towards Staphylococcus aureus but not Pseudomonas areruginosa.

Keywords*:* Chlorhexidine gluconate, Savlon, Hibiscrub, Purit, bactericidal kinetics

INTRODUCTION

Chlorhexidine gluconate (CHG) is a bisbiguanide cationic compound widely used as

*Corresponding author:
E-mail: igboyega@yahoo.com
Phone: 2348033524399

antiseptics/disinfectants because it is relatively rapid acting, non-toxic and with residual adherence to skin surfaces. It is bactericidal or bacteriostatic against a wide range of Gram-positive and Gram-negative bacteria but more active on the former than the latter (Matrindale, 1996; Imperial Chemical Industries (IC1), 1973). A concentration of 1:2000000 inhibit *Staph. aureus* whereas 1:50000 inhibits *Ps. aeruginosa* (Scott *et al,* 2004). There are many reports that Gram-negative bacteria, especially *Pseudomonas, Proteus* and *Providencia* species were not only less susceptible, but are getting more resistant to CHG and have actually been isolated from dilute solutions of CHG (Scott *et al*., 2004; Bordon *et al*.,1967; Stickler *et al*.,1987; Onaolapo, 1990; Ogunsola *et al*., 2002). A significant number out of 443 clinical strains of Gram-negative bacteria were not inhibited by 0.02-0.05%^{w}/v, which is the concentration normally used for disinfection, and *Pseudomonas, Proteus* and *Providenicia* species specifically required $\geq$0.1%^{w}/v (Mengistu *et al*., 1998).

Consequently, efforts are continuously directed towards improving the potency of chlorhexidine, especially against Gram-negative bacteria. Hence CHG products formulated with additives such as cetrimide, alcohols and surfactants are many and commercially available (Richards *et al*., 1973; Wilson *et al*.,1990; Russel *et al*., 1977). It has been suggested that the resultant effects of such additives on the bacteriostatic and bactericidial activities of the final product should be investigated (Lowbury *et al*., 1973).

The present study was undertaken to compare the bactericidal kinetics of the three CHG products: Hibiscrub (ICI/Zeneca, UK), Salvon Antiseptics (Johnson & Johnson, UK) and Purit (CAPL, Nigeria) in order to determine the resultant effect of the added agents. A Chlorhexidine gluconate solution B.P. (FeF Chemicals, UK) (containing 20% CHG in water), without any additive was used as control solution. The percentage content of the CHG in the preparations was ascertained by non-aqueous titrimetric assay procedure described in B.P. 1998 (British Pharmacopoeia, 1998.

MATERIALS AND METHODS

Disinfectants*:*
The Chlorhexidine gluconate formulations used in this study have the following composition: Hibiscrub® (chlorhexidine gluconate 4.0w/v, isopropyl alcohol, and lauryl dimethamine oxide), Savlon® (chlorhexidine gluconate 0.3% w/v, cetrimide 0.3% w/v, *n*-propyl alcohol 2.84% w/v), Purit® (chlorhexidine gluconate 0.3% w/v, cetrimide 3.0% w/v). The manufacturing and expiry dates, storage conditions and other manufacturers' specifications were strictly considered for the products.

Preparation of disinfectant working solution*:*
The CHG concentration in each of the formulations and the control solution was adjusted to 0.1% w/v by diluting with sterile distilled water (SDW), to obtain a working solution for each brand and control solution.

Microorganisms*:*
Clinical isolates of the following bacteria; *Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, and Staphylococcus aureus* were obtained from University College Hospital (UCH) while standard strains *Pseudomonas aeruginosa NCTC 6750* and *Staphylococcus aureus NCTC 6571* were obtained fromPharmaceutical Microbiology Laboratory, University of Ibadan, Ibadan, Nigeria, They were maintained on nutrient agar slopes at 4^0C in the laboratory.

Media*:*
All bacteria were cultured on nutrient agar (No. 2), nutrient broth (pH 7.4) and Mueller-Hinton agar (Oxoid, UK).

Other additives*:*
Tween-80 and Lecithin (BDH, UK)

Susceptibility Testing*:*
The susceptibility of bacterial isolates to CHG products was determined by using agar dilution method following standard procedure (British standards, 1984; Lennette *et al*., 1988) as follows. Geometric serial dilution of each product was made from the working solution (0.1% w/v CHG), to produce 0.1 to 0.0004% CHG solutions. 1ml of each dilution was mixed with 9 ml of molten Mueller-Hinton agar in 50mm diameter Petri dish. The agar was allowed to set, dried briefly at 37^oC and inoculated with the test organisms by surface spreading at inoculum size of 10^6 -10^7 cells per ml (optical density = 0.7 at 540 nm). As a control, the organisms were also inoculated on agar containing no CHG. All tests were carried out in triplicates, and plates were then incubated at 37^oC for 24 hours.

Bactericidal Kinetics*:*
The time-survival curve method was used to evaluate the bactericidal kinetics of the products and control solution on *Ps. aeruginosa* and *Staph. aureus* using standard procedure (Lennette et al., 1988; B.P, 1998) as follows. Each organism prepared as overnight culture containing 10^7 cells per ml (0.1ml) was added to the working disinfectant solution (0.1% CHG, 10 ml) of each product. At time intervals of 0, 30, 60, 90, 120, 210 and 240 minutes, 1.0 ml aliquot of the reaction mixture was transferred to 10 ml tubes containing a mixture of tween-80 (5 ml) and lecithin (1.5 ml) as inactivator (B.P., 1998). Further serial dilutions were done in SDW and the numbers of viable cells were determined by dilution plate counting on nutrient agar after incubation of plates at 37^oC for 24 hours. The limit of detection was 10^2 CFU per ml.

Mathematical Modeling and Statistical Analysis*:*
Bactericidal kinetics was modeled by one-phase exponential decay curve fitting analysis. A global model that analyzes together, the family of data sets for each of the four preparations was adopted. The constraint specified for the analysis was that the plateau is shared among the data sets and must be >0. In addition, the best-fit rate constant, K, is set as >0.
Best-fit rate constants (K, min[-1]) was computed as Mean±SEM by non-linear regression analysis, with 95% confidence interval (CI). Half-life $(t_{1/2})$ was computed by the experession $t_{1/2}= 0.693/K$, typical for a

first-order exponential decay. The K values for the various preparations were compared by 1-way analysis of variance (ANOVA). Statistical significance was defined as P<0.05. was performed by GraphPad Prism Version 4.01 for Windows (GraphPad Software, San Diego CA, USA, www.graphpad.com).

The curve fitting analysis and 1-way ANOVA were performed by GraphPad Prism Version 4.01 for Windows (GraphPad Software, San Diego CA, USA, www.graphpad.com)

RESULTS

The minimum inhibitory concentrations (MIC) of the various formulations against four microorganisms are shown in Table 1. Figure 1 shows the exponential decay curve fitting of the bactericidal kinetics data. The best-fit rate constants obtained from the bactericidal kinetics of the formulations against the two model organisms are shown in Table 2. Statistically significant difference in the best–fit rate constant is indicated by superscripts. Savlon showed a statistically significant increase in the bactericidal kinetics against *Staph. aureus*, (P<0.0001) while Hibiscrub showed an increase in the bactericidal kinetics against *Ps. aeruginosa*, which is not statistically significant (P>0.05). The best-fit rate constants were used to compute best-fit half-life for each brand. Half-life was; 76.5, 65.5, 66 and 74 (min) for Control solution, Hibiscrub®, Savlon ®and Purit® respectively, against *Ps. aeruginosa*. The corresponding values obtained against *Staph. aureus* are 51.0, 61.7, 29.3, and 49.0 (min)   (95%   CI).

Table 1
Minimum inhibitory concentrations (MICs) of chlorhexidine gluconate products on test organisms.

	MIC (%v/v)			
Test Organism	**Hibiscrub®**	**Savlon®**	**Purit®**	**Control solution**
Staph. aureus	0.0008	0.0008	0.0008	0.0016
Pr. mirabilis	0.0031	0.0063	0.0063	0.0063
E. coli	0.0031	0.0063	0.0063	0.0063
Ps. aeruginosa	0.0063	0.0125	0.0250	0.0600

Table 2
Curve fitting and rate constant

Products		Rate constant K (min^{-1})		
	Best-fit value	**95% CI**	**Std Error**	**R^2**
Pseudomonasaeruginosa.*				
Control	0.00906	0.00710-0.0110	0.000953	0.937
Hibiscrub®	0.0106	0.00873-0.0124	0.000903	0.962
Savlon®	0.0105	0.00884-0.0122	0.000810	0.97
Purit®	0.00919	0.00697-0.0114	0.00108	0.925
Staphylococcus aureus*				
Control	0.0136	0.0113 – 0.0159	0.00108	0.974
Hibiscrub®	0.0112	0.00866 - 0.0138	0.00122	0.954
Savlon®	0.0236 ^{a}	0.0201 - 0.0271	0.00163	0.986
Purit®	0.0141	0.0117 - 0.0166	0.00113	0.974

Curve fitting was performed by global model of non-linear regression analysis, with the following constraints and statistics:
Constraints: Plateau is shared and must be >0, K is >0, _Global shared parameter:_ Plateau = 0.598% , _Goodness of fit:_ DF=27, R^2 = 0.947, Absolute sum of squares = 2116, S$_{y.x}$ = 8.85, p > 0.05 (1-way ANOVA)
**Constraints:* Plateau is shared and must be >0, K is >0, _Global shared parameter:_ Plateau = 3.99%, _Goodness of fit:_ DF=15, R^2 =0.974, Absolute sum of squares = 733, S$_{y.x}$ = 6.99, p <0.0001 (1-way ANOVA), *a* Superscripted item indicates brand that is significantly different from the control and the other two formulations at p<0.05 probability level (1-way ANOVA).
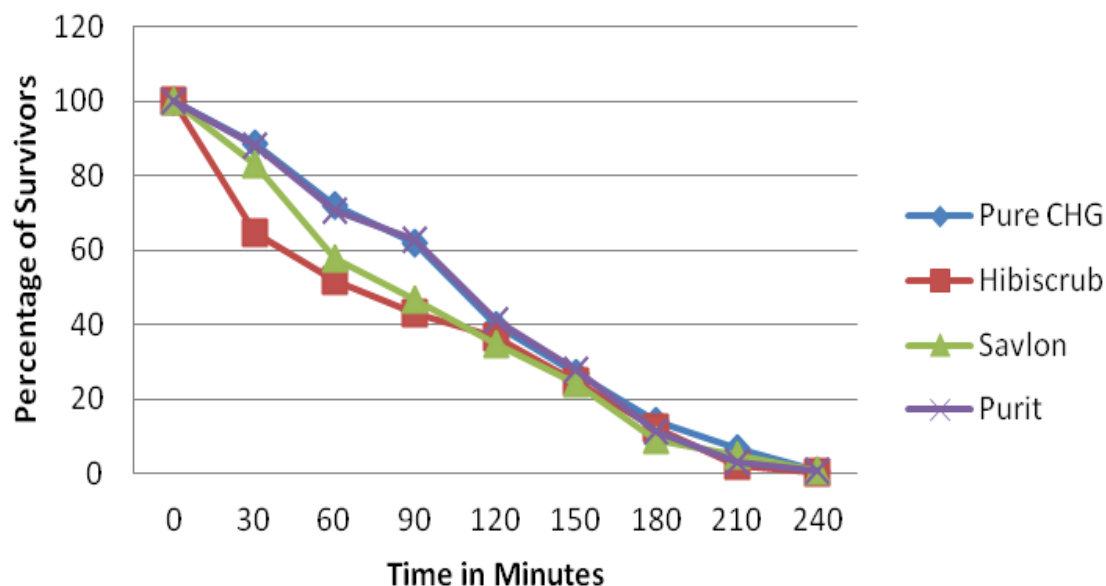
Figure 1a:
Bacterial kinetics of chlorhexidine gluconate formulations against *Pseudomonas aeruginosa*
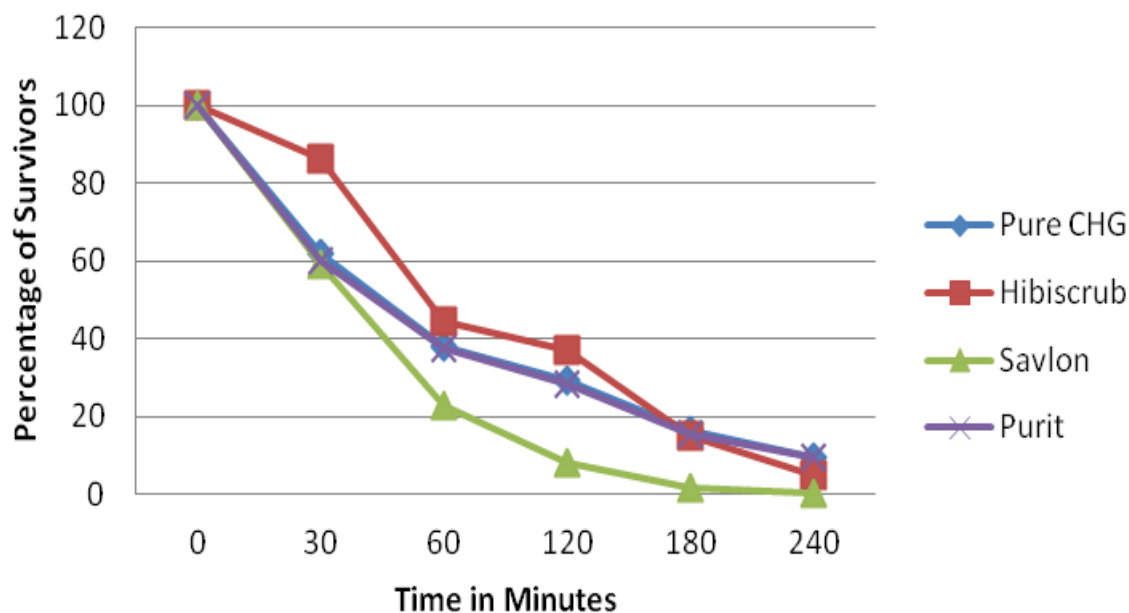

Figure 1b:
Bacterial kinetics of chlorhexidine gluconate formulations against *Staphylococcus aureus*

DISCUSSION

The bactericidal kinetics of all the products showed an initial rapid kill followed by prolonged survival of the remaining microbial cells which is typical of CHG and quaternary ammonium compounds (Senior, 1972). This initial rapid kill is one of the essential qualities of CHG that made it useful in preoperative disinfection of skin surfaces and other clinical applications (Imperial Chemical Industries (IC1), 1973). The effect of additives on the bactericidal kinetics followed a different pattern in Gram-negative *Ps. aeruginosa* compared with Gram–positive *Staph. aureus*.

Hibiscrub which has surfactant and alcohol in its formulation showed the highest rate of initial kill on *Ps. aeruginosa* ($t_{1/2}$ = 65.5min). The onset of action was

fastest killing up to 40% of population in 30 min while the other formulations could only kill less than 20% of *Ps. aeruginosa* population. This improved activity is probably due to the presence of surfactant in Hibiscrub formulation which is absent in Savlon® and Purit®. According to Russel and Furr, any membrane-acting biocide that contains surfactant is able to disaggregate the cell wall or remove the Mg^{2+} ions of *Ps. aeruginosa,* thus giving chlorhexidine direct access to attack the bacterial membrane (Lowbury *et al*., 1973). The presence of alcohol may further enhance the penetrability of chlorhexidine into the microbial cell. This may explain why the alcohol containing CHG products (Hibiscrub and Savlon) showed higher rate of biocidal activity than products without alcohol (Purit® and Pure CHG). The results were similar to those reported on oral pathogens (Herrera *et al*., 2003). However, the increased rate of kill of surfactant containing Hibscrub, relative to the other brands is not statistically significant (P>0.05) as all the formulations showed almost similar kill kinetics after 2h.

In contrast, Savlon which contain cetrimide and alcohol in its formulation showed the highest rate of kill ($t_{1/2}$ = 29.3 min) against Gram positive *Staph. aureus*, relative to Hibiscrub® and Purit®. This suggests that the main enhancement of bactericidal kinetics on *Staph. aureus* is the presence of alcohol in the formulation rather than the presence of certimide (component of Purit® and Savlon®) or perhaps a synergistic effect between cetrimide and alcohol. It was noted that the onset of action at 30min was faster with Savlon and Purit (40% of *Staph. aureus* population killed) than within Hibiscrub® (<13% of *Staph aureus* population killed). The lowest bactericidal kinetics of Hibiscrub® on *Staph. aureus* may be a counter-effect of surfactant in slowing down bactericidal activity by micellar formation. This is consistent with the suggestion of Lowbury and Lilly that when QAC's are formulated with detergents, the activity of the final formula must be confirmed by microbiological tests (British Pharmacopoeia, 1998). The presence of alcohol in Savlon (without surfactant) produced the highest cidal rate on *Staph. aureus* which is statistically significant (P <0.0001).

Considering the MIC value obtained (Table 1), all the products have an enhanced bacteriostatic activity on all the tested organisms compared with the control. As expected from previous analyses (Richards *et al*.,1973; Russel *et al.,*1977; Baker *et al*., 1987), Gram negative bacteria (*Ps. aeruginosa, Pr. Mirabilis, and E. coli)* were less susceptible to CHG product than Gram positive *Staph. aureus* (Table 2). The use of tween-80 and lecithin as inactivator for CHG has an additional advantage of overcoming the clumping of cells during viable counts, which is a common problem with the

QAC and CHG products (British Pharmacopoeia, 1998). The results correlated with various "in use" in vitro and in vivo tests (McLure et al., 1992; Slots et al., 1991; Orjajarvi, 1976; Ayliffe et al., 1988; Lowbury et al., 1974). The differences in the bactericidal kinetics, as shown by the half life and rate constants (Table 2), of the products within the first 60 min were noticeable but not significant on *Ps. aeruginosa*. But Savlon showed a higher bactericidal kinetics that was significantly different than the activity of the control solution and the other two brands towards *Staph. aureus*.

Overall, the study shows that the choice of additives in the formulation of chlorhexidine antiseptic solutions significantly alters the kinetics and overall bactericidal effect of CHG towards Gram positive *Staphylococcus aureus* but not on Gram negative *Pseudomonas aeruginosa*.

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