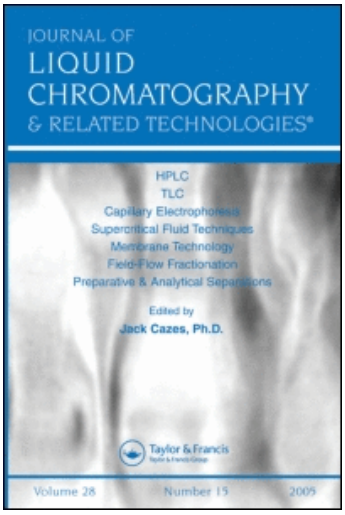

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LIQUID CHROMATOGRAPHIC ANALYSIS OF CHLORHEXIDINE AT SPECIFIC SITES IN THE SALIVA FILM AFTER APPLICATION OF A TOOTH-BONDED DELIVERY SYSTEM

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ABSTRACT

This paper describes the use of an HPLC assay to investigate whether a chlorhexidine-containing tooth-bonded delivery system produces antibacterial concentrations in the surrounding saliva film.

Saliva (<1.5 µl) was collected from ten sites in the mouth at eight times over a period of four days following application of tooth-bonded delivery systems in two dentally healthy subjects. Analysis of chlorhexidine concentrations in these samples showed a non-uniform distribution of chlorhexidine in the saliva film. Antibacterial concentrations were produced in the area immediately surrounding the delivery system (20 ± 4 and 28 ± 12 µg/ml for Subjects One and Two respectively) whereas concentrations at more distant sites remained low.

INTRODUCTION

Chlorhexidine is an antibacterial agent used in the treatment of plaque-initiated gum diseases such as gingivitis and periodontitis. It has a wide spectrum

of antibacterial activity [1, 2] and has demonstrated effectiveness as an agent for supra-gingival plaque control [3-5]. It is usually administered as a mouthrinse or topical solution and its effectiveness is, in part, due to its ability to reversibly bind to the tissue surfaces in the mouth which can provide antibacterial effects for up to 24 hours in some individuals [6, 7]. However, for sustained anti-plaque effects, twice daily administration is usually recommended [8] and therefore good patient compliance is required. Some problems identified with the use of chlorhexidine mouthrinses include the bitter taste imparted by the high drug concentration (0.2 %w/v) and tooth discolouration that occurs with prolonged use [9-11]. Also, mouthrinses have been shown to have little or no effect on the subgingival microflora as the drug cannot penetrate to this site [12]. To improve the delivery of chlorhexidine for the treatment of gum diseases, systems that employ smaller quantities of chlorhexidine and deliver the drug directly to diseased sites have been investigated. To date these systems have been evaluated *in vivo* by analysis of the rate at which drug is released from the delivery system along with assessment of clinical and microbiological effects [13-22]. Analysis of drug concentrations has been limited to measurement of concentrations in total saliva [23] because of the lack of chlorhexidine assays with suitable sensitivity which would allow determination in the small samples, typically <1.5 µl, that can be collected from some sites in the mouth.

This paper describes the use of a recently developed assay [24] to determine chlorhexidine concentrations in the saliva film at different distances from a tooth-bonded delivery system.

MATERIALS AND METHODS

Chemicals and reagents

Chlorhexidine diacetate B.P. was purchased from ICI Chemicals (Wellington, New Zealand). Poly(ε-caprolactone) was purchased from Polysciences (Warrington, U.S.A.). The internal standard, benzethonium chloride was AnalaR grade purchased from Sigma Chemical Co. (St Louis, MO, USA). Acetonitrile,

and glacial acetic acid were HPLC grade purchased from Ajax Chemicals Pty. Ltd. (Auburn, N.S.W., Australia) and sodium laurylsulphate was HPLC grade purchased from BDH chemicals Ltd (Poole, England). Sodium hydroxide was AnalaR grade purchased from BDH chemicals. Deionised water was produced with a Millipore Milli-Q system (Bedford, MA, U.S.A.).

Equipment

A Mettler five decimal place analytical balance (Mettler AT201) was used to determine the mass of samples collected from the saliva film.

A Spectra Physics HPLC system was used for the analysis of chlorhexidine in saliva samples. It comprised a SP8800/8810 ternary pump, a Spectra System UV 2000 dual wavelength detector, a SP4400 Chromjet integrator and a Rheodyne injector with a 50 µl sample loop. The stainless steel column, 10 cm x 2.1 mm i.d. was packed with 5 µm C18 ODS-B Exsil purchased from HiChrome Ltd. (Berkshire, England).

Preparation of the chlorhexidine delivery system

Chlorhexidine diacetate was dissolved in water and sufficient sodium hydroxide was added to increase the pH to 12. The precipitate (chlorhexidine base) was collected, dried then recrystallised twice from methanol. The final product was dried to constant weight in a vacuum oven, then sieved using Endecott sieves (Endecott Ltd., London, England) and the particle size fraction 63-125 µm was collected.

Duplicate films of poly(ε-caprolactone) containing 20%w/w chlorhexidine were prepared by solvent evaporation. Poly(ε-caprolactone) (0.96 g) was dissolved in dichloromethane (8 ml) and chlorhexidine (0.24 g) was added as a powder, stirred, then poured into an aluminium ring (7.6 cm diameter) placed on a silanised glass plate. Dichloromethane was evaporated at 25°C for 24 hours, then a vacuum was applied for a further 12 hours. The resulting films were stored in a desiccator with silica gel, at room temperature, until required.

Characterisation of the chlorhexidine delivery system

Drug loading of films was determined by cutting discs (0.5 cm diameter) and dissolving the poly(ε-caprolactone) in chloroform (0.5 ml), then extracting the chlorhexidine into 1%v/v glacial acetic acid (5 ml) by shaking for 20 minutes. The aqueous and organic phases were separated by centrifugation at 2500 rpm using a Megafuge 1.0R centrifuge (Heraeus Sepatech), then chlorhexidine concentrations in the aqueous portions were determined by UV spectroscopy (λ =254nm).

In vitro release of films was determined by cutting five discs (0.5 cm diameter) from each film and attaching them to individual teflon discs with a silicone adhesive (Bostick RTV sealant). These were immersed in sodium citrate/sodium hydroxide buffer (5 ml, pH 6.6, 0.1 M sodium citrate) and placed in a shaking water-bath (Grant Instruments Ltd.) at 37°C and 100 oscillations per minute. At sampling times, which were determined so that the chlorhexidine concentration in the release medium did not exceed 15% of its maximum solubility, the buffer was removed and replaced with fresh pre-warmed buffer. Chlorhexidine released was measured by UV absorbance spectroscopy (λ =254nm). Calibration curves for chlorhexidine in sodium citrate/sodium hydroxide buffer were linear over the range of chlorhexidine concentrations 1 to 16 μg/ml chlorhexidine (R^2>0.99). Control experiments showed that poly(ε-caprolactone) or the silicone adhesive did not interfere with the assay.

Attachment of the chlorhexidine delivery system and determination of chlorhexidine concentrations in the saliva film

Ethical approval for this part of the study was obtained from the Southern Regional Health Authority (Otago, New Zealand).

One section was cut from each film to approximately 2.5 x 4 mm and trimmed to allow attachment to the buccal surface of the left lower first molar in two dentally healthy subjects. Sections were weighed then attached to the tooth surface using a dental adhesive system (Scotchbond, 3M Pharmaceuticals). The subjects did not brush their teeth or eat breakfast prior to collection of morning samples and refrained from using toothpastes, mouthrinses or brushing the tooth to which the delivery system was attached during the study.

Saliva (<1.5 μl) was collected on Periopaper strips (Harco Electronics, Winnipeg, Canada*) from the saliva film at ten sites in the mouth prior to and one (t1) and three (t2) hours after attachment of the chlorhexidine-containing film, then again on the morning of the following four days (t3, t5 t6 and t8) and in the afternoon on the second (t4) and fourth (t7) days. Chlorhexidine concentrations were determined using the HPLC method previously described [24]. At each sampling time teeth were observed for the presence of plaque. Film sections were removed from the teeth after saliva samples had been collected on the morning of the fifth day and the remaining chlorhexidine was determined by dissolving the poly(ε-caprolactone) in chloroform (0.5 ml) then extracting the chlorhexidine into 1%v/v glacial acetic acid (2 ml). Chlorhexidine in the aqueous portion was determined by absorbance spectroscopy (λ=254nm).

RESULTS AND DISCUSSION

Characterisation of the chlorhexidine delivery system

The drug load of films, as determined by extraction of chlorhexidine, did not differ from the theoretical loading calculated from the weight of starting materials by greater than ±1.5%. Duplicate films contained 19.9 ± 0.4 (mean ± s.e.m., n=4) and 20.3 ± 0.8 (n=5) %w/w chlorhexidine. *In vitro* release profiles are shown in Figure 1. Films released 33.0 ± 0.9 (mean ± s.e.m., n=5) and 31.1 ± 0.7 (n=5) of the drug load over four days and the difference between films was not significant ($p>0.05$).

Chlorhexidine concentrations in the saliva film after bonding of the chlorhexidine delivery system to a tooth

The sections attached to teeth weighed 1.4 mg and contained approximately 280 μg chlorhexidine. They released 24 and 48% of their drug loads over the study in Subjects One and Two respectively. The consistancy of *in vitro* release (Figure 1) would suggest that this variation in the percentage released *in vivo* was

*current supplier is IDE Interstate (Amityville, U.S.A.)

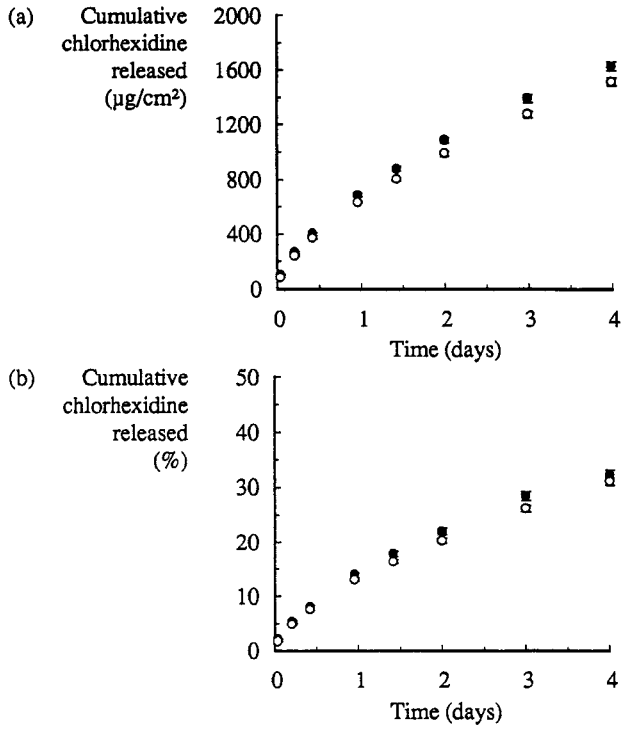
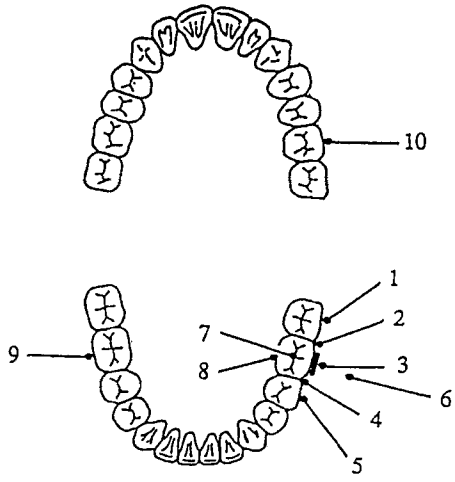


FIGURE 1: *In vitro* release profiles for chlorhexidine release from films containing 20%w/w chlorhexidine in poly(ε-caprolactone). Film 1 (•) and Film 2 (o). Error bars represent the standard error of the mean (n=5).

due to differences in conditions within the mouths of the subjects rather than differences in the film sections.

Sites in the oral cavity from which saliva was collected are shown in Figure 2 and the chlorhexidine concentrations measured at these sites, for times t1 to t8, are shown in Figure 3. Typically, the highest chlorhexidine concentrations were measured directly below the tooth-bonded delivery system (Site 3). Concentrations at this site were 20 ± 4 and 28 ± 12 (mean ± s.e.m., n=8) μg/ml for Subjects One and Two respectively while lower concentrations were measured at the more distant sites. Considerable variability was, however, observed in the



Site	Description of site
1	Buccal gingival margin of the lower left second molar
2	Buccal interdental space between the lower left first and second molars
3	Buccal gingival margin of the lower left first molar *(i.e. immediately below the tooth-bonded delivery system)*
4	Buccal interdental space between the lower left second pre-molar and the first molar
5	Buccal gingival margin of the lower left second pre-molar
6	Lower left buccal sulcus
7	Occlusal surface of the lower left first molar
8	Lingual gingival margin of the lower left first molar
9	Buccal gingival margin of the lower right first molar
10	Buccal gingival margin of the upper left first molar

FIGURE 2: Sites in the oral cavity for saliva collection.

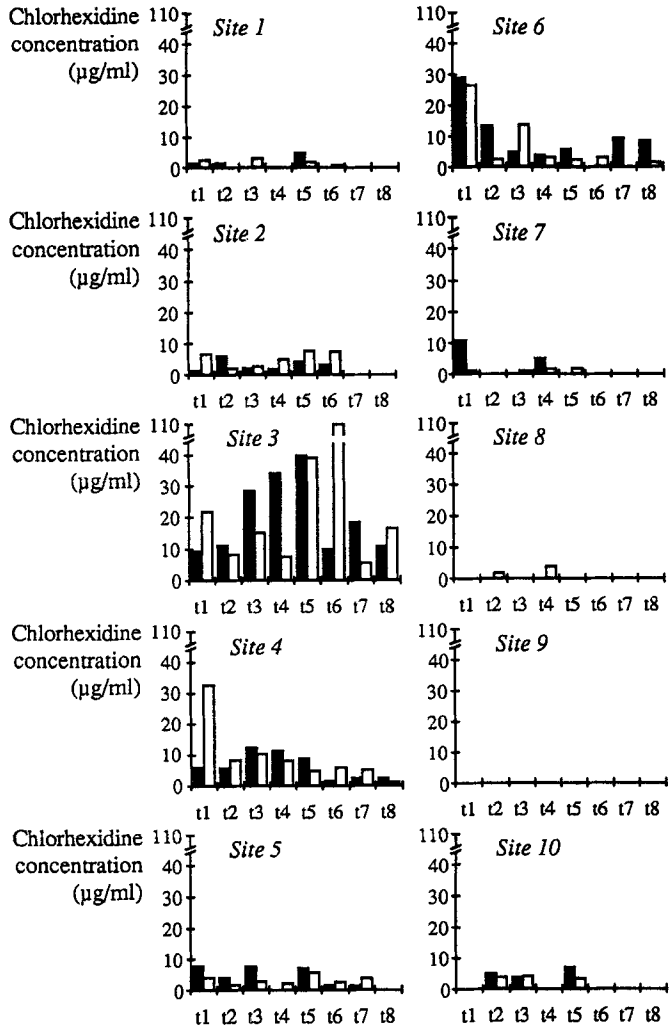


FIGURE 3: Chlorhexidine concentrations in the saliva film at Sites 1 to 10 (times t1-t8) in Subject One (■) and Subject Two (□).

concentrations measured at Site 3. This variability was not unexpected as the subjects continued normal oral function throughout the study, with the exception that they did not brush their teeth or eat breakfast prior to collection of the morning samples and refrained from using toothpaste or brushing the tooth to which the delivery system was attached. Normal oral functions such as movement of the cheek on the film surface and mastication, is likely to affect the drug release from the delivery system as well as clearance of the released drug. These processes will vary between and within subjects and could contribute to the variability in both the percentage drug released from the film sections *in vivo* and the saliva chlorhexidine concentrations at individual sites. It is interesting to note that despite a lack of control over subjects oral function the chlorhexidine concentrations measured immediately below the delivery system (Site 3) were in the order of those reported to inhibit the growth of plaque bacteria *in vitro* [2, 25, 26]. Examination of tooth surfaces throughout the study confirmed the localised anti-plaque effect of this tooth-bonded delivery system as no plaque was observed on the buccal side of the left lower first molar in either subject. Subject Two did show some plaque accumulation a tooth away, on the buccal side of the left lower second molar. In the absence of oral hygiene plaque would be expected to accumulate over the period of this study, however it appears that chlorhexidine released from the tooth-bonded delivery system was sufficient to prevent this occurring in areas immediately adjacent to the delivery system.

CONCLUSION

Measurement of chlorhexidine concentrations in the saliva film surrounding a tooth-bonded delivery system showed that effective concentrations were maintained immediately adjacent to the delivery system for a period of four days. At other, more distant, sites chlorhexidine concentrations were lower, thus indicating the tooth-bonded delivery system may be useful for inhibiting plaque-growth at specific sites in the mouth where gum disease exists. This may avoid the side effects that relate to high saliva chlorhexidine concentrations evident with conventional delivery systems.

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