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Synergistic antifungal interaction between miconazole nitrate and chlorhexidine acetate

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Abstract

Antifungal interaction between miconazole nitrate and chlorhexidine acetate was investigated prior to development of a new bioadhesive lozenge for the more effective treatment of oral candidosis. In vitro susceptibility of a laboratory isolate of *Candida albicans* to these drugs alone and in combination was examined. Minimum lethal concentration (MLC) was the most reliable method of determining susceptibility. A significant synergistic relationship was established, with maximum effect in the ratio miconazole nitrate/chlorhexidine acetate of 2:1. Antifungal activity was not affected by the presence of Cremophor RH 40 as a solubilizing agent for miconazole nitrate.

Keywords: Antifungal interaction; Miconazole nitrate; Chlorhexidine acetate; Synergy; Isobologram

1. Introduction

Oral candidosis is a fungal infection which affects many patients suffering from immuno-suppressed states including those with AIDS, denture stomatitis or the side-effects of cancer chemotherapy or radiotherapy. The primary etiological agent is *Candida albicans*, which because of high frequency switching is known to exhibit increasing resistance to all established antifungal agents such as the mono-(*N*)-substituted imidazole group (miconazole, ketoconazole and clotrimazole) and nystatin (Gallagher et al., 1992). Reports of resistance to newer triazoles such as fluconazole and itraconazole in patients with oral candidosis are increasing also (Cameron et al., 1993; Johnson et al., 1995), which resistance may be attributable to reduced uptake of the drug due to changes in membrane sterol composition, mutation of cytochrome P-450 resulting in decreased binding affinity for azoles and excess production of cytochrome P-450. A possible way of overcoming this resistance is the use of combinations of anti-

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fungal agents with preferable synergistic action in advanced drug delivery systems, which ensure high local concentrations in the mouth. The efficacy of antifungal therapy is related to the time the treated microorganism is above the minimum inhibitory concentration, which effect can be achieved locally in the mouth using improved drug delivery systems unlike existing conventional lozenges, gels and mouthwashes, which are rapidly cleared from the oral cavity. The minimum inhibitory concentration of miconazole nitrate has been reported to vary from 0.1 to 25 μg/ml (Odds, 1988). The main reason for reported differences in sensitivity may be interlaboratory variation due to inoculum level, temperature of incubation, etc, rather than difference in resistance of *Candidal* strains.

For the in vitro evaluation of antifungals, a number of parameters may be assessed including minimum inhibitory concentration (MIC), minimum lethal concentration (MLC), inhibitory concentration (IC), relative inhibition factor (RIF), disc diffusion, two-dimensional scatterplots and hyphal elongation. Both intra- and inter-laboratory MICs vary considerably. Problems include variability in subjective interpretation during visual examination where growth is only partially inhibited, as well as objective variation in inoculum size, medium composition, pH, incubation temperature, duration of incubation and partial growth inhibition. MLCs may be determined by removing an aliquot from each MIC tube demonstrating no visible growth and plating on solid growth medium. After incubation, the plates prepared from the MIC tube containing the lowest drug concentration showing ≤ 5 colonies may be considered to be the MLC (Casadevall et al., 1993).

Berenbaum (1978) devised a simple method for the measurement of synergy (or antagonism) with combinations of any number of antimicrobial agents. The concentration of each of n agents that produces some 'specific effect' (e.g. MIC or MLC) is found. A reference combination made up of $1/n$ of each of these concentrations is titrated to find a dilution that produces the specific effect. The nature of the effect is determined either algebraically or geometrically. In the algebraic method, when the sum of the fractional inhibitory concentration of each agent is 1, the combination is additive; when the sum is <1, the combination is synergistic; and when the sum is >1, the combination is antagonistic. In the geometric method, a graph is constructed with the axes representing antimicrobial agent concentration on linear scales. When the combination is additive, the isobole (the line joining the points that represent all combinations with the same effect, including equally effective concentrations of the agents alone) is straight. A concave line is obtained for synergistic combinations, and a convex line for antagonistic combinations. Odds (1982) used the method to study the interaction between miconazole, ketoconazole, flucytosine and amphotericin B, and more recently Gallagher (1994) used the method to show a synergistic effect between chlorhexidine and either ketoconazole or fluconazole. Chlorhexidine and clotrimazole in combination were shown also to have a synergistic effect by Nair and Chien (1996) as assessed by fractional inhibitory concentrations. Also a synergistic relationship has been demonstrated between miconazole and fluconazole (Mikami et al., 1992).

This project is concerned with the evaluation of the antifungal interaction of miconazole nitrate and chlorhexidine acetate prior to incorporation into a novel bioadhesive lozenge to ensure the extended delivery of both agents in the desired ratio. Miconazole nitrate acts by inhibition of cytochrome P-450$_{(14DM)}$, inhibition of essential fatty acid biosynthesis, direct interaction with lipid bilayers and reduced adherence of *C. albicans* to mucosa, whereas chlorhexidine acetate acts by direct interaction with cellular components such as lipoprotein membranes and nucleic acids, and decreased adherence of *C. albicans* to buccal epithelial cells associated with inhibition of hyphal development. Consequently because of differences in mode of action, the compounds might be expected to have synergistic activity. A major formulation disadvantage of miconazole nitrate is its very poor aqueous solubility (3.7×10^{-4} M in water, Pedersen et al., 1993) and whereas chlorhexidine acetate is soluble 1 in 55 of water, it has a most unpleasant taste.

2. Materials and methods

2.1. Materials

Candida albicans (Department of Microbiology, University of Dublin), chlorhexidine acetate (ICI), Cremophor® RH 40 (BASF), dimethyl sulphoxide (DMSO, Aldrich), miconazole nitrate (Sigma), Sabouraud's dextrose agar (SDA), Sabouraud's dextrose broth (SDB) (Oxoid), and glass-distilled water were used. All reagents were GPR unless otherwise indicated.

2.2. In vitro susceptibility testing

C. albicans was grown overnight on SDA at 37°C. It was suspended in SDB, the number of cells/ml determined in triplicate using an improved Neubauer haemocytometer (Hudson and Hay, 1989) and diluted with SDB to contain 1×10^5 cells per 4.5-ml containing tube. Miconazole nitrate or chlorhexidine acetate were dissolved in DMSO, using a final appropriate serial dilution into SDB to ensure that when 0.5 ml was transferred to the 4.5-ml inoculated tubes for incubation at 35°C for 24 h; the final concentration of DMSO was not more than 1% (Beggs, 1984). The drug concentration ranged from 0.039–20 μg/ml and the inoculum level was 2×10^4 cells/ml. The effect of Cremophor® RH 40, 160 μg/ml, on the activity of miconazole nitrate was examined similarly. Control tubes containing either no drug or inoculum were treated also and all experiments were duplicated.

After incubation for each of the drugs, the MIC (lowest drug concentration that displayed no growth on visual examination) was determined and scored according to Fromtling et al. (1993), where 0 is optically clear, 1+ is slightly hazy, and 2+, 3+ or 4+ is prominent, slight or no decrease in turbidity as compared to drug-free control. This method is to be recommended for decreasing subjective variability. The MLC (the lowest drug concentration that showed ≤ 5 colonies following subculture of incubated tubes) was found by removing 0.1-ml aliquots from each of the MIC tubes displaying no growth and spreading on sterile SDA plates (20 ml) prior to incubation at 37°C for 24 h. A Carey 3E UV Visible spectrophotometer was used to find the inhibitory concentration (IC_F). The % transmission at 540 nm of each of the incubated tubes was measured against a tube containing only growth medium. The lowest drug concentration which satisfied the following equation was defined as the IC_F:

$$\%T \geq [\%T_{\text{control}} + F(100 - \%T_{\text{control}})]$$

where $\%T$ is percent transmission, control is drug-free tube, and F is a selected fraction less than 1. This equation defines a fraction of inhibition (as set by F) as a function of the turbidity in the drug-free control tubes.

The effect of incubation time was considered by comparing tubes incubated for 24 h at 35°C with those vortexed after 24 h and further incubated to 48 h.

To test for interaction between miconazole nitrate and chlorhexidine acetate, duplicate serial dilutions with replication were made from the reference combination (X′) containing 1/2 the MLCs of both drugs along the line X′0 until a combination X was found that had the same effect as the equally effective concentrations of each of the antifungals used alone (Fig. 1). The initial experiment to find the individual MLCs for

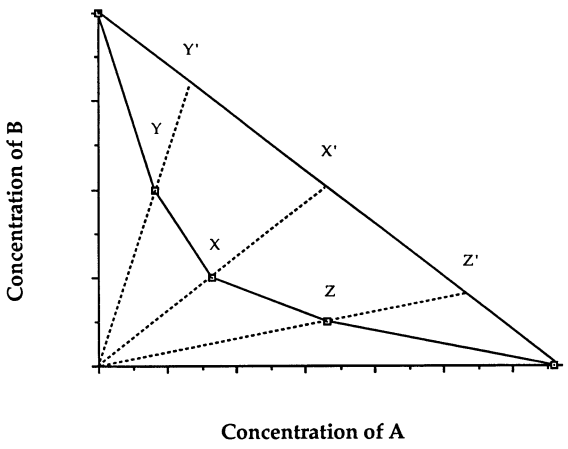

Fig. 1. Isobologram demonstrating synergy between drug A and drug B, where X, Y and Z are the concentrations of A and B in combination that have the same effect as X′, Y′ and Z′, the equally effective concentrations when A and B are titrated alone.

Table 1
Visual scoring of MIC for miconazole nitrate after incubation for 24 and 48 h

Drug concentration (μg/ml)	24-h Incubation		48-h Incubation	
	Series 1	Series 3	Series 1	Series 3
20	0	0	0	0
10	0	0	0	0
5	0	1+	0	1+
2.5	1+	1+	1+	1+
1.25	1+	2+	2+	2+
0.625	1+	2+	2+	2+
0.313	1+	2+	2+	2+
0.156	2+	2+	2+	3+
0.078	2+	2+	2+	3+
0.039	2+	2+	3+	3+

0, optically clear; 1+, slightly hazy; 2+, prominent decrease in turbidity as compared to drug-free control; 3+, slight decrease in turbidity as compared to drug-free control; 4+, no decrease in turbidity as compared to drug-free control.

each drug was repeated in parallel on each occasion, as were the control tubes, because experimental variables may affect reproducibility of results. Furthermore, serial dilutions were made in duplicate on two occasions along the lines Y′0 (containing 3/4 MLC of miconazole nitrate and 1/4 MLC of chlorhexidine acetate) and Z′0 (containing 1/4 MLC of miconazole nitrate and 3/4 MLC of chlorhexidine acetate) until the combinations Y and Z were found that had the same effect as the equally effective concentrations of each of the antifungals alone, in order to confirm the shape of the isobole.

3. Results and discussion

3.1. Preliminary studies to assess in vitro susceptibility

Having considered the literature on factors affecting the in vitro assessment of antifungal agents and in particular guidelines issued by the US National Committee for Clinical Laboratory Standards (Galgiani et al., 1992), a protocol for the testing was devised to include a number of desirable features. Initially, miconazole nitrate and chlorhexidine acetate were considered separately. A Sabouraud's dextrose broth dilution method was used to determine MIC by visual assessment, as it appeared that the agar component of Sabouraud's dextrose agar antagonized the antifungal activity of chlorhexidine acetate. When sterile absorbent paper discs were dipped in solutions of chlorhexidine acetate and placed on inoculated solid medium, no zones of inhibition were apparent after incubation at 37°C for 24 h, whereas their incubation in broth medium caused inhibition of *Candida* growth. The inoculum level used (2×10^4 cells/ml) was similar to that used by previous workers (Pfaller et al., 1990; Casadevall et al., 1993). Tubes were incubated at 35°C according to recommended guidelines. Because of the lack of agreement as regards duration of incubation, a duplicate series of tubes was incubated for a 24-h period, and a separate duplicate series for 48 h.

The results for each of the antifungals are shown in Tables 1 and 2. Duplicate experiments (series 1 and 2 and series 3 and 4) performed on the same occasion showed excellent agreement and hence the results from series 1 and series 3 are shown only. For miconazole nitrate it was seen that the MIC varied from 5–10 μg/ml and the duration of incubation did not appear to affect the result. For chlorhexidine acetate, the MIC varied from 2.5–5 μg/ml with the duration of incubation not being significant. Any variability in MIC values was probably mainly due to slight differences in inoculum levels.

Table 2
Visual scoring of MIC for chlorhexidine acetate after incubation for 24 and 48 h

Drug concentration (μg/ml)	24-h Incubation		48-h Incubation	
	Series 1	Series 3	Series 1	Series 3
20	0	0	0	0
10	0	0	0	0
5	0	0	0	0
2.5	0	2+	0	2+
1.25	1+	3+	4+	3+
0.625	2+	4+	4+	4+
0.313	2+	4+	4+	4+
0.156	4+	4+	4+	4+
0.078	4+	4+	4+	4+
0.039	4+	4+	4+	4+

0, optically clear; 1+, slightly hazy; 2+, prominent decrease in turbidity as compared to drug-free control; 3+, slight decrease in turbidity as compared to drug-free control; 4+, no decrease in turbidity as compared to drug-free control.

Because of the subjective error associated with visual assessment of MIC, the MLC was also determined. Subcultures from the series of tubes containing miconazole nitrate incubated for 24 h resulted in MLCs that varied from 10–20 μg/ml, whilst that from tubes incubated for 48 h resulted in a consistent MLC of 10 μg/ml for all four series of tubes. It was interesting to note that the MLC for miconazole nitrate (10–20 μg/ml) was higher than the MIC (5–10 μg/ml), confirming the fungistatic nature of this drug. When chlorhexidine acetate was examined, slightly more variable results were obtained for MLC, values ranging from 2.5–5 μg/ml for tubes incubated for both 24 and 48 h prior to subculture.

The results of inhibitory concentration determinations are shown in Fig. 2. Because of rapid sedimentation of *Candida* cells, accurate spectrophotometric analysis was difficult. $IC_{0.5}$ is the most commonly reported inhibitory concentration (Galgiani et al., 1987; Hughes et al., 1988). However the tubes containing drug concentrations at the lower end of the range examined (0.039–1.25 μg/ml) gave very similar % transmission values and consequently it was not possible to accurately determine $IC_{0.5}$. Consequently $IC_{0.75}$ values were determined as there was a greater difference in transmission readings at the higher concentrations. The $IC_{0.75}$ was 5 μg/ml for tubes containing miconazole nitrate regardless of incubation time, while that for chlorhexidine acetate was 2.5 μg/ml after 24 h and 5 μg/ml after 48 h incubation.

When the mean % transmission at 540 nm was plotted against visual MIC score for miconazole nitrate (Fig. 3), good correlation was obtained ($r^2 = 1.0$ and 0.95 for 24 and 48 h incubation, respectively). Scores 1+ and 2+ were difficult to interpret consistently as seen by their larger standard deviations for % transmission. This was similar to the findings of Scott et al. (1995), who reported correlation results of visual scores from microtiter plates with mean optical density measurements at 492 nm, where MIC scores of 2+ and 3+ showed maximum variance. When chlorhexidine acetate was considered (Fig. 4), an excellent linear relationship between visual scores and % transmission was observed for the 24-h incubation period ($r^2 = 0.96$). However the results from 48 h incubation did not follow a good linear relationship. This was related to the subjective assessment of MIC, where the 1+, 2+ and 3+ data points were based on low numbers of data points.

According to the method of Berenbaum (1978) for investigating a possible synergistic relationship between two antifungals, the concentration of each of the agents that produces some 'specific effect' must be found first. Whilst the MIC values found for both miconazole nitrate and chlorhexidine acetate against *C. albicans* are in good agree-

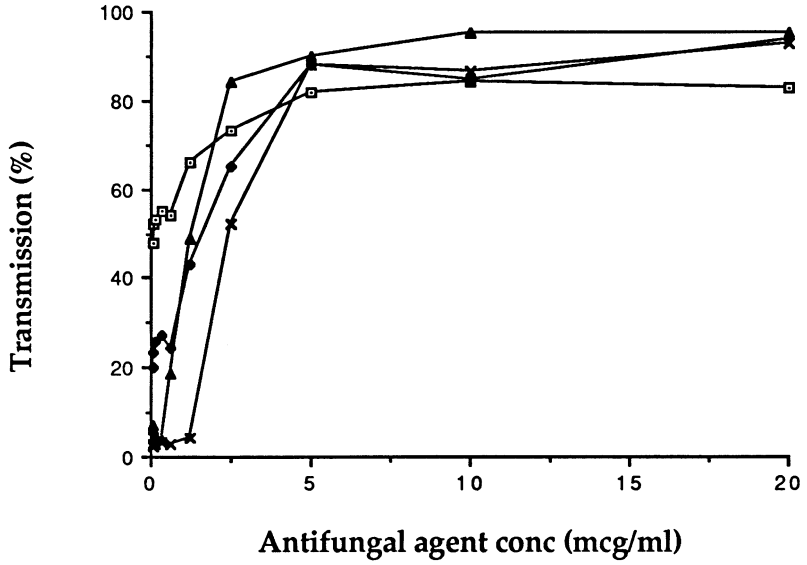

Fig. 2. Plot showing mean % transmission at 540 nm for a series of broth macrodilution tubes containing miconazole nitrate incubated for 24 (– □ –) and 48 h (– • –) or chlorhexidine acetate incubated for 24 (– ▲ –) and 48 h (– × –).

ment with widely cited literature values, such determinations are subjective based and liable to individual bias. Of the two quantitative measurements, MLC would appear more accurate, because of the greater associated error in calculating IC_F from % transmission due to rapid *Candida* cell sedimentation. It was decided therefore that for investigations of synergistic interaction, the 'specific effect' would be MLC after 48 h incubation. The MLC of miconazole nitrate and chlorhexidine acetate were taken as 10 and 5 μg/ml, respectively, the higher concentration of the range for the latter compound to better ensure a lethal action.

3.2. Interaction between miconazole nitrate and chlorhexidine acetate

The existence of a synergistic interaction between miconazole and chlorhexidine has not been reported previously, though each agent alone has been confirmed to be effective against *C. albicans*. Incubation of the combined agents resulted in a MLC value of 0.74 μg/ml miconazole nitrate and 0.37 μg/ml chlorhexidine acetate. These results were expressed arithmetically to find the fractional inhibitory concentration for miconazole nitrate (FIC_{mn}) and chlorhexidine acetate (FIC_{ca}), where the concentrations of each antifungal in the combination that produces the 'specific effect' [MN_c] and [CA_c] are expressed as a fraction of the concentration that produces the same effect when the antifungal is used alone [MN_a] and [CA_a], i.e.

$$FIC_{mn} = \frac{[MN_c]}{[MN_a]}$$

and

$$FIC_{ca} = \frac{[CA_c]}{[CA_a]}$$

Σ FIC is the sum of the fractional inhibitory concentrations, and when this value is 1 the combination is additive, >1 antagonistic and <1 synergistic according to Berenbaum (1978). Scott et al. (1995) adopted a more cautious approach to their interpretation of Σ FIC when investigating interactions between fluconazole and ibuprofen, sodium salicylate or propylparaben. They stated that where Σ FIC <0.5 synergy existed; >0.5 to <4 indicated additivity or indifference, whereas ≥ 4 denoted antagonism. For the combination of miconazole nitrate and chlorhexidine acetate ex-

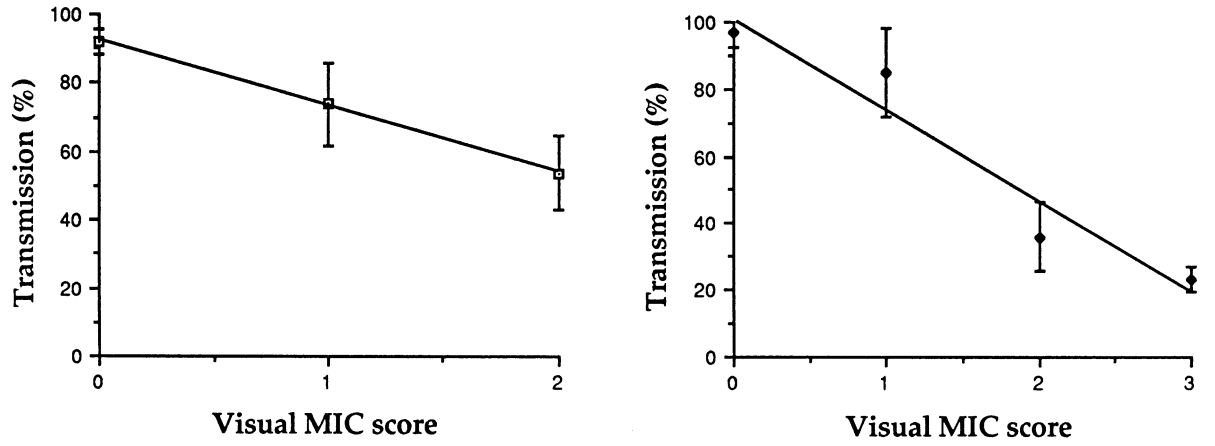

Fig. 3. Correlation of visual MIC score with mean % transmission at 540 nm for miconazole nitrate macrodilutions incubated for 24 h (□), where $n = 10$, 12 and 17 for 0, 1+ and 2+ scores, respectively; and 48 h (◆), where $n = 10$, 5, 17 and 8 for 0, 1+, 2+ and 3+ scores, respectively. Bars represent ±1 S.D.

amined above, the Σ FIC was determined to be 0.148, well below the value of 0.5, and is a strong indication of synergistic interaction between the two drugs.

According to Greenwood (1995), although a Σ FIC value below 1 should theoretically indicate synergy, in practice partial antibacterial effects below the MIC often cause apparent deviations from additivity when true synergy is absent. He advised caution be exercised in the interpretation of such results and acceptance of a synergistic relationship only when more than a 4-fold reduction in the MIC of at least one component was seen. The combination examined in this study for the 'specific effect' MLC showed that this was reduced from 10 to 0.74 μg/ml for miconazole nitrate and from 5 to 0.37 μg/ml for chlorhexidine acetate, indicating more than 4-fold decrease for both antifungals and strong synergy between the compounds.

A geometric representation of the interaction involving two more reference combinations was performed again in duplicate and repeated on a separate occasion, containing either 7.5 μg/ml miconazole nitrate with 1.25 μg/ml chlorhexidine acetate (combined MLC of 1.492 μg/ml miconazole nitrate and 0.0249 μg/ml chlorhexidine acetate) or 2.5 μg/ml miconazole nitrate with 3.75 μg/ml chlorhexidine acetate (combined MLC of 0.373 μg/ml miconazole nitrate and 0.583 μg/ml chlorhexidine acetate). These points were plotted as shown in Fig. 5, confirming that marked synergy between the two compounds existed over a wide concentration range.

The most likely mechanism for the synergy observed between miconazole nitrate and chlorhexidine acetate is enhanced cellular permeability of one or more of the compounds, as altered membrane permeability appears the most relevant common mode of action. Cremophor RH 40 is a solubilizing agent shown by us in preliminary studies to be capable of enhancing the dissolution of miconazole nitrate and which it was intended to utilize in the design of a novel bioadhesive lozenge employing both antifungal agents. However, an in vitro susceptibility study carried out showed that the antifungal activity was not affected by the presence of this surface-active agent.

In vitro evidence of a synergistic interaction does not necessarily mean that the same effect will be seen in vivo (Denyer et al., 1985). An important consideration is to ensure comparable pharmacokinetics in order that the compounds are released in vivo in the ratio that has been shown to interact in vitro. In vitro profiles to be reported by us for spray-dried forms of the drugs indicated release of miconazole nitrate/chlorhexidine acetate

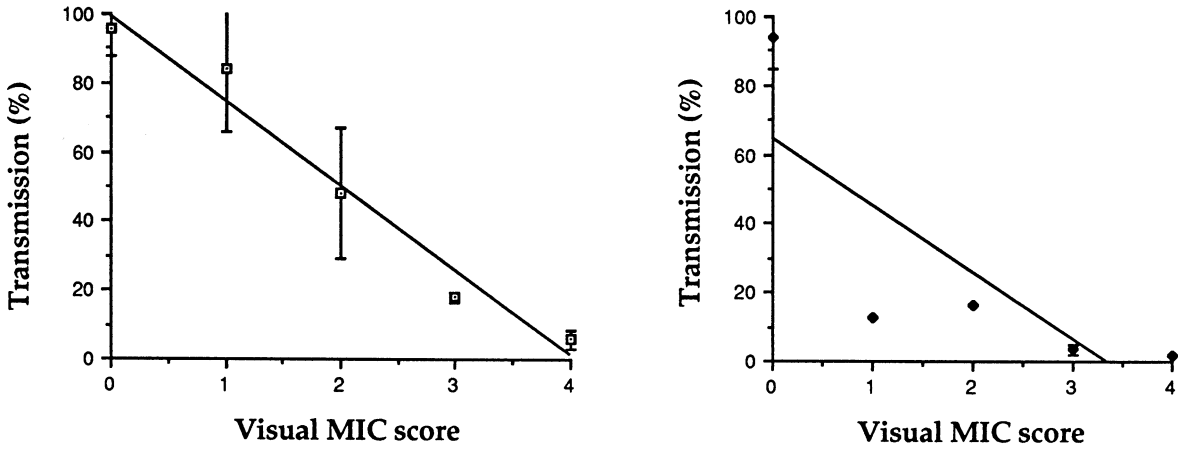

Fig. 4. Correlation of visual MIC score with mean % transmission at 540 nm for chlorhexidine acetate macrodilutions incubated for 24 h (□), where $n = 14$, 3, 2, 2 and 17 for 0, 1+, 2+, 3+ and 4+ scores, respectively; and 48 h (◆), where $n = 16$, 1, 1, 2 and 18 for 0, 1+, 2+, 3+ and 4+ scores, respectively. Bars represent ±1 S.D.

roughly in the ratio of 2:1 over a prolonged period. While this ratio demonstrated maximum synergistic effect in vitro, significant interaction was observed all along the isobole, leading to the conclusion that even if the optimum 2:1 ratio was not achieved in vivo, marked synergistic interaction should still occur between miconazole nitrate and chlohexidine acetate, of advantage for the intended application in a new bioadhesive lozenge to be reported later.

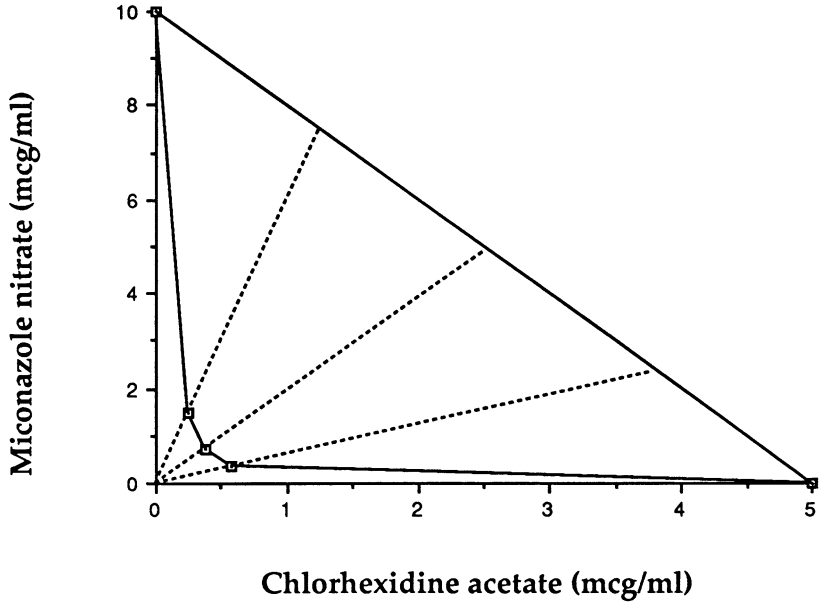

Fig. 5. Geometric depiction of synergy between miconazole nitrate and chlorhexidine acetate against *C. albicans*.

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