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Chitosan-based buccal mucoadhesive bilayer tablets enhance the bioavailability of tizanidine hydrochloride by bypassing the first-pass metabolism

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ABSTRACT

Tizanidine hydrochloride (TZN) is an antimuscarinic agent used in the treatment of pain-related spasms, multiple sclerosis, and stroke-related spasticity. It has low oral bioavailability (40 %) due to excessive first-pass metabolism in the liver. The aim of this project was to enhance the systemic bioavailability of TZN by developing buccal mucoadhesive bilayer tablet formulations using chitosan salts with molecular weights of 136 kDa (7 cP) and 169 kDa (10 cP). Structural characterisation of chitosans and their salts was performed by FTIR, ^{1}H NMR, DSC, TGA, and XRD analyses. Soluble chitosan salts, chitosan glutamate and chitosan chloride, for the adhesive layer, and insoluble ethyl cellulose for the impermeable backing layer were selected as polymers to fabricate the buccal tablets by direct compression method. The tablets containing TZN demonstrated high swelling and good mucoadhesion characteristics as well as released all the drug within 8 h. While TC10, formulated with 10 cP chitosan chloride, showed the highest swelling properties, TG10, prepared with 10 cP chitosan glutamate, exhibited the highest mucoadhesion. Chitosan glutamate tablets (TG7 and TG10) demonstrated better mucoadhesive characteristics and stability than chitosan chloride ones (TC7 and TC10) in the buccal medium based on the results of swelling and drug release studies. The permeability studies performed by Franz diffusion cell demonstrated that the amount of TZN passing through the bovine buccal mucosa was between 13.4 and 14.4 %. Stability studies conducted at 5 ± 2 °C, 25 ± 2 °C and 40 ± 2 °C for 6 months showed that no changes in the content uniformity and pH were observed. The *in vivo* comparative bioavailability studies in female New Zealand rabbits were performed and TZN-containing buccal mucoadhesive bilayer tablets fabricated with both chloride (TC10) and glutamate (TG10) salts of chitosan demonstrated three times higher bioavailability than the commercial TZN product (Sirdalud® oral tablet) administered by the gastrointestinal route.

1. Introduction

The orotransmucosal drug delivery, which includes sublingual, buccal, and soft palatal routes, has emerged as an alternative application route for improving the systemic bioavailability of drugs [1,2]. Among these, the buccal route offers significant advantages such as allowing rapid and direct delivery into blood circulation bypassing first-pass metabolism, and providing a larger surface area than the sublingual route [3,4].

In recent years, numerous investigations have been conducted on buccal administration of various dosage forms, including tablets [5,6], patches [7,8], films [9,10], wafers [11,12], and hydrogels [13,14]. Buccal tablets are one of the well-researched dosage forms that enable prolonged drug delivery due to their ability to remain in the mouth for an extended period without disintegrating. Moreover, the buccal tablets do not pose a problem for speaking and drinking as they are smaller and thinner than the conventional tablets [15]. However, the elimination of a part of the active ingredients from the buccal mucosa through saliva poses a significant challenge for orotransmucosal drug delivery [16]. To address this issue, buccal bilayer tablets can be formulated with water-insoluble polymers such as ethyl cellulose [17,18]. The impermeable backing layer containing hydrophobic substance ensures

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unidirectional drug release, in other words, the active ingredient is absorbed only through the buccal mucosa [19].

Bioadhesive polymers used to fabricate buccal tablets enhance the systemic bioavailability of the drug by allowing the dosage form to remain on the buccal mucosa for the desired period. Chitosan is a biodegradable, biocompatible, mucoadhesive, and low-toxic polymer produced by partially deacetylating chitin. This makes it an attractive option for researchers interested in mucosal drug delivery due to its properties. It demonstrates mucoadhesive properties through ionic interaction with the negative charge of mucin glycoproteins due to its cationic structure. Chitosan is a weak base that dissolves by protonating its primary amine group in an acidic medium [20–26]. Water-soluble chitosan salts such as chitosan glutamate, chitosan lactate, and chitosan chloride are the preferred polymers for preparing buccal bioadhesive formulations. The buccal dosage forms prepared with these polymers are highly effective in providing prolonged drug release due to their good swelling and bioadhesive characteristics [27–29].

TZN is an antispasticity agent used in the treatment of pain-related spasms, multiple sclerosis, and stroke-related spasticity [30,31]. The oral bioavailability of TZN, which is highly exposed to the first-pass mechanism in the liver, is about 40 % [32]. The most common side effect of TZN, which has a narrow therapeutic index, is sedation which may cause somnolence, especially at the beginning of the treatment [33]. The daily dose of TZN might be increased up to 36 mg depending on the course and severity of the disease and 2–4 mg of it is generally used three times a day [34].

Researches were conducted on buccal formulations to improve the bioavailability of TZN. In a previous study of our group, the systemic effect of TZN was increased by developing chitosan-based buccal monolayer patch formulations [35]. However, due to the fact that the patches lose their integrity faster than tablets and the rapid swelling of chitosan, the monolayer design of the films makes buccal tablets attractive if longer-term remaining on the mucosa is aimed. Buccal tablet formulations of TZN have been reported in the literature. However, these studies were limited to characterisation, drug release, and *ex vivo* permeability studies. Shanker et al. [36] developed TZN-containing buccal bilayer tablet formulations, which included HPMC and CMC as mucoadhesive polymers and ethyl cellulose as an impermeable layer. They investigated the effect of different penetration enhancers on the permeability of TZN. Shivanand et al. [37] prepared buccal bilayer tablets of TZN using Carbopol, HPMC, and CMC. The formulation containing Carbopol and HPMC was identified as the optimal tablet for buccal administration based on the results of characterisation and drug release studies.

The objective of this work was to fabricate buccal mucoadhesive bilayer tablet formulations using chitosan salts to achieve unidirectional drug release, thereby improving the bioavailability of TZN and reducing the systemic side effects. The physicochemical properties and bioadhesive characteristics of the buccal tablets were determined, and *in vitro* drug release studies and permeability studies using bovine buccal tissue were performed. Also, *in vivo* bioavailability studies were conducted in New Zealand rabbits to compare the pharmacokinetic parameters obtained with the mucoadhesive bilayer tablets and the commercial product (Sirdalud® oral tablet) administered by the buccal and the gastrointestinal route, respectively.

2. Materials & methods

2.1. Materials

Low molecular weight chitosans (7 cP and 10 cP, 70–80 % deacetylated) were kindly donated by Primex, Iceland. The molecular weights of 7 cP and 10 cP chitosans were determined as 136 kDa and 169 kDa, respectively, by gel permeation chromatography in-house. Tizanidine hydrochloride (TZN) was purchased from TCI Chemicals, Japan. Ammonium acetate, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDAC), ethyl cellulose, glutamic acid, hydrochloric acid, mucin (type III), phosphate buffer tablet (pH 7.4) and sodium hydroxide were purchased from Sigma, USA. Calcium chloride (anhydrous), and pH 6.8 phosphate buffer tablet were obtained from Merck, USA. Magnesium stearate from Doğa İlaç, Türkiye, and azelaic acid from Fluka, USA were purchased. The dialysis membrane (Visking Dialysis Tubing, diameter 28 mm, 12–14 kDa) was purchased from Serva Electrophoresis GmbH, Germany. Membrane filters (0.22 μm and 0.45 μm of PTFE membranes, 0.22 μm of Nylon membrane, Millex, Merck-Millipore, Darmstadt, Germany) were obtained from Millipore, USA. Ketamine hydrochloride (Ketasol) from Richter Pharma Ag, Austria, and Xylazine hydrochloride from Bayer, Germany were purchased. Commercial Tizanidine hydrochloride tablet (Sirdalud® oral tablet 2 mg) was purchased from Novartis, Türkiye. All other chemicals were of pharmaceutical grade.

2.2. Preparation of chitosan salts

Chitosan was dissolved in purified water in a 1:1 M ratio with either hydrochloric acid or glutamic acid [38,39] by stirring at the rate of 300 rpm min^{-1} on a magnetic stirrer (Mr-Hei Standard, Heidolph, Germany) at 25 °C for 24 h. Then, the solution was dialysed using the dialysis membrane against purified water for 72 h to remove all unreacted acid residues. The solution was then filtered using a 0.22 μm membrane filter to remove all insoluble materials and freeze-dried (SP Scientific, VirTis Advantage, Suffolk, UK) at −35 °C for 48 h. The freeze-dried polymers were milled by a knife mill to reduce their size, passed through the sieve with a pore size of 0.25 mm, and stored under vacuum at 4 °C until further use.

The polymers prepared with chitosan of 7 cP and hydrochloric acid or glutamic acid were named CC7 and CG7, while those prepared with chitosan of 10 cP were named CC10 and CG10, respectively.

2.3. Characterisation of chitosan and chitosan salts

2.3.1. Fourier-transform infrared (FTIR) analysis

FTIR spectra of chitosan and its salts were obtained by utilizing KBr discs (sample/KBr = 1/200, w/w) on an FTIR Spectrophotometer (Cary 630, Agilent, USA) in the range of 4000–400 cm^{-1} [35].

2.3.2. Proton nuclear magnetic resonance (^1H NMR) analysis

^1H NMR analysis was conducted using a Nuclear Magnetic Resonance Spectrometer (Bruker Avance III, Germany). The analyses of chitosan and its salts were carried out in D_2O, at 400 MHz and 25 °C. Phase and baseline correction of the ^1H NMR spectra was performed using the software Mestrenova v14.1.2–25024 (Mestrelab Research SL, Spain) [35].

2.3.3. Powder X-ray diffraction (XRD) analysis

XRD patterns of chitosan and its salts compressed into tablet-shaped discs were obtained using a Philips PANalytical X'Pert Pro X-ray Powder Diffractometer (Netherlands) with Cu Kα radiation ($\lambda = 1.5406$ nm) at 40 mA and 45 kV with a scanning speed of 1 min^{-1} from $2\theta = 5$ to $2\theta = 60$ [40].

2.3.4. Differential scanning calorimetry (DSC) analysis

Differential scanning calorimetry (DSC) analyses of chitosan and its salts were performed using a heat-flux type DSC (DSC 214 Polyma, Netzsch, Germany). High-purity calibration standards of the metals indium (In), tin (Sn), and zinc (Zn) were used for the temperature and heat flow calibration of the instrument. Approx 4–6 mg of samples were heated from 20 to 300 °C in an aluminium crucible using the following parameters: the heating rate of 5 °C min^{-1} and under a nitrogen flow of 60 mL min^{-1} [41].

2.3.5. Thermogravimetric analysis (TGA)

Thermogravimetric analyses (TGA) were performed on chitosan and its salts, weighing approx 30 mg, using a TGA instrument (Setaram, France) in the temperature range of 25–600 °C at a heating rate of 10 °C min^{-1} in flowing nitrogen gas [41].

2.4. Fabrication of buccal mucoadhesive bilayer tablets

Buccal mucoadhesive bilayer tablets (Table 1) were fabricated by the direct compression method with some modifications according to previous studies [42,43]. To prepare the bioadhesive layer of the tablets (80 mg), chitosan salt as a bioadhesive polymer, magnesium stearate as a lubricant, and TZN were mixed in a cubic blender for 10 min. The powder mixture was compressed at a force of 50 kg cm^{-2} (49 bar) using an eccentric tablet machine equipped with an 8 mm diameter flat-face punch (Tablet Press Machine, Yeniyurt, Türkiye). To fabricate the double-layer tablet, ethyl cellulose (40 mg), the impermeable layer polymer, passed through the sieve with a pore size of 0.25 mm and added onto the bioadhesive layer placed in the die. Then, the final compression was performed at a force of 240 kg cm^{-2} (235.4 bar) using an 8 mm diameter flat-face punch on the same tableting machine.

2.5. Physicochemical characterisation of buccal mucoadhesive bilayer tablets

The diameter and thickness of the tablets were determined using a digital micrometre (Guanglu, China). Ten replicates were run for each formulation.

The weight uniformity of formulations was performed with 10 tablets from each batch using an electronic balance (PA413, Ohaus Pioneer, Switzerland).

The determination of the pH of the buccal formulations was conducted as described by Shirsand et al. [43]. The tablet was kept in a Petri dish (Ø = 3 cm) having 5 mL of phosphate buffer (pH 6.8) at room temperature. The cap of the dish was closed, and the pH value was measured using a pH meter (Inolab 720, WTW, Germany) after 2 h. The experiments were carried out five times for each batch.

To determine the friability of the buccal tablets, 20 tablets from each formulation were precisely balanced and the total weight was detected (M_1). The tablets were placed in the plexiglass drum of the friabilitor (PTF 20E, Pharmatest, Germany) and allowed to rotate at a speed of 25 rpm min^{-1} for 4 min [44]. Finally, the buccal tablets were reweighed (M_2). The friability (%) was calculated with the following formula (Equation (1)).

$$\text{Friability (\%)} = [(M_1 - M_2)/M_1] \times 100 \quad \text{(Equation 1)}$$

The hardness of the tablets was measured using a hardness tester (PTB 311E, Pharmatest, Germany) (n = 10). The mean and the standard values were calculated.

2.6. Swelling index

The initial weights of three tablets from each batch were weighed separately (M_1). The buccal tablets were individually placed in a Petri dish (Ø = 3 cm) having 5 mL of phosphate buffer (pH 6.8) to allow swelling at 37 ± 0.5 °C. The tablets were taken from the Petri dishes at specified time intervals (30 min–6 h) and carefully dried with cellulose filter paper. The swollen tablets were reweighed (M_2). The swelling index (%) was calculated using the equation below (Equation (2)) [35].

$$\text{Swelling index (\%)} = (M_2 - M_1) / M_1 \times 100 \quad \text{(Equation 2)}$$

2.7. Drug content uniformity

Five tablets were powdered in a mortar. 120 mg powder was transferred to a beaker containing 50 mL of phosphate buffer (pH 6.8) and stirred for 15 min. The volume was completed to 200 mL with the same buffer. One mL of the sample was taken and filtered through a 0.22 μm membrane filter. TZN amount was determined by the high-performance liquid chromatography (HPLC) method. The studies were carried out five times for each batch.

HPLC (1100 series, Agilent, USA) analyses were performed using a C18 column (150 × 4.6 mm, 5 μm, Interstil, GL Sciences, Japan) at 25 °C column temperature. The flow rate was set as 1 mL min^{-1} and the volume of injection was adjusted to 10 μL. Acetonitrile and 0.1 M of ammonium acetate mixture (v/v 15:85) were used as the mobile phase. The UV detector was set at 228 nm [45]. The method was fully validated according to the International Conference on Harmonization guidelines [46]. The HPLC method was used in the studies of content uniformity, drug release, and *ex vivo* permeability.

2.8. Mucoadhesion studies

TA-XT*Plus* Texture Analyser equipped with a 5 kg load cell (Stable Micro Systems, Haslemere, Surrey, UK) was used in *ex vivo* mucoadhesion studies [20,47]. The fresh bovine buccal mucosa obtained from the slaughterhouse was stored at −30 °C. Before the studies, the mucosa was thawed for 1 h, placed between the plexiglass plates, and fitted by its screws. The surface of the buccal mucosa was hydrated with 75 μL phosphate buffer (pH 6.8) containing 2 % of mucin. The impermeable layer surface of the tablet was firmly adhered to the probe (SNSP/10, Ø: 10 mm) using double-sided adhesive tape. The tablet and the mucosa were contacted for 120 s with a force of 1 N at a speed of 0.5 mm s^{-1}. Then, the probe was moved upwards at a speed of 0.5 mm s^{-1}. The peak of mucoadhesive strength (N cm^{-2}) and the work of adhesion (mJ cm^{-2}) were determined from the area under the detachment curve using Equation (3). The experiments were conducted at room temperature and performed four times. The mean and standard deviation values were calculated.

$$\text{Work of adhesion (mJ cm}^{-2}) = AUC_{1\text{-}2} / \pi r^2 \quad \text{(Equation 3)}$$

$AUC_{1\text{-}2}$: Area under the curve of the force-distance profile.

πr2: The surface area of the tablet

2.9. In vitro drug release studies

The release studies were performed in a beaker containing 200 mL phosphate buffer (pH 6.8) at 37 ± 0.5 °C on a magnetic stirrer (RT15, IKA, Germany) at a speed of 50 rpm [35]. The impermeable surface of the buccal tablet was stuck on a glass plate (2 × 2 cm^2) using

Table 1
The composition of buccal mucoadhesive bilayer tablets.

Formulations	TZN (mg)	Chitosan chloride (CC7) (mg)	Chitosan chloride (CC10) (mg)	Chitosan glutamate (CG7) (mg)	Chitosan glutamate (CG10) (mg)	Magnesium stearate (mg)	Ethyl cellulose (mg)
TC7	2.288	76.112	–	–	–	1.6	40
TC10	2.288	–	76.112	–	–	1.6	40
TG7	2.288	–	–	76.112	–	1.6	40
TG10	2.288	–	–	–	76.112	1.6	40

cyanoacrylate glue. The glass plate was left in the beaker at a right angle. The sample (0.5 mL) was collected from the beaker at specified time intervals (30 min–8 h) and filtered using a 0.45 μm PTFE membrane filter. Also, the fresh medium was added to the beaker as soon as the sample was taken. The samples were analysed by the HPLC method. The studies were conducted in quintuplicate.

The drug release from buccal tablets was compared to each other using the similarity factor (f_2). The f_2 value, calculated using Equation (4), greater than 50 refers to the similarity of the release profiles between two formulations [48,49].

$$f_2 = 50 \times \log\left\{\left[1 + \frac{1}{n}\sum_{n=1}^{n}(R_t - T_t)^2\right]^{-0.5} \times 100\right\} \quad \text{(Equation 4)}$$

t: Time.

R_t: Drug release percent of reference formulation at t time
T_t: Drug release percent of test formulation at t time
n: Number of time points

The release kinetics of TZN from the formulations were analysed using the zero-order, first-order, Higuchi and Hixson-Crowell kinetic models [50] as well as Korsmeyer-Peppas semi-empirical model [47] using Equation (5).

$$M_t/M_\infty = k_{KP}t^n \quad \text{(Equation 5)}$$

M_t/M_∞: The fractional amount of drug release at time t.

k_{KP}: The release rate constant
n: The diffusional exponent

The value of n, which clarifies the type of drug release mechanism, and k_{KP} were calculated using a linear regression graph of log Mt/M∞ and log t. For the cylindric tablets, the value of n indicates non-Fickian release when it is between 0.45 and 0.89, the Fickian release mechanism when it is equal to 0.45, and zero-order release mechanism when it is 0.89 [47].

2.10. Permeability studies

The assays were conducted on bovine buccal tissue using a Franz diffusion cell (1.77 cm^2 of membrane area) in keeping with earlier research with some modifications [51,52]. The acceptor compartment (12 mL) was filled with phosphate buffer (pH 7.4). The buccal tissue was settled between the acceptor and the donor compartment, and the neck of the cell was clamped with a metal clip. After the donor compartment was wetted with one mL of the buffer (pH 6.8), the buccal tablet was posited on the mucosa with the bioadhesive layer facing the mucosa. The studies were conducted at a stirring rate of 300 rpm at 37 ± 0.5 °C. The samples (1 mL) were collected at predetermined times (30 min–24 h) and filtered using a PTFE membrane filter (0.45 μm). After each sample was taken, the acceptor compartment was completed with fresh phosphate buffer (pH 6.8). The samples were analysed by the HPLC method. The permeability studies were conducted in triplicate for each formulation. Permeability coefficient (K_p) was calculated using Equation (6). The slope of the linear portion of the curve was estimated as flux (J_{ss}). The x-intercept of the linear portion of the curve showing the cumulative amount of drug permeated versus time under steady-state circumstances was used to compute the lag time (h) [53].

$$K_P = J_{ss} / C_v \quad \text{(Equation 6)}$$

C_v: Amount of drug placed in the donor compartment.

K_p: Permeability coefficient

Moreover, the amount of TZN that accumulated in the mucosa was determined. To this end, the buccal tissue was cut into pieces with scissors and placed in a glass tube containing 20 mL of PBS (pH 6.8). The samples were mixed using a high-shear mixer (Silent Crusher M, Heidolph, Germany) at 15.000 rpm for 10 min, followed by vortexing after completion to 50 mL by PBS. They were then centrifuged at 4 °C and 14,000 rpm for 30 min using a cooling centrifuge (3–18 KS, Sigma, Germany). The supernatant was filtered through a membrane filter and one mL was transferred to a glass vial for analysis using HPLC.

2.11. Stability studies

The buccal tablets were placed in an amber bottle and tightly closed. The bottles were stored at 5 ± 2 °C, 25 ± 2 °C and 40 ± 2 °C for 6 months. The pH and the content uniformity values of the tablets were detected. Also, the physical appearances of the tablets were visualised. The studies were carried out three times for each formulation.

2.12. In vivo bioavailability studies

The *in vivo* studies were performed using female New Zealand rabbits (2.5–3 kg) housed at 60 ± 5 % of relative humidity and 22 ± 1 °C under a 12:12 light-dark (LD) cycle. All experiments were carried out according to European Community regulations on animal research and approved by Istanbul Medipol University Ethical Council (No: 78/2015).

Buccal tablets with a diameter of 8 mm and a weight of 30 mg (20 mg of adhesive layer and 10 mg of impermeable layer) were re-prepared by the direct compression method so that they were large enough not to induce discomfort in the rabbit's mouth. Also, the dose of TZN, which does not cause toxicity in rabbits, was determined to be 0.572 mg (a quarter of the human dose). Also, the commercial oral product (Sirdalud® tablet (2 mg), Novartis, Türkiye) was milled and the powder including a sufficient amount of TZN (0.572 mg) was suspended.

The rabbits were divided into three groups with 5 animals in each group (n = 5). The suspended commercial product was administered to the rabbits by gastric gavage (the first group). Before administering the buccal tablets, the rabbits were anaesthetised by intramuscular (im) injection of xylazine hydrochloride (5 mg kg^{-1}) and ketamine hydrochloride (35 mg kg^{-1}) (the second and the third groups). The buccal tablets were applied by lightly pressing to the buccal tissue of the anaesthetised rabbits. The anaesthesia was sustained with half of the initial dose of ketamine through im injection when necessary to prevent rabbits from swallowing buccal tablets.

The blood samples were taken to the tubes with EDTA at specified intervals of time (0.5–24 h) by placing a catheter in the ear's central artery. The tubes were centrifuged at 4000 rpm for 4 min. The supernatant was taken from the tube and frozen at −20 °C until the HPLC analysis. The samples were analysed by HPLC at 228 nm after being filtered by a 0.22 μm nylon membrane filter.

HPLC system including a gradient pump, a thermostable column department, and a UV detector, was procured by LC-2010C, Shimadzu, Japan. The studies were conducted using a C18 column (5 μm, 150 × 4.6 mm, Interstil, GL Sciences, Japan). The volume of injection and the flow rate were adjusted to 100 μL and 1 mL min^{-1}, respectively. Also, the detector temperature was adjusted to 30 °C. The mobile phase included acetonitrile and 0.1 M ammonium acetate mixture (v/v 15:85) [45]. The linearity of the HPLC method was evaluated between 2 and 200 ng mL^{-1} of TZN solutions. The method was fully validated according to the ICH guidelines.

2.13. Statistical analyses

The results were statistically assessed using the one-way analysis of variance (ANOVA). The data were presented as the mean ± SD. $p < 0.05$ was evaluated as statistically significant.

3. Results & discussion

The present study aimed to fabricate TZN-containing buccal mucoadhesive tablets using the low molecular weight of chitosan salts, which can degrade more readily compared to high molecular weight ones, to improve the systemic bioavailability of the drug by buccal route compared to its oral administration.

3.1. Characterisation of chitosan and chitosan salts

3.1.1. Fourier-transform infrared (FTIR) analysis

The formation of chitosan chloride salts (CC7, CC10) and chitosan glutamate salts (CG7, CG10) was confirmed by FTIR.

As seen in FTIR spectra of chitosan-hydrochloric acid salt (CC7, CC10) (Fig. 1), the broad and small peak among the characteristic absorption peaks of C7 and C10 with a max at 1577 cm^{-1} resulting from the bending vibration of the $–NH_2$ group in the chitosan molecule, appeared as a sharp peak at 1531 cm^{-1} due to the $–NH_3^+$ group formed by its quaternisation with HCl. In addition, the disappearance of the peaks resulting from the stretching vibration of the $–CH_2OH$ groups in the chitosan molecule in the 1300-1430 cm^{-1} region and the elongation of the peak at approx 1067 cm^{-1} resulting from the stretching vibration of the C–O–C bond indicates that intramolecular condensation occurs during quaternisation [54,55].

As seen in FTIR spectra of chitosan-glutamic acid salt (CG7, CG10) (Fig. 1), instead of the broad small peak resulting from the bending vibration of the $–NH_2$ group in the C7 and C10, with its max at 1577 cm^{-1}, the small sharp peak belonging to the N–H deformation and C–N stretching vibrations from the (–CONH) bond could be observed at 1552 cm^{-1}. The small peak of –NH stretching vibration in the formed amide structure at max 2931 cm^{-1} and the broad shoulders and large sharp peaks, which belong to the deformation of $–NH_3^+$ and $–COO^-$ groups resulting from glutamic acid (max at 2106 and 1400 cm^{-1}) could be observed. On the other hand, the absorption peak with its max at approx 1715 cm^{-1} due to the stretching vibration of the C═O bond of the –COOH groups in glutamic acid and the peaks resulting from the stretching vibration of the $–CH_2OH$ groups in C7 and C10 (1300-1430 cm^{-1} region) could not be observed. In addition, the peak elongation at approx max 1067 cm^{-1} resulting from the stretching vibration of the C–O–C bond, which indicates intramolecular condensation during the reaction [55,56].

3.1.2. Proton nuclear magnetic resonance (^1H NMR) analysis

Formations of CC7, CG7, CC10, and CG10 were confirmed by ^1H NMR spectral analysis (Fig. 2). The methyl (−NHCOC$\boldsymbol{H}_3$−) proton signals in C7 and C10 spectra were detected at 1.99 ppm. The H2–H2′ signals in the glucosamine unit were approx at 2.70 ppm, which corresponds to a range of 2.50–3.10 ppm. Many signals within the range of 3.6–4.15 ppm correspond to the hydrogens of H3–H6. The signals observed at 4.76 and 5.11 ppm were attributed to H1–H1′ hydrogen atoms within the glucosamine unit. An intense broad singlet at 8.38 ppm was identified and ascribed to an uncharged group with a single bond NH_2/NH on the backbone of C7 and C10. Furthermore, N-deacetylation degrees of C7 and C10 were calculated using ^1H NMR technique (C7-D_{deac}: 80 %, C10-D_{deac}: 80.4 %) [57].

The ^1H NMR spectrums of CC7, CG7, CC10, and CG10 are shown in Fig. 2. The identified intense broad signal at 8.38 ppm, which belongs to an uncharged group with a single bond NH2/NH on the backbone of pure chitosans (C7 and C10), was not detected in the spectrum of any of the chitosan salts. New signals from CG7 and CG10 salts appeared at 2.50 ppm and 2.58 ppm assigned to $OCCH_2–CH_2$ groups and at 2.21 ppm and 2.30 ppm assigned to $OCCH_2–CH_2–CH$ groups after glutamate modification [58]. All spectral changes indicate that the expected modifications of chitosan with chloride and glutamate occurred. Furthermore, the deacetylation degree values of the chitosan salts were almost 100 % as evidenced by the absence of the peak at 1.99 ppm, which corresponds to the $–NHCOCH_3$- group in the chitosan structure. The FTIR and ^1H NMR results demonstrated the successful completion of all modification reactions on chitosan.

3.1.3. Powder X-ray diffraction (XRD) analysis

XRD patterns of chitosans (C7 and C10) as well as their chloride (CC7, CC10) and glutamate (CG7, CG10) salts were evaluated comperatively. Powder XRD patterns are presented in Fig. 3. Crystallinity degree (Deg_{Cr}) and index value (Cr_{In}) results, which were calculated with Equations (7) and (8), are given in Table 2.

$$Deg_{Cr} = \int I_{Cr} d\theta \Big/ \int I_o d\theta = F_{Cr} \Big/ (F_{Cr} + F_{am}) \quad \text{(Equation 7)}$$

where I_o represents the corrected diffractogram's total intensity following the subtraction of the parasitic background; I_{cr} is the intensity of the crystalline scattering; F_{cr} is the area of the crystalline scattering; F_{am} is the area of the amorphous scattering.

$$Cr_{In} = (I_O - I_{am}) / I_O \quad \text{(Equation 8)}$$

where I_o is the height of the (020)-peak and I_{am} is the height of amorphous scattering at $2\theta = 16°$ [59].

The XRD pattern exhibits prominent, well-defined, and symmetrical diffraction peaks at low 2θ angles. As seen in the XRD pattern of the C7

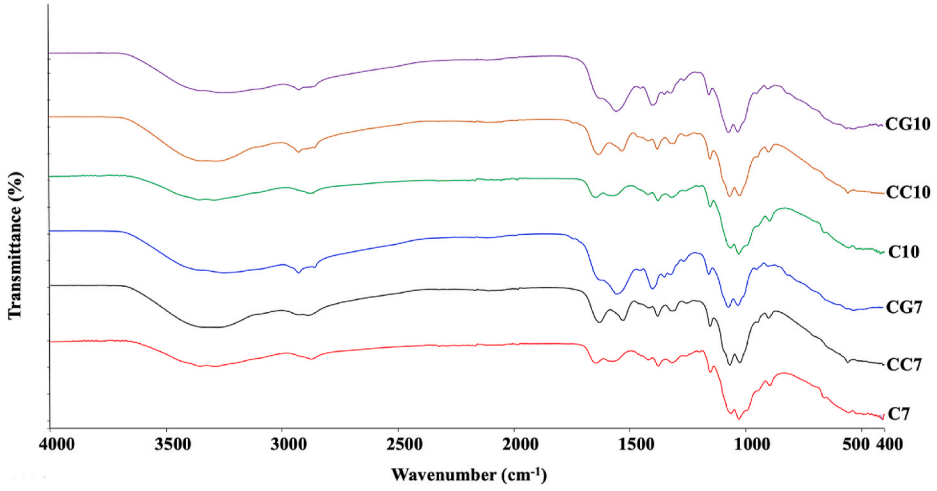

Fig. 1. FTIR spectra of pure chitosan polymers (C7 and C10) and chitosan salts (CC7, CC10, CG7 and CG10).

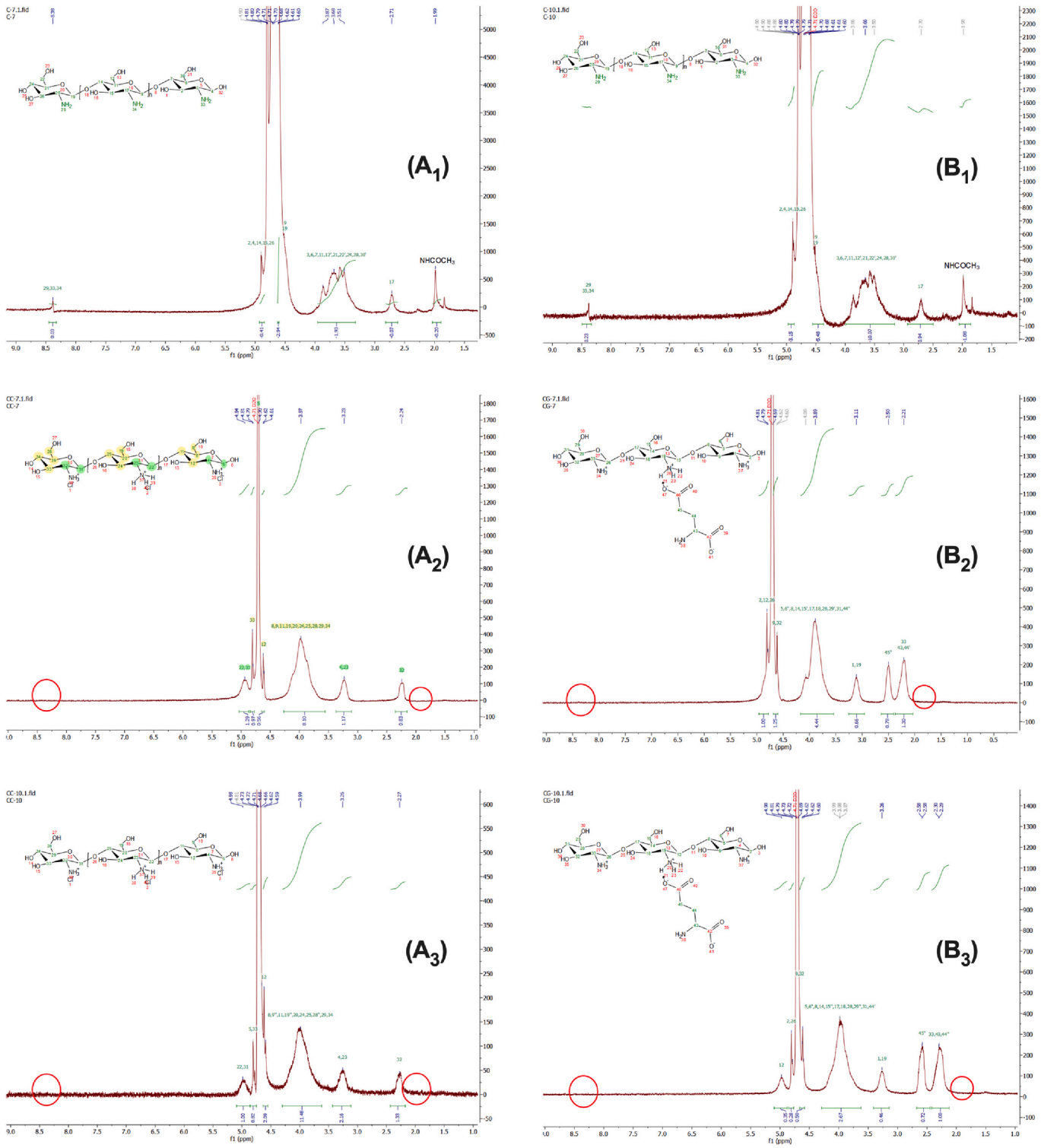

Fig. 2. ^{1}H NMR spectra of pure chitosan polymers (C7 (A_1) and C10 (B_1)) and chitosan salts (CC7 (A_2), CC10 (A_3), CG7 (B_2), and CG10 (B_3)).

and C10 samples (Fig. 3), the main weak peak near $2\theta = 9.94°$, corresponding to d = 0.8845 nm, and the main strong peak near $2\theta = 19.95°$, corresponding to d = 0.4457 nm, were attributed to diffraction from the (010)&(100) and (020) crystalline lattice planes, respectively. As seen in the CC7, CG7, CC10, and CG10 XRD diffractions, it was clearly seen that after the modification reactions, the weak peak corresponding to (010)&(100) diffraction disappeared, while the intensity of the strong peak corresponding to (020) diffraction decreased. Similarly, the crystallinity degree and crystallinity index results in Table 2 were lower in modified products. The reason for this is due to the increase in amorphous structures on the chitosan molecule as a result of the modification made with hydrochloric acid and glutamic acid [59].

3.1.4. *Differential scanning calorimetry (DSC) analysis*

The DSC thermograms of chitosans (C7, C10) and their salts are shown in Fig. 4. C7 and C10 showed Tg temperatures at 56.17 °C and 73.66 °C, respectively. The Tg temperatures of CG7 and CG10, which are glutamic acid salts of chitosan, decreased to 45 °C due to a reduction in

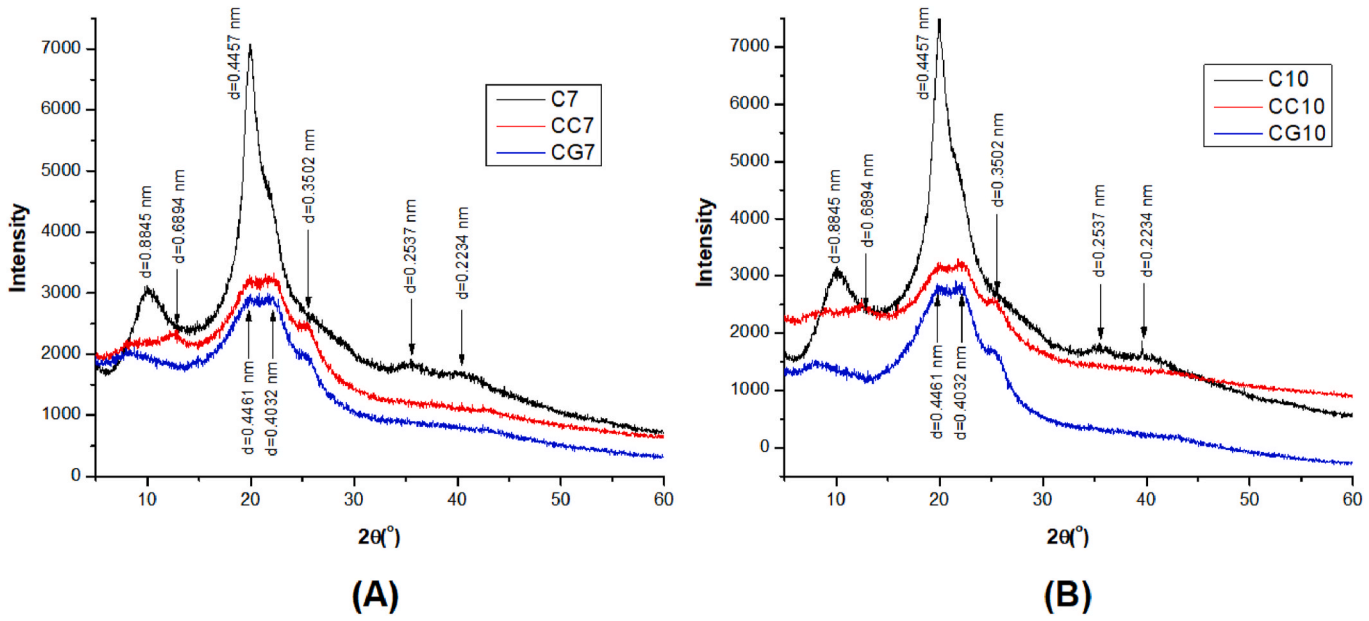

Fig. 3. Powder XRD Patterns of (A) C7 and its salts (CC7 and CG7); (B) C10 and its salts (CC10 and CG10).

Table 2
Deg_{Cr} and Cr_{In} of pure chitosan polymers (C7 and C10) and chitosan salts (CC7, CC10, CG7 and CG10).

	C7	CC7	CG7	C10	CC10	CG10
Deg_{Cr}	0.882154	0.803176	0.796611	0.848162	0.836109	0.818161
Cr_{In}	0.634074	0.318773	0.305927	0.646689	0.260481	0.462734

crystallinity. Furthermore, water loss was observed at temperatures of 99.88 °C and 109.09 °C in C7 and C10, respectively. In CC7 and CC10, these temperatures were higher, at 115 °C and 125 °C, respectively. However, CG7 and CG10 exhibited a sharp endothermic peak due to their amorphous nature at 153.82 °C and 166.93 °C, respectively.

3.1.5. Thermogravimetric analysis (TGA)

The TGA-DTG curves of chitosans (C7, C10) and their salts (CC7, CC10, CG7, CG10) are presented in Fig. 5. The thermograms and thermal transitions of chitosans and their salts were similar among themselves; that is, the thermal properties between C7 and C10, CC7 and CC10, and CG7 and CG10 were almost the same. Analysis of these curves revealed the existence of four different degradation stages for C7 and C10. In the temperature range of approx 25–130 °C, corresponding to the initial phase, a visible decrease in weight of approx 5 % was observed as a result of the evaporation of physically adsorbed water molecules in these polymers. The second stage occurred at about 130–300 °C, with a weight loss of about 15 %. The third stage occurred approx 300–415 °C with a weight loss of approx 55 %. CC7 and CC10 polymers showed almost the same thermal properties as C7 and C10 polymers. While the initial stage, which was 5 % weight loss temperature in CC7, occurred between 25 °C and 130 °C, this part occurred as only 2.5 % weight loss in CC10. In the second stage, at approx 130–238 °C, there was a weight loss of approx 10 % for CC7, while this rate was only 2.5 % for CC10. The third stage occurred approx at 238–320 °C with a weight loss of approx 40 %. In CG7 and CG10 polymers, unlike chitosan chloride salts, the degradation process took place in 5 stages. While the initial stage, which was a 5 % weight loss temperature in CG7 and CG10, occurred between 25 °C and 110 °C, the second stage occurred at about 110–190 °C, with a weight loss of about 10 %. The third stage occurred at approx 190–300 °C with a weight loss of approx 40 %. The fourth stage occurred at approx 300–450 °C with a weight loss of approx 50 %. As a result, the organic mass decomposed primarily in the second and third stages (for CG7 and CG10, it also occurred in the fourth stage). After completing these stages at approx 600 °C, the final degradation stage was detected. The findings were compatible with the previous studies [35,56,60–62].

3.2. Fabrication and characterisation of buccal tablets

The buccal tablet formulations were designed with a double layer to ensure the unidirectional drug release towards the buccal mucosa. The buccal bilayer tablets were prepared by pre-compressing the first layer of the bioadhesive polymer and active agent, followed by a final compression after adding ethyl cellulose, a hydrophobic polymer, to one side of the first layer (Fig. 6). In literature, ethyl cellulose has been used as an impermeable backing layer polymer in the preparation of buccal bioadhesive formulations [17,42,63,64]. The tablet formulations were designed in a diameter of 8 mm, a thickness of 2 mm, and a weight of 120 mg to avoid any discomfort in the mouth.

Bilayer tablets were assessed for quality attributes like weight uniformity, diameter, and friability to determine the compatibleness of the formulations for buccal delivery. The results are given in Table 3. The weight of the buccal tablets was determined to be between 119 and 120 mg, with a diameter of 7.99–8.00 mm and a thickness of 2.01–2.29 mm, respectively. The tablets prepared with CG polymers were slightly thicker than those prepared with CC ($p < 0.001$). The pH of tablet formulations administered buccally should not cause any irritation in the mouth [65]. The pH values of the buccal tablets ranged from 6.068 to 6.428, indicating their suitability for buccal administration.

The buccal tablet should have a hardness of at least 30 N to prevent it from breaking apart during administration [66]. The hardness values ranged from 75.56 N to 85.44 N. In the hardness experiments, only the impermeable layers of the buccal tablets were broken, while the bioadhesive layer containing the chitosan salt remained intact and did not crumble. This is because the bioadhesive layer became like paper

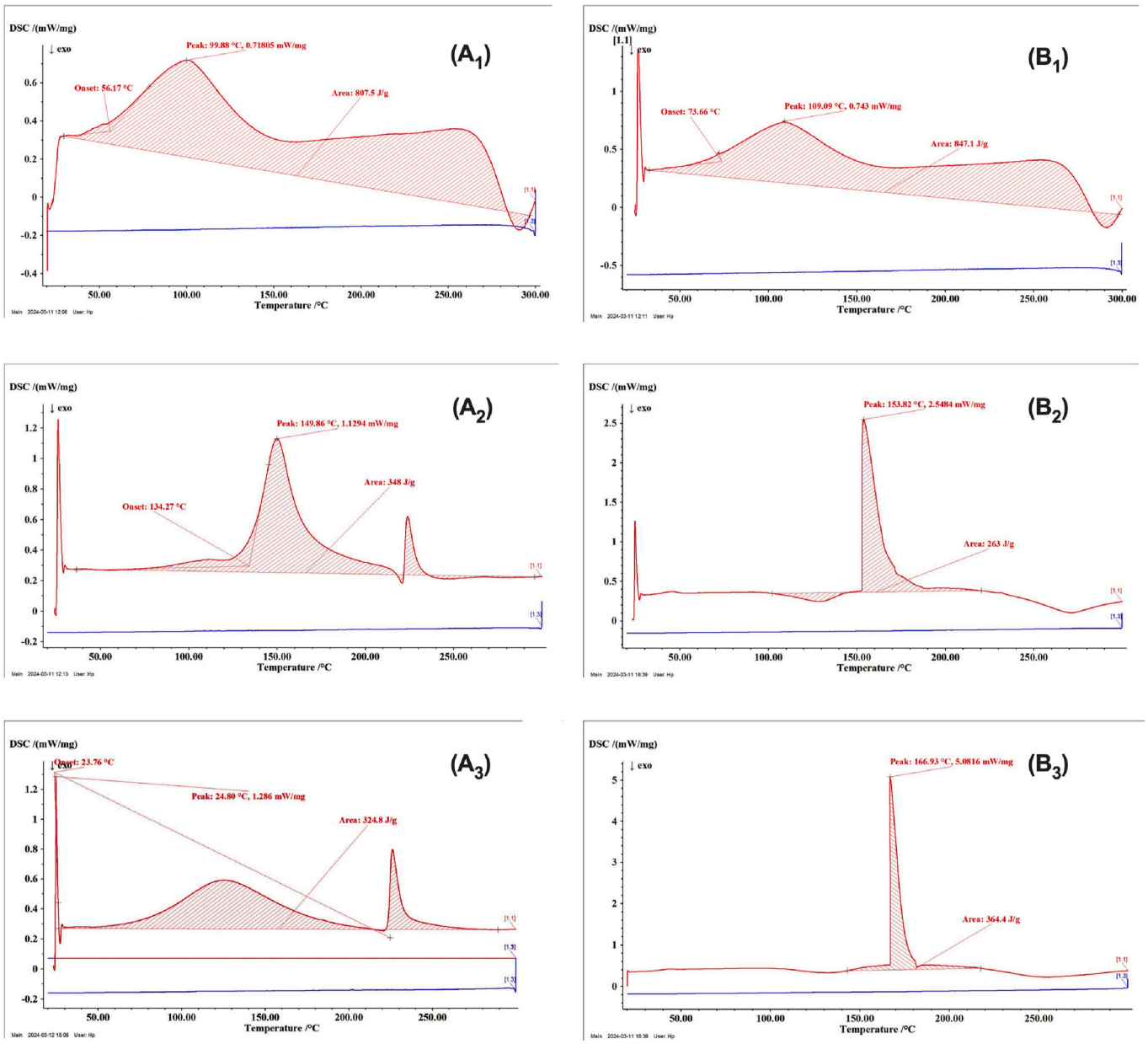

Fig. 4. DSC Analysis of pure chitosan polymers (C7 (A_1) and C10 (B_1)) and chitosan salts (CC7 (A_2), CC10 (A_3), CG7 (B_2), and CG10 (B_3)).

cardboard after the tablet compression. The results indicated that the hardness of the tablet formulations was appropriate.

In addition, the maximum mean weight loss from 20 tablets for each formulation did not exceed 1 % during the friability test of all the buccal tablets. The weight loss values for the tested formulations ranged between 0.13 % and 0.80 %, which is considered acceptable according to pharmacopoeial specifications [44]. The results of content uniformity varied between 79.29 % and 101.09 %.

Hydration of a mucoadhesive polymer is essential for the relaxation and interpenetration of the polymer chains [67]. Buccal formulations are expected to demonstrate good swelling abilities, indicating strong mucoadhesive properties upon application. Fig. 7 shows that the percentage of swelling ranged from 271.71 % to 496.85 % at the 6th h. TC10 and TG7 had the highest and lowest swelling percentages, respectively. However, all formulations demonstrated significant swelling properties. The results indicated a statistically significant difference in the swelling percentages of the tablets ($p < 0.01$).

Chitosan derivatives, which are the second most abundant polysaccharide after cellulose [68], have been used to develop various bioadhesive tablet formulations due to their excellent swelling properties [69–71]. According to our findings, tablets prepared with chitosan salts of higher molecular weight (169 kDa) exhibited greater swelling than those made with 136 kDa of chitosan salts ($p < 0.01$). Moreover, tablets formulated with CC demonstrated a greater swelling index in comparison to tablets prepared with CG of the same molecular weight ($p < 0.01$). This may have been caused by the fact that the chitosan salts contain different chemical groups.

Huanbutta et al. [27] stated that the swelling degree of chitosan salt was directly affected by its molecular weight and the type of salt used. The matrix tablets prepared with chitosan glycolate and CG exhibited the highest and lowest swelling values, respectively. The presence of free carboxylic acid and amine groups in the chemical structure of glutamic acid can cause interactions and attractive forces between polymer chains, resulting in a reduction in polymer swelling. Our study observed that the higher swelling of CC tablets may be attributed to the absence of functional acid groups inducing interactions of polymer chains in CC

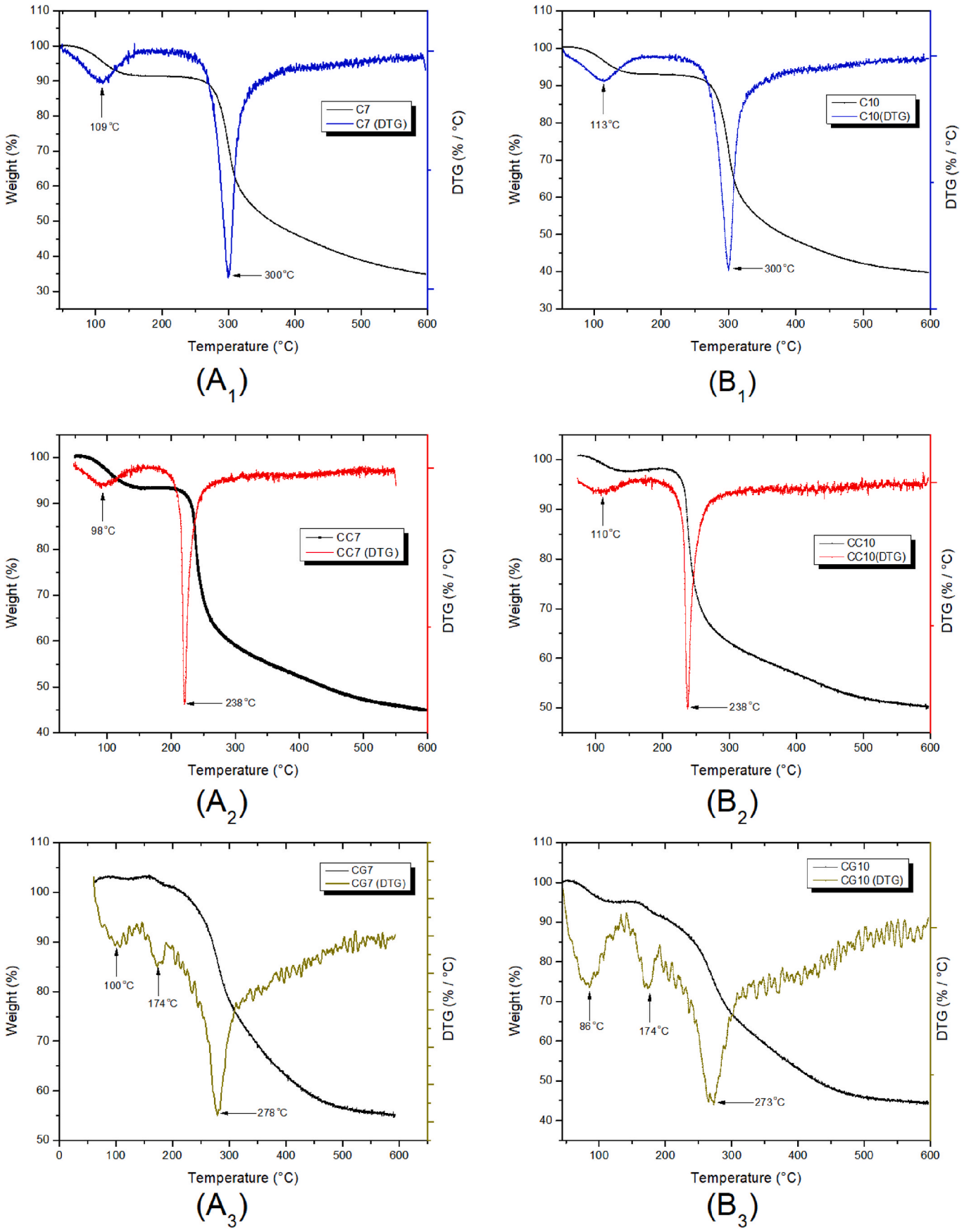

Fig. 5. TGA-DTG curves of pure chitosan polymers (C7 (A_1) and C10 (B_1)) and chitosan salts (CC7 (A_2), CC10 (A_3), CG7 (B_2), and CG10 (B_3)).

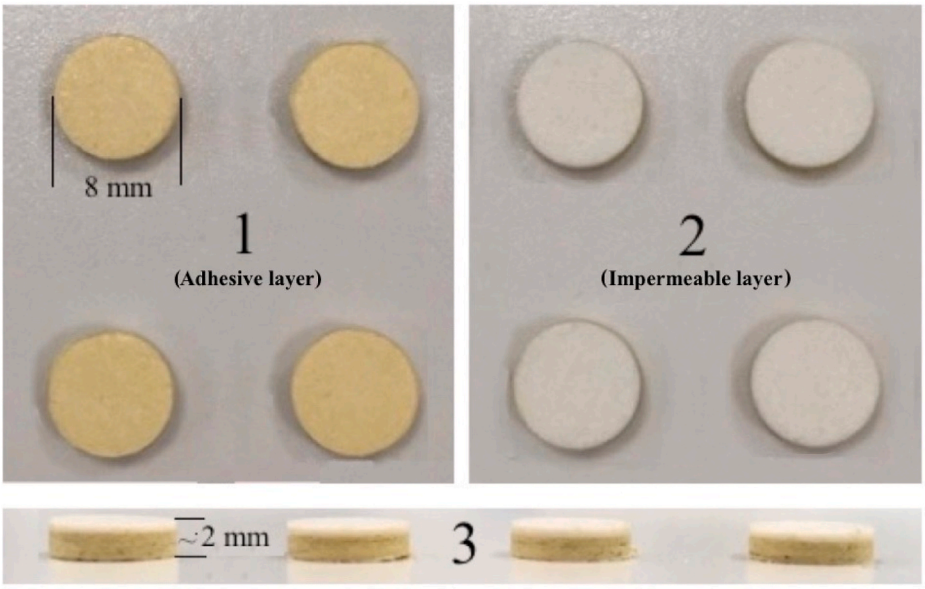

Fig. 6. The images of buccal bilayer tablet formulations containing TZN. *1:* The image of the adhesive layer of the tablet; *2:* The image of the impermeable (backing) layer; *3:* The side view of the tablet.

Table 3
The characterisation of buccal bilayer tablets.

Formulations	Weight uniformity (mg ± SD)	Diameter (mm ± SD)	Thickness (mm ± SD)	pH (±SD)	Friability (%)	Hardness (N ± SD)	Drug content uniformity (% ± SD)
TC7	119.5 ± 1.0	7.99 ± 0.02	2.01 ± 0.01	6.192 ± 0.021	0.13	80.13 ± 4.38	79.29 ± 4.13
TC10	119.5 ± 1.1	7.99 ± 0.01	2.01 ± 0.01	6.148 ± 0.023	0.25	84.41 ± 6.02	88.80 ± 1.13
TG7	119.0 ± 0.8	8.00 ± 0.01	2.29 ± 0.01	6.068 ± 0.019	0.80	81.07 ± 5.59	89.18 ± 1.92
TG10	120.0 ± 1.0	8.00 ± 0.01	2.28 ± 0.01	6.428 ± 0.015	0.59	85.44 ± 4.92	101.09 ± 3.77

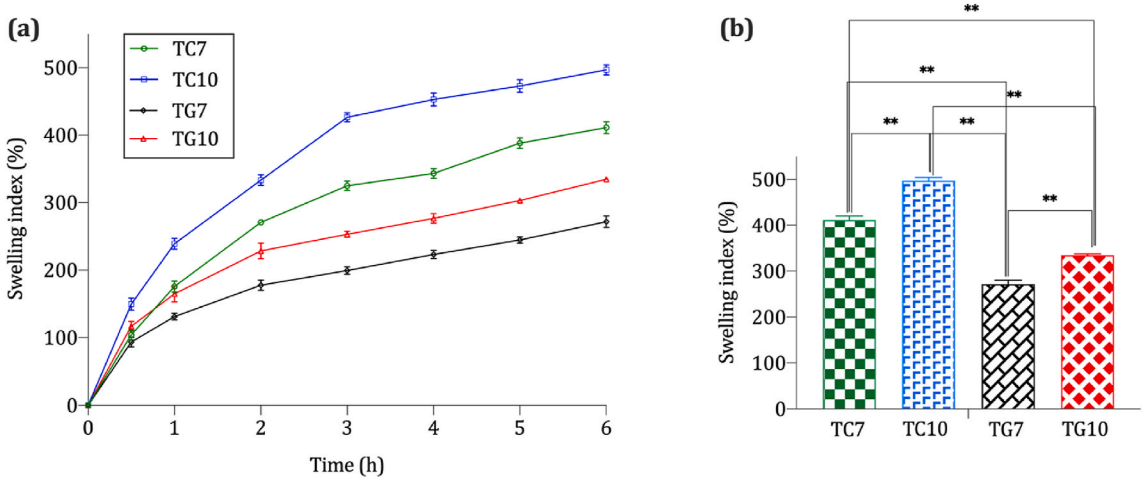

Fig. 7. The results of the swelling studies performed for 6 h ($n = 3$). *(a)* The change in the swelling index (%) of the tablets over time, *(b)* The comparison of the cumulative swelling index (%) of the tablets at 6th h (**$P < 0.01$).

structure relative to CG. Likewise, in another study, Tangsadthakun et al. [72] observed that the swelling of polymer-based scaffolds increased with an increase in the molecular weight of chitosan. The findings obtained in our study are in line with the existing literature.

3.3. Mucoadhesion studies

The ability of the polymer to adhere to and interact with the mucosa is known as mucoadhesiveness [5]. The bioavailability of the drugs is directly influenced by the ability of the formulations to adhere to mucosal surfaces. Thus, drug delivery systems applied to mucosal

surfaces should possess satisfactory bioadhesive properties to remain at the application site for a sufficient time.

The bioadhesive characteristics of the buccal tablets were evaluated on the bovine buccal tissue using the texture analyser in the present study. The mucoadhesive strength of the tablets ranged from 2.341 to 3.516 N cm^{-2}, while the work of adhesion values varied from 2.801 to 7.526 mJ cm^{-2} (Fig. 8). Formulations prepared with CG10 or CC10 polymers exhibited significantly higher mucoadhesiveness compared to those formulated with the same polymers of 7 cP ($p < 0.01$). Mucoadhesion is a complex process involving physical and chemical interactions between the mucosa and the polymer and can be explained by various theories including wetting, adsorption, electronic interaction, and diffusion [73]. The mucoadhesive ability of positively charged chitosan is primarily based on the electrostatic interaction between its primary amine groups and the sialic acids located at the terminal ends of mucin glycoproteins [74]. The amine groups present in the chitosan structure form hydrogen bonds with the carboxyl groups of sialic acid [75]. Furthermore, the linear structure of chitosan allows for physical entanglement with mucous due to its chain flexibility [76]. The interaction between the mucin and the polymer, as well as the mucoadhesion ability, are directly affected by the molecular weight of chitosan [77]. The increase in molecular weight of the polymer enables greater swelling, resulting in stronger physical entanglement through hydrogen and van der Waals bonding between mucin glycoproteins and polymer molecules, leading to higher mucoadhesion [78,79]. Bravo-Osuna et al. [80] synthesised thiolated chitosan-poly(isobutyl cyanoacrylate) based nanoparticles using low molecular weight chitosan polymers (20 and 100 kDa) for nasal application. They stated that the derivatives prepared with higher molecular weight chitosan improved mucoadhesion as it increased the interpenetrating ability of the mucus chain during the attachment process. In another study, Honary et al. [81] demonstrated that the mucoadhesion of prednisolone-loaded formulations increased significantly with an increase in the molecular weight of chitosan. Huanbutta et al. [27] found that the swelling properties of matrix tablets prepared with chitosan salts increased as the molecular weight of chitosan increased (45–200 kDa). Our findings were in line with the literature.

In this work, as seen in Fig. 8, when compared to the formulations prepared with the same molecular weight chitosan salts, the mucoadhesiveness of tablets fabricated with CG polymer was higher than those of CC-based tablets ($p < 0.01$). This may be due to the ability of free amine groups on glutamic acid structure, which enhances the positive charge density of the chitosan salt, to form hydrogen bonds with negatively charged glycoproteins, thereby enhancing mucoadhesion [82]. Also, the larger molecular structure of CG provided greater chain flexibility and entanglement, resulting in increased mucoadhesion. It was reported that chain flexibility has a positive effect on adhesion [83,84]. Karava et al. [85] synthesised two different derivatives of chitosan (chitosan-2-acrylamido-2-methyl-1-propanesulfonic acid (chitosan-AAMPS) and chitosan-[2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (chitosan-MEDPS). The mucoadhesion was found to be associated with the electronic interaction of the new functional groups that were attached to chitosan. Chitosan-AAMPS exhibited a statistically higher swelling index than chitosan-MEDPS but lower mucoadhesive ability. On the other hand, chitosan-MEDPS, which has a lower swelling ability, exhibited stronger mucoadhesive strength due to more ionic interactions between tertiary amine and SO_3^- groups on the structure of MEDPS's monomer and mucus glycoproteins. Larger molecular structure of chitosan-MEDPS also contributed to a higher interpenetration ability and physical entanglements, resulting in higher mucoadhesion. The thioethylamidine conjugate of chitosan synthesised by Kafedjiiski et al. [86] demonstrated enhanced mucoadhesive capability while exhibiting no significant change in swelling characteristics. These results suggest that the thiol groups in the structure of the chitosan derivative enhanced adhesion. As a result, our findings are consistent with previous reports.

3.4. In vitro drug release studies

The release studies of buccal tablets were maintained for 8 h as their bioadhesive layers were almost completely disintegrated in the release medium by the end of this period. It was observed that CC-based tablets started to disintegrate more rapidly than CG-based tablets due to the swelling behaviour of the polymer salts. As in the swelling studies (Fig. 7), TC7 and TC10 formulations absorbed more water in the dissolution medium and swelled more than TG7 and TG10, causing the tablets to disintegrate faster. The results of drug release studies demonstrated (Fig. 9) that the cumulative release from TC10 formulation at 4th h was statistically significant compared with those from other formulations ($p < 0.05$).

To reveal the similarity between the release profiles of the formulations, the similarity factor (f_2) between the formulations was calculated (Table 4). The release profiles of the tablets prepared with the same molecular weight chitosan were not similar ($f_2 < 50$). Although the release rate enhanced with increasing molecular weight of chitosan, it was observed that the release profile of tablets prepared with the same type of polymer was similar ($f_2 > 50$), and the difference in the drug release profiles occurred due to the type of chitosan salts. The researchers found that the caffeine release profiles from the matrix tablets fabricated with different chitosan salts, chitosan lactate and CG, were not similar [87]. In another study, Cerchiara et al. [39], stated that the release profiles of chitosan-based formulations containing vancomycin

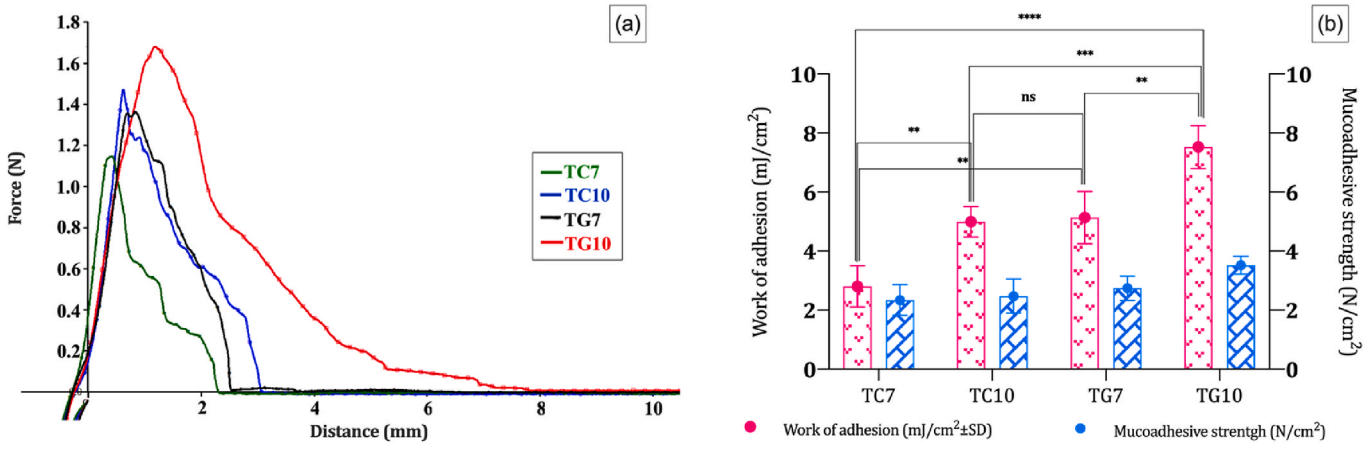

Fig. 8. Mucoadhesive characterisation of the buccal bilayer tablets ($n = 4$). *(a)* Mucoadhesion graphs of buccal tablets from the texture analyser, *(b)* Comparison of the findings obtained in the mucoadhesion study (ns: not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

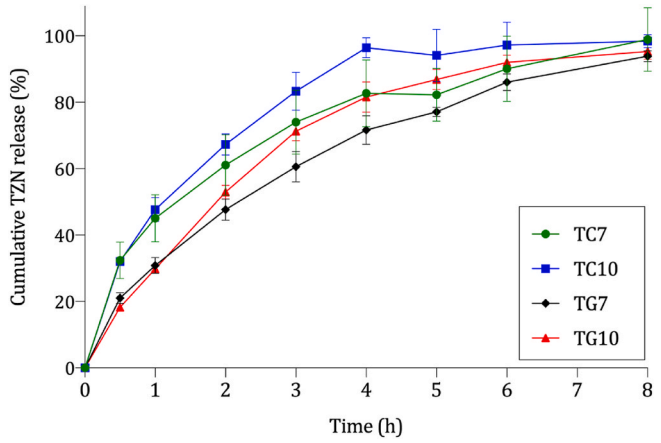

Fig. 9. TZN release profiles from the buccal bilayer tablets ($n = 5$).

Table 4
The similarity factor (f_2) of buccal bilayer tablets.

Formulations	f_2	Similarity
TC7 – TC10	53.23	Similar
TC7 – TG7	48.81	Dissimilar
TC7 – TG10	52.52	Similar
TC10 – TG7	36.77	Dissimilar
TC10 – TG10	44.34	Dissimilar
TG7 – TG10	56.30	Similar

varied depending on the chitosan salt used. It was observed that the swelling ability and viscosity of the chitosan salts affected the release rate, and the chitosan lactate, which had the highest swelling ability, achieved the highest release percentage.

In our work, as in the studies mentioned, the type of chitosan salt had a direct effect on the release profile of TZN from the tablets. In addition, the fact that CC-based tablets were thinner ($p < 0.001$) may also have an effect on these results.

Increasing the thickness of the matrix tablets fabricated with water-swellable polymers can lead to a delay in drug release due to an increase in the swollen gel layer that controls the diffusion of the drug during dissolution studies [88].

The fact that CC-based tablets had a faster release profile compared to CG-based tablets due to their thinner structure, faster and higher swelling ability, and faster erosion and disintegration properties, indicates that our results are in line with the literature.

The release profiles of the chitosan tablets were kinetically evaluated using the Zero order, First order, Higuchi, and Hixson-Crowell kinetic models. The Higuchi kinetic model was found to have the highest r^2 value for all formulations, as shown in Table 5. In line with the previous studies [89–91], the high correlation obtained by Higuchi's model indicates that the release of the drug from the swollen matrix tablet is controlled by diffusion. However, Korsmeyer-Peppas model was applied to investigate the release kinetics of the tablet formulations. The values of r^2 and n were calculated using the release data. The n values ranged from 0.458 to 0.770 (Table 5), indicating that the drug release conformed to the non-Fickian release kinetics and was time-dependent. Moreover, the drug release from the formulations was controlled by swelling, diffusion, and erosion mechanisms [47].

Al-Ani et al. [92] formulated mucoadhesive buccal tablets containing chlorhexidine and found that drug release was controlled by relaxation and swelling or erosion of the matrix tablet. In a separate study, Çelik [93] demonstrated that the release mechanisms of risperidone buccal mucoadhesive tablets were diffusion through polymer matrices and erosion of the polymer chains. Vijayaraghavan et al. [94] noted comparable release characteristics in buccal tablets containing propranolol that were based on chitosan and locust bean gum. Our findings were in accordance with the aforementioned studies.

3.5. Permeability studies

Although TC7 formulation showed a release profile similar to that of TC10 and TG10, it released TZN faster than TG7. However, it was not selected for further studies due to its poor bioadhesive properties compared to all the other formulations. The permeability study results are shown in Table 6. While the formulation with the highest amount of drug permeated (K_{24}: Total amount of TZN passing per unit area in 24 h) was TC10 (112.22 ± 5.03 μg cm^{-2}), the TG10 formulation showed the lowest permeability (100.22 ± 15.05 μg cm^{-2}) at the end of 24 h. Also, the amount of TZN accumulated in the buccal mucosa ranged from 7.97 % to 14.84 %. Although TG7 and TG10 provided higher TZN accumulation than TC10, there was no significant difference between the percent of drug permeated (%, $t_{0\text{-}24}$) of all formulations. In addition, the difference between the flux values of TC10, TG7, and TG10 was also not significant ($p > 0.05$). The penetration abilities of the formulations were similar when evaluated in terms of the total amount of TZN accumulated and passed through the mucosa. The lag time of TC10, which had the fastest release profile and the highest swelling properties, was lower than the others ($p < 0.01$). The lag time indicates that the drug has started to accumulate in the mucosa and has not yet reached an amount that can diffuse into the acceptor compartment. The low lag time of TC10 was an important advantage, which could mean that the drug could pass through the mucosa and reach the acceptor compartment more quickly.

TC10 formulation disintegrated slightly at the end of the 8th h and almost completely at the end of the 24 h due to its greater swelling. On the contrary, TG7 and TG10 retained their integrity over 24 h and, despite the swelling of the core tablet, the integrity of the ethyl cellulose barrier layer was not altered and did not separate from the adhesive layer (Fig. 10). This indicates that CG-based tablets can remain in the buccal cavity for a prolonged period of time without disintegration, despite continuous salivary secretion.

Shanker et al. [36] prepared TZN-containing buccal tablets with polymers of cellulose derivatives and sodium deoxylactate salt as a penetration enhancer. *Ex vivo* permeability studies performed using porcine buccal tissue showed that the percent of drug permeated (%) ranged from 7.47 % to 62.73 % after 6 h. The high level of drug permeability from the mucosa was explained by the use of sodium deoxylactate salt, which increases intercellular and intracellular transitions. In the present study, the reason for the lower permeability values than in the aforementioned study could be that the bovine buccal mucosa is keratinised compared to the porcine buccal tissue [51,95].

TG10 was judged to be the most suitable formulation for *in vivo*

Table 5
The release parameters that characterise the mechanism of TZN release from the mucoadhesive tablet formulations.

Formulation	Zero order (r^2)	First order (r^2)	Higuchi (r^2)	Hixson-Crowell (r^2)	Korsmeyer-Peppas k_{KP}	n	(r^2)
TC7	0.8919	0.7934	0.9712	0.8292	1.6500	0.9995	0.458
TC10	0.9811	0.9171	0.9997	0.9433	1.6705	0.9984	0.535
TG7	0.9366	0.8221	0.9921	0.8671	1.4969	0.9993	0.595
TG10	0.8492	0.7261	0.9483	0.7719	1.4858	0.9980	0.770

Table 6
Ex vivo permeability parameters of TZN-containing buccal bilayer tablets ($n = 3$).

Formulations	Flux (J_{ss}) (μg $cm^{-2}h^{-1}$±SD)	K_8 (μg cm^{-2}±SD)	K_{24} (μg cm^{-2}±SD)	K_P * 10^{-2} (cm h^{-1})	lag time (h±SD)	r^2 (±SD)	Drug permeated from buccal tissue (t_{0-8}) (%±SD)	Drug permeated from buccal tissue (t_{0-24}) (%±SD)	Drug accumulated in buccal tissue (% ±SD)
TC10	7.959 ± 0.805	49.29 ± 6.85	112.22 ± 5.03	6.279	2.478 ± 0.263	0.998 ± 0.001	6.11 ± 0.64	14.40 ± 0.30	7.97 ± 2.06
TG7	4.333 ± 1.075	25.26 ± 6.72	109.47 ± 12.90	5.703	2.923 ± 0.176	0.985 ± 0.001	2.74 ± 0.85	13.40 ± 1.17	14.84 ± 1.33
TG10	4.348 ± 1.091	23.53 ± 5.81	100.22 ± 15.05	6.539	3.172 ± 0.028	0.983 ± 0.005	3.14 ± 1.06	13.93 ± 3.28	10.16 ± 2.41

K_8: Total amount of TZN passing through per unit area in 8 h. K_{24}: Total amount of TZN passing through per unit area in 24 h. K_P: Permeability coefficient.

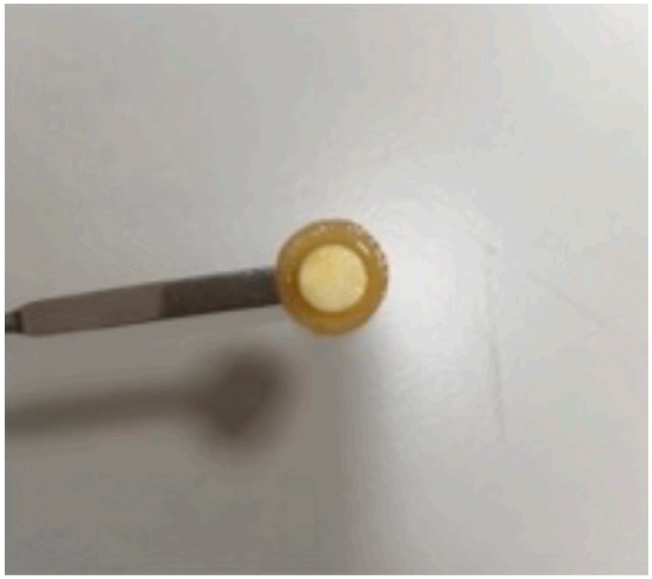

Fig. 10. The image of TG10 buccal bilayer tablet after 24 h *ex vivo* permeability studies.

studies as it was able to maintain physical integrity in the buccal medium for a longer period of time according to swelling, *in vitro* release, and *ex vivo* permeability findings and had the highest mucoadhesive characteristics. TC10 was also selected as a second formulation for *in vivo* studies due to its higher swelling, and faster release and permeability properties than TG7 formulation.

3.6. Stability studies

The results of the stability studies of TC10 and TG10 formulations selected for *in vivo* studies are shown in Table 7. No changes in the physical appearance of the buccal tablet formulations were observed during the 6-month stability studies conducted in the refrigerator (5 °C), at room temperature (25 ± 2 °C, 60 % RH), and under accelerated conditions (40 ± 2 °C, 75 % RH). No significant difference in pH and drug content was observed under the same conditions ($p > 0.05$).

3.7. In vivo bioavailability studies

In vivo studies were conducted in New Zealand rabbits using TC10 and TG10 buccal bilayer tablets as well as the commercial product (Cp). Three pharmacokinetic parameters recognised by the international drug regulatory authority, the maximum drug concentration in the blood after administration (C_{max}), the time the drug reaches the maximum concentration in the blood (T_{max}), and the drug bioavailability as a demonstration of total drug exposure across time (AUC: the area under the blood plasma drug concentration and time curve) were determined

Table 7
The results of stability studies for six months of TC10 and TG10 buccal bilayer tablets ($n = 3$).

Temperature	Time (months)	TC10		TG10	
		Content uniformity (%±SD)	pH (±SD)	Content uniformity (%±SD)	pH (±SD)
5 °C	**0.**	90.60 ± 0.16	6.136 ± 0.025	100.24 ± 2.81	6.428 ± 0.015
	1.	86.22 ± 3.61	6.216 ± 0.023	99.02 ± 1.50	6.456 ± 0.027
	3.	86.72 ± 2.45	6.224 ± 0.035	99.34 ± 0.86	6.470 ± 0.032
	6.	87.76 ± 2.17	6.237 ± 0.028	99.16 ± 1.26	6.494 ± 0.029
25 °C	**0.**	90.95 ± 1.26	6.130 ± 0.019	100.45 ± 2.07	6.438 ± 0.015
	1.	90.39 ± 2.00	6.196 ± 0.016	99.48 ± 4.04	6.450 ± 0.033
	3.	89.43 ± 1.74	6.242 ± 0.035	99.54 ± 2.70	6.458 ± 0.021
	6.	89.30 ± 1.90	6.279 ± 0.029	99.38 ± 2.94	6.481 ± 0.019
40 °C	**0.**	90.25 ± 0.39	6.134 ± 0.016	99.47 ± 2.07	6.436 ± 0.070
	1.	88.62 ± 2.99	6.198 ± 0.012	98.56 ± 0.92	6.438 ± 0.024
	3.	86.36 ± 2.73	6.218 ± 0.025	100.19 ± 2.49	6.444 ± 0.019
	6.	87.13 ± 2.17	6.240 ± 0.021	99.26 ± 2.12	6.460 ± 0.031

in the *in vivo* studies [96].

As expected, TG10 was observed to adhere more easily and significantly better to the buccal tissue of the rabbit compared to TC10 due to its high bioadhesiveness. The buccal tablets began to degrade slightly in the buccal tissue between the 6th and 8th h. Buccal formulations maintained their integrity for longer in *in vivo* studies compared to *in vitro* drug release studies. This was due to slower swelling caused by less salivary secretion and motility under *in vivo* conditions. It was observed that TC10, which has a higher water absorption capacity, swelled more than TG10 in the rabbit buccal tissue. Fig. 11a demonstrates that the C_{max} of TC10 and TG10 buccal tablets was 158.87 ± 15.43 ng mL^{-1} and 132.60 ± 37.99 ng mL^{-1}, respectively, while the C_{max} of orally administered Cp was 36.36 ± 1.54 ng mL^{-1}.

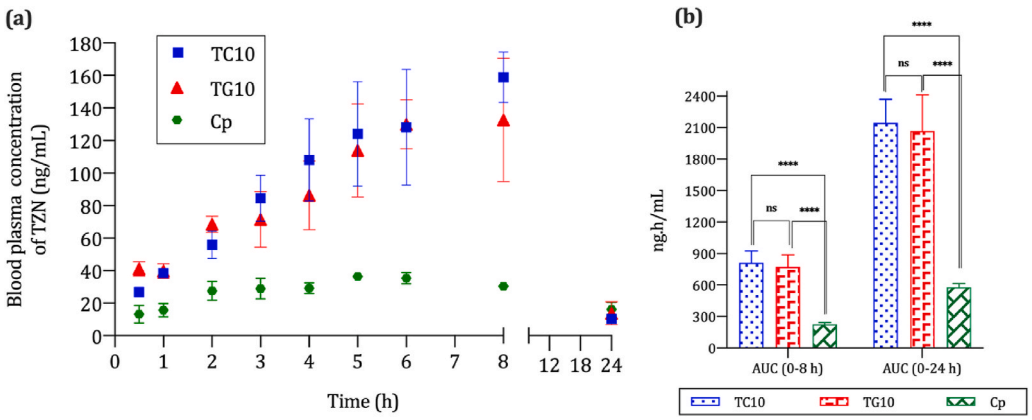

Fig. 11. Result of *in vivo* bioavailability studies using New Zealand rabbits ($n = 5$) *(a)* plasma profiles of TZN-containing buccal bilayer tablets and commercial product (C_P), *(b)* $AUC_{0\text{-}8}$ and $AUC_{0\text{-}24}$ of buccal bilayer tablets and commercial product (C_P) (****$P < 0.0001$).

Furthermore, although the T_{max} of C_p was 5 h, the plasma drug concentration of buccal tablets continued to rise for 8 h. This might be because TC10 and TG10 buccal tablets remained on the buccal tissue for approx 6–8 h without disintegrating and continued to release the TZN. As a result of $AUC_{0\text{-}8}$, the buccal mucoadhesive formulations improved the bioavailability of TZN by approx 3 times compared to orally administered C_p. Although the AUC values of the buccal tablets and the Cp showed a statistically significant difference ($p < 0.0001$) (Fig. 11b), there was no significant difference in the bioavailability of TC10 and TG10 ($p > 0.05$).

Making the tablet formulations bilayer by adding ethyl cellulose minimised the loss of TZN from the buccal region to the gastrointestinal tract. Bilayer formulations, consisting of an impermeable layer containing a water-insoluble substance and an adhesive layer containing a mucoadhesive polymer, are often preferred for buccal drug delivery to ensure unidirectional drug release. In recent years, pharmacokinetic studies have demonstrated the efficacy of bilayer formulations [16,97,98].

Buccal drug delivery offers an attractive opportunity to improve the systemic bioavailability of active substances with low oral bioavailability due to the advantages of the buccal region, which includes a rich vascular network and sufficient drug application area, and eliminates the hepatic first-pass metabolism [99].

Numerous studies have shown that buccal formulations offer higher bioavailability and extended drug release compared to oral administration, as evidenced by *in vivo* studies. El-Mahrouk et al. [100] demonstrated that chitosan lactate-based wafers containing TZN, which has low oral bioavailability due to exposure to the first-pass effect, doubled the drug's bioavailability. Zewail et al. [5] stated that the lornoxicam-containing buccal tablets provided an earlier and extended response compared to the commercial oral product. Buccal delivery has the potential to enhance the drug bioavailability and may also reduce the frequency of drug administration due to the prolonged release. In addition, systemic side effects of drugs can be reduced by decreasing the drug dose compared to oral administration [67]. A study showed that buccal formulations containing propafenone, which has a short plasma half-life, provided a longer duration in the buccal region, resulting in higher bioavailability and a reduction in the frequency of administration compared to the reference oral tablet [101]. However, to achieve these positive results, it is necessary to use mucoadhesive polymers that can provide the desired retention time on the mucosa. Chitosan, a mucoadhesive polymer used in this study, is a reliable penetration enhancer that is commonly used in the preparation of various types of buccal formulations. The ability of chitosan to affect buccal tissues is likely to be due to its interference with lipid organisation in the epithelium, as well as its ability to repackage epithelial cells to the basal membrane and partially disorganise desmosomes [102]. In our previous study, a chitosan-based buccal mucoadhesive monolayer patch of TZN was developed. *In vivo* studies revealed that the buccal patch significantly enhanced the bioavailability of TZN. It was found that chitosan, a good mucoadhesive polymer, was very favourable in increasing permeability in the buccal region [35].

In this work, buccal tablet formulations formulated with chitosan salts of TZN, which undergoes hepatic first-pass metabolism and therefore has low oral bioavailability, exhibited higher bioavailability compared to the commercial oral product by providing a long residence time and good penetration into the rabbit buccal area. Furthermore, this enhanced bioavailability may allow the drug to be administered at a lower dose, thereby reducing the systemic side effects of the TZN and improving patient compliance. The developed TZN-containing buccal bilayer formulations are remarkable as a promising alternative to orally administrated TZN due to their extended release.

4. Conclusions

In the present study, buccal mucoadhesive bilayer tablet formulations of TZN with poor oral bioavailability due to the first-pass metabolism were fabricated using chitosan salts (CG and CC).. Characterisation studies demonstrated that chitosan salts were successfully prepared. The tablets produced with higher molecular weight chitosan salt demonstrated better swelling and bioadhesive properties. Besides, tablets based on CG (TG7 and TG10) had higher bioadhesive properties, while CC-based tablets (TC7 and TC10) had a higher swelling index. Due to the swelling characteristics, TC7 and TC10 released the drug faster. According to the results of the permeability tests, the amount of TZN passing through the bovine buccal mucosa ranged from 13.40 % to 26.36 %. *In vivo* studies revealed that TC10 and TG10 provided about three times better bioavailability than the orally administrated commercial product. As a result, TZN-containing buccal tablets formulated with chitosan polymers provided a promising approach for clinical studies due to their better pharmacokinetic findings compared to the commercial product. Additionally, these results showed that the dose of TZN could be reduced, thereby improving the patient's quality of life and reducing systemic side effects.

CRediT authorship contribution statement

Muhammet Davut Arpa: Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Neslihan Üstündağ Okur:** Validation, Supervision, Methodology, Investigation. **Mehmet Koray Gök:** Formal analysis, Methodology, Validation, Writing – original draft. **Erdal Cevher:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project

administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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