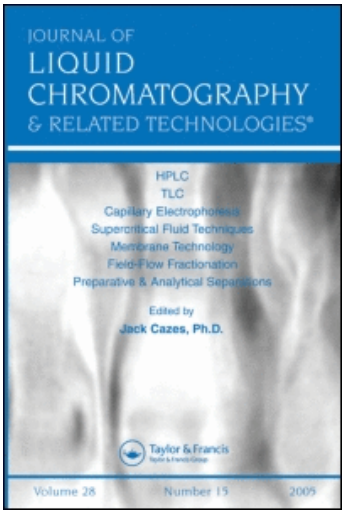

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) ASSAY FOR THE DETERMINATION OF CHLORHEXIDINE IN SALIVA FILM

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ABSTRACT

An HPLC assay for the determination of chlorhexidine in small samples (<1 µl) of saliva is described. A base deactivated reverse phase C-18 narrow bore column (ODS-B Exsil) was used for the analysis. Saliva samples were collected on Periopaper strips and chlorhexidine was extracted with 0.1 ml mobile phase. The optimal mobile phase comprised 55 %v/v acetonitrile, 0.2 %v/v glacial acetic acid, 7 mM sodium laurylsulphate and column temperature was maintained at 55°C. Benzethonium was included as the internal standard and a dual wavelength UV absorbance detector was used to analyse chlorhexidine at 254 nm and benzethonium at 275 nm. Triplicate standard curves were linear for the range of concentration 1 to 15 ng/sample ($R^2 > 0.99$). If 0.5 µl of saliva is collected then 2 to 30 µg/ml chlorhexidine can be measured. The intra-assay variability, determined from repeated injections of quality control standards containing 1, 3.75 and 12.5 ng chlorhexidine/tube was 12.9, 4.4 and 1.5% respectively.

INTRODUCTION

Chlorhexidine is a bisdiguanide antiseptic discovered in 1956 [1]. It has a broad spectrum of antimicrobial activity and has been shown to be effective against plaque bacteria [2-5]. When given as a mouthrinse the drug is distributed throughout the oral cavity and its effectiveness is, in part, due to its ability to reversibly bind to the tissue surfaces in the mouth and drug can be detected in the saliva for up to 24 hours in some individuals [6-9]. Analysis of the chlorhexidine concentration usually involves collection of samples from the saliva pool and average concentrations are reported. 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 [10, 11]. Attempts to overcome these problems have resulted in the development of delivery systems that employ smaller quantities of chlorhexidine and deliver the drug to specific sites in the mouth [12-14]. It is therefore important to monitor the chlorhexidine concentration at the desired site of action to assess the effectiveness of the delivery system.

The major problem encountered in analysis of drugs at some sites in the mouth is the small amount of fluid available for sampling. The volume and thickness of the saliva film vary throughout the mouth and an average thickness of 0.07 to 0.1 mm has been reported by Collins and Dawes [15]. In periodontal pockets the volume of fluid depends on the severity of inflammation and an average volume of 0.5 µl has been reported at diseased sites compared to 0.04 µl at healthy sites [16]. Filter paper strips are used to collect these small samples, but the drug must be extracted and diluted to provide sufficient volume for injection in HPLC analysis [17-19]. HPLC assays have been reported for the determination of chlorhexidine in pharmaceutical preparations [20, 21] or biological fluids [22-25], but these are not sensitive enough to allow analysis of chlorhexidine in the saliva film or periodontal pocket.

We report an HPLC assay for the determination of chlorhexidine using a reverse phase base deactivated C-18 narrow bore column and show that it may be used to determine the chlorhexidine concentration in the saliva film at the gingival margin after administration of a 0.2 %w/v mouthrinse.

MATERIALS AND METHODS

Chemicals and reagents

Chlorhexidine diacetate B.P. was purchased from ICI Chemicals (Wellington, New Zealand). The internal standard, benzethonium chloride was AnalaR grade purchased from Sigma Chemical Co. (St Louis, MO, USA). Acetonitrile, methanol and glacial acetic acid were HPLC grade purchased from Ajax Chemicals Pty. Ltd. (Auburn, N.S.W., Australia) and sodium laurylsulphate and triethylamine were HPLC grade purchased from BDH chemicals Ltd (Poole, England). Deionised water was produced with a Millipore Milli-Q system (Bedford, MA, U.S.A.).

Equipment

A Spectra Physics HPLC system was used comprising 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).

Drummond microcap tubes (2 µl) were purchased from Drummond Scientific Co. (Broommall, PA, U.S.A.). Periopaper strips were purchased from Harco Electronics Ltd. (Winnipeg, Canada). Polypropylene centrifuge tubes (Eppendorf) were purchased from Salmond-Smith Biolab (Christchurch, New Zealand). Saliva sample volumes were calculated from masses determined using a five decimal place Sartorious analytical balance and a saliva density of 1.002 - 1.012 g/ml [26].

Optimisation of the HPLC method for chlorhexidine

The mobile phase was pumped at a rate of 0.5 ml/min for all analyses. The effects of changes in the concentration of ion-pairing agent, sodium laurylsulphate

and acetonitrile on the retention and separation of chlorhexidine and benzethonium were investigated. Samples contained 1 μg/ml chlorhexidine and 10 μg/ml benzethonium in mobile phase.

Extraction of chlorhexidine from filter paper strips

A 10 μg/ml chlorhexidine solution was prepared in deionised water. Samples of about 0.5 μl were measured with Drummond microcap tubes and placed onto Periopaper strips. The exact volume of each sample was calculated from the length of liquid in the Drummond microcap tube. The Periopaper strips consist of a strip of filter paper attached to a plastic tag. The plastic tags were removed and the filter paper strips were placed in 0.6 ml polypropylene tubes (Eppendorf). 0.1 ml of the extracting solution, which comprised 55 %v/v acetonitrile, 0.2 %v/v glacial acetic acid and 7 mM sodium laurylsulphate was added to each tube. These were vortexed for one minute, sonicated for 20 minutes (Bransonic ultrasonic bath, 100 W, 55 000 Hz), then vortexed again prior to analysis (extracts). Controls were prepared by direct addition of the 0.5 μl samples into 0.1 ml of extracting solution and were treated in the same way as the extract samples. The internal standard (benzethonium) was included in the extraction solution at a concentration of 2 μg/ml and both the chlorhexidine and benzethonium peaks were analysed at 254 nm. Extraction and control experiments were performed in triplicate and the percentage extracted was calculated by comparison of the peak height ratio after correction for the sample volume. In addition, a similar dilution of the original chlorhexidine solution was performed using larger volumes i.e. 10 μl in 2 ml (standards).

Preparation of chlorhexidine standards

Standard curves were prepared, on three days, for the extraction of chlorhexidine from Periopaper strips to validate the assay procedure. For each standard curve triplicate chlorhexidine solutions (100 μg/ml in deionised water) were prepared and diluted to give standards with chlorhexidine concentrations of 2, 5, 10, 20 and 30 μg/ml in deionised water. These were diluted 1 in 200 (10 μl

in 2 ml) with mobile phase containing the internal standard (2 μg/ml benzethonium). 0.1 ml was transferred to 0.6 ml polypropylene tubes (Eppendorf) containing one piece of filter paper (cut from a Periopaper strip) per tube. The tubes were pre-rinsed with methanol. 50 μl of each standard was injected into the HPLC and chlorhexidine peaks were analysed at 254 nm while the internal standard peaks were analysed at 275 nm using a dual wavelength detector. Peak height ratios were calculated and results expressed as the amount of chlorhexidine per tube (1, 2.5, 5, 10 and 15 ng/tube chlorhexidine). Within day accuracy and precision were determined by preparation of three quality control solutions containing 2, 7 and 25 μg/ml chlorhexidine. These were processed in the same way as the standards and analysed repeatedly (n=5) in a random order throughout the first standard curve. The chlorhexidine concentrations were determined from the standard curve and the mean, coefficient of variation and percent deviation from the theoretical concentration were calculated. Between day variation was assessed by comparison of the slope and intercept of the three standard curves prepared on three separate days.

Measurement of the chlorhexidine concentration in the saliva film after administration of a 0.2 %w/v mouthrinse

The chlorhexidine concentration in the saliva film at the gingival margin was measured in two subjects after administration of a 0.2 %w/v chlorhexidine mouthrinse. The mouthrinse was prepared in deionised water and 10 ml was rinsed around the oral cavity for one minute. Samples were collected on Periopaper strips at the gingival margin on the buccal side of the upper second premolars (left = BL and right = BR) and the lingual side of the lower second premolars (left = LL and right = LR) immediately prior to application of the mouthrinse then at 0.5, 3, 6 and 8 hours post-mouthrinse.

The sample volume was measured by mass and samples were stored at 4°C until analysis. Periopaper strips were placed in 0.6 ml polypropylene tubes (Eppendorf) and weighed using a five place analytical balance (w_1). To collect a sample the Periopaper strip was removed and placed at the sampling site in the oral cavity, then the plastic tag was cut and the filter paper strip returned to the

Eppendorf tube. The plastic tag was retained and weighed again with the Eppendorf tube containing the saliva sample (w_2). The sample volume was calculated as the weight difference (w_2 - w_1) multiplied by the density of saliva, which was taken to be 1.002-1.012 g/ml (total saliva) [26].

Standards at concentrations of 1 and 15 ng/tube were prepared in triplicate as above and used to determine the slope and intercept of the standard curve at the time of sample analysis. Samples were prepared by addition of 0.1 ml of the extracting solution to the tube containing the sample on Periopaper and vortexed, then sonicated for 20 minutes (Bransonic ultrasonic bath) and vortexed again before analysis. Quality control samples of concentrations 2, 7 and 25 µg/ml were prepared as previously and analysed in a random order throughout the run.

RESULTS AND DISCUSSION

Optimisation of the HPLC method for chlorhexidine

Good peak shape was obtained for chlorhexidine when the base deactivated column (ODS-B Exsil) was used. In comparison, normal reverse phase materials such as C-18 ODS Hypersil produced poor peak shape with significant tailing. Initially, these effects were overcome with addition of a competitive amine, triethylamine, into the mobile phase [27]. However some tailing remained and the minimum quantifiable concentration of this earlier assay was 0.1 µg/ml. This would allow measurement of chlorhexidine at concentrations greater than 10 ng/tube (or 20 µg/ml in 0.5 µl saliva) which was not good enough as the minimum inhibitory concentration of chlorhexidine for some oral pathogens is 8 µg/ml [28]. Inclusion of the hydrophobic ion-pairing agent, sodium laurylsulphate into the mobile phase increased the retention of chlorhexidine and improved the peak shape. The effects of alterations in the concentration of sodium laurylsulphate is shown in Figure 1. Separation of chlorhexidine and benzethonium peaks could be obtained with a mobile phase comprising 55 %v/v acetonitrile, 0.2 %v/v glacial acetic acid and 5 mM sodium laurylsulphate. This separation could be maintained and the analysis time reduced from 8 to 4 minutes if the acetonitrile concentration was increased to 60 % v/v.

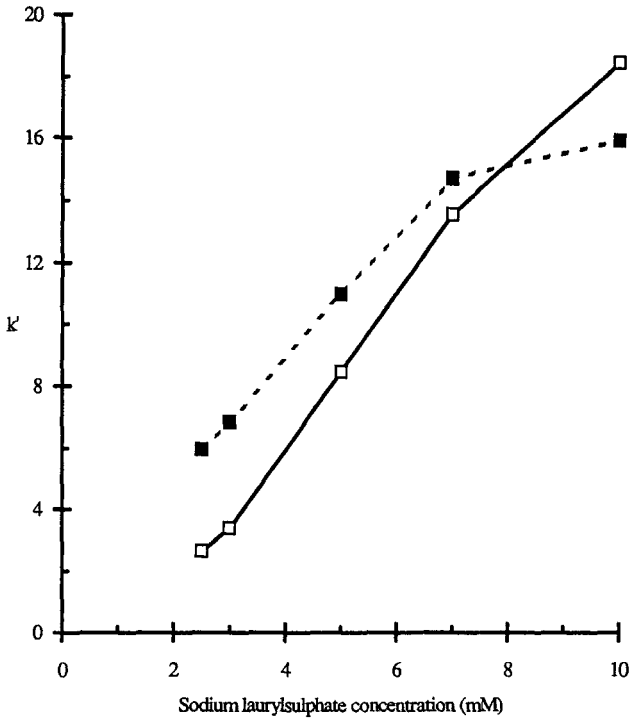

FIGURE 1

Effect of alterations in the concentration of sodium laurylsulphate on the retention of chlorhexidine —□— and benzethonium - - ■ - - . Acetonitrile concentration = 55 %v/v, glacial acetic acid concentration = 0.2 %v/v.

When dilute chlorhexidine solutions were prepared in acidic conditions (50 ng/ml chlorhexidine in 1 %v/v glacial acetic acid) a decrease in the chlorhexidine peak height was noticed. These solutions were stored in 1.5 ml polypropylene tubes and it was thought that the chlorhexidine may have adsorbed to the container surfaces. Table 1 shows the changes in chlorhexidine peak height with time for solutions in 1 %v/v glacial acetic acid. The loss of chlorhexidine was not prevented by addition of triethylamine (0.25 %v/v). It was thought that addition of a competitive amine would prevent or reduce adsorption if it bound to the same sites as chlorhexidine, but as a reduction in peak height of similar magnitude was observed to that seen in 1 %v/v glacial acetic acid it would appear

TABLE 1

Variation in chlorhexidine peak height for solutions containing 50 ng/ml chlorhexidine in 1 %v/v glacial acetic acid stored in 1.5 ml polypropylene Eppendorf tubes.

Solvent	Time (h)	Chlorhexidine peak height mean ± s.e.m. (n=2)
1 %v/v glacial acetic acid	0	606 ± 37
	3	186 ± 28
	5	ND
with 5 mM sodium laurylsulphate	0	1280 ± 60
	3	1290 ± 60
	5	1330 ± 110
with 0.25 %v/v triethylamine	0	524 ± 4
	3	88 ± 19
	5	ND

ND = not determined.

the amine groups are not responsible for this interaction. Addition of 5 mM sodium laurylsulphate to the solution prevented the chlorhexidine loss and the peak height remained stable for at least five hours. It is possible that ion-pairing of chlorhexidine and sodium laurylsulphate prevents the interaction between the polypropylene tube and the ionised chlorhexidine.

Extraction of chlorhexidine from filter paper strips

Table 2 shows the peak height ratios for chlorhexidine extracted from Periopaper strips. Although there was no significant difference between the peak

TABLE 2

Extraction efficiency for extraction of chlorhexidine from Periopaper strips

Solution	Peak height ratio mean ± s.e.m. (n=3)
extracts	2.19 ± 0.05*
controls	2.06 ± 0.11*
standards	2.10 ± 0.12*

* no significant differences $p>0.05$

height ratios when analysed using an ANOVA ($p>0.05$), the peak height ratio for the controls tended to be lower than that for extracts or standards. This may be due to the difficulty removing the sample from the Drummond microcap tube in the absence of a Periopaper strip. If the peak height ratios for extract samples is compared with the standard solutions, 100 percent extraction was achieved.

Analysis of standards and extracts

Some interference peaks were extracted from the Periopaper strips and these were separated from the chlorhexidine and internal standard peaks by adjustment of the column temperature and mobile phase composition. The increased temperature caused sharpening of peaks and decreased the retention time. Optimal conditions for analysis of chlorhexidine and benzethonium in the presence of Periopaper strips was a mobile phase comprising of 55 %v/v acetonitrile, 0.2 %v/v glacial acetic acid and 7 mM sodium laurylsulphate and a column temperature of 55°C. Standard curves were linear over the range of chlorhexidine concentrations 2 to 30 μg/ml ($R^2 > 0.99$). These gave final amounts of chlorhexidine after extraction of 1 to 15 ng per tube. The within day variability and accuracy and the between day variability are shown in Table 3.

TABLE 3

a) Within day reproducibility and accuracy

Chlorhexidine concentration (ng/tube)	Mean observed concentration	n	C.V. (%)	Accuracy (%)
1	1.13	4	12.4	113
3.75	3.67	5	4.4	97.8
12.5	12.2	5	1.5	97.7

b) Between day variability

Standard curve	R^2	Slope ± std. error	Intercept ± std. error
Day 1	0.995	0.109 ± 0.001	-0.024 ± 0.014
Day 2	0.998	0.101 ± 0.001	0.058 ± 0.009
Day 3	0.999	0.1003 ± 0.0007	0.051 ± 0.006

Measurement of the chlorhexidine concentration in the saliva film after administration of a 0.2 %w/v mouthrinse

The chlorhexidine concentration in saliva, at the gingival margin of the second pre-molars, is shown in Table 4 and typical chromatographs for saliva samples are shown in Figure 2 and Figure 3. The chlorhexidine concentration was generally higher at the gingival margin of the upper second pre-molars (BL and BR) and this was expected as these samples were taken from the buccal side, whereas samples from the lower second pre-molars were taken from the lingual side (LL and LR). It is possible that chlorhexidine was more rapidly diluted at these lower sites as they are closer to the major salivary glands and a greater mixing of the saliva by the tongue would be expected. These factors have been shown by Dawes and Weatherell [29] to be important in the distribution and clearance of fluoride in the oral cavity. A large variation was observed in the chlorhexidine concentrations

TABLE 4

Chlorhexidine concentration (μg/ml) in the saliva film at the gingival margin at four sites within the mouth after rinsing with a 0.2 %w/v chlorhexidine solution

	Subject one					Subject two				
	pre-rinse	post-rinse (h)				pre-rinse	post-rinse (h)			
Site*		0.5	3	6	8		0.5	3	6	8
BL	0	81	12	-	-	0	194	40	9	27
BR	0	ND	20	6	6	0	94	29	25	22
LL	0	19	23	-	-	0	26	28	30	10
LR	0	15	26	-	-	0	71	23	9	18

* BL = buccal gingival margin on the upper left second pre-molar.
BR = buccal gingival margin on the upper right second pre-molar.
LL = lingual gingival margin on the lower left second pre-molar.
LR = lingual gingival margin on the lower right second pre-molar.

- = chlorhexidine less than 1 ng/tube.
ND = not determined.

achieved at different sites in each subject and between subjects. This was particularly apparent in the first post-rinse sample and may be due to differences in the degree of mixing and rates of clearance from the individual sites. Subject one ate a meal between the samples at times three and six hours and this may be responsible for the lack of chlorhexidine at six and eight hours post rinse. In contrast, subject two did not eat over the eight hour period and concentrations of between 10 and 27 μg/ml were measured eight hours after rinsing. These values are similar to those obtained when radiolabelled chlorhexidine was used [7]. The advantage of this method is that radiolabelled chlorhexidine is not required and the

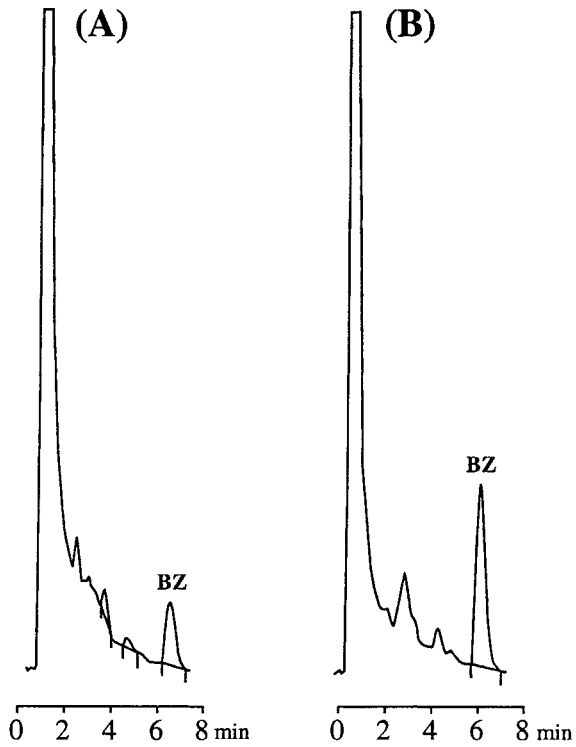

FIGURE 2

Typical chromatograph of saliva extracts. Blank saliva sample, Subject 1 (sampling time = pre-mouthrinse; sample site = buccal gingival margin on the upper right second pre-molar; saliva volume = 0.72 µl). Detector wavelengths; (A) = 254 nm and (B) = 275 nm. BZ = internal standard (2 µg/ml benzethonium).

concentration at different sites can be determined. It was interesting to note that chlorhexidine was not able to be detected in samples taken from the gingival margin in the subject who ate during the post rinse period. This may indicate that the gingival margin clearance is accelerated by eating and although chlorhexidine may be detected in the saliva pool there may be sites within the mouth where an effective concentration is not maintained. Further studies are being undertaken to confirm this and to determine the clinical significance.

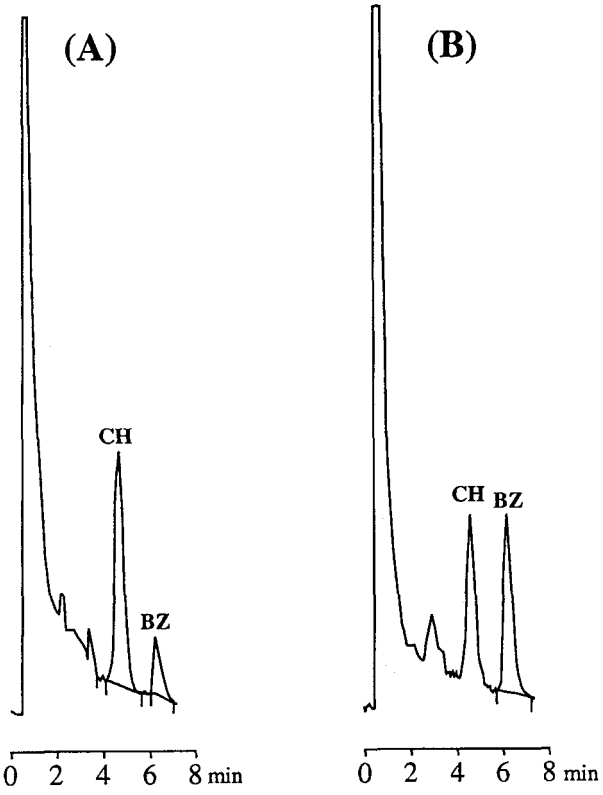

FIGURE 3

Typical chromatograph of saliva extracts. Subject 2 (sampling time = 3 hours post-mouthrinse; sample site = buccal gingival margin on the upper right second pre-molar; saliva volume = 0.46 µl). Detector wavelengths; (A) = 254 nm and (B) = 275 nm. CH = chlorhexidine; BZ = internal standard (2 µg/ml benzethonium).

CONCLUSION

This assay allows measurement of chlorhexidine concentrations greater than 2 µg/ml in 0.5 µl saliva. Since the minimum inhibitory concentration of most oral pathogens is greater than 8 µg/ml [28] it allows monitoring of individual sites within the mouth and determination of the effectiveness of chlorhexidine delivery systems in producing and maintaining effective antibacterial concentrations.

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REFERENCES

1. Rose, F.L. and Swain, G. Bisdiguanides having antibacterial activity. J. Chem. Soc., 4422-4425 (1956).

2. Hennessey, T.D. Antibacterial properties of Hibitane. J. Clin. Periodont., 4, 36-48 (1977).

3. Netuschil, L., Reich, E. and Brecx, M. Direct measurement of the bactericidal effect of chlorhexidine on human dental plaque. J. Clin. Periodontol., 16, 484-488 (1989).

4. Wade, W.G. and Addy, M. In vitro activity of a chlorhexidine-containing mouthwash against subgingival bacteria. J. Periodontol., 60, 521-525 (1989).

5. Addy, M., Jenkins, S. and Newcombe, R. The effect of some chlorhexidine-containing mouthrinses on salivary bacterial counts. J. Clin. Periodontol., 18, 90-93 (1991).

6. Davies, R.M., Børglum Jensen, S., Rindom Schiøtt, C., Löe, H. The effect of topical application of chlorhexidine on bacterial colonization of the teeth and gingiva. J. Periodont Res., 5, 96-101 (1970).

7. Bonesvoll, P., Lökken, P., Rölla, G. and Paus, P.N. Retention of chlorhexidine in the human oral cavity after mouth rinses. Archs. Oral Biol., 19, 209-212 (1974).

8. Bonesvoll, P. Retention and plaque-inhibiting effect in man of chlorhexidine after multiple mouth rinses and retention and release of chlorhexidine after toothbrushing with a chlorhexidine gel. Arch. Oral Biol., 23, 295-300 (1978).

9. Bonesvoll, P. and Olsen, I. Influence of teeth, plaque and dentures on the retention of chlorhexidine in the human oral cavity. J. Clin. Periodontol., 1, 214-221 (1974).

10. Flötra, L., Gjermo, P., Rölla, G. and Waerhaug, J. Side effects of chlorhexidine mouth washes. Scand. J. Dent. Res., 79, 119-125 (1971).

11. Eriksen, H.M., Nordbø, H., Kantanen, H. and Ellingsen, J.E. Chemical plaque control and extrinsic tooth discoloration. A review of the possible mechanisms. J. Clin. Periodontol., 12, 345-350 (1985).

12. Addy, M., Rawle, L., Handley, R., Newman, H.N. and Coventry, J.F. The development and *in vitro* evaluation of acrylic strips and dialysis tubing for local drug delivery. J. Periodontol., 53, 693-699 (1982).

13. Friedman, M. and Golomb, G. New sustained release dosage form of chlorhexidine for dental use. J. Periodont. Res., 17, 323-328 (1982).

14. Mirth, D.B., Bartkiewicz, A., Shern, R.J. and Little, W.A. Development and *in vitro* evaluation of an intra-oral controlled-release delivery system for chlorhexidine. J. Dent. Res., 68, 1285-1288 (1989).

15. Collins, L.M.C. and Dawes, C. The surface area of the adult human mouth and thickness of the salivary film covering the teeth and oral mucosa. J. Dent. Res., 66, 1300-1302 (1987).

16. Hattingh, J. and Ho, E. The concentration of proteins in human gingival crevicular fluid. J. Periodont. Res., 15, 90-95 (1980).

17. Britt, M.R. and Pohlod, D.J. Serum and crevicular fluid concentrations after a single oral dose of metronidazole. J. Periodontol., 57, 104-107 (1986).

18. Higashi, K., Seike, M., Mitani, Y., Morisaki, K., Hayashi, S., Kitamura, M., Fujimoto, N., Kimura, S., Ebisu, S. and Okada, H. Concentration of ofloxacin in human gingival crevicular fluid after oral administration of Tarivid®. J. Periodont. Res., 24, 409-411 (1989).

19. Stolze, K. Concentration of metronidazole in periodontal pockets after application of a metronidazole 25% dental gel. J. Clin. Periodontol., 19, 698-701 (1992).

20. Bauer, M., Degude, C. and Mailhe, L. Simultaneous determination of chlorhexidine, tetracaine and their degradation products by ion-pair liquid chromatography. J. Chromatogr., 315, 457-464 (1984).

21. Richard, A., Elbaz, M. and Andermann, G. Determination of 4-chloroaniline and chlorhexidine digluconate by ion-pair reversed-phase high-performance liquid chromatography. J. Chromatogr., 298 , 356-359 (1984).

22. Huston, C.E., Wainwright, P. and Cooke, M. High-performance liquid chromatographic method for the determination of chlorhexidine. J. Chromatogr., 237, 457-464 (1982).

23. Gaffney, M.H. and Cooke, M. Improved method for the determination of chlorhexidine in urine. J. Chromatogr., 306, 303-313 (1984).

24. Brougham, L.R., Cheung, H. and Pittman, K.A.. Sensitive high-performance liquid chromatographic method for the determination of chlorhexidine in human serum and urine. J. Chromatogr., 383, 365-373 (1986).

25. Wainwright, P. and Cooke, M. Direct determination of chlorhexidine in urine by high-performance liquid chromatography. Analyst, 111, 1343-1344 (1986).

26. Lentner, C (Ed). Units of Measurement, Body fluids, Composition of the body, Nutrition. In: Geigy Scientific Tables, Ciba-Geigy Ltd., Basle, Vol. 1, 114-122 (1981).

27. Medlicott, N.J., Jones, D.S., Tucker, I.G. and Holborow, D. Preliminary release studies of chlorhexidine (base and diacetate) from poly(ε-caprolactone) films prepared by solvent evaporation. Int. J. Pharm., 84, 85-89 (1992).

28. Stanley, A., Wilson, M. and Newman, H.N. The *in vitro* effects of chlorhexidine on subgingival plaque bacteria. J. Clin. Periodontol., 16, 259-264 (1989).

29. Dawes, C. and Weatherell, J.A. Kinetics of fluoride in the oral fluids. J. Dent Res., 69, special issue, 638-644 (1990).

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