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Research Article

In Vitro Characterization of Chitosan Gels for Buccal Delivery of Celecoxib: Influence of a Penetration Enhancer

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Abstract. Celecoxib (Cx) shows high efficacy in the treatment of osteoarthritis and rheumatoid arthritis as a result of its high specificity for COX-2, without gastrolesivity or interference with platelet function at therapeutic concentrations. Besides of anti-inflammatory effects, Cx also has a potential role for oral cancer chemoprevention. For these conditions, oral administration in long-term treatment is a concern due to its systemic side effects. However, local application at the site of injury (*e.g.*, buccal inflammation conditions or chemoprevention of oral cancer) is a promising way to reduce its toxicity. In this study, the *in vitro* characterization of mucoadhesive chitosan (CHT) gels associated to Azone® was assessed to explore the potential buccal mucosal administration of Cx in this tissue. Rheological properties of gels were analyzed by a rheometer with cone-plate geometry. *In vitro* Cx release and permeability studies used artificial membranes and pig cheek mucosa, respectively. Mucoadhesion were measured with a universal test machine. CHT gels (3.0%) containing 2.0% or 3.0% Az showed more appropriate characteristics compared to the others: pH values, rheology, higher amount of Cx retained in the mucosa, and minimal permeation through mucosa, besides the highest mucoadhesion values, ideal for buccal application. Moreover, the flux (J) and amounts of drug released decreased with increased CHT and Az concentrations. CHT gels (3.0%) associated with 2.0% or 3.0% Az may be considered potential delivery systems for buccal administration of Cx.

KEY WORDS: azone; buccal mucosa; celecoxib; chitosan; *in vitro* permeability; *in vitro* release; mucoadhesion; rheology.

INTRODUCTION

Celecoxib (Cx) or 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide (Fig. 1) is the first synthesized non-steroidal anti-inflammatory drug (NSAID) able to selectively inhibit cyclooxygenase-2 (COX-2) activity, with significantly less toxicity. It shows high efficacy in the symptomatic treatment of osteoarthritis and rheumatoid arthritis in long-term therapy (1). Recently, research studies have indicated a potential role of Cx in chemoprevention of various cancers including oral cancer (2–6). Another study elected Cx as one of the drugs of choice to target COX-2 in oral epithelial cells by topical application in oral cancer chemoprevention using animal models due to its suitable physico-chemical and biochemical properties (7,8). In general, the chemopreventive action mechanism is due to COX-2 inhibition and its influence in apoptosis and angiogenesis (9).

However, toxic systemic side effects (increased risk of cardiovascular effects with continued use) limit its use for long periods of time, after oral administration (4,10). For this reason, in the treatment of localized inflammatory conditions or chemopreventive treatment by buccal administration of these drugs turns out to be a better strategy, reducing the risk of systemic toxicity while preserving the treatment efficacy. The exploration of Cx administration by other routes as buccal (3,4,8) or transdermal (11,12) has been explored to reformulate this drug for use in several inflammatory conditions.

Nowadays, Cx is the only drug COX-2 inhibitor currently available in the market, the others being withdrawn from the market due to serious side effects. To date, there are no mucoadhesive formulations for buccal application and the only dosage form presently available commercially is as capsule. However, in the development of buccal delivery systems, the highest challenge is the retention of formulation on the site of action since oral saliva and mechanical activities quickly remove it from the buccal mucosa area (13,14). The adhesive polymers like chitosan (CHT) can overcome this

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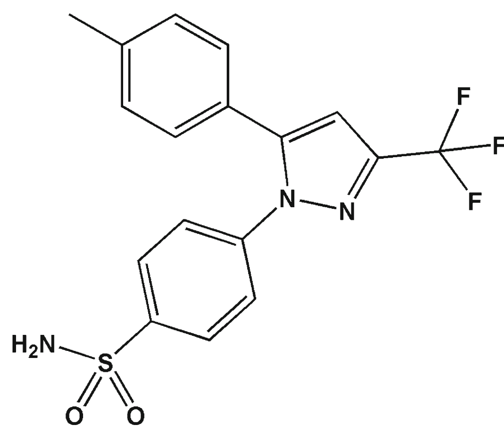

Fig. 1. Chemical structure of celecoxib

limitation by retaining the formulation in the site of action and optimizing the local effect. Moreover, CHT-based delivery systems like CHT gels are considered for sustained delivery of drugs (15,16). The cationic polyelectrolyte nature (positively charged amine groups) of CHT chemical structure could produce mucoadhesive characteristics through a strong electrostatic interaction with mucus or a negatively charged mucosal surface (17,18).

Mucosal delivery of Cx could be an interesting strategy to avoid systemic side effects (8) because the buccal epithelium is the target of the chemoprevention effect in oral cancer and other inflammation conditions of oral mucosa. While the mucosae of the oral cavity are generally considered more permeable than the skin (19), the buccal mucosa still acts as a barrier and may limit the penetration of a drug administered. Moreover, Cx is highly lipophilic (log *P* value of 4.21) and could show low retention in this tissue. In order to improve drug delivery to this route, the tissue may be treated with penetration enhancers (PE). The retention of other lipophilic drugs as estradiol (20) and triamcinolone acetonide (21) in mucosal tissue was improved by association with PE as Azone® (Az) or laurocapram, a derivative of the ethoxylated nonionic surfactant Span 20 in buccal mucosa local therapy (20,21). Increased drug retention on the buccal mucosa by its capacity to overcome the mucous barrier layer, or increased drug partition to the target tissue, is one of the described mechanisms for the Az effect. Consequently, it improves availability of drugs at the site of action by retention of lipophilic drugs (20). Moreover, since CHT also shows some penetration enhancer activity (13,22,23), the Cx availability to the mucosal tissue could be overcome by an additive effect with Az.

To date, no Cx buccal formulation is available, and its ability to penetrate buccal mucous tissue has not been investigated. The aim of the present work was to develop a novel alternative for Cx delivery—buccal delivery systems associated to Azone®—and evaluate the influence of both chemical enhancer and CHT concentrations on gels properties (pH, reology, and mucoadhesion). Moreover, we aim to study the influence of Az and CHT concentrations on Cx *in vitro* release and *in vitro* permeability (retention in the mucosa and permeation through mucosa) to gage the potential of these formulations as buccal delivery systems. For the present purpose, an ideal formulation would lead to improved drug retention at the site of action, minimizing the transbuccal delivery to avoid systemic effects.

MATERIALS AND METHODS

Chemicals

Celecoxib (purity 99.4%) was obtained by extraction of commercial capsules of Celebra® 200 mg (Pfizer, Brazil) according to methodology described previously (24). HPLC grade acetonitrile and lactic acid (PA) were purchased from Tedia, USA. Azone was from NetQem, USA and chitosan (MMW 190,000–310,000 Da) 85% deacetylated from Sigma Aldrich, USA. Analytical reagent grade monobasic potassium phosphate, sodium hydroxide, ethanol, sodium lauryl sulfate, polysorbate 20, and polysorbate 80 were purchased from Vetec, Brazil. Highly purified water was prepared by using the Millipore Milli Q plus purification system. The artificial hydrophobic membrane (cellulose nitrate) was purchased from Millipore, USA.

Methods

Preparations of CHT Gels

Pure chitosan gels (chitosan gels without drug or adjuvants) were obtained by dispersion of appropriate amounts of CHT in 1% aqueous lactic acid (stirred under mechanical agitation until homogenization) to yield 1.0%, 2.0%, and 3.0% (*w*/*w*) gels. Gels were loaded with weighed amounts of Az, the penetration enhancer (1.0%, 2.0%, and 3.0% *w*/*w*), and Cx to a final concentration of 2.0% (*w*/*w*) using ethanol as co-solvent (minimum volume). Concentrations of CHT, Cx, and Az reported in Table I are expressed as percentages of weight/weight (% *w*/*w*). Each polymer concentration had its respective control, that is, gels containing Cx in the absence of the enhancer Az. For *in vitro* release studies, the control contained only 2.0% Cx in absolute ethanol. No insoluble particles were observed after preparation of the gels.

Physico-chemical Characterization of Formulations: pH and Rheological Measurements

Formulation pHs were determined in a potentiometer fitted with a DME-CV4 electrode. Oscillatory measurements were carried out at 25°C in a rotational rheometer (model DV-III) (Brookfield) equipped with a cone-plate geometry and #CP52 spindle. Samples were placed in the cylinder and the internal rotating spindle rotated in a crescent angular velocity (1 rpm to 20 rpm), initially disrupting the system, which was then reorganized by a decreasing angular velocity. All measurements were done at room temperature. CHT gels containing Az in different concentrations were evaluated through a Rheocalc v1.01 program in order to determine the effects of both components on gel viscosities (apparent viscosities) and other parameters such as flow index, consistency index, pseudoplasticity, and thixotropy.

Table I. Rheological Parameters and pH Values in Formulations Containing Different Concentrations of CHT, Cx, and Az Compared to Respective Controls

Formulation number	Formulation*	Flow index	Consistency index	Apparent viscosity (mPa)	Thixotropy (mPa/s)	pH value*
0	Cx (2.0%) in absolute ethanol	ND	ND	ND	ND	ND
1	CHT pure gel 1.0%	ND	ND	58.98	−11,796	3.2±0.15[e]
2	CHT gel 1.0%+Cx (2.0%)	ND	ND	41.28	−86.50	4.6±0.10[f]
3	CHT gel 1.0%+Cx (2.0%)+Az 1%	ND	ND	ND	ND	4.6±0.12[g]
4	CHT gel 1.0%+Cx (2.0%)+Az 2%	ND	ND	ND	ND	4.4±0.13
5	CHT gel 1.0%+Cx (2.0%)+Az 3%	ND	ND	ND	ND	4.6±0.15
6	CHT pure gel 2.0%	0.89[a]	1,897[a]	481.67[a]	−192.67[a]	4.4±0.11[h]
7	CHT gel 2.0%+Cx (2.0%)	0.74	1,366	198.57	599.63	5.0±0.10
8	CHT gel 2.0%+Cx (2.0%)+Az 1%	0.98[b]	523.9[b]	171.04[b]	163.18[b]	5.1±0.15[i]
9	CHT gel 2.0%+Cx (2.0%)+Az 2%	0.88	678.1	165.14	194.63	5.0±0.16
10	CHT gel 2.0%+Cx (2.0%)+Az 3%	1.03	441.7	3169.08	96.33	5.1±0.11
11	CHT pure gel 3.0%	0.64[c]	14,450[c]	1,490.23[c]	497.40[c]	5.9±0.10
12	CHT gel 3.0%+Cx (2.0%)	0.39	13,068	574.07	3,688.27	5.8±0.12
13	CHT gel 3.0%+Cx (2.0%)+Az 1%	0.41[d]	8,116[d]	375.51[d]	1,950.27[d]	5.8±0.16[j]
14	CHT gel 3.0%+Cx (2.0%)+Az 2%	0.46	9,381	513.13	3,275.36	5.8±0.13
15	CHT gel 3.0%+Cx (2.0%)+Az 3%	0.41	12,987	570.14	3,898.58	5.8±0.10

ND not determined parameters

*All determinations of rheological parameters (flux index, consistency index, apparent viscosity, and thixotropy) and pH were analyzed using $n=3\pm SD$ for each formulation). One-way ANOVA test

[a] Statistically significant for CHT pure gel 2.0% (#6) compared to formulation #7 or #11 (***$p<0.001$)

[b] Statistically significant (***$p<0.001$) compared with formulations #9 and #10

[c] Statistically significant (***$p<0.001$) compared with formulation #12

[d] Statistically significant ($p>0.05$) among formulations #13, #14, and #15

[e] Statistically significant for CHT pure gel 1.0% (#1) compared to all other formulations (***$p<0.001$)

[f] Statistically significant (***$p<0.001$) compared with formulations #6 and #11

[g] Not statistically significant ($p>0.05$) among formulations #3, #4, and #5

[h] Statistically significant (***$p<0.001$) compared with formulation #11

[i] Not statistically significant ($p>0.05$) among formulations #8, #9, and #10

[j] Not statistically significant ($p>0.05$) among formulations #13, #14, and #15

HPLC Analysis

Cx amounts were quantified by a HPLC method, which was developed and validated (linearity, selectivity, inter- and intra-day precision, accuracy, detection limit, and quantification limit) using a HPLC Shimadzu model LC-20 AT equipment containing a photodiode array detector (λ 254 nm). Separation was performed on a C8 reverse phase Shim-pack column (Shimadzu) CLC (5 μm) 250×4 mm at room temperature (25°C). Acetonitrile/0.02 M phosphate buffer pH 7.4 (70:30, *v*/*v*) mixtures were used as the mobile phase at a flow rate of 1 mL/min. A Cx analytical stock solution was prepared (1,000 μg/mL) by dissolving the analyte in acetonitrile. Samples of the calibration curve at concentrations of 1, 10, 20, 30, and 50 μg/mL were obtained by successive dilutions of Cx analytical stock solution using acceptor solution (phosphate buffer 0.02 M pH 7.4 containing Tween 20 at 1.5% *w*/*w*) as diluent.

Cx retention time was 10.0 min and the assay was linear for concentrations between 1 and 50 μg/mL with a correlation coefficient (*r*) of 0.999. Precision and intermediate precision were evaluated, respectively, by intra-day and inter-day repeatability. The results show RSD values lower than 2% for all concentrations levels tested, thus, below values suggested by current legislation (RSD<5%) and revealing appropriate repeatability and intermediate precision. Recovery results for the three Cx concentrations tested (80%, 100%, and 120%) showed values in the range of 98–102%, indicative of appropriate accuracy (7). LOD and LOQ values obtained from parameters in the calibration curve were 0.04 μg/mL and 0.12 μg/mL, respectively. These values are considered appropriate for analytical assays. No unidentified peaks were detected by HPLC.

In Vitro Drug Release Studies

Using membrane models to characterize *in vitro* kinetics of drug release from CHT gel formulations into receptor media may serve as a comparative tool for the development of topical formulations for drug diffusion from gel matrices. *In vitro* drug release studies were carried out in a diffusion system, as previously described (25), constituted by a glass water bath (maintained at 37°C) mounted on a six-point stirring plate. Each of the six beakers fitted inside the bath contained the acceptor solution, 100 mL of phosphate buffer 0.02 M (pH 7.4) added of 1.5% (w/v) Tween 20 to ensure *sink* conditions and were stirred with a magnet bar at 500 rpm. The solubility of Cx in the receptor medium was 473 μg/mL. Stainless steel cylindrical gel donor compartments set over the beakers were separated by an artificial cellulose nitrate membrane (lipophilic) having an exposed surface area of 1.13 cm^2. The formulations (0.2 g) were introduced into the donor compartment, and at each time point (0, 1, 2, 4, 6, 8, 10, 12, and 24 h), 1 mL of the acceptor solution was withdrawn, followed by the reposition of an equal volume of medium. The collected fractions were filtered through 0.45-μm membranes (filtering unity; Millipore Corporate Headquarters, Billerica, MA, USA) and the filtrates were analyzed by HPLC as described before to determine the amount of Cx diffused into the acceptor solution. The amounts of Cx released in each time point was calculated according to Eq. (1): $Q_{\mathrm{real},t} = (C_{\mathrm{measured},t} \times V_{\mathrm{r}} + (V_{\mathrm{a}} \times \Sigma^{n-1} C_{\mathrm{a}})$, where Q_{real} = real value at time *t*, $C_{\mathrm{measured},\,t}$ = concentration measured in the sample at time *t*, V_{r} = volume of the diffusion cell, V_{a} = volume of the removed sample, and $\Sigma^{n-1} C_{\mathrm{a}}$ = sum of concentrations of Cx (μg/mL) determined at sampling intervals 1 through n^{-1}. The amount of released Cx was divided by the exposed area (μg/cm^2) and the results plotted as a function of time (h) and presented as averages±SD of six experiments (*n*=6) for each group.

The flux of drug across the membrane (*J*) was calculated from the slope in the linear portion of the plot (1–24 h) and expressed as micrograms per square centimeter per hour. The determination of release kinetics was done by linear regression analysis of the *xy* scatter chart applying three models: (1) zero-order kinetics—amount released per unit area (μg/cm^2) *versus* time (h), (2) Higuchi kinetics—amount released per unit area (μg/cm^2) *versus* the square root of time (h), and first-order kinetics—log of amount released per area (μg/cm^2) *versus* time (h).

In Vitro Permeability Studies

Pig Cheek Mucosa. The mucosa obtained from the slaughterhouse shortly after the death of the animal (UFRRJ, Seropédica, RJ) was cleaned, separated from underlying tissue (muscle, fat, and skin), and frozen at −20°C until use.

In Vitro Permeation Studies. The studies were conducted in the same vertical diffusion system described for *in vitro* release experiments but the artificial membrane was substituted by the fresh pig cheek mucosa. All other experimental conditions (donor phase, acceptor medium, collection times of samples, and bath temperature) were maintained as before. The tests were performed for all formulations with six replicates for each formulation. Statistical analysis was carried out using one-way ANOVA test (Tukey's multiple comparison). The determination of *in vitro* permeation kinetics for this experiment was done as described before.

In Vitro Retention Studies

Twenty-four hours after the *in vitro* permeation experiments, the pig cheek mucosae were taken from the donor compartment and the excess formulation removed with the aid of cotton soaked in water. The areas corresponding to permeation were cut out, divided into small pieces, and placed in Falcon tubes. For Cx extraction, an aliquot of 5.0 mL of ethanol was added to each tube and samples were submitted with crushing to an ultra-Turrax for 1 min. The supernatant was filtered twice through filter paper and a disposable filter unit (0.45 μm pore, Millipore®). The Cx retained in the mucosa after 24 h was quantified by HPLC. The tests were performed with six replicates for each formulation. The recovery of Cx from mucosa tissue after extraction procedures was about 90.0%.

In Vitro Mucoadhesive Strength

The study of mucoadhesive properties was carried out by the quantitative method of tensile strength described previously (9). The method measures the force required to break the adhesive bonds between mucosa and the polymer in formulations. A universal testing machine Model 2000 MEM (Emic

Manufacturers) with 10 kgf load cell and velocity t=1 mm/min, contact area of 400 mm^2, and initial contact force of 0.2 N for 3 min was used for the tests. A bath system was assembled, having a base of Plexiglas slides drawer type attached to it, to ensure that the specimens stay submerged in the liquid medium (artificial saliva) during the test. The pig mucosal tissue was adhered to Plexiglas slides with cyanoacrylate adhesive, immersed in the bath system, and attached to the drawers. The test formulation was spread on filter paper and was attached to a steel support with annular top opening where the hook of traction was seated. The support containing the formulation was put in contact with the mucosa submerged in a bath of artificial saliva, and this was slowly moved, recording the tensile strength along the displacement. The measurement of mucoadhesion was given by the force of maximum tension obtained during the displacement, which coincides with the force required to break the bioadhesive links. The tests were performed with six replicates for each formulation.

Statistical Analysis

Results are reported as means±SD. Data were statistically analyzed by one-way ANOVA (followed by Tukey's multiple comparison test). The level of significance was set at $p < 0.05$.

RESULTS

Physico-chemical Characterization of Formulations

Values of pH in gels, pure or loaded with Cx and Az, are shown in Table I. For pure gels (formulations #1, #6, and #11), the pH increases as CHT concentrations vary from 1.0% to 3.0%. Addition of Cx (formulations #2 and #7) to 1.0% or 2.0% CHT gels results in pH values slightly higher compared to pure gels. No significant changes in pH values were observed in additions to 3.0% CHT (#12). In general, gels containing 2% Cx and Az (1% to 3%) showed pHs around 4.6, 5.0, and 5.9, respectively, for CHT concentrations of 1.0%, 2.0%, and 3.0% showing that the addition of Az did not significantly affect pH values of formulations containing different concentrations of CHT and Cx. Rheological parameters for all formulations are shown in Table I. The flow and consistence indexes for formulations #1 to #5 were not measured under conditions of the assay due to the low viscosity of 1% CHT. In general, pure gels with increasing CHT concentrations (formulations #6 and #11) show significant increases in the consistency index, apparent viscosity and thixotropy, but not in the flow index.

The addition of 2% Cx to the pure gels with increasing CHT concentrations (formulations #2, #7, and #12) decreased the flow and consistency indexes and apparent viscosities, but increased thixotropy. Gels presenting flow index values lower than 1.0 are considered pseudoplastic, and the lower the flow index value, the more pronounced is this behavior. Then, the decreased flow index values in the presence of Cx imply an increased pseudoplastic property of the gels. So, formulations containing 3.0% CHT (#12 to #15) showed a better pseudoplastic behavior due to lower flow index values (around 0.4). The further addition of increasing concentrations of Az to 3.0% CHT gels (formulations #13, #14, and #15) positively influenced flow and consistency index, besides apparent viscosity when compared to addition to 2.0% CHT gels (formulations #8, #9, and #10). Among all the formulations tested, those without Az (#12) or containing 2.0% and 3.0% Az (#14 and #15) associated with 3.0% CHT gel showed the best pseudoplastic behavior (low flow index value) and higher thixotropy (no significant difference between them).

In Vitro Drug Release Studies

Figure 2 shows *in vitro* release of Cx from all formulations. Release of Cx in ethanol (formulation #0), Fig. 2a, is higher (2263.73 μg/cm^2) and at a higher flux J (Table II) compared to other controls (formulations #2, #7, and #12). It also shows that increased CHT concentrations promoted slower Cx release up to 24 h, characterizing CHT gels as sustained delivery systems. The drug slower release is related to lower flux (J) values as observed for 2.0% or 3.0% CHT concentrations. Figure 2b, c, and d shows *in vitro* Cx release from CHT gels at concentrations of 1.0%, 2.0%, and 3.0%, respectively, before and after addition of increasing Az concentrations. They show that the association of increasing concentrations of both CHT (1.0–3.0%) and penetration enhancer Az (1.0–3.0%) decreases Cx release and flux values (J) from formulations. The lower flux was observed for formulations CHT at 1.0%, 2.0%, and 3.0% containing 3.0% Az (Fig. 2b, c, and d representing formulations #5, #10, and #15, respectively). There is no significant difference in J values between formulations #10 and #15.

Linear coefficients (r) obtained in each kinetic model evaluated the *in vitro* release kinetics. The model that showed the highest r value was chosen for *in vitro* release experiments, revealing pseudo-first-order kinetics for Cx liberation from ethanol and a zero-order kinetics for all formulations containing CHT in different concentrations in the absence or presence of Az. Different concentrations of CHT and Az did not influence the release kinetics model observed, but influenced the release flux (J) values and consequently the amount of drug released.

In Vitro Permeability (Permeation and Retention) Studies Using Pig Cheek Mucosa

Table II also shows the amount of Cx permeated through pig mucosa after 24 h from 1.0%, 2.0%, and 3.0% CHT formulations, in the absence and presence of Az at different concentrations. The amount of Cx permeated through the mucosa was considered low (below 60 μg/cm^2) for all formulations. Increasing the concentration of CHT (1.0% to 3.0%) decreased the amount of permeated Cx (μg/cm^2) and the flux J. The lowest amounts of permeated Cx were obtained from 1.0%, 2.0%, and 3.0% CHT gels associated with 2.0% or 3.0% Az (no significant difference). The lowest flux values (J) were shown from formulations 2.0% and 3.0% CHT containing or not Az.

The kinetic model obtained for *in vitro* permeation studies was zero-order kinetics for all formulations containing CHT in different concentrations in the absence or presence of Az.

In vitro retention studies of Cx in porcine cheek mucosa were performed after the end of the permeation studies (Fig. 3) showing that for formulations containing 1.0% CHT, increasing Az concentrations (1.0% to 3.0%) decreased the amounts of Cx retained in the tissue, but the differences were not significant. On the other hand, at polymer concentrations of 2.0% and 3.0%, tissue retention increases with increasing

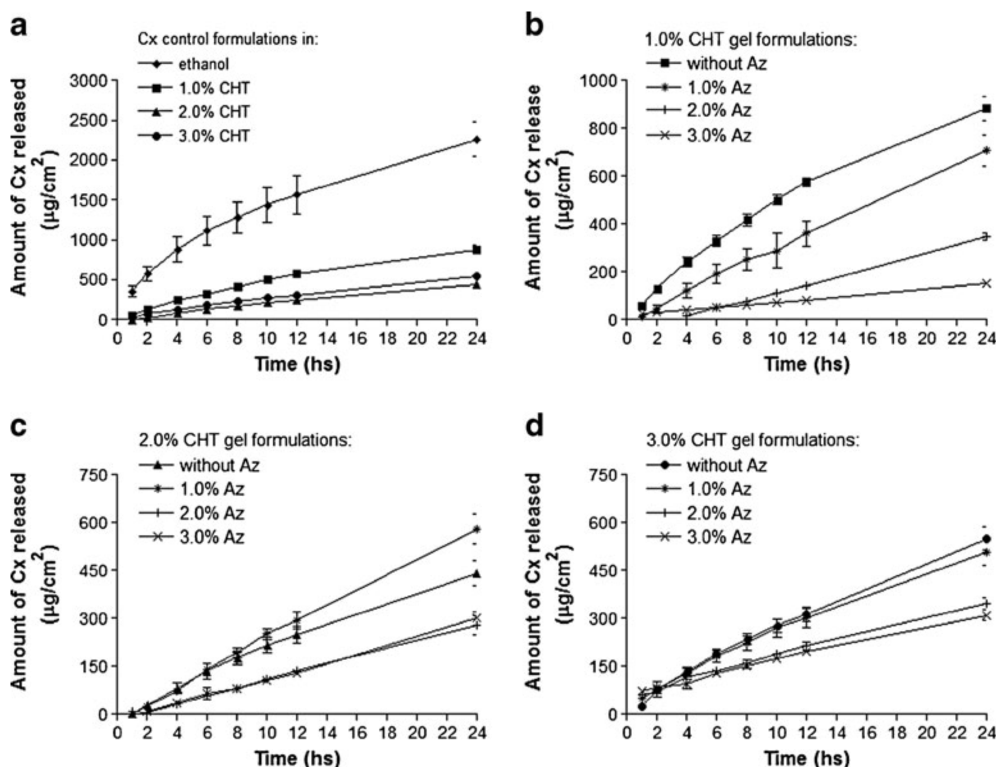

Fig. 2. *In vitro* release profiles of Cx (2.0% *w/w*) **a** from CHT gels in absence of Az, **b** from 1.0% CHT control gels and in presence of 1–3% Az, **c** from 2.0% CHT control gels and in presence of 1–3% Az, and **d** from 3.0% CHT control gels and in presence of 1–3% Az. Data represent the mean of six independent determinations±SD. Significant values for $p<0.05$. Statistical test: one-way ANOVA test (Tukey's multiple comparisons). Values considered significant after 24 h between groups: **a** Cx (2.0%) in ethanol *versus* control gels 1.0%, 2.0%, or 3.0% CHT (***$p<0.001$); **b** 1.0% CHT gels without Az *versus* all 1.0% CHT gels containing Az (***$p<0.001$), among all 1.0% CHT gels containing Az (***$p<0.001$); **c** 2.0% CHT gels without Az *versus* all 2.0% CHT gels containing Az (***$p<0.001$); **d** 3.0% CHT gels without Az *versus* 2.0% CHT gels containing 1.0% Az (**$p<0.01$) and 2.0% CHT gels containing 2.0–3.0% Az (***$p<0.001$)

concentrations of Az, with higher values observed for 3% CHT associated to 3% Az (625.54 µg/cm^2).

In Vitro Mucoadhesive Strength

The results presented in Fig. 4 show that increasing the polymer concentration in gels without Az (formulations #2, #7, and #12) causes an increase in tensile strength. Formulations containing 3.0% CHT had values of mucoadhesion around two times higher those formulations of CHT 1% or 2%. However, addition of Az (1–3%) did not alter the mucoadhesion initial values in any of the CHT concentrations (differences not significant).

DISCUSSION

The present study proposed an innovative strategy for the use of Cx and emphasized the potential of CHT-based delivery systems for the release of this class of drugs since no mucoadhesive dosage forms of this type are currently found in the market.

Chitosan, a cationic polysaccharide containing free amino groups (pK_a=6.5), is insoluble in aqueous neutral or basic solutions (26), but soluble in acid ones with a charge density dependent on pH and the deacetylation degree. Amino group protonation, which convert glucosamine units into soluble R-NH3$^+$ forms (27), promote polymer solubilization in acid pHs. Increased polymer concentrations increase the number of free amino groups that may undergo protonation, thus significantly reducing the number of free protons and increasing pH values as observed in Table I. The formulations containing 3.0% CHT show pH values around 5.9, close to buccal pH (5.5–7.4) (28) and therefore suitable for buccal application.

Rheological studies are important in formulation development especially when certain characteristics should be present such as easiness in product removal from packaging and application, adequate spreading, and smooth texture on the application site. Chitosan hydrogels may show pseudoplastic or plastic behavior depending both on type and content of added substances and also on interactions between polymer and additions. The lowest flow index determined in formulations containing CHT 3.0% (#12 to #15) shows the influence of polymer concentrations in pseudoplastic properties (Table I). In general, this property favors the local action of drugs, which remain longer in the free form, show increased bioavailability and, consequently, a gain in local effect. This pseudoplastic behavior for chitosan hydrogels has already been reported in other studies (29,30). Our results showed that increased viscosity in formulations is proportional to chitosan content. This can be explained considering the numerous intermolecular bonds that occur between polymer chains responsible for viscosity and thickness of the gels (30). Although Cx influenced the

Table II. Flux (*J*) Values (μg cm^2 h^{-1}) of 2.0% Cx of the *In Vitro* Release Studies and Amounts of Cx (*Q*) Permeated (μg/cm^2) Through Mucosa and its Respective flux *J* after 24 h of *In Vitro* Permeation Studies from Different Formulations

Formulation number	*In vitro* release flux (*J*) (μg/cm^2 h^{-1})±SD and its correlation coefficient (*r*)	Amount of Cx permeated (*Q*) (μg/cm^2)±SD and flux (*J*) (μg/cm^2/h)±SD after 24 h of *in vitro* permeation studies
0	J=433.11±88.41[a] (r=0.9978)	Q=107.8±7.9[e] J=5.3±1.30
2	J=34.99±6.99[b] (r=0.9850)	Q=56.68±3.23[f, g] J=0.66±0.16
3	J=29.98±3.26 (r=0.9810)	Q=35.50±0.97 J=0.43±0.10
4	J=15.18±1.81 (r=0.9982)	Q=31.16±0.96 J=0.39±0.11
5	J=4.87±1.27[c] (r=0.9952)	Q=46.19±5.71 J=0.87±0.12
7	J=16.80±5.08 (r=0.9936)	Q=46.87±5.48[h] J=0.40±0.00
8	J=25.37±2.08 (r=0.9984)	Q=32.54±3.10 J=0.13±0.00
9	J=12.99±2.34 (r=0.9980)	Q=27.09±1.10 J=0.13±0.00
10	J=13.08±0.94[d] (r=0.9926)	Q=27.50±0.80 J=0.13±0.00
12	J=22.09±1.47 (r=0.9920)	Q=27.15±0.80 J=0.25±0.00
13	J=20.93±3.09 (r=0.9948)	Q=26.32±1.35 J=0.11±0.00
14	J=13.18±2.22 (r=0.9923)	Q=25.31±2.23 J=0.11±0.00
15	J=10.49±0.72 (r=0.9917)	Q=28.32±1.02 J=0.75±0.20

[a] Means±SE of the results in six experiments are shown (one-way ANOVA test)
Statistically significant for control Cx in absolute ethanol (#0) compared to control gels (#2, #7, and #12) (***p<0.001)
[b] Formulation #2 shows a statistically significant difference compared with formulations #7 (***p<0.001) and #12 (*p<0.05)
[c] Statistically significant (***p<0.001) compared with formulation #10 or #15
[d] Not statistically significant (p>0.05) compared to formulation #15
[e] Statistically significant (***p<0.001) compared with all other formulations
[f] Statistically significant (**p<0.01) compared with formulation #12
[g] Statistically significant (*p<0.1) compared with formulations #3, #4, and #5
[h] Statistically significant (**p<0.01) compared with formulations #9 and #10

rheological behavior of formulations, decreasing apparent viscosity, it contributed to the increase of the pseudoplastic and thixotropic properties. As an advantage in terms of rheological properties, the addition of Az (2.0% and 3.0%) to the gels containing Cx did not change significantly the apparent viscosity in 3.0% CHT gels (#14 and #15) compared to #12 (without Az).

Thixotropy or time-dependent change in viscosity (28) is a desirable property for pharmaceutical formulations due to the flexibility requirements of drug delivery (31). Thixotropic topical formulations deform during application, *i.e.*, they become more fluid and consequently the spreading on the application site is easier. At the end of application, drug drainage is prevented by recovery of the initial viscosity. Thixotropic behavior is characterized when gel restructuring occurs slowly. In general, the higher thixotropy of semisolid products improves shelf-life and topical application. Thixotropy can be influenced by several factors in a gel system: pH, temperature, polymer modification or combinations, addition of cations or anions, and polymer concentrations (31). It is shown in this study that thixotropy of pure gels (#1, #6, and #11) increased with increasing concentration of polymer CHT. Furthermore, thixotropy was more markedly increased by addition of Cx (#2, #7, and #12) as compared to pure gels, and the highest values were obtained in gels #12 to #15. Excipients such as lecithin, sodium chloride, and glycerol may be used to produce viscous thixotropic gels with enhanced stability (32). The penetration enhancer Az was chosen as adjuvant in the present investigation because it is effective at low concentrations and can increase tissue retention of various drugs (33). Evaluating the effects of increasing concentrations of Az on CHT gels thixotropy, it was shown that addition of Az to 2.0% CHT gels (#8, #9, and #10) decreased the property but did not affect 3% CHT (#14 and #15) compared to gels in the absence of Az. Therefore, formulations #14 and #15 present both pseudoplastic and thixotropic properties besides higher apparent viscosity values when compared with other formulations containing Az.

Considering buccal application, more viscous pharmaceutical dosage forms have the advantage of a slow flow index, which minimizes intoxication risks by accidental swallowing. Based on the best rheological results, it was possible to select suitable formulations containing 3.0% CHT associated with 2.0 or 3.0% Az as those presenting good apparent viscosity, pseudoplastic, and thixotropy characteristics.

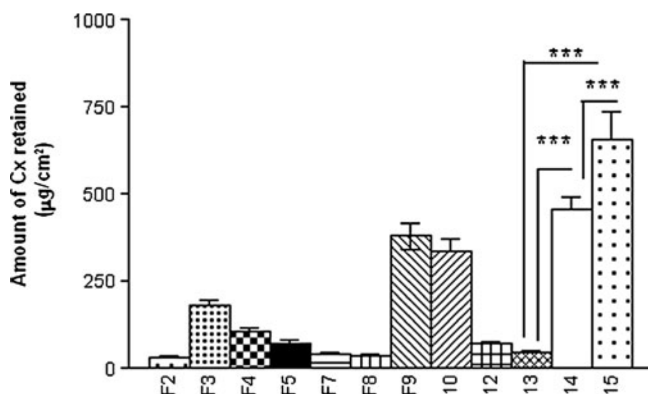

Fig. 3. Amount of Cx retained (μg/cm^2) in pig cheek mucosa after 24 h of *in vitro* permeation from formulations of Cx 2.0% (*w*/*w*) in CHT 1.0%, 2.0%, and 3.0% in the absence of Az (control formulations F2, F7, and F12) and in presence of different concentrations of AZ (1.0% to 3.0%). Significant values for $p<0.05$. Statistical test: one-way ANOVA test (Tukey's multiple comparisons). Values considered significant (***$p<0.001$) between groups: F13 *versus* F14, F13 *versus* F15, and F14 *versus* F15

The potential of CHT gels as drug sustained release systems has been suggested in the literature (34,35), but this is the first time that this was tested for sustained release of Cx also in the presence of Az. It is known that *in vitro* release profiles for chitosan gels depend on temperature, chitosan deacetylation degree, molecular weight, and substances present. Moreover, in drug delivery systems, time-dependent changes in viscosity provide pharmaceutical formulations with flexible rheological manifestations, which subsequently affect the release profile of loaded drugs. Figure 2a shows that gels with increasing concentrations of polymer CHT (1.0% to 3.0%) have decreased release of Cx and lower flow (*J*) in 24 h (Table II). This is probably related to increased gel viscosity in high polymeric concentrations, characterizing these gels as delivery systems. In general, the total amount of released Cx was inversely proportional to CHT content. In a similar study, other authors showed the same relationship between CHT content and drug release profile (30,34).

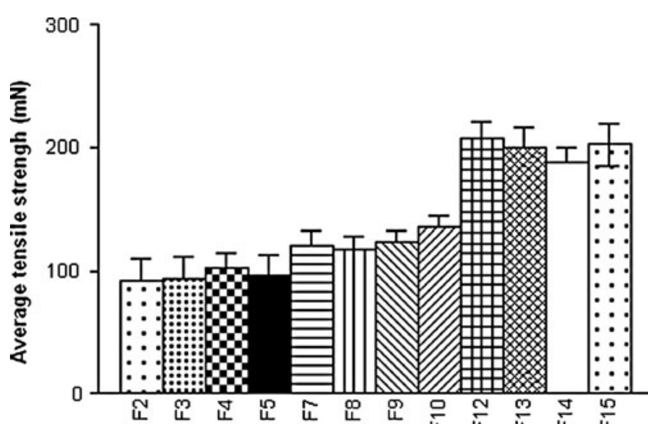

Fig. 4. Mucoadhesion measurements—tensile forces (mN) in CHT control gels (without Az) and CHT 1.0–3.0% (*w*/*w*) gels containing 1.0–3.0% (*w*/*w*) Az. Mean values and standard deviations ($n=6$) for each group. Significant difference for $p<0.05$. Statistical test: one-way ANOVA test (Tukey's multiple comparisons). Values considered significant between groups 1.0% CHT (F2 to F5) and 3.0% CHT (F12 to F15) gels (***$p<0.001$) and between groups 2.0% CHT (F7 to F10) and 3.0% CHT (F12 to F15) gels (***$p<0.001$) with or without Az

The further addition of a lipid component as a penetration enhancer (Az 3.0%) more significantly decreases Cx release and flux (*J*) from 1.0%, 2.0%, and 3.0% CHT gels, (Fig. 2b–d and Table II), respectively, probably due to the increased drug solubility in this system. Indeed, as shown in Table I, addition of 3.0% Az to gels (formulations #10 and #15) did not significantly affect viscosity of gels when compared to those without Az. This could mean that reduced drug release is not related to increased viscosity, but due to the solubilization of the lipophilic drug in the penetration enhancer.

Az, a surfactant, may decrease the thermodynamic activity of drugs in the delivery system, although it is probable that thixotropy also plays a part in the dynamics of release.

Based on all results from *in vitro* release studies, it is possible to conclude that formulations containing 3.0% CHT added of 2.0% (#14) or 3.0% (#15) Az show low *in vitro* release flux (*J*) values (no significant differences between them) compared with the respective control (#12) and that drug release occurs slowly, characterizing a sustained release system. On the other hand, it is interesting to verify that 1.0% CHT associated to Az 3.0% (#5) among all formulations showed the lowest *in vitro* release flux values ($J=4.87$ μg/cm^2). However, formulations with 1.0% CHT did not have appropriate physico-chemical properties for local application. Similarly, 2.0% CHT gels (formulations #9 and #10) showed *in vitro* release *J* values similar to formulations #14 and #15 (Table II), but did not have appropriate rheological parameters (Table I). In brief, 3.0% CHT gels containing Az (2.0% and 3.0%) showed higher thixotropy values (#14 and #15) and other suitable rheological parameters (low flow index, high consistency index, and high apparent viscosity) besides low *in vitro* release Cx flux (*J*) values.

All CHT formulations in different concentrations (1.0% to 3.0%) both in the absence or presence of Az in different concentrations (1.0% to 3.0%) showed zero-order kinetics for both *in vitro* drug release studies and *in vitro* permeation studies. Pharmaceutical dosage forms that have zero-order profiles release equal amounts of drug per time unit, constituting one of the best options for drug sustained release. This could be explained by increased gel network complexity as the polymer concentration increases. The mechanism of chitosan gel formation is not known exactly, but it is clear that the length of chitosan chains and the degree of reacetylation are important.

In vitro permeation and retention studies were carried out in order to evaluate if the formulations in this study are promising for Cx buccal application, in terms of higher local retention and lower permeation through the buccal mucosa. The results shown in Table II demonstrate that, similar to the Cx *in vitro* release studies, increasing the concentration of pure CHT (formulations #2, #7, and #12) decreases the amount Cx permeated (μg/cm^2) and the permeation flux *J* through the tissue. In this case, it is probable that increased viscosity (Table I) of such formulations decrease the amount of Cx released and consequently the amount of permeated drug. The addition of Az further decreased the amount of permeated Cx (μg/cm^2) (Table II); the lowest permeation values were observed for 2.0% and 3.0% CHT gels

(associated with 1.0–3.0% Az). Such reductions, again, must be related to increased drug solubilization by Az in the gel, not by increases in viscosity as stated above. Interestingly, the flux (J) of Cx permeation slightly increased only for the CHT gels at 1.0% and 3.0%, in the presence of Az 3.0% (formulations #5 and #15), compared to the gels without Az (formulations #2 and #12).

Anyway, for all tested formulations, Cx permeation and its flux J through mucosa was minimal, which is an advantage for local application since it avoids the side effects of systemic administration.

Other studies have shown that the presence of Az reduces the *in vitro* transbuccal flux of drugs, *i.e.*, increases the reservoir function of the oral mucosa for lipophilic drugs (20,21), which could explain the significant tissue accumulation of Cx in the current study. That is, the reduction in flow was associated with increased uptake in tissue, suggesting that Az increased the reservoir capacity of the oral mucosa. Recent studies (36) also showed that the presence of 5% Az did not significantly modify the permeation of a drug through buccal mucosa.

According to reports in the literature, one of the mechanisms of action for Az is the increased uptake of the drug in the buccal mucosa, *i.e.*, it promotes the permeability of certain compounds by increasing partition in the buccal mucosa. As observed in Fig. 3, the highest tissue retention of Cx were observed for 3.0% CHT gel containing 2.0% and 3.0% Az (8.8 and 13.1 times, respectively, compared to 3.0% CHT gel without Az), thus, characterizing these formulations as topical delivery systems with low systemic absorption, ideal for the application of Cx in the oral mucosa. Additionally, the highest values for tissue retention were observed for formulations containing 3% CHT associated to 3% Az, suggesting a possible synergistic effect between CHT and Az to promote the penetration of the drug in the oral mucosa.

The action of CHT as penetration enhancer in oral mucosa has been described as a relatively mild and reversible effect on the morphology of the epithelial mucosa. One explanation for the enhancing effect observed may be due to the bioadhesive nature of chitosan, which increases drug retention at the application site (reservoir effect). It was demonstrated that the interaction between polymer and mucin, responsible for the mucoadhesive bond formation is involved with the mechanism of penetration enhancement (37). The interpenetration of polymer and mucin probably weakens the epithelial barrier, partially undoing the structure of the extracellular matrix and intercellular connections. Chitosan may also act in the organization of intercellular lipid layer that forms the barrier of the oral epithelium, reflecting a direct permeabilizing effect (38). Thus, the enhancing effect of chitosan occurs either by direct action in the disorganization of the lipid barrier of the oral epithelium or by mucoadhesion that promotes increased retention of formulation on the mucosal surface. This represents a clinical advantage since the elimination of the formulation by salivary flow can be reduced (21). In addition, early studies have demonstrated a reservoir function of the buccal mucosa (39,40) resides in the superficial epithelial layers of the tissue. It has been suggested that these layers become saturated with drug; however, the reservoir nature of this region can be enhanced with the use of chitosan (22). In a similar manner, in the current study, we can speculate that Az was able to enhance the reservoir capacity of the buccal mucosa for Cx, having a synergistic effect with CHT.

In general, there are several other classes of chemical enhancers for buccal drug delivery: surfactants (sodium lauryl sulfate, sodium dodecyl sulfate, *etc.*), bile salts (sodium glycocholate, sodium fusidate, *etc.*), fatty acids (oleic acid, lauric acid, *etc.*), inclusion complexes (cyclodextrins), chelators (ethylene diamine tetra acetic acid, citric acid), polymers (chitosan), cod liver oil extracts, and lysalbinic acid (41). However, depending on concentrations applied, these enhancers can potentially cause swelling, irritation, lipid extraction, or ulceration of the tissue (42).

Early literature did suggest that Az produces minimal irritation on mucous membranes or skin when applied neat (43); however, more recent studies have demonstrated that Az has the potential to cause irritation and some tissue damage when applied to the skin (44,45), rectal mucosa (46), and cornea (47,48).

Az is generally used at low concentrations (1–5% *v*/*v*) and its enhancer activity may be increased by using co-solvents including propyleneglycol; however, it does not show genotoxic, teratogenic, or embryotoxic effects (49). Its use for dermal administration may cause mild irritation, but for oral mucosa in low concentrations as studied in the work (1–3%) this effect may be smaller. It is also reported to be non-irritant and non-allergenic (50) so that its use was patented in various transdermal formulations (51). Furthermore, one can clearly see in the "RESULTS" section that Az increased retention of the drug at the site of application compared to the formulation in the absence of it, causing minimal transbuccal absorption, what is desirable for treatment at the site of inflammation/cancer chemoprevention in the buccal mucosa.

Mucoadhesion is a desirable characteristic of formulations intended for application to mucous membranes, which must be maintained in the presence of adjuvants. The interaction of the polymer with the mucosa is dependent on concentration besides molecular weight and degree of deacetylation characterizing a direct proportional relation as observed in Fig. 4. The increase in mucoadhesion by increasing polymer concentration may be explained by a greater number of positively charged amino groups available to interact with the negative charges of the mucosal surface forming adhesive bonds. The 3% CHT formulation (with or without Az) showed higher tensile strength (approximately two times higher compared to 1% or 2% CHT), and therefore the greatest mucous adhesion. Statistically, there was a significant difference ($p<0.001$) for formulation 3.0% CHT compared with other concentrations.

In a recent study (31), the strength of mucoadhesion was evaluated in gels of oral NSAIDs already on the market and values in the range of 380–502 mN found for porcine cheek mucosa. Another study (52), using gels containing chitosan (0.5% to 2.0%) for vaginal application, showed values of tensile strength in the range 43–93 mN. Our results showed average values from 90 to 200 mN, using CHT concentrations of 1.0–3.0%, confirming that high mucoadhesive properties were achieved in the presence of additives like Cx and Az.

CONCLUSIONS

Our results have elected 3.0% CHT gels associated with 2.0% or 3.0% Az (#14 and #15) as potential Cx delivery systems

(a slow and sustained release), having the ideal physicochemical and rheological properties (pH, pseudoplasticity, consistency index, apparent viscosities, and thixotropy) necessary for application on buccal tissue. These formulations increased drug retention at this site by higher mucoadhesion and mucosa retention, which acts as a deposit for the continuous and gradual absorption of drug. It is possible to conclude that these formulations may be explored for administration of Cx for buccal mucosa delivery in inflammatory conditions or chemopreventive treatment of tumors in this tissue.

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