The Effect of Formulation on the Antimicrobial Activity of Cetylpyridinium Chloride in Candy Based Lozenges

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Purpose. The purpose of this investigation was to determine the influence on the antimicrobial activity of cetylpyridinium chloride of the various components of the formulation of each of six candy based lozenges.
Methods. *In vivo* activity was investigated using six volunteers by determining the reduction in colony forming units recoverable from the oropharynx after sucking each lozenge separately on different days. *In vitro* determinations investigated the relative activity of aqueous solutions of the lozenges, the effect on activity of additional active ingredients, pH and lozenge base ingredients against separate inocula of each of the test organisms *Staphylococcus aureus, Streptococcus pyogenes* and *Candida albicans.*
Results. Both *in vivo* and *in vitro* results showed that the pH of the dissolved lozenge solution was the single most influential readily adjustable formulation parameter which significantly influenced the activity of cetylpyridinium chloride activity in candy based lozenges.
Conclusions. Lozenges containing cetylpyridinium chloride as the active ingredient should be formulated at a pH greater than 5.5.

KEY WORDS: cetylpyridinium chloride; lozenge formulation; pH; antimicrobial activity; *in vivo; in vitro.*

INTRODUCTION

Forty years ago lozenges containing cetylpyridinium chloride (CPC)[3] were shown to have antibacterial activity (1). Subsequent reports of the efficacy and of the effect of formulation on the antimicrobial activity of lozenges have been few but have confirmed the activity of CPC formulated in lozenges and have indicated that the antimicrobial activity is greater in candy based lozenges then in tablet based lozenges (2–6). The present investigation was undertaken to evaluate the effect on the antimicrobial activity of CPC of the excipients used in the formulation of candy based lozenges.

MATERIALS AND METHODS

Staphylococcus aureus NCTC 10788, *Streptococcus pyogenes* NCTC 10867 and *Candida albicans* NCTC 3179 were obtained from the National Collection of Type Cultures, Colindale, London. Tween 80, lecithin and benzyl alcohol (BZA) from BDH, Poole, England; cetylpyridinium chloride (CPC), menthol (MT), benzocaine (BZC), L-ascorbic acid (sodium salt) and sucrose (SU) from Sigma, Poole, England; eucalyptus oil (EO), ascorbic acid (AA) and liquid glucose (LG) from Thornton and Ross, Huddersfield, England. Nutrient broth, Sabouraud broth, Todd-Hewitt broth, blood agar base, and fabricated horse blood were obtained from Oxoid, Basingstoke, England. The manufacturer and composition of each lozenge and the pH of the lozenge solutions in saliva and distilled water plus the pH of aqueous CPC solutions containing the various active ingredients contained in the lozenges are given in Table 1.

Measurement of pH

The pH of each antimicrobial solution was determined at 20–22°C using a Mettler Delta 320 pH meter.

In Vivo Evaluations: Antimicrobial Activity in Oral Cavity

Six proprietary lozenges were tested on six separate days using the same six volunteers throughout the investigation (Figure 1). A 24 h wash out was allowed between tests. This was more than adequate because it had been shown previously that the antimicrobial effect of a single lozenge did not last more than 2 h (4). The identity of the lozenge was unknown to the volunteers who were independently given the same brand of lozenge to suck on the same day. A lozenge was sucked and the lozenge saliva solution collected as described previously (2). The pH value for each lozenge saliva solution was measured. The effect of sucking a lozenge on the number of microorganisms recoverable from the oral cavity before and after sucking a lozenge was determined as described previously (4). A standard 10 sec gargle followed by a 20 sec vigorous rinse of the mouth was carried out by each subject using 10 ml of sterile water and 0.15 ml quantities of these washings were immediately mixed with 0.15 ml quantities of inactivation broth consisting of Nutrient broth containing 0.125% w/v lecithin and 3% w/v Tween 80 (4–6). Ten μl of each mixture was then diluted and 0.1 ml quantities plated in triplicate on blood agar plates using the microtitre counting method and incubated at 37°C for 48 h (6).

In Vitro Evaluation: Inoculum Preparation

S.aureus and *Str.pyogenes* were cultured at 37°C for 18 h and *C.albicans* at 25°C for 48 h using Nutrient broth, Todd Hewitt broth and Sabouraud broth respectively. The cultures were then centrifuged (4000 × g, 10 min, 4°C). The cell pellets were washed with sterile 0.9% w/v sodium chloride, recentrifuged and resuspended in sodium chloride. The cell numbers were adjusted to approximately 1.56×10^{11}, 4.48×10^{9}, and 6.65×10^{9} organisms per ml for *S.aureus, Str.pyogenes*, and *C.albicans* respectively.

Determination of Antimicrobial Activity

The microtitre colony forming units (CFU) counting method was validated for each test organism as described by Richards & Xing (6). A volume of 0.15 ml sterile distilled water (control) and each of the antimicrobial solutions was

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[3] ABBREVIATIONS: AA: ascorbic acid; CPC: cetylpyridinium chloride; BZA: benzyl alcohol; BZC: benzocaine; EO: eucalyptus oil; LG: liquid glucose; MT: menthol; SU: sucrose.

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Table 1. The pH of Lozenge Solutions in Saliva, Lozenge Solutions in Distilled Water, and Aqueous CPC Solutions Plus the Various Active Ingredients Contained in the Lozenges

Lozenge	Volume and pH of lozenge saliva solution*		pH of 10 ml aqueous lozenge solution	Composition and pH of 10 ml aqueous solution of active ingredients	
	ml	pH		composition	pH
Merocets#	14.65 ± 1.17	5.72 ± 0.04	5.44	CPC 1.4 mg CPC 1.4 mg	6.12
Merocaine#	14.97 ± 0.73	6.17 ± 0.05	5.36	BZC 10 mg CPC 1.4 mg	6.83
Merothol#	15.17 ± 0.64	6.77 ± 0.07	6.12	MT 6 mg CPC 1.4 mg	6.52
				EO 5 mg CPC 1.4 mg	6.24
Merovit#	18.22 ± 1.95	4.93 ± 0.06	4.38	AA 120 mg CPC 1.4 mg	4.28
				Na ascorbate 140.6 mg CPC 1.33 mg	7.18
Cepacol**	14.40 ± 0.74	6.11 ± 0.05	5.70	BZA 6 mg	6.21
Lemsip##	23.72 ± 3.40	4.16 ± 0.11	3.92	CPC 2.5 mg	6.12

*Mean of six subjects. All lozenges dissolved within 8.2 to 9.8 min.
#Marion Merrell Dow, U.K.
##Reckitt & Coleman Australia.
**Marion Merrell Dow Thailand.

mixed with 0.15 ml of cell suspension to yield final concentrations of approximately 8×10^9, 1.76×10^8, and 2.0×10^8 per ml for *S.aureus, Str.pyogenes* and *C.albicans* respectively. After 10 min contact time at 20–22°C, 10 µl of each culture was inactivated and diluted for counting. Each dilution was transferred in triplicate to blood agar plates. The bacteria were incubated at 37°C for 48 h and the yeast incubated at 25°C for 72 h before counting the CFU.

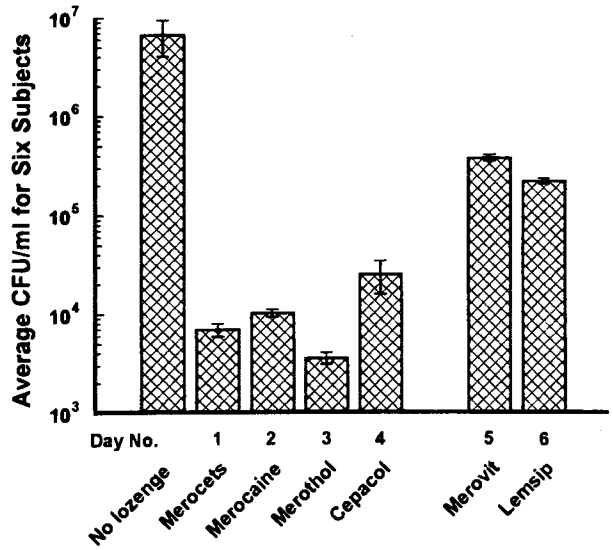

Fig. 1. Average counts with standard deviations of CFU recovered from the oropharynx of six subjects before and after sucking each of six lozenges on six separate days. Tukey's Wholly Significant Difference Analysis showed that a significant difference existed between the activity of both Merovit and Lemsip lozenges and the activity of the other four lozenges.

Comparison of the Activity of Lozenge Solutions in Distilled Water

The test solutions were prepared by dissolving each of the test lozenges separately in 10 ml volumes of sterile distilled water. Sterile distilled water was the control. Plating was performed at a contact time of 10 min. The 10 ml volume was chosen because it is within the previously determined range of volumes (8–20 ml) produced by people when sucking and dissolving each of a series of lozenges in the mouth (4) and the 10 min contact time was chosen because it represents the approximate time for the lozenges to dissolve and release CPC in the mouth (Table 2).

Effect of Additional Ingredients on Antimicrobial Activity of CPC

The reduction in CFU for inoluca of *S.aureus* and *Str.pyogenes* was compared for 10 ml aqueous solutions containing CPC 1.4 mg; CPC 1.4 mg plus BZC 10 mg; CPC 1.4 mg plus MT 6 mg; CPC 1.4 mg plus EO 5 mg, CPC 1.4 mg plus AA 120 mg, and CPC 1.4 mg plus BZA 6 mg. Ten ml aqueous solutions containing CPC 2.5 mg alone and plus the same concentrations of the above lozenge ingredients were also evaluated using *C.albicans* as the test organism. Dilutions and plating out were performed at a contact time of 10 min.

Effect of pH on Antimicrobial Activity of CPC

The reduction on bacterial CFU with time was determined for 10 ml aqueous solutions containing CPC 1.4 mg adjusted

with HCl to pH 3.5, 4, 4.5, 5, 5.5, 6, and 7. Ten ml aqueous solutions containing the higher concentration of CPC 2.5 mg were also adjusted to the above pH values and the reduction of CFU with time determined using *C.albicans* as the test organism. Counts were determined after a contact time of 10 min.

Effect of Lozenge Base Constituents on Antimicrobial Activity of CPC

The reduction of CFU with time was also determined for 10 ml aqueous solutions containing CPC 1.4 mg; CPC 1.4 mg plus LG 1 g (50 percent lozenge weight); CPC 1.4 mg plus SU 1 g (50 percent lozenge weight); CPC 1.4 mg plus LG 1g/SU 1g; CPC 1.4 mg plus LG 1g/SU 1g/BZC 10 mg. Ten ml aqueous solutions containing CPC 2.5 mg alone and plus the same concentrations of the candy base excipients as those above were also evaluated with *C.albicans* as the test organism. Counts were determined after a contact time of 10 min.

Statistics

The difference in activity between pairs of antimicrobial solutions within a particular test group, e.g., the activity of the six aqueous lozenge solutions against a particular test organism, was tested for significance using Tukey's Wholly Significant Difference (WSD) test at the 5 percent significance level.[7] The WSD was calculated as shown below:[8]

$$WSD = q(k, v)\sqrt{\frac{ErrorMS}{n}}$$

WSD represents the 'Wholly Significant Difference' between means at the 5 percent level of significance; *ErrorMS* indicates the pooled error mean square; *n* indicates the number of readings in each mean; *k* represents the number of means under test; *v* represents the number of degrees of freedom associated with the ErrorMS; *q(k,v)* is the quantile of a studentized range distribution. Values of q(k,*v*) at the 5 percent level are given in Table 11 of "Statistical Tables".[7] A significant difference at the 5 percent level existed when the difference between the means in a particular group tested was greater than the calculated WSD value. This test is available in commercially available statistical packages (SPSS and Minitab) and is also known as Tukey's Honestly Significant Difference test. The test reduces the possibility of false positives compared with pairwise 't' tests.

RESULTS

In Vivo

The volume of saliva produced dissolving the various lozenges over a similar time (8.2 to 9.8 min); the pH of the lozenge saliva solutions and the pH of equivalent concentrations of CPC solutions containing various lozenge secondary active ingredients are given in Table 1. It is seen that saliva has a slight buffering capacity modifying the pH compared with the distilled water solutions by an average of 0.47. The antibacterial activity of each of the six lozenges against the normal aerobic bacterial flora of the oropharynx of each of six subjects is given in Figure 1. The activities of Merovit and Lemsip lozenges reduced the average bacterial count by approximately 1.5 log cycles but this activity was significantly less than the activities of Merothol, Merocets, Merocaine, and Cepacol. The reduction in count produced by these latter lozenges ranged between 3.3 log cycles for Merothol and 2.5 log cycles for Cepacol.

In Vitro

The *in vitro* investigations were performed to evaluate the effect of lozenge base materials and secondary active ingredients on the activity of CPC.

Comparison of Aqueous Lozenge Solutions

Figure 2 compares the activities of the six lozenges separately dissolved in 10 ml of sterile distilled water. Merovit and Lemsip solutions are seem to have significantly less activity against *S.aureus* and *Str.pyogenes* (but not against *C.albicans*) than the other four lozenges.

Effect of Additional Active Ingredients

The effect of BZC, MT, EO, AA, and BZA on the activity of CPC is given in Figure 3. AA caused a significant reduction in activity against each test organism and BZC caused a non statistically significant reduction in activity against the test bacteria. MT enhanced CPC activity against both the bacterial test strains and BZA enhanced activity against *Str.pyogenes* but in neither case was the enhanced activity significant.

Effect of pH

Figure 4 shows that a gradual reduction of CPC activity occurred as the pH was reduced but the significant reduction of CPC activity is seen to occur between pH 5.0 and pH 5.5 with each test organism.

Effect of Lozenge Base Constituents

Figure 5 shows that the addition of either LG or SU at concentrations present in lozenge formulations did not significantly reduce the activity of CPC against the test organisms. The addition of both LG and SU and the addition of SU plus LG plus BZC significantly reduced the activity of the test solutions of CPC against the test organisms.

DISCUSSION

It is seen from the results that there is consistency between the *in vivo* and *in vitro* determinations of the antibacterial activity of the lozenges. In both situations Merothol, Merocets, Merocaine and Cepacol were more active than Lemsip and Merovit.

The latter two lozenges were the only two lozenges which gave a pH of less than 5.5 when dissolved in saliva. Merovit (pH 4.93) contains AA and Lemsip (pH 4.16) contains natural lemon. Thus although Lemsip contains by far the highest concentration of CPC of all the lozenges tested it is together with Merovit less active *in vivo* and *in vitro* than the four lozenges formulated at higher pH. Therefore pH is seen to be a critical parameter in the formulation of candy based antimicrobial lozenges having CPC as the active agent. These lozenges should be formulated at a pH greater than 5.5. It should be noted

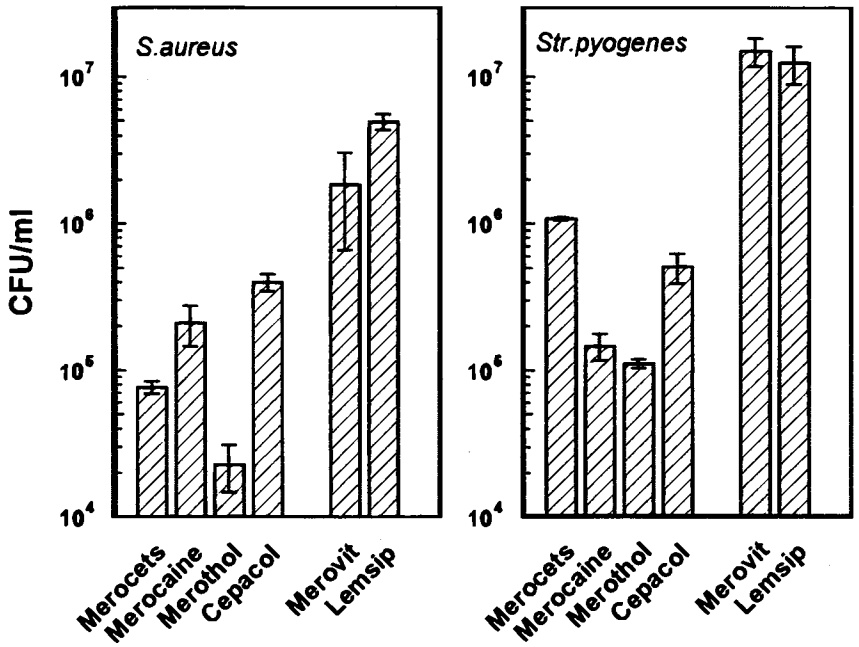

Fig. 2. Kill produced by 10 ml lozenge solutions in distilled water after 10 min contact at 20–22°C with bacterial cell suspensions. The approximate final inocula of test organisms were *S. aureus* 8.0×10^9 CFU/ml and *Str.pyogenes* 1.76×10^8 CFU/ml. Tukey's Wholly Significant Difference Analysis showed that a significant difference existed between both Merovit and Lemsip lozenge solutions compared with the other four lozenge solutions.

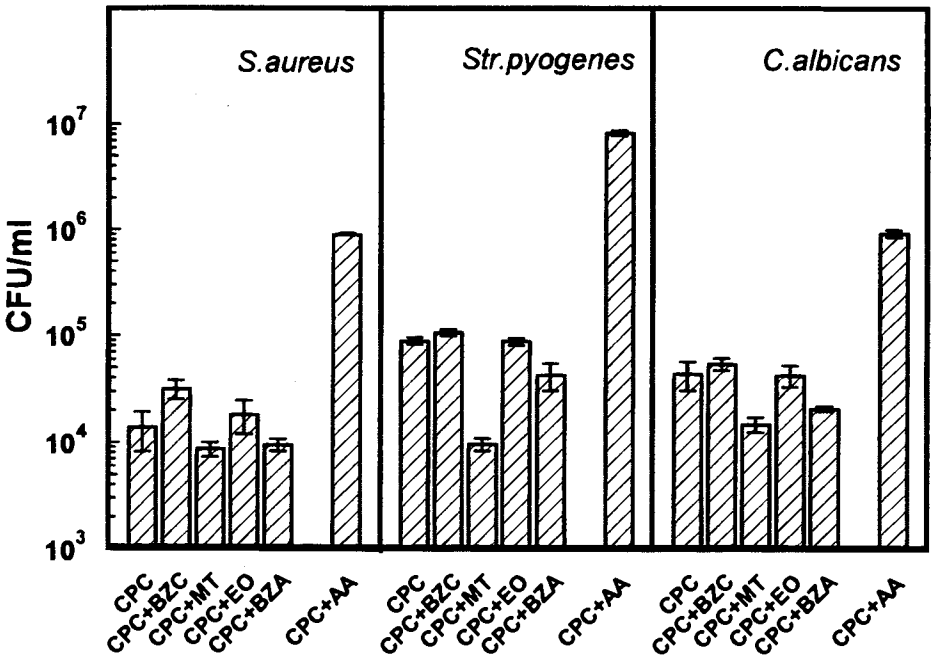

Fig. 3. Kill at 20–22°C of *S.aureus, Str.pyogenes* and *C.albicans* suspensions after 10 min contact with 10 ml aqueous solutions CPC (1.4 mg/10 ml for bacteria, 2.5 mg/10 ml for yeast) and CPC plus an additional ingredient. The approximate final inocula of test organisms were *S.aureus* 8.0×10^9 CFU/ml, *Str.pyogenes* 1.76×10^8 CFU/ml, and *C. albicans* 2.0×10^8 CFU/ml. The concentrations of additional ingredient were BZC 10 mg/10 ml, ME 6 mg/10 ml, EO 5 mg/10 ml, AA 120 mg/10 ml, BZA 6 mg/10 ml. Tukey's Wholly Significant Difference Analysis showed that a significant difference existed between CPC plus AA and CPC plus the other additional ingredient.

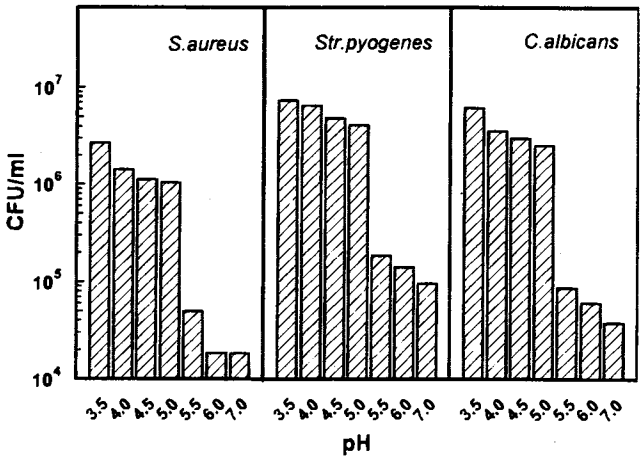

Fig. 4. Kill at 20–22°C of *S.aureus, Str.pyogenes* and *C.albicans* suspensions after 10 min contact with 10 ml aqueous solutions CPC (1.4 mg for bacteria, 2.5 mg for yeast), at different pHs. The approximate final inocula of test organisms were *S.aureus* 8.0×10^9 CFU/ml, *Str.pyogenes* 1.76×10^8 CFU/ml and *C.albicans* 2.0×10^8 CFU/ml. Tukey's Wholly Significant Difference Analysis showed that a significant difference existed between pH 3.5, 4.0, 4.5, and 5.0 compared with 5.5, 6.0, and 7.0.

that the increase in activity of CPC at increasing pH was not considered to be due to a CPC ionisation effect because CPC is ionised at all the pH values (9). The increased activity of CPC at higher pH values is thought to result from an increased ionisation of the bacterial surface groups which provides more negatively charged sites of interaction for the cationic antimicrobial agent (10).

None of the additional active ingredients significantly enhanced the activity of CPC. BZA is the only additional ingredient which has been reported to have marked antibacterial activity (11). The concentration 1 percent, used in that investigation, was far in excess of the 0.06 percent concentration used in this investigation.

The candy base consisting of both SU and LG also significantly reduced the activity of CPC. This would have affected all six lozenge formulations similarly. The addition of SU plus LG is an intrinsic consequence of having a candy based lozenge formulation. Despite the two sugars reducing the antibacterial activity the overall activity of candy based lozenge formulations with a pH greater than 5.5 is still quite marked (2.5–3.3 log cycle reduction in CFU in the *in vivo* evaluation). Thus the candy based formulations result in products with an acceptable level of antibacterial activity.

However, none of the lozenge formulations exhibited marked activity against the high inoculum of *C.albicans* used in this evaluation although it is seen from Figure 5 that CPC 2.5 mg in 10 ml water produced a marked reduction of approximately 3.7 log cycles in CFU at 10 min contact. This indicates that the production of a lozenge containing CPC and possessing activity against *C.albicans* should be possible.

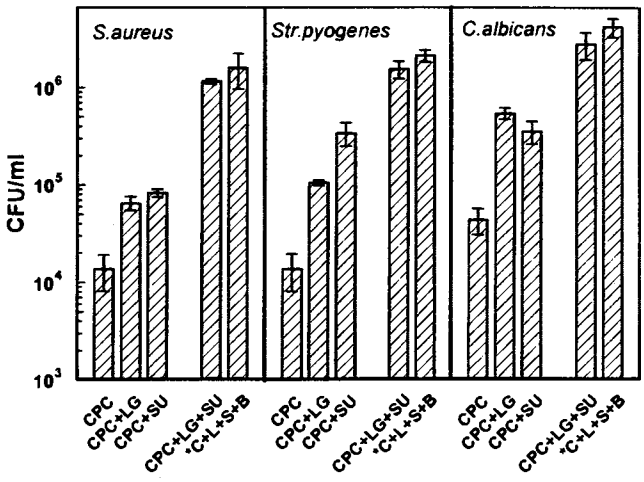

Fig. 5. Kill at 20–22°C of *S.aureus, Str.pyogenes* and *C.albicans* suspensions after 10 min contact with 10 ml aqueous solutions containing CPC (1.4 mg for bacteria, 2.5 mg for yeast) and CPC plus additional ingredients. The approximate final inocula of test organisms were *S.aureus* 8.0×10^9 CFU/ml, *Str.pyogenes* 1.76×10^8 CFU/ml and *C. albicans* 2.0×10^8 CFU/ml. The concentrations of additional ingredients were LG 1 g/10 ml, SU 1g/10 ml, and BZC 10 mg/10 ml. Tukey's Wholly Significant Difference Analysis showed that a significant difference existed between both CPC + LG + SU and * CPC + LG + SU + BZC compared with CPC alone, CPC + LG and CPC + SU.

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