AAPS PharmSciTech 2007; 8 (3) Article 75 (http://www.aapspharmscitech.org).

Micromatricial Metronidazole Benzoate Film as a Local Mucoadhesive Delivery System for Treatment of Periodontal Diseases

Received: February 21, 2007; Final Revision Received: April 16, 2007; Accepted: April 19, 2007; Published: September 14, 2007

Amal Hassan El-Kamel,[1] Lubna Y. Ashri,[1] and Ibrahim A. Alsarra[1]

[1]Department of Pharmaceutics, Faculty of Pharmacy, King Saud University, Riyadh, Kingdom of Saudi Arabia

ABSTRACT

The main objective of this study was to develop a local, oral mucoadhesive metronidazole benzoate (MET) delivery system that can be applied and removed by the patient for the treatment of periodontal diseases. Mucoadhesive micromatricial chitosan/poly(ε-caprolactone) (CH/PCL) films and chitosan films were prepared. Thermal behavior, morphology, and particle size measurements were used to evaluate the prepared films. The effect of different molar masses of CH and different ratios of medium Mwt molar mass chitosan (MCH):PCL on water absorption, in vitro bioadhesion, mechanical properties, and in vitro drug release was examined. In vivo performance of the selected formulation was also evaluated. Differential scanning calorimetry examination revealed that MET existed mainly in amorphous form. Under microscopic examination, PCL microparticles were homogeneously dispersed in the films. The use of different molar masses of CH and different ratios of (MCH):PCL affected the size of the entrapped particles. Addition of PCL significantly decreased percentage water uptake and bioadhesion force compared with pure CH film. With regard to mechanical properties, the 2-layered film containing 1:0.625 MCH:PCL had the best tensile properties. At fixed CH:PCL ratio (1:1.25), the slowest drug release was obtained from films containing high molar mass CH. On the other hand, the 2-layered film that consisted of 1:0.625 MCH:PCL had the slowest MET release. In vivo evaluation of the selected film revealed that metronidazole concentration in saliva over 6 hours ranged from 5 to 15 μg/mL, which was within and higher than the reported range of minimum inhibitory concentration for metronidazole. A significant in vitro/in vivo correlation under the adopted experimental conditions was obtained.

KEYWORDS: Metronidazole benzoate, chitosan, poly(ε-caprolactone), periodontal, mucoadhesive, correlation.

Corresponding Author: Amal Hassan El-Kamel, Department of Pharmaceutics, Faculty of Pharmacy, King Saud University, Kingdom of Saudi Arabia, PO Box 22452, Riyadh 11495. Tel: +9664-4067419; Fax: +9661-4067419; E-mail: amalelkamel@yahoo.com

INTRODUCTION

Periodontal diseases are localized infections and inflammatory conditions that are associated with anaerobic Gram-negative bacteria[1] and affect teeth-supporting structures.[2] The aim of current periodontal therapy is to remove the bacterial deposits from the tooth surface and to shift the pathogenic microbiota to one compatible with periodontal health.[3] Therapeutic approaches include surgical techniques and mechanical debridement of the root surfaces and/or treatment of the infection with systemic or local antibiotics.[4] Local delivery of antimicrobial agents is becoming more prevalent since it leads to higher concentration of the drug at the intended site of action using a lower dose with an associated reduction in side effects relative to systemic administration. Mucoadhesive tablets,[2] dentifrices, mouth rinses, dental gels, fibers, compacts, injectable semisolid systems,[3] irrigation devices,[5] films,[6] inserts,[7] and microspheres[8] are local delivery systems that have been tried in the treatment of periodontal diseases. The application and removal of these delivery systems may require surgery procedures and, sometimes, the incisions are supported by the use of cyanoacrylate glue.

Metronidazole is a front-line chemotherapeutic agent for treating infections by anaerobic bacteria such as *Porphyromonas gingivalis* because of the low minimum inhibitory concentration (MIC) required.[9]

Chitosan has some antibacterial activities,[10] and it is a promising bioadhesive material at physiological pHs. However, adhesion failure may occur when overhydration converts the chitosan gel network to slippery mucilage.[11] Therefore, addition of other types of biodegradable polymers in the delivery system may provide some control over the swelling of chitosan and thereby prevent adhesion failure. Biodegradable polymers are biocompatible, are easy to formulate into different devices, and have good mechanical properties.[11]

The main objective of this study was to develop a local, oral, mucoadhesive, controlled-release metronidazole benzoate (MET) delivery system that can be applied to the buccal mucosa and removed by the patient for the treatment of periodontal diseases. To reach this objective, mucoadhesive micromatricial chitosan/poly(ε-caprolactone) (CH/PCL) films and mucoadhesive chitosan films were prepared. The influence of formulation parameters on the physicochemical properties of the prepared films and release characteristics of MET was

examined. Evaluation of the selected MET film in vivo was also performed.

Materials and Methods

The different grades of chitosan used in this study were purchased from Aldrich (St Louis, MO) and include low molar mass CH (LCH) (viscosity, 275.9 cps; degree of deacetylation [dd], 80.5%), medium molar mass CH (MCH) (viscosity, 755.8 cps; dd, 76.58%), and high molar mass CH (HCH) (viscosity, 2064 cps; dd, 70.3%). The viscosity of 1% solution CH in 1% acetic acid was measured by a Brookfield viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA) as stated by the manufacturer. PCL was purchased from Absorbable Polymers International (Pelham, AL) with inherent viscosity of 1.24 dL/g. MET was provided by Alexandria Pharmaceuticals (Alexandria, Egypt). All reagents and chemicals used for chromatography were of high-performance liquid chromatography (HPLC) grade. All other chemicals and organic solvents were of reagent grade.

Preparation of Micromatricial Chitosan/Poly (ε-caprolactone) Films

CH/PCL films containing MET were prepared by the emulsification/casting/solvent evaporation technique.[11] In this technique, PCL and MET were dissolved in 3.2 mL of dichloromethane. This organic phase was emulsified into an aqueous phase using an Ultra Turrax homogenizer IKA model no. T18 basic (IKA Works Inc., Wilmington, NC) at 10 000 rpm. The aqueous phase was composed of 0.2 g CH dissolved in 15 mL 1% vol/vol acetic acid and contained 4% wt/wt glycerine as a plasticizer and 0.2% wt/wt Tween 80 as an emulsifier. The formed oil in water (o/w) emulsion was cast into Teflon plates with a diameter of 6.3 cm and left to dry in an oven at 37°C ± 0.5°C overnight. Complete drying and solvent evaporation were ensured by thermogravimetric analysis (TGA). Microparticles were formed by evaporation of dichloromethane and precipitation of PCL. The amount of MET was 0.24 g per plate in all formulations. The composition of various prepared formulations is shown in Table 1. In the case of the 2-layered film, a second layer of 2% wt/vol pure MCH solution in 10 mL of 1% vol/vol acetic acid was cast on the surface of the 1:0.625 wt/wt MCH:PCL film before it dried completely.

Preparation of Chitosan and Poly(ε-caprolactone) Films

Chitosan (0.2 g) was dissolved in 15 mL 1% vol/vol acetic acid containing 4% glycerine. For MCH films containing drug, 0.24 g MET was suspended in the mixture. In the case of PCL films, PCL (0.2-0.5 g) was dissolved in 3.2 mL dichloromethane. Films were cast into Teflon plates with a diameter of 6.3 cm and dried in an oven at 37°C ± 0.5°C. The solvent was evaporated, leaving PCL film behind.

Table 1. Composition of Different Prepared Formulations and Melting Point and Enthalpy of MET in Different Films*

Film Code	CH:PCL Ratio (wt/wt)	T_m (°C)	Enthalpy ΔH (J/g)
MET	—	99.0	107
MCH+MET	—	—	—
Pure LCH†	—	—	—
Pure MCH†	—	—	—
Pure HCH†	—	—	—
PCL†	—	—	—
MCH:PCL	1:0.625	91.9	17.1
LCH:PCL	1:1.25	91.2	27.4
MCH:PCL	1:1.25	90.7	21.7
HCH:PCL	1:1.25	91.4	23.5
MCH:PCL	1:2	90.1	6.00
MCH:PCL	1:2.5	91.2	13.7
2-layered MCH:PCL	1:0.625 (with a second layer of pure 2% wt/wt MCH in 1% acetic acid)	92.7	40.0

*MET indicates metronidazole benzoate; CH, chitosan; PCL, poly (ε-caprolactone); MCH, medium molar mass chitosan; LCH, low molar mass chitosan; and HCH; high molar mass chitosan.
†Films containing no metronidazole benzoate.

Thermal Analysis Studies

Differential scanning calorimetry (DSC) was performed using a PerkinElmer DSC (Shelton, CT). Film samples of 3 to 5 mg were sealed in aluminum pans and heated from 40°C to 150°C at a heating rate of 5°C/min under a constant flow of nitrogen gas. TGA was performed using a TGA7 thermogravimetric analyzer (PerkinElmer, Norwalk, CT) to determine the residual solvent level in films.[12] Films were heated from 25°C to 100°C at a heating rate of 5°C/min.

Morphological Characterization and Particle Size Measurements of Microparticles Entrapped in the Prepared Films

Morphological characterization of different prepared films was studied using scanning electron microscopy (SEM) (Jeol JSM-6060, Jeol Ltd, Tokyo, Japan). Film samples were gold-sputtered under vacuum and visualized at an acceleration voltage of 80 kV.

The particle size of the microparticles entrapped in different films was evaluated using an advanced biological microscope with digital camera at a magnification power of ×100 (Moticam 2000 USB, 2.0 megapixels, Motic China Group Co, Ltd, Xiamen, China). Four hundred particles were measured and analyzed using Motic Images Advanced software (Version

3.2, Motic). The average diameters of the particles were calculated. The polydispersity index (*PI*), which indicates the width of particle size distribution and is defined as the relative standard deviation, was calculated using the following equation[13]:

$$PI = s/\bar{R} \quad (1)$$

where $\bar{R}$ is the mean radius and s is the standard deviation.

Water Absorption Capacity

Circular samples, with a surface of ~2.3 cm^2, of different films were allowed to swell on the surface of agar plates prepared in simulated saliva[14] (2.38 g Na_2HPO_4, 0.19 g KH_2PO_4, and 8 g NaCl per liter of distilled water adjusted with phosphoric acid to pH 6.7) and kept in an incubator maintained at 37°C ± 0.5°C. At preset time intervals (0.25, 0.5, 1, 2, 3, and 4 hours), samples were weighed (wet weight) and then left to dry for 7 days in a desiccator over anhydrous calcium chloride at room temperature; then the final constant weights were recorded. Water uptake (%) was calculated using the following equation[15]:

$$\text{Water Uptake } (\%) = \frac{w_w - w_f}{w_f} \times 100 \quad (2)$$

where w_w is the wet weight and w_f is the final weight. The swelling of each film was measured at least 3 times.

In Vitro Bioadhesion

The determination of the mucoadhesive force of the prepared films was performed using an Instron universal testing machine equipped with a 5-kg load cell (model 8500, digital control, Instron Co, Canton, MA). Two equal cylindrical metallic supports with a circular surface of 1.7 cm in diameter were constructed. A circular patch of the film with a surface area equal to that of the metal support was attached to the upper support. The external side of a chicken pouch (isolated from a white hybrid chicken) was attached to the lower support using cyanoacrylate adhesive. The surfaces of both the film and the chicken pouch were wetted with simulated saliva; then the 2 surfaces were brought immediately into contact with an initial force of 1.5 N for 3 minutes. The whole experiment was performed at room temperature and a relative humidity of 50%. The lower support was withdrawn at a speed of 20 mm/min. During the experiment, the force was recorded as a function of displacement until the break point.[16] Data collection and calculation were performed by dedicated software (Instron series IX, version 8.32.00). The peak detachment force was used to evaluate the bioadhesive force of the films.[17] PCL film was used as a negative control, while pure CH film was used as a positive control. Each measurement was repeated at least 4 times.

Mechanical Properties

Mechanical properties of the prepared films were evaluated using an Instron universal testing machine (model 8500 digital control, Instron) equipped with a 5-kg load cell. Film strips (20×10 mm) were held between 2 clamps. The thickness of each strip was measured before each run using an electronic digital caliper (Ultra-Cal Mark III, Fred V. Fowler Co, Newton, MA). The force and elongation were measured when the films broke. The resulting profiles were analyzed using dedicated software (Instron series IX, version 8.32.00). The following equations were used to calculate the tensile strength (*TS*),[18] elongation at break (*EB*),[14] and elastic modulus (*EM*) of the films:

$$TS(\mathrm{N/mm^2}) = \frac{\text{Breaking Force}}{\text{Cross-sectional Area}} \quad (3)$$

$$EB(\%) = \frac{\text{Increase in Length}}{\text{Original Length}} \times \frac{100}{\text{Cross-sectional Area}} \quad (4)$$

$$EM(\mathrm{N/mm^2}) = \frac{\text{Force at Corresponding Strain}}{\text{Cross-sectional Area}} \times \frac{1}{\text{Corresponding Strain}} \quad (5)$$

In Vitro Release Studies

Pieces of film (≈1 × 1 cm) equivalent to 5 mg of drug were placed in stoppered 100-mL conical flasks. The dissolution medium was used under sink conditions, using 30 mL simulated saliva kept at 37°C ± 0.5°C. The conical flasks were shaken in a thermostated horizontal shaker at 50 rpm. At predetermined intervals, samples of dissolution fluid (2 mL) were removed and analyzed using UV spectroscopy (Genesys 5 spectrophotometer, Thermo Fisher Scientific Inc, Waltham, MA) at 310 nm. A UV standard curve was constructed over a concentration range of 8 to 20 μg/mL. The presence of other formulation ingredients did not interfere with the spectroscopic assay. Each experiment was performed in triplicate.

Release data were kinetically analyzed using Equation 6[19]:

$$\frac{M_t}{M_\infty} = \mathrm{kt^n} \quad (6)$$

where M_t corresponds to the amount of drug released in time t and M_∞ is the total amount of drug released after infinite time; k denotes a constant; and n is the release exponent indicating the type of drug release mechanism.

Weight Loss Study

A piece of film (1 × 1 cm) was weighed (W_i) and immersed in 30 mL simulated saliva at 37°C ± 0.5°C. After 7 hours of immersion, the piece of film was removed and kept in a desiccator over anhydrous calcium chloride for 7 days prior to being reweighed (W_f). The amount of drug released in the medium after 7 hours (W_r) was analyzed spectrophotometrically at 310 nm. Each experiment was performed in triplicate. The weight loss was calculated according to the following equation:

$$\text{Weight Loss } \% = \frac{(W_i - W_r) - W_f}{W_i} \times 100 \qquad (7)$$

In Vivo Evaluation of the Selected Formulation

Fourteen healthy adult volunteers (3 males, 11 females), aged between 20 and 30 years old, participated in the study after signing informed consents. The study was approved by the King Saud Ethical Committee and conducted in accordance with the Declaration of King Saud University. The volunteers were required to rinse their mouth with water before 2 pieces of the selected film (each 0.5 × 2 cm) equivalent to 20 mg of MET were placed, one on each side of their buccal cavity. The volunteers were deprived of food and drink during the evaluation period. They were asked to record the residence time of the film on the buccal mucosa in the oral cavity, which was taken as the time for the film to dislodge completely from the buccal mucosa. Samples of saliva were collected after 0.25, 0.5, 1, 2, 3, 4, 5, and 6 hours of film application. (Blank saliva samples had been harvested from volunteers before film application.) Three hundred microliters of methanol were added to 100-μL saliva samples. The drug was assayed by HPLC.[20] The HPLC system was equipped with a gradient pump (LC-10AT VP Schimadzu, Kyoto, Japan) and a UV detector (SPD-10A VP Schimadzu) adjusted at 317 nm. The mobile phase consisted of a mixture of (25:75 vol:vol) methanol and 0.1 M sodium acetate (pH 4.8). The column used was a reversed phase Nova-Pak C_{18} (4.6 × 150 mm with a particle size of 4 μm, Waters Co., Milford, MA). The flow rate was 1 mL/min. Standard solutions for the calibration curve were prepared by spiking 80 μL of human saliva with 20 μL of MET stock solutions to prepare the following concentrations: 1, 2, 3, 5, and 8 μg/mL. Methanol (300 μL) was added as a protein precipitant. The tubes were vortex-mixed for 10 seconds and centrifuged at 6000 rpm for 10 minutes at 5°C. An aliquot of 20 μL of the supernatant of each standard concentration was injected onto the HPLC column in triplicate.

Statistical Analysis

Data were analyzed by 1-way analysis of variance (ANOVA) using SPSS statistical package (version 10, 1999, SPSS Inc, Chicago, IL). Statistical differences yielding $P \leq .05$ were considered significant. Duncan or Tukey's multiple-comparison post hoc tests were applied when necessary.

Results and Discussion

Thermal Analysis Studies

Thermograms recorded from 4°C to 150°C for pure LCH, MCH, and HCH were similar and showed no thermal events. Endothermic sharp peaks of pure MET and PCL were recorded at 99°C and 57.8°C, respectively. In all prepared film formulations, PCL showed a broader endotherm at 57.5°C ± 1.3°C, as shown in Figures 1 and 2. On the other hand, the MET endotherm shifted to a lower temperature (91.3°C ± 0.8°C) with a smaller melting enthalpy (ΔH = 6-40 J/g) relative to pure drug ΔH (107 J/g), as shown in Table 1. This finding may indicate that the drug existed in the formulations mostly in an amorphous form. In addition, the presence of other ingredients in the film may have lowered the chemical potential of the crystalline drug, causing ΔH values to decrease.[21] TGA revealed that all prepared films had no traces of dichloromethane.

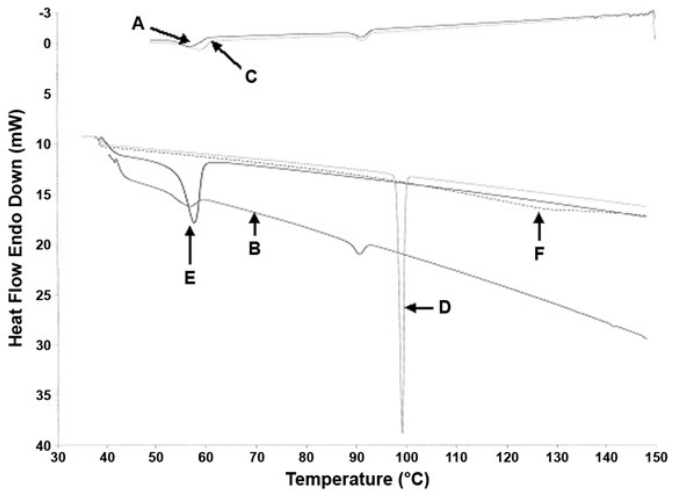

Figure 1. DSC thermograms of films containing 1:1.25 various molar mass CH:PCL: (a) LCH:PCL, (b) MCH:PCL, and (c) HCH:PCL in comparison with (d) MET, (e) PCL, and (f) CH (LCH, MCH, and HCH). DSC indicates differential scanning calorimetry; CH, chitosan; PCL, poly(ε-caprolactone); LCH, low molar mass chitosan; MCH, medium molar mass chitosan; HCH, high molar mass chitosan; and MET, metronidazole benzoate.

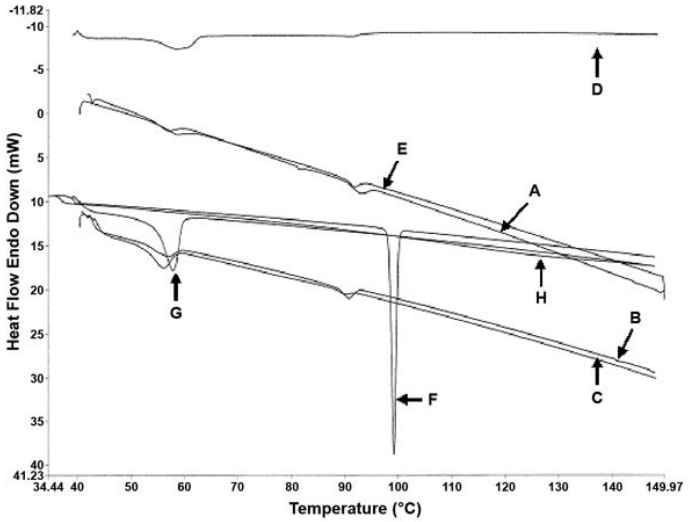

Figure 2. DSC thermograms of films containing various ratios of MCH:PCL: (a) 1:0.625, (b) 1:1.25, (c) 1:2, (d) 1:2.5, and (e) 2-layered 1:0.625 in comparison with (f) MET, (g) PCL, and (h) MCH. DSC indicates differential scanning calorimetry; MCH, medium molar mass chitosan; PCL, poly(ε-caprolactone); and MET, metronidazole benzoate.

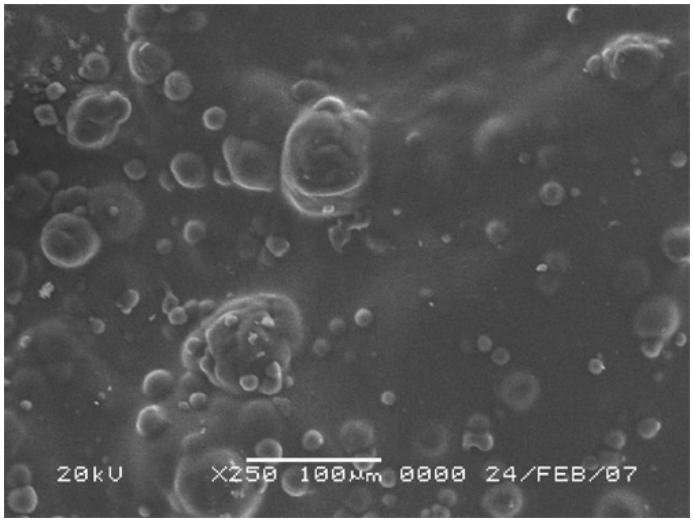

Figure 3. Scanning electron photomicrograph of one of the micromatricial MCH:PCL films containing MET. Magnification ×250. MCH indicates medium molar mass chitosan; PCL, poly (ε-caprolactone); and MET, metronidazole benzoate.

Morphological Characterization and Particle Size Measurements of Microparticles Entrapped in the Prepared Films

All films that contained CH only had a uniform thickness of 140 to 190 μm (Table 2) and were transparent. The 2-layered film was thicker (880 μm) than all 1-layered films (300-590 μm), as shown in Table 2, and all were opaque.

For all films, PCL microparticles were evident and homogeneously dispersed, as shown in the SEM photomicrograph (Figure 3), and the outer surface of the films was rough.

The mean size of entrapped particles in films containing 1:1.25 LCH:PCL, MCH:PCL, and HCH:PCL was 31 μm (PI = 0.40), 42 μm (PI = 0.57), and 77 μm (PI = 0.29), respectively, as shown in Table 2. This finding implies that the molar mass of CH had a noticeable influence on the size of entrapped PCL particles. This could be due to the increased

Table 2. Thickness, Particle Size, Polydispersity Index, and Mechanical Properties of Films Containing 1:1.25 Different Molar Mass Chitosan and Different MCH:PCL wt/wt Ratios (mean ± SD, n = 3)*

Formulations	Film Thickness (μm)	Particle Size (μm)	PI	TS (MPa)	% EB (% mm^{-2})	EM (MPa)
1:1.25 LCH:PCL	300 ± 30	31.0	0.40	0.184 ± 0.01	3.98 ± 0.34	0.89
1:1.25 MCH:PCL	590 ± 20	42.0	0.57	0.503 ± 0.09	5.32 ± 0.83	2.31
1:1.25 HCH:PCL	350 ± 20	77.0	0.29	0.232 ± 0.01	3.97 ± 0.44	1.15
1:0.625 MCH:PCL	590 ± 30	43.8	0.82	1.12 ± 0.11	5.15 ± 0.18	4.82
1:2 MCH:PCL	470 ± 30	51.0	0.71	0.470 ± 0.17	1.74 ± 0.15	5.68
1:2.5 MCH:PCL	440 ± 10	74.5	0.55	0.420 ± 0.05	2.74 ± 0.97	3.59
1:0.625 2-layered MCH+MET	880 ± 20	—	—	1.49 ± 0.06	4.51 ± 0.85	5.41
LCH	230 ± 30	—	—	1.05 ± 0.19	25.10 ± 4.23	1.84
MCH	190 ± 00	—	—	0.819 ± 0.15	25.88 ± 4.89	1.67
HCH	180 ± 10	—	—	1.38 ± 0.07	16.24 ± 0.69	4.83
	140 ± 30	—	—	1.14 ± 0.19	21.43 ± 3.75	2.83

*MCH indicates medium molar mass chitosan; PCL, poly(ε-caprolactone); PI, polydispersity index; TS, tensile strength; % EB, percentage elongation at break; EM, elastic modulus; LCH, low molar mass chitosan; HCH; high molar mass chitosan; and MET, metronidazole benzoate.

viscosity of CH solution as the molar mass increased. Consequently, the efficiency of homogenization decreased, leading to the formation of larger PCL particles.

The measured mean particle size of embedded particles in the film formulations containing different ratios of MCH: PCL was 43.8 μm (PI = 0.82), 42 μm (PI = 0.57), 51 μm (PI = 0.71), and 74.5 μm (PI = 0.55) for 1:0.625, 1:1.25, 1:2, and 1:2.5 wt/wt MCH:PCL, respectively, as listed in Table 2. The PI calculated for all films ranged between 0.55 and 0.82, which indicated a wide particle size distribution. It is noticeable that the largest particles were measured for the film that contains the highest ratio of PCL. The high ratio of PCL may increase the overall viscosity of the homogenized mixture. Consequently, the efficiency of homogenization may decrease, leading to the formation of large particles.

Water Absorption Capacity

The swelling results were expressed in terms of percentage water uptake at 37°C. The effect of 2 main factors, CH molar mass and MCH:PCL ratio, on the percentage water uptake was investigated. One-way ANOVA revealed that there was no statistically significant difference in percentage water uptake by 1:1.25 wt/wt different molar mass CH:PCL films. This result was in agreement with Roldo et al,[22] who found no correlation between the molar mass of CH and its swelling behavior.

Figure 4 shows the swelling behavior of various films containing different ratios (wt/wt) of MCH:PCL in comparison to pure MCH film and MCH films containing MET. The water uptake by the prepared films can be ranked as follows: 1:0.625 two-layered ~ 1:0.625 ~ 1:1.25 ~ 1:2 ~ 1:2.5 < MCH+MET < pure MCH. The results showed that the percentage water uptake of the prepared formulations ranged from 144% to 512%. The equilibrium state was obtained within about 2 hours. The highest percentage hydration was obtained for pure CH owing to its hydrophilic nature as a result of the presence of a free amino group in its structure.[23] The swelling ability of MCH+MET or all films containing PCL decreased because of the hydrophobic effect exerted by PCL contents in film. This result was in agreement with what was documented by Perugini et al,[11] who reported a decrease in film swelling upon addition of the biodegradable polymer polylactide-co-glycolide to CH film. However, at the examined ratios of PCL, there was no significant decrease ($P > .05$) in water uptake as the ratio of PCL increased in the film.

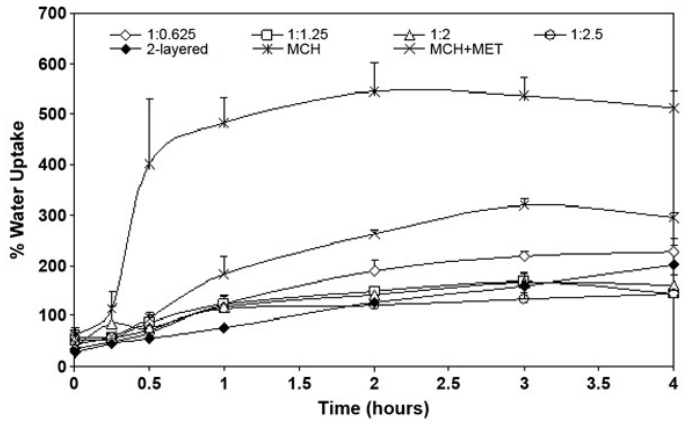

Figure 4. Percentage water uptake for films containing different ratios of MCH:PCL in comparison with plain MCH film and MCH containing MET. MCH indicates medium molar mass chitosan; PCL, poly(ε-caprolactone); and MET, metronidazole benzoate. Error bars represent ± SD, n = 3.

In Vitro Bioadhesion

In this study, the mucoadhesive strength was determined by measurement of the force of detachment or force of adhesion. These parameters are the most frequently studied adhesive properties.[24] Chitosan was documented to produce good adhesion to the mucosal surface, possibly because of the cationic nature of the polymer, which allows electrostatic interaction with the negatively charged mucus in addition to the physical entanglement of the polymer chains with the mucus.[25]

Plain films containing different molar masses of CH had forces of adhesion of 10.5 ± 1, 9 ± 0, and 8.4 ± 1 N for LCH, MCH, and HCH, respectively. Statistical analysis revealed that there was no significant difference ($P > .05$) in bioadhesion force between the films. These results were in agreement with Ikinci et al.[26] On the contrary, Roldo et al[22] showed that the maximum detachment force of MCH was higher than that of both LCH and HCH.

Similarly, no statistically significant difference was observed in bioadhesion force for films containing 1:1.25 wt/wt various molar mass CH:PCL and different ratios of MCH:PCL examined in this study. However, pure MCH and MCH containing MET films showed statistically higher ($P < .05$) bioadhesive force in comparison with films containing different ratios (wt/wt) of MCH:PCL, as shown in Figure 5. Statistical analysis revealed that the presence of MET has no significant influence on the bioadhesion force of the prepared MCH films containing MET.

Mechanical Properties

The tensile testing provides an indication of the strength and elasticity of the film, which can be reflected by the parameters TS,[18] EM, and percentage EB.[14] The effects of CH molar mass and MCH:PCL ratios (wt/wt) on mechanical properties of the prepared films were examined.

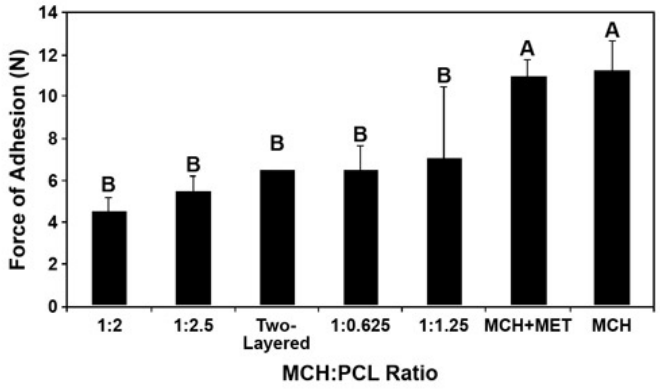

Figure 5. Bioadhesion force of films containing different ratios of MCH:PCL in comparison with plain MCH film and MCH containing MET. Similar letters denote no statistically significant differences, a > b. MCH indicates medium molar mass chitosan; PCL, poly(ε-caprolactone); and MET, metronidazole benzoate. Error bars represent ± SD, n = 4.

Effect of Different Molar Masses of Chitosan

The mechanical properties of various plain CH films and films containing 1:1.25 wt/wt different molar mass CH:PCL are listed in Table 2. One-way ANOVA of TS and EM data of the prepared plain CH films revealed the following rank order: MCH > HCH > LCH. Concerning % EB, the 3 films differed significantly ($P \leq .05$); the highest % EB was obtained for LCH, followed by HCH, and finally MCH. These results were inconsistent with those reported by Sarasam and Madihally,[21] who found that increasing the molar mass of CH would cause an increase in the % EB and a decrease in the TS and EM of the films. This inconsistency in results could be due to many factors, such as the difference in CH molar mass or the grade and degree of deacetylation of the used chitosan.

Film containing 1:1.25 wt/wt MCH:PCL showed the highest TS and differed significantly from film containing 1:1.25 wt/wt LCH:PCL and film containing 1:1.25 wt/wt HCH:PCL. However, there was no statistically significant difference in TS between 1:1.25 wt/wt LCH:PCL and 1:1.25 wt/wt HCH:PCL. These 3 films showed no statistically significant difference in % EB. On the other hand, the EM of the 3 films was in the following rank order: MCH:PCL > HCH:PCL > LCH:PCL.

It was concluded from the above results that the formulation containing 1:1.25 wt/wt MCH:PCL was the strongest and the most flexible as indicated by the values of % EB (5.32% mm^{-2}) and EM (2.31 MPa). Consequently, it was expected to be suitable for preparing buccal films.

Effect of Different Ratios of Medium Molar Mass Chitosan:Poly(ε-caprolactone)

Table 2 shows the mechanical properties of various film preparations containing different ratios (wt/wt) of MCH:PCL. One-way ANOVA followed by Duncan post hoc test revealed the following order for TS: plain MCH ~ 1:0.625 two-layered > 1:0.625 ~ MCH+MET > 1:1.25 ~ 1:2 ~ 1:2.5. In other words, there was no direct relationship between TS and PCL ratio. This finding was in disagreement with Sarasam and Madihally,[21] who documented that as the ratio of PCL increased TS also increased. This controversy could be due to the difference in the method adapted in blending CH with PCL. Additionally, in this study, PCL was present in the form of microparticles that could reduce the consistency of the films and cause discontinuities in their internal structure and variation in the strength of the film matrix.[27] It was noted that the highest TS (1.49 MPa) was obtained for 1:0.625 wt/wt MCH:PCL 2-layered film, indicating a strong film. The increased TS for the 2-layered film could be due to the low ratio of PCL (in other words, more film homogeneity) as well as the presence of a second pure MCH layer.

Concerning % EB, Duncan post hoc test revealed the following order: 1:0.625 two-layered ~ 1:0.625 ~ 1:1.25 > 1:2 ~ 1:2.5. All films containing different ratios (wt/wt) of MCH:PCL except 1:2 and 1:2.5 could be classified as strong with moderate % EB,[28] falling in the range of 4.51% to 5.32% mm^{-2}. The % EB of 1:2 and 1:2.5 films was very low, having a range of 1.74% to 2.74% mm^{-2}, which indicated brittle films.[27] This finding can be attributed to the presence of PCL microparticles in higher ratio. On the other hand, the % EB of MCH +MET (25.1% mm^{-2}) was significantly higher than that of all MCH:PCL films. All prepared films had low EM (0.8-5.68 MPa), as shown in Table 2. Two-layered film containing 1:0.625 wt/wt MCH:PCL had a high EM (5.4 MPa) relative to almost all other films, indicating relatively high resistance to deformation under stress.

In summary, the 2-layered film containing 1:0.625 wt/wt MCH:PCL had the best tensile properties among all prepared films, as it had the highest TS and relatively high EM and % EB. Consequently, it could be considered strong and flexible, which makes it a good candidate for further studies.

In Vitro Release Studies

The MET UV standard curve was linear over a concentration range of 8 to 20 μg/mL, with a determination coefficient >0.9998. Regression analysis of the data of the calibration curve revealed that the values of the coefficient of regression (b) were highly significant ($P \leq .0001$). Intraday and interday precision and accuracy data were generated and coefficients of variation were calculated and found to be less than 0.6%. Tests of significance among several regression lines revealed an insignificant difference between the slopes of calibration curves (9 replicates) ($F_{8,27} = 1.03$). This finding indicated high reproducibility of the standard calibration curve.

The effect of 3 different molar masses of CH on the release of MET from the prepared micromatricial film at a CH:PCL ratio of 1:1.25 wt/wt was examined. Statistical analysis followed by Tukey's test revealed that the rank order of the amount of MET released from the prepared films was LCH:PCL ~ MCH:PCL > HCH:PCL. This behavior was predictable, taking into account the direct relationship between the molar mass of CH and the viscosity of its solution. By increasing the viscosity of the polymer, the diffusion of the drug through the formed gel layer into the release medium was retarded.[29] The high polymer viscosity may also affect the size of particles formed by reducing the homogenization efficiency, leading to the formation of larger PCL microparticles, as indicated by the particle-size analysis studies. Therefore, the exposed surface area is reduced and the release of the entrapped drug is decreased.

Figure 6 shows the effect of various ratios of MCH:PCL (wt/wt) on the release characteristics of MET from the prepared films. One-way ANOVA followed by Tukey's test revealed the following rank order: 1:0.625 ~ 1:1.25 ~ 1:2 > 1:2.5. It was obvious that the slowest release was obtained from films containing the highest ratio of PCL. This result could be attributed to the higher viscosity of the emulsified PCL solution used in this formulation. Consequently, relatively large PCL particles were formed (74.5 μm).

The film that consisted of 1:0.625 wt/wt MCH:PCL was chosen as the formulation because, from an economic point of view, it had the lowest PCL amount and released ~64% of the drug in ~7 hours. In addition, it had the highest TS among the examined formulations. To improve its adhesive properties, a second layer of pure MCH was cast onto its surface to form a 2-layered film. The release of MET from the 2-layered film was significantly lower ($P \leq .0001$) than that of the corresponding 1:0.625 wt/wt MCH:PCL 1-layered film. This result could be due to the presence of a second layer of CH that acts as an additional barrier for drug release.

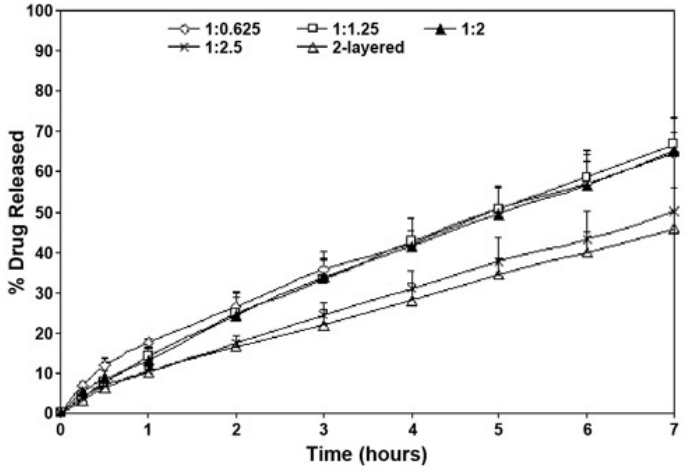

Figure 6. Effect of different ratios of MCH:PCL on the release of MET from the prepared films. MCH indicates medium molar mass chitosan; PCL, poly(ε-caprolactone); and MET, metronidazole benzoate. Error bars represent ± SD, n = 4.

To investigate more precisely the effect of the CH/PCL blend on the release of MET, the results were analyzed according to the Peppas equation. The calculated kinetic release parameters and determination coefficients (R^2) are summarized in Table 3.

In general, the values of the determination coefficient obtained after fitting the data to the Peppas equation were high (≥0.997). The release behavior of MET from the prepared film formulations was anomalous (non-Fickian), as indicated by the values of n, which varied from 0.65 to 0.89. The results of weight loss were consistent with the predicted release kinetic behavior. A weight loss ranging from 43.8% to 58.4% was observed for various examined film formulations after 7 hours of incubation in simulated saliva, as shown in Table 3. Since the weight loss indicated the extent of surface erosion and/or polymer degradation, it could be concluded that the process of polymer degradation, surface erosion, drug dissolution, and water uptake would occur simultaneously during the release of MET from films. Similarly, Sarasam et al[30] reported an initial weight loss in the PCL-CH blend due to CH, after which no significant changes were observed. These results were consistent with the literature, in which many authors have generally observed that n departs from Fickian diffusion for poorly soluble drugs and that the rate-limiting factor for release is the erosion of the hydrophilic matrix.[31]

As a conclusive result of bioadhesion, mechanical properties, and in vitro release studies for the 2-layered 1:0.625 wt/wt MCH:PCL film, this film was selected as the formulation to be evaluated in vivo.

In Vivo Studies

In vivo evaluation of the selected film (2-layered 1:0.625 wt/wt MCH:PCL) on healthy volunteers revealed that the film did not cause discomfort, the taste was acceptable, and no severe salivation was observed. Placement of the film in contact with cheek mucosa made it possible for the volunteer to speak; however, food and drink were restricted over the duration of the experiment to minimize the local clearance of drug from saliva and the interindividual variability.

The preliminary studies performed to measure in vivo adhesion time indicated that the 2-layered film containing 1:0.625 wt/wt MCH:PCL had an in vivo bioadhesion time of 2.15 ± 0.4 hours compared with about 10 minutes for other films. The great difference among the adhesion time of films could be due to their composition, because the 2-layered film containing 1:0.625 wt/wt MCH:PCL had a second pure chitosan layer that could prolong its adhesion time. It is worth mentioning

Table 3. Percentage Weight Loss of Films After 7-Hour Incubation in Simulated Saliva and Kinetic Parameters of MET Release From Different Films Containing MET*

Formulations	% Weight Loss ± SD (n = 3)	Peppas Parameters $M_t/M_\infty = kt^n$		
		R^2	k	n
1:0.625 MCH:PCL	50.1 ± 1.9	0.9983	0.175	0.6532
1:1.25 LCH:PCL	58.4 ± 1.0	0.9984	0.159	0.7828
1:1.25 MCH:PCL	55.2 ± 0.4	0.9994	0.133	0.8372
1:1.25 HCH:PCL	57.8 ± 1.4	0.9977	0.111	0.8042
1:2 MCH:PCL	50.4 ± 2.1	0.9992	0.162	0.7509
1:2.5 MCH:PCL	47.6 ± 0.3	0.9977	0.108	0.7639
1:0.625 2-layered	43.8 ± 1.9	0.9970	0.098	0.7774
MCH+MET	—	0.9984	0.102	0.8927

*M_t indicates amount of drug released in time t; M_∞, total amount of drug released after infinite time; K, release constant; n, release exponent indicating the type of drug release mechanism; MET, metronidazole benzoate; MCH, medium molar mass chitosan; PCL, poly(ε-caprolactone); LCH, low molar mass chitosan; and HCH, high molar mass chitosan.

that the plain CH layer was the layer that was sticking to the mucosal surface.

The HPLC standard curve of MET was linear over the calibration range of 1 to 8 μg/mL with a determination coefficient of 0.9996. Regression analysis of the data from the calibration curve revealed that the values of the coefficient of regression (b) were highly significant ($P \leq .0001$). Coefficients of variation calculated from intraday and interday accuracy data were less than 7.3%.

Figure 7 shows the salivary profile of MET over 6 hours after buccal application of the selected film. Many interindividual variations were observed in C_{max} (32.9 ± 12.6 μg/mL), T_{max} (3.64 ± 1.9 hours), and area under the salivary drug concentration curve (AUC_{0-6}) (114.99 ± 50 μg·h/mL). This finding was probably due to the variation in the individuals with respect to the salivary flow rate, which influences film hydration and clearance of drug in the mouth. The oral anatomy and the individuals' movement pattern of the tongue could also have caused variations.[32]

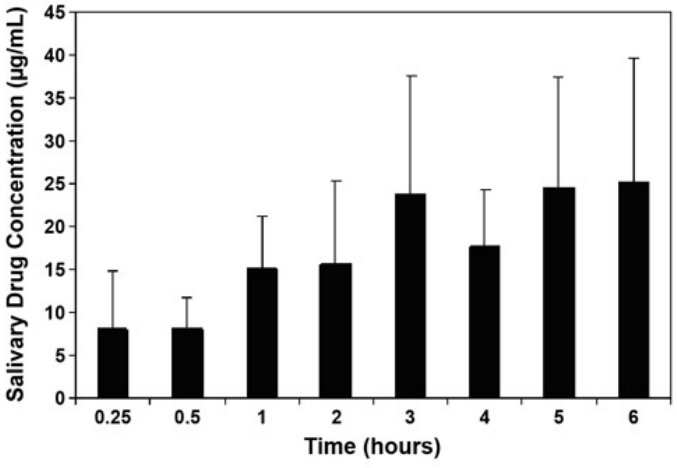

Figure 7. Mean salivary concentration of MET after application of 2-layered 1:0.625 MCH:PCL film (equivalent to 20 mg of MET) to 14 healthy volunteers. MET indicates metronidazole benzoate; MCH indicates medium molar mass chitosan; and PCL, poly(ε-caprolactone); Error bars represent ± SD, n = 14.

The calculated equivalent amount of metronidazole (microbiologically active form)[33] released from the selected film over a period of 0.25 to 6.0 hours ranged from 5 to 15 μg/mL. This range was within and higher than the range of MIC reported for metronidazole against the anaerobic bacteria responsible for periodontal diseases (≈0.1-8 μg/mL).[34] It is notable that the amount of drug ingested with swallowed saliva was not calculated.

In an attempt to correlate the pharmacokinetics (PK) of MET and its action (pharmacodynamics, PD) on target pathogens (*P gingivalis),* the PK/PD relation between C_{max} and MIC was calculated. Considering that the MIC of metronidazole toward *P gingivalis* ranged from ≤0.03 to 2 μg/mL,[35] the calculated C_{max}-to-MIC ratios ranged from 10.3 to 685, which indicated high PD efficacy for MET using a dose of 20 mg.[36]

In an attempt to find a correlation between in vitro and in vivo data, the concentration of drug released in vitro and the salivary drug concentration in vivo were plotted at the same time points over 6 hours. A positive linear correlation was found, with a determination coefficient (R^2) of 0.96 and Pearson coefficient of 0.980 ($P < .01$). Consequently, salivary drug concentration can be predicted from in vitro release studies under the adapted experimental conditions using the following equation without the need for expensive in vivo studies:

$$y = 6.1056 + 10.427\,x \qquad (8)$$

where x is the concentration of drug released in vitro (mg/mL), and y is the salivary drug concentration (μg/mL).

This significant correlation may also indicate that the mechanism controlling the drug release is the same in vitro and in vivo.

CONCLUSION

The developed mucoadhesive film was satisfactory in terms of drug release, and mechanical and bioadhesion properties. In addition, it allowed local delivery of the drug in a concentration higher than the metronidazole MIC against anaerobic bacteria responsible for periodontal diseases, over 6-hour periods with high PD efficacy. Moreover, by comparing the applied dose to the reported systemic dose (20 vs 300 mg), a remarkable reduction of the single drug dosage was achieved (~15-fold). In addition, the proposed formulation is not to be professionally delivered, so it requires little or no patient compliance for success.

ACKNOWLEDGMENTS

The authors are grateful to the College of High Education and the Research Centre at King Saud University. The authors also thank King Abdulaziz City for Science and Technology for its financial support.

REFERENCES

1. Piovano S. Bacteriology of most frequent oral anaerobic infections. *Anaerobe.* 1999;5:221–227.

2. Perioli L, Ambrogi V, Rubini D, et al. Novel mucoadhesive buccal formulation containing metronidazole for the treatment of periodontal disease. *J Control Release.* 2004;95:521–533.

3. Schwach-Abdellaoui K, Vivien-Castioni N, Gurny R. Local delivery of antimicrobial agents for the treatment of periodontal diseases. *Eur J Pharm Biopharm.* 2000;50:83–99.

4. Vyas SP, Sihorkar V, Mishra V. Controlled and targeted drug delivery strategies towards intraperiodontal pocket diseases. *J Clin Pharm Ther.* 2000;25:21–42.

5. Brackett MG, Drisko CL, Thompson AL, Waller JL, Marshall DL, Schuster GS. Penetration of fluids into periodontal pockets using a powered toothbrush/irrigator device. *J Contemp Dent Pract.* 2006; 7:30–39.

6. Ahuja A, Ali J, Rahman S. Biodegradable periodontal intrapocket device containing metronidazole and amoxycillin: formulation and characterisation. *Pharmazie.* 2006;61:25–29.

7. Barat R, Srinatha A, Pandit JK, et al. Niridazole biodegradable inserts for local long-term treatment of periodontitis: possible new life for an orphan drug. *Drug Deliv.* 2006;13:365–373.

8. Samati Y, Yuksel N, Tarimci N. Preparation and characterization of poly (D,L-lactic-co-glycolic acid) microspheres containing flurbiprofen sodium. *Drug Deliv.* 2006;13:105–111.

9. Liebana J, Castillo AM, Alvarez M. Periodontal diseases: microbiological considerations. *Med Oral Patol Oral Cir Bucal.* 2004;9:82–91.

10. Zheng LY, Zhu JF. Study on antimicrobial activity of chitosan with different molecular weights. *Carbohydr Polym.* 2003;54:527–530.

11. Perugini P, Genta I, Conti B, Modena T, Pavanetto F. Periodontal delivery of ipriflavone: new chitosan/PLGA film delivery system for a lipophilic drug. *Int J Pharm.* 2003;252:1–9.

12. USP. *US Pharmacopeia National Formulary.* vol. 21. Rockville, MD: USP; 1999.

13. Patty PJ, Frisken BJ. Direct determination of the number-weighted mean radius and polydispersity from dynamic light-scattering data. *Appl Opt.* 2006;45:2209–2216.

14. Peh KK, Wong CF. Polymeric films as vehicle for buccal delivery: swelling, mechanical, and bioadhesive properties. *J Pharm Pharm Sci.* 1999;2:53–61.

15. Baro M, Sanchez E, Delgado A, Perera A, Evora C. In vitro-in vivo characterization of gentamicin bone implants. *J Control Release.* 2002;83:353–364.

16. Ponchel G, Touchard F, Duchene D, Peppas NA. Bioadhesive analysis of controlled-release systems, I: fracture and interpenetration analysis in poly(acrylic acid)-containing systems. *J Control Release.* 1987;5:129–141.

17. Wong CF, Yuen KH, Peh KK. Formulation and evaluation of controlled release Eudragit buccal patches. *Int J Pharm.* 1999; 178:11–22.

18. Padula CP, Colombo G, Nicoli S, Catellani PL, Massimo G, Santi P. Bioadhesive film for the transdermal delivery of lidocaine: in vitro and in vivo behavior. *J Control Release.* 2003;88:277–285.

19. Ritger PL, Peppas NA. A simple equation for description of solute release, II: Fickian and anomalous release from swellable devices. *J Control Release.* 1987;5:37–42.

20. Metz P, Kohlhepp SJ, Gilbert DN. Study of different off-line sample processing procedures and the measurement of antibiotic and antiviral levels in human serum by high-performance liquid chromatography. *J Chromatogr B.* 2002;773:159–166.

21. Sarasam A, Madihally SV. Characterization of chitosan-polycaprolactone blends for tissue engineering applications. *Biomaterials.* 2005;26:5500–5508.

22. Roldo M, Hornof M, Caliceti P, Bernkop-Schnurch A. Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: synthesis and in vitro evaluation. *Eur J Pharm Biopharm.* 2004;57: 115–121.

23. Wenling C, Duohui J, Jiamou L, Yandao G, Nanming Z, Xiufang Z. Effects of the degree of deacetylation on the physicochemical properties and Schwann cell affinity of chitosan films. *J Biomater Appl.* 2005; 20:157–177.

24. Needleman IG, Smales FC. In vitro assessment of bioadhesion for periodontal and buccal drug delivery. *Biomaterials.* 1995;16:617–624.

25. Harding SE. Trends in mucoadhesive analysis. *Trends Food Sci Tech.* 2006;17:255–262.

26. Ikinci G, Senel S, Akincibay H, et al. Effect of chitosan on a periodontal pathogen Porphyromonas gingivalis. *Int J Pharm.* 2002; 235:121–127.

27. Dhanikula AB, Panchagnula R. Development and characterization of biodegradable chitosan films for local delivery of paclitaxel. *AAPS J.* 2004;6:Article 27.

28. Martin AN, ed. Polymer science. In: *Physical Pharmacy: Physical Chemical Principles in the Pharmaceutical Sciences.* 4th ed. Philadelphia, PA: Lea & Febiger; 1993:575–578.

29. Lorenzo-Lamosa ML, Remunan-Lopez C, Vila-Jato JL, Alonso MJ. Design of microencapsulated chitosan microspheres for colonic drug delivery. *J Control Release.* 1998;52:109–118.

30. Sarasam AR, Krishnaswamy RK, Madihally SV. Blending chitosan with polycaprolactone: effects on physicochemical and antibacterial properties. *Biomacromolecules.* 2006;7:1131–1138.

31. Colombo P, Santi P, Bettini R, Brazel CS. Drug release from swelling controlled systems. In: Wise DL, ed. *Handbook of Pharmaceutical Controlled Release Technology.* vol. 9. New York, NY: Marcel Dekker; 2000:183–209.

32. Khanna R, Agarwal SP, Ahuja A. Mucoadhesive buccal tablets of clotimazole for oral candidiasis. *Drug Dev Ind Pharm.* 1997; 23:1–7.

33. Mody SSB, Mody PD, Doshi MM, inventors. Pharmaceutical dental formulation for topical application of metronidazole benzoate and chlorhexidine gluconate. US patent 6 017 516. January 25, 2000.

34. Parfitt K. *Martindale: The Complete Drug Reference.* London, UK: Pharmaceutical Press; 1999.

35. Milazzo I, Blandino G, Musumeci R, Nicoletti G, Lo Bue AM, Speciale A. Antibacterial activity of moxifloxacin against periodontal anaerobic pathogens involved in systemic infections. *Int J Antimicrob Agent.* 2002;20:451–456.

36. Mckellar QA, Sanchez Bruni SF, Jones DG. Pharmacokinetic/ pharmacodynamic relationships of antimicrobial drugs used in veterinary medicine. *J Vet Pharmacol Ther.* 2004;27:503–514.