Pharm Methods, 2016; 7(2): 75-88
A multifaceted peer reviewed journal in the field of Pharm Analysis and Pharmaceutics
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Original Article

Development and Characterization of Buccal Film of Candesartan

Dipak Rajaram Malpure[1], Sharada Laxman Deore[2*]

[1]Department of Pharmaceutical Sciences, Shri Jagdish Prasad Jhabarmal Tibrewala University, Vidyanagari, Jhunjhunu, Rajasthan INDIA.
[2]Govt. College of Pharmacy, Amravati-444604 MS INDIA.

ABSTRACT

Candesartan is potent antihypertensive drug of class angiotensin II receptor antagonist. But it exhibits poor water solubility and extensive first pass metabolism. Present research deals with development of candesartan buccal film. Optimisation of buccal film was done by design expert. Optimised concentration range selected for development of trial batches of candesaratanbuccal films. Mucoadhesivebuccal films of candesartan were prepared by solvent casting technique using chitosan, HPMC, gelatin and EDTA as permeation enhancer. Prepared buccal films evaluated for various pharmaceutical parameters, stability studies, in-vitro and *ex-vivo* evaluation parameters performed. In-vitroangiotensin II receptor antagonist studies were also performed. Results showed improved bioavaibility of candesartan through buccal films.

Keywords: Candesartan, Angiotensin II receptor antagonist, Buccal film, Box-Behnken desig. Mucoadhesive strength.

Correspondence:
Sharada L Deore,
Govt. college of Pharmacy, Amravati-444604 MS INDIA.

E-mail: sharudeore_2@yahoo.com
DOI : 10.5530/phm.2016.7.12

INTRODUCTION

Antihypertensive candesartan drug exhibits poor water solubility. Candesartan cilexetil is prodrug of candesartan, an antihypertensive angiotensin II receptor antagonist that on action of esterase enzyme present in the intestinal wall hydrolyses to active candesartan moiety in gastrointestinal tract.[1] Prodrug form of candesartan has not overcome poor oral bioavailability, approximately raised 40% from 15% in humans. The reasons for candesartan's low bioavailability and low absorption are low water solubility and efflux by drug resistance pumps in the gastrointestinal tract. Wide research is being carried out in design and development of systems which could increase absorption, bioavaibility of poorly water soluble and extensive first pass metabolism prone drug-candesartan[2] This research provided unique and simple mucoadhesive system, which is alternative to the other conventional types of drug delivery systems of antihypertensive drug-candesartan.

MATERIALS AND METHODS

Candesartan was obtained as a gift sample from Glen mark Pharmaceuticals Limited, Mumbai. HPMCK4M was obtained as a gift sample from Ajanta Pharma, Aurangabad, Gelatin (Type B) was obtained as a gift sample from Sigma Aldrich and Chitosan was obtained as a gift sample from Himedia.

Pre-formulation studies

Pre-formulation studies or preliminary studies to generate supportive data to understand physicochemical behavior of a drug and necessary modifications to design develop and evaluate dosage form.TLC, UV max, calibration curve and excipient compatibility by using FTIR were performed.

Optimization of Formulation

Formulation optimization process was carried out using a Box-Behnken design, as it requires few runs with three or four variables. Here three variables at three levels (Table 1) were studied using total 17 runs.[3] Layout of the Box-Behnken design isrepresented in Table 2. Effect of three factors X1 (HPMC), X2 (Chitosan) and X3 (Gelatin) on mucoadhesive strength of film and percentage drug release in 6 hr were studied by Box-Behnken design. A set of points lying at the midpoints of each edge of the multidimensional design cube as well as replicated center points were utilized to construct mathematical models and response surfaces using Design Expert® software (Version 9.0.6, Stat-Ease Inc., Minneapolis, MN, USA).[4]

Development of buccal film formulation

Candesartan buccal films were prepared by solvent casting method[5] using five different combinations of hydrophilic and hydrophobic polymers (HPMCK-4M, chitosan, and gelatin). Different concentrations and ratios of polymer solution were selected based on Box-Behnken design, prepared as mentioned in Table 4**.** The selected polymeric solution Glycerin, PEG 400 or PG was added and stir on a magnetic stirrer at low rpm until homogenous clear solution formed. The drug (*Candesartan* in 10 mL of Chitosan solution of 1% v/v glacial acetic solution) was added to the above solution. The homogenous and air bubbles free solution obtained by high-speed mechanical stirrer. Then solution poured into a circular Petridis. Plates were initially dried at room temperature in a hot air oven. The dried film were carefully removed and checked for any cracks and cut into 2x2 cm diameter film using specially fabricated stainless steel cutter.

Table 1: Box-Behnken design variables and responses

Independent variables			
	−1	0	1
X1 = HPMC	800	1000	1200
X2 = CHITOSAN	50	100	150
X3 = Gelatin	2	4	6
Dependent variables			
R1= Cumulative drug release			
R2 = Mucoadhesive strength			

Table 2: Layout of the Box-Behnken design

		Factor 1	Factor 2	Factor 3	Response 1	Response 2
Std	Run	A:HPMC	B:Chitosan	C:Gelatin	R1	R2
		mg	mg	mg	CDR	Mucoadhesive strength
6	1	1200	100	2	80.67	31.43
10	2	1000	150	2	92.22	32.76
17	3	1000	100	4	86.55	35.22
4	4	1200	150	4	93.2	27.07
7	5	800	100	6	94.5	27.03
3	6	800	150	4	91.67	20.79
15	7	1000	100	4	92.35	36.89
1	8	800	50	4	94.45	30.44
5	9	800	100	2	84.79	30.63
8	10	1200	100	6	87.96	33.86
9	11	1000	50	2	91.85	34.97
2	12	1200	50	4	78.78	32.04
16	13	1000	100	4	81.56	35.88
13	14	1000	100	4	87.77	36.07
12	15	1000	150	6	90.82	30.56
14	16	1000	100	4	82.33	31.08
11	17	1000	50	6	92.49	37.74

Characterization of buccal film

Appearance

Visual inspection of developed film formulation can provide results of desired organoleptic properties like color, flavor, and taste. Now-a-days, e-tongue software are useful to determine taste of formulation. Uniformity in color and odor along with good taste brings patient acceptability.[6] The general appearance and elegance of film was identified visually, which include shape, color, presence of an odor, taste, surface texture etc.

Weight variation studies

The individual weight of 3 samples (2x2 cm) of each formulation was determined using an analytical balance. The results were analyzed for mean and standard deviation. The weight of the film was determined using a digital balance.[7] The individual weight of 3 samples (2x2 cm) of each formulation was determined using an analytical balance.

Thickness and Diameter

The thickness of 3 patches (2x2 cm) of each formulation was measured using Digital thickness measurement apparatus (Figure 3.7) and the results were analyzed for mean and standard deviation. Three films from each batch were used, and an average value was calculated. Film was selected random from individual formulation and thickness was measured.

Percent moisture absorption

The buccal films were weighed accurately and placed in the desiccators containing 100 ml of saturated solution of aluminum chloride up to 86% relative humidity. After 3 days, the films were taken out and weighed. Percent moisture absorption determined by formula: final weight – initial weight/initial weight * 100.

Percent moisture loss

The buccal films were weighed accurately and kept in desiccators containing anhydrous calcium chloride. After 3 days, the patches were taken out and weighed. The percentage moisture absorption and moisture loss were calculated using the formula: Initial weight - final weight /initial weight X 100

Surface pH

pH of film should be near to 7 or neutral to get absorb through oral mucosa without irritation and toxic effects. Film dissolved in suitable solvent is used to determine surface pH-by-pH meter. The surface pH of the film was determined in order to investigate the possible side effects; since an acidic or alkaline pH may cause irritation to the buccal mucosa.[8] The buccal patch was allowed to swell by keeping it in contact with 5 ml distilled water for one hour at room temperature. The surface pH was measured by placing a pH paper on the surface of the swollen film. The experiment was performed and the average valueswere calculated.

Folding endurance

Folding endurance of 3 films of each batch was determined by repeatedly folding one film at the same place up to 200 times till it broke or folded, which is considered satisfactory to reveal good patch properties.[9]

Tensile Strength

It requires specially designed apparatus as shown in Figure 3.8. Tensile strength (TS) is the maximum stress applied to a point at which the film specimen breaks and can be computed from the applied load at rupture as a mean of three measurements and cross sectional area as described from the following equation: Breaking force (N)/Cross sectional area (mm^2)[7]

Swelling Index

Buccal film units were weighed individually, W1, and placed separately on 2% agar gel plates and incubated at $37^0C \pm 1^0C$. At every 30 minutes regular intervals, the films were removed from the gel and adhering gel was removed carefully with tissue paper. The weight of the swollen film was W2.[8] Percentage swelling was calculated using the formula.

$$S.I = \frac{W2 \quad W1}{W1} \quad 100$$

Mean of three determinations was considered. (n=3) Where, S.I = Swelling Index; W2 = Weight of swollen film after time t; W1 = Weight of film before placing in beaker.

Drug Content uniformity

Drug content uniformity was determined by dissolving the buccal film (10 mm in diameter) from each batch by homogenization in 100 ml of an isotonic phosphate buffer (pH 6.8) for 6 h under occasional shaking. The 5ml solution was taken and diluted with isotonic phosphate buffer pH 6.8 up to 20 ml, and the resulting solution was filtered through a 0.45 mm What man filter paper. The drug content was then determined after proper dilution at 271 nm using an UV-spectrophotometer.[9] Percent drug content was calculated by experimental drug content/theoretical drug content X 100.

Differential Scanning Calorimetry (DSC) studies

DSC measures the enthalpies associated with transitions and chemical reactions and determines the temperature at which these processes occur. The method is used for the identification and characterization of materials. The change in enthalpy of candesartan measured by DSC, Metler, Toledo.[10]

Morphological analysis by Scanning Electron Microscopy (SEM)

The outer macroscopic structure of the buccal film was investigated by Scanning Electron Microscopy with a S4800 TYPE II scanning electron microscope (Hitachi high technologies , Japan), operating at 15kV. The sample was fixed on a SEM-stub using double-sided adhesive tape and then coated with a thin layer of gold. The outer macroscopic structure of the nME as investigated by Scanning Electron Microscopy with a scanning electron microscope (FEI, the Netherlands), operating at 15kV. The sample was fixed on a SEM- stub using double-sided adhesive tape and then coated with a thin layer of gold.[11-12]

Determination of in- vitro bio adhesion strength

Mucoadhesive strength was determined by using modified physical balance method (Figure 3.11 and 3.12), for which goat stomach mucosa was collected from local slaughter house and stored in saline solution. Mucosa layer was stick on the glass slide using double sided sticker which was already stuck on the bottom of 100ml beaker, and this beaker was placed in 1L of beaker. The mucosal and film surface was wetted with few drop of 0.01 N HCl and on the left pan film 50 gm weight was placed for 5 min to allow the initial contact of mucoadhesion. Then drop wise water was added in beaker of right pan till the detachment of tablet from the mucous membrane was observed.[7] Then weight of water present in right pan beaker was determined, using following formula: Mucoadhesive Strength (gm) = (Weight of beaker +Weight of water) - Weight of empty beaker. After determination of mucoadhesive strength, force of adhesion was calculated using formula, Force of Adhesion (N) = (Mucoadhesive Strength)/1000×9.81

Determination of ex-vivo mucoadhesion time

The ex vivo residence time of *Candesartan* films was evaluated by assessing the time required for these films to detach from goat buccal mucosal membrane fixed in a well stirred beaker. The goat buccal mucosa was fixed on the internal side of a beaker with cyanoacrylate glue. The film (2x2 cm) was wetted with 50 µl of phosphate buffer pH 6.8 and was pasted to the goat buccal tissue by applying a light force with fingertip for one minute. The beaker was filled with 250 ml phosphate buffer pH 6.8 and kept at 37 ± 0.5°C. After 2 min the beaker was magnetically stirred at 50 rpm stirring rate to simulate the buccal cavity environment.[3,4,8-10] The time taken for the patch to completely erode or detach from the mucosa was observed as the ex vivo mucoadhesion time. The experiment was performed in triplicate and the results were analyzed for mean and standard deviation.

In-vitro drug release studies

Since performing bio-studies on every manufactured batch is impractical and costlier affair, formulators must rely on in-vitro testing to ensure batch-to-batch uniformity and consistency in bioavailability among developed formulations. Dialysis membrane (Himedia), 200 µm in thickness, pH 5.8 to 8 and porosity 2.4 nm was used as a artificial membrane for preliminary in-vitro studies because of simplicity, homogeneity and uniformity. Dialysis membrane is actually regenerated seamless cellulose tubing wherein the membrane is partially permeable, having molecular weight cut off between 12,000 to 14,000. This ideal for mimicking in-vivo permeation studies.[11]

Activation of Dialysis Membrane: The dialysis membrane tubings were washed in running water for 3-4 hours to remove glycerol followed by treatment of tubing with sodium sulfide solution (0.3% w/v) at 80°C for 1 min to remove sulfur compounds. Washed with hot water (60°C) for 2 min, followed by acidification with a 0.2% (v/v) solution of sulfuric acid, then rinse with hot water to remove the acid. Then the dialysis membranes were dipped overnight in the diffusion medium before dialysis for thorough wetting of the tubing.

Experimental: Franz diffusion cell (Figure 3.13) having 10 mm diameter and 16 ml capacity was used to study *in-vitro* diffusion of buccal film. Dialysis membrane (Himedia) of molecular weight of 12000–14000 kDa was used as diffusion membrane. Before experiment, pieces of dialysis membrane were soaked in phosphate buffer (PB) pH 6 for 24 hrs. Then phosphate buffer pH 6 was added to diffusion cell to fill it and then dialysis membrane was mounted on cell and attached at the brim of donor compartment with the help of glue to avoid any leakage. Rubbers were used to connect both donor and accepter chamber. After 20 min of pre-incubation time, 10 mg of buccal film was placed in the donor chamber. Then for next 4 hr, samples were periodically withdrawn from the receptor compartment with simultaneous replacement of same amount of fresh phosphate buffer solution. The withdrawn solutions were further assayed at 280 nm by a spectrophotometer.

Biological evaluation

Ex-vivo permeation studies

Ex- vivo skin permeation study was performed by using a Franz diffusion cell with a receptor compartment capacity of 13 ml the receptor compartment of diffusion cell was filled with phosphate buffer pH 6.8 goat buccal mucosa membrane was mounted between the donor and receptor compartment. The formulated film of 2×2cm diameter was cut and placed over the goat buccal mucosa membrane. The donor compartment was then placed and fixed over it with the help of rubber bandages. The whole assembly was placed on a magnetic stirrer, and the solution in the receptor compartment was continuously stirred. The temperature was maintained at 37 ± 2°C. Samples of 1 ml were withdrawn at time intervals of 1, 2, 3, 4, 5, and 6 hr and were analyzed at 271 nm spectrophotometrically for drug content against blank. The receptor phase was replenished with an equal volume of phosphate buffer each time the sample was withdrawn.[12] The percentage of the released drug was calculated.

Pharmacokinetics study

In vitro dissolution has been recognized as an important element in drug development. To analysis the mechanism for the release and release rate kinetics of the formulated dosage form, the data obtained from conducted studies was fitted into Zero order, First order, Higuchi matrix, Korsmeyer-Peppas and Hixson Crowell model. (Table 4) Best-fit model

Table 3: Summary of results of quadratic model for regression analysis of responses Y1, Y2,

Source	SD.	R^2	Adjusted R^2	Predicted R^2	% CV
Y1	0.45	0.6736	0.540	0.6112	0.46
Y2	1.88	0.9159	0.807	0.817	0.87

Table 3: Formulae for various film formulations

Ingredients (mg)	Formulation Code				
	F1	F2	F3	F4	F5
Candesartan	18	18	18	18	18
HPMC K4M	1000	900	800	700	1200
Chitosan	50	100	100	150	-
Gelatin	4	-	2	6	6
Glycerine	2	3	-	-	-
EDTA	50	20	20	20	20
1% Glacial acetic acid	10	10	10	10	10
Purified water	Q.S	Q.S	Q.S	Q.S	Q.S

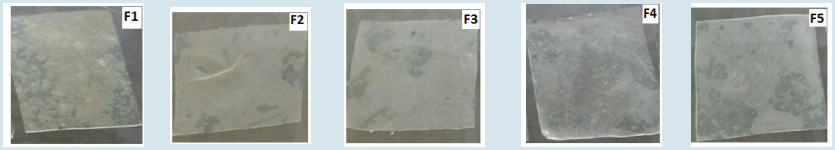

Table 4: Statistical analysis of formulations

Code	Zero order	First order	Hixon- Crowell	Higuchi plot	Korsmeyer-Peppas		Best fit model
	R	R	R	R	R	Release Exponent (n)	
F1	0.9873	0.7389	0.8501	0.3892	0.3969	0.6489	Zero order
F2	0.9599	0.7274	0.8831	0.3672	0.9684	0.7745	Higuchi
F3	0.8801	0.6570	0.7699	0.6089	0.9201	1.0836	Higuchi
F4	0.8379	0.6894	0.7704	0.4807	0.9302	1.0238	Higuchi
F5	0.8536	0.6793	0.7702	0.8995	0.4865	1.1933	Higuchi

Table 5:Korsmeyer-Peppas model

Code	R	Release Exponent (n)	Transport Mechanism
F1	0.7098	0.9074	Super case Transport
F2	0.6704	1.0283	Super case Transport
F3	0.3799	1.2869	Super case Transport
F4	0.7298	0.9801	Super case Transport
F5	0.8198	0.8422	Anomalous

Table 6: Physicochemical evaluation of formulation F-3 during stability studies at 40 ± 2°C/ 75 ± 5% RH

Parameter	0 Days	30 Days	45 Days	60 Days
Thickness (mm)	0.180± 0.36	0.180± 0.36	0.180± 0.36	0.180± 0.36
Folding Endurance (times)	>200	>200	>200	>200
Surface pH	6.5 ± 0.034	6.5 ± 0.036	6.5 ± 0.034	6.5 ± 0.034
Swelling behavior (%)	110	110	110	110
Tensile Strength (MPa)	7.068± 0.124	7.068± 0.124	7.068± 0.124	7.068± 0.124
Drug content (%)	97±0.7	97±0.72	97±0.70	97±0.79

Table 7: Summary of Results of Pharmaceutical Evaluation of Various Candesrtan-Chitosan Formulae

Code	Mass Uniformity (mg)	Thickness (mm)	Surface pH	Folding Endurance (Times)	Tensile Strength (MPa)	Swelling Index 60 min	Percent moisture absorption	Percent moisture loss	Average % Drug Content ± SD	In-vitro drug release After 350 min	Bio-adhesive Strength (gm)	Ex- vivo mucoadesion time (min)	ACE inhibition activity (%)
F -1	35.2± 1.003	0.110± 0.059	6.5± 0.42	>200	8.219± 0.105	97	4.3± 0.06	4.3± 0.032	91± 0.31	74.37	46.77± 0.720	92± 2.951	76.43± 0.490
F -2	30.6± 0.684	0.157± 0.033	6.5± 0.51	>200	8.103± 0.028	109	3.2± 0.84	3.4± 0.45	92± 0.082	79.66	35.02±0.034	102 ± 2.842	70.21±0.083
F -3	22.1± 1.103	0.127± 0.078	6.5 ± 0.37	>200	7.068± 0.124	112	6.8± 1.13	2.6± 0.04	97± 0.74	96.21	49.25± 0.082	121± 1.987	89.44± 0.03
F -4	32.4± 0.007	0.140± 0.010	6.0± 0.12	>200	4.537± 0.173	106	4.6± 1.07	3.2± 0.07	88± 0.59	80.12	34.41± 0.833	91 ± 2.923	74.41± 0.065
F -5	43.2± 0.892	0.183± 0.066	6.0± 0.32	>200	3.965± 0.013	99	3.4± 1.82	5.7± 0.2	79± 0.49	81.25	39.07± 0.134	94 ± 2.956	79.71± 0.105

can selected by comparing the r-values obtained. This expression applies to pharmaceutical dosage form such as films, where the dissolution occurs in planes that are parallel to the drug surface if the film dimensions diminish proportionally, in such a manner that the initial geometrical form keeps constant all the time. To study the release kinetics, data obtained from *in vitro* drug release studies were plotted as cube root of drug percentage remaining in matrix *versus* time.[10-12]

Ex-vivo muco irritation studies

Ex-vivo muco irritation of optimized buccal films F-3 was performed by using a fresh sheep buccal mucosa was purchased from local slaughter house immediately after slaughter (sheep buccal mucosa was used for the histological examination within 2 h). Histological examination was performed to evaluate the pathological changes in cell morphology and tissue structure during administration of bucco adhesive films. The epithelial tissues of mucosa were fixed in 10% neutral buffered formalin for 2 h, washed with distilled water upto 1 h and dehydrated with graded ethanol (60%, 80%, 90%, 95% and 100%). Then it is treated with xylene for permeation and embedded with liquid paraffin. After 8 h the samples were cut in 4 µm thick sections on a microtome with a surgical blade and conveniently colored with eosin.[13] The photograph of both controlled untreated and candesartan buccal film subjected to simple diffusion in sheep buccal mucosa.

In-vitro ACE Inhibitory Activity

The ACE inhibitor activities of optimized film F-3 formulation were performed as described. Twenty five micro liters of sample solution and 75 µl of 0.1 M sodium borate buffer (pH 8.3) containing 5.83 m Mhippuryl-L-histidylL-leucine as substrate and 1.0 M NaCl in an Eppendorf tube were pre incubated at 37˚C for 5 min.[14] The mixture was incubated with 25 µl of 0.1 M sodium borate buffer (pH 8.3) containing 1 mU ACE and 1.0 M NaCl at 37˚C for 60 min. After the reaction was stopped by the addition of 125 µl of 1.0 M HCl, the resulting hippuric acid was extracted with 750 µl of ethyl acetate by violently mixing for 15 s. After centrifugation at 6000 rpm for 3 min, 500 µl of the upper layer was transported into the other tube and evaporated at 80˚C for 2 h. The hippuric acid was dissolved in 500 µl of distilled water, and then the absorbance was measured at 228 nm. The IC50 value was defined as the concentration of the sample required to inhibit 50% of the ACE activity.[15] All analyses were carried out in triplicate samples. The percent inhibition of enzyme activity was calculated as follows: % inhibition = hippuric acid (control) – hippuric acid (sample)/ hippuric acid (control) * 100[16]

Stability Studies

Stability study in human saliva

The stability study of films was performed in natural human saliva. Samples of human saliva were collected from 10 humans (ages 18-40 years) and filtered. The films were placed in petriplate containing 5 ml of human saliva and put in a temperature controlled oven at 37°C ± 0.2°C for 6 h. The films were examined for changes in morphology and physical stability at definite time intervals. The prepared formulation was placed in natural human saliva containing petridish and these were checked regularly for the appearance, color, shape and physical stability.[17] the results were indicate there is no change in the film physical properties hence the prepared formulation is more stable during administration or placed in the buccal cavity throughout the period.

Physical stability

The optimized formulations, F-3 were subjected to stability testing for periods of 3 months at room temperature to simulate patient usage conditions and Refrigerator condition (4^{0}C). During 3 months of storage, the formulations were examined periodically after 1, 2, and 3 months for

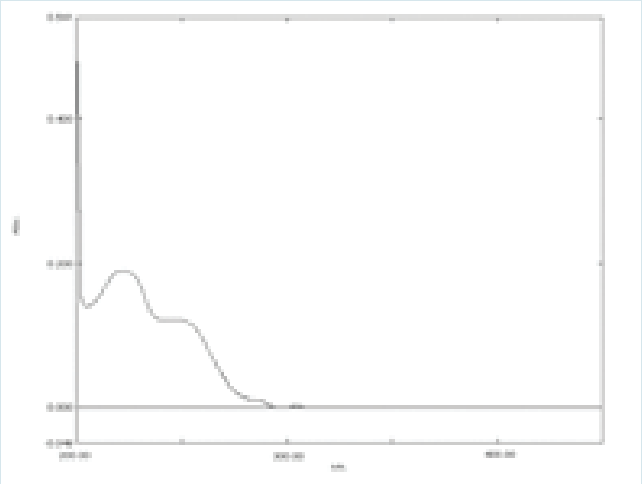

Figure 1: UV Absorption Maxima of Candesartan.

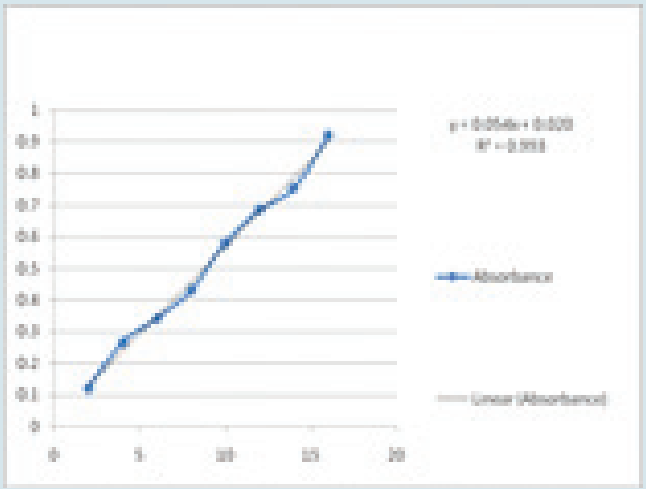

Figure 2: Calibration Curve of Candesartan Equation is Y = mx + C So, Equation of regressed line: Y= 0.054x + 0.020 R^2 = 0.993.

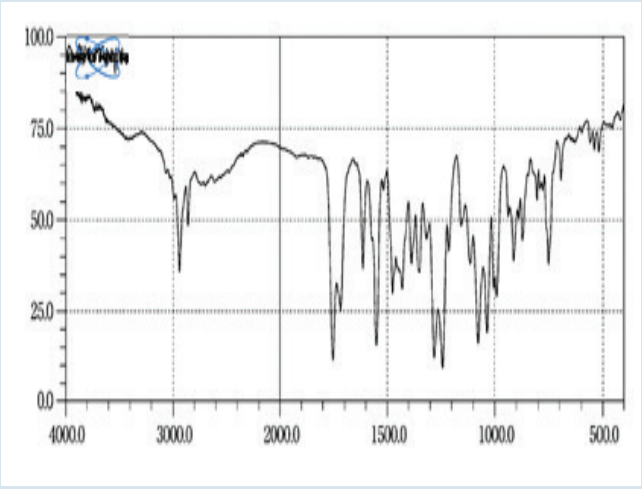

Figure 3: FTIR spectrum of Candesartan.

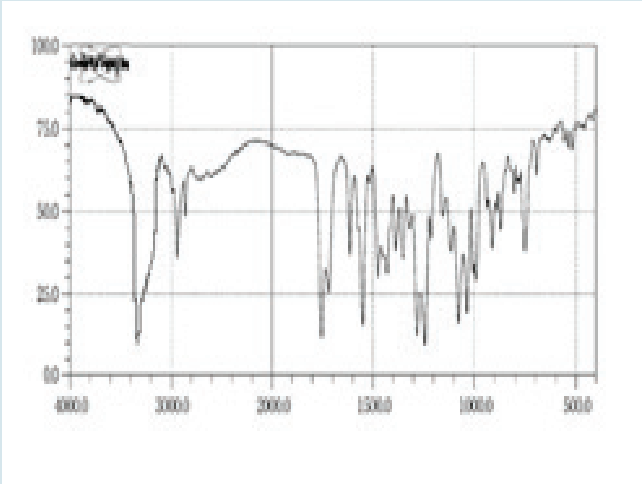

Figure 4: FTIR spectrum of Chitosan.

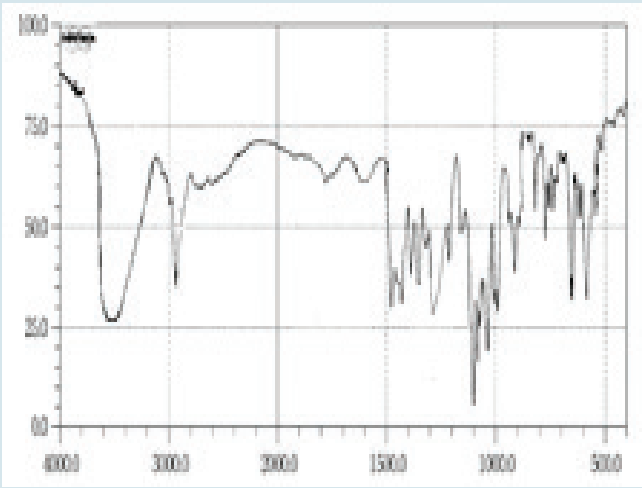

Figure 5: FTIR spectrum of HPMC.

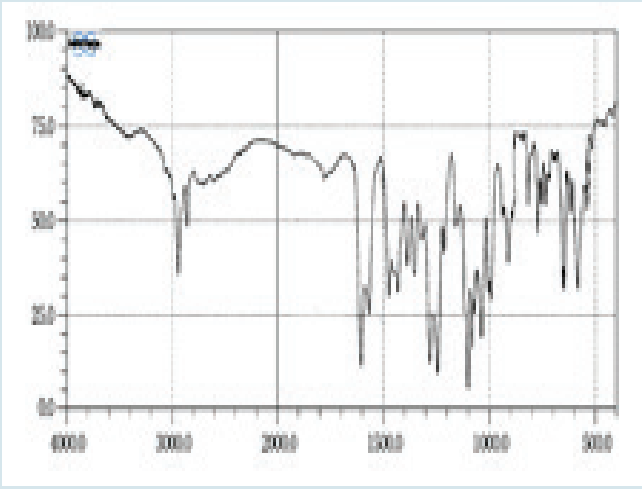

Figure 6: FTIR spectrum of Placebo film without Candesartan (HPMC + Chitosan + PG + EDTA).

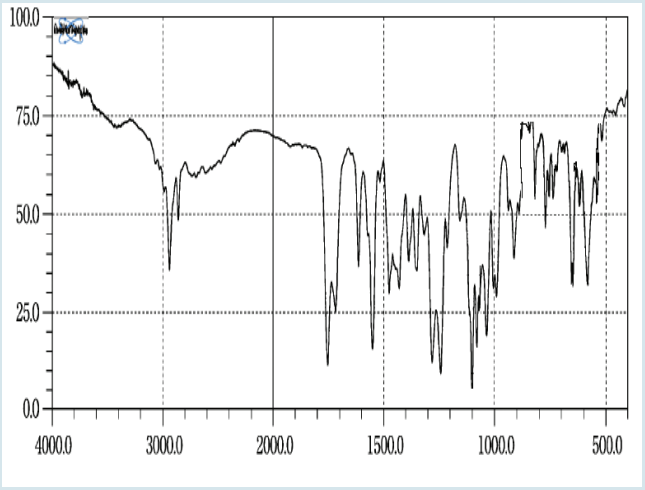

Figure 7: FTIR spectrum of Buccal film Candesartan + HPMC + Chitosan + PG + EDTA.

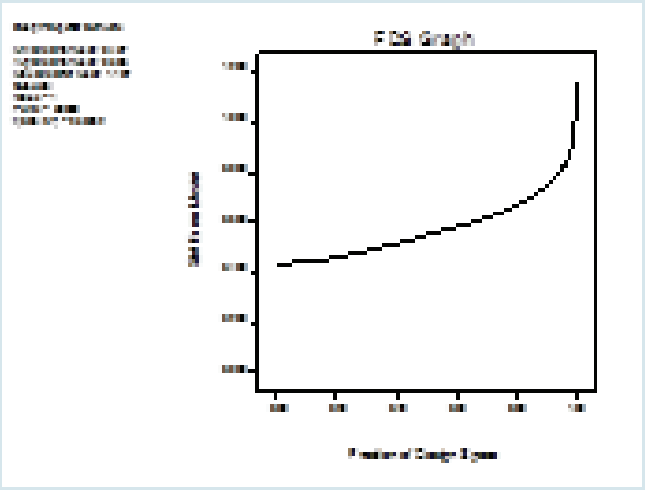

Figure 8: FDS graph of Box behnken design for optimisation of candesartan buccal film.

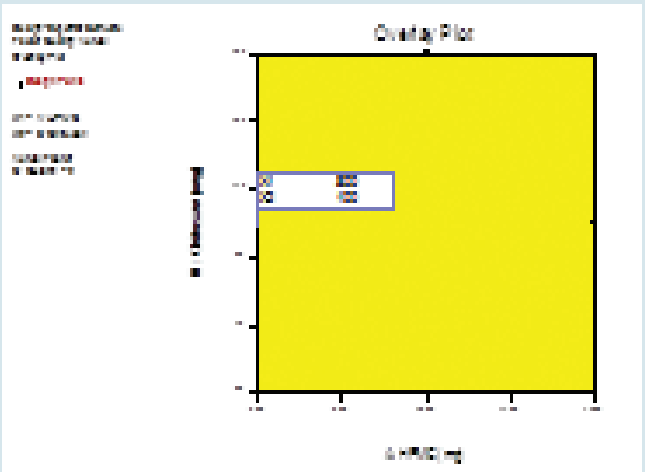

Figure 9: Optmised batch suggested by overlay graph.

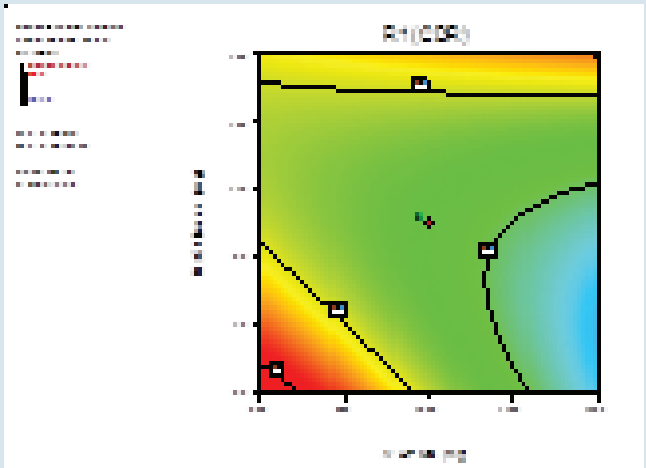

Figure 10: Cantour plot for response R1- Cumulative Drug Release.

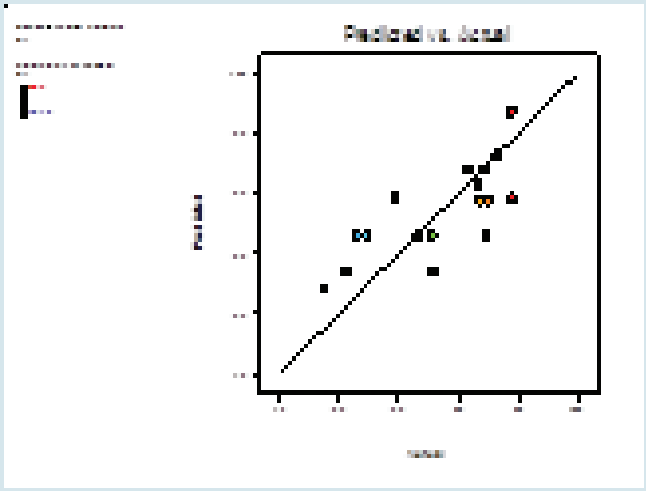

Figure 11: Prediction plot for response R1- Cumulative Drug Release.

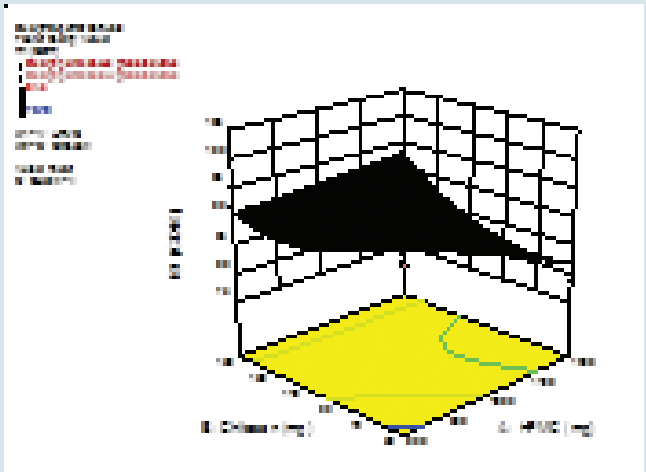

Figure 12: Surface response plot for response R1- Cumulative Drug Release.

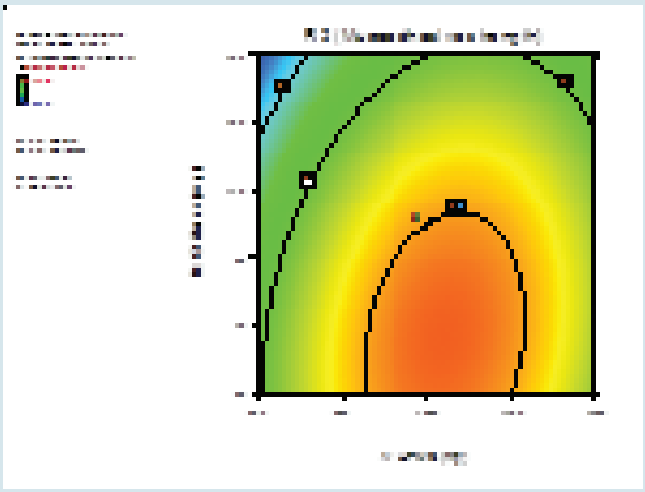

Figure 13: Cantour plot for response R2- Mucoadhesive strength.

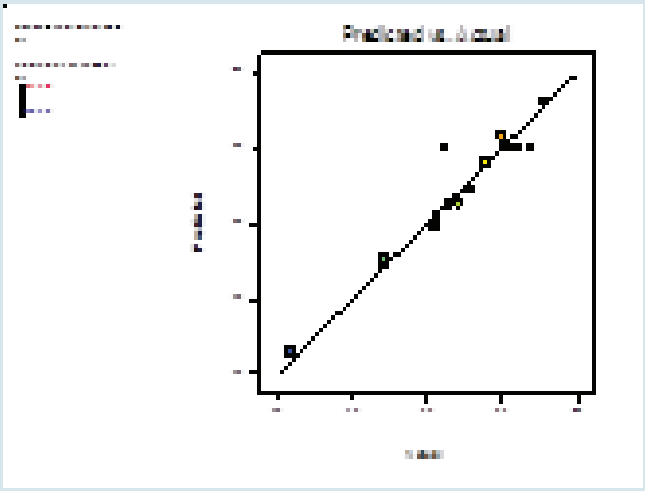

Figure 14: Prediction plot for response R2- Mucoadhesive strength.

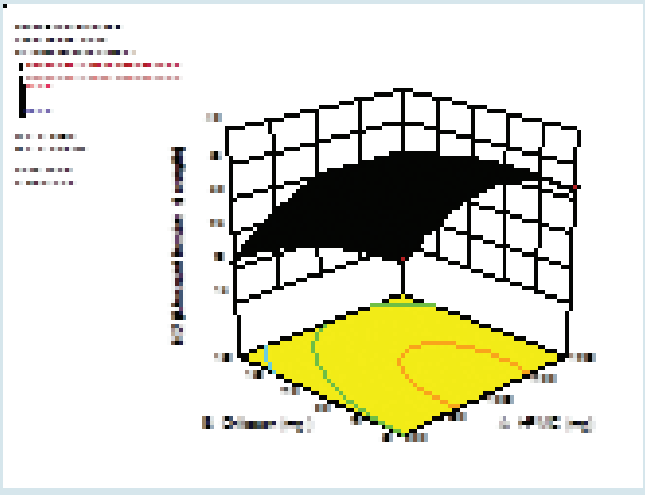

Figure 15: Surface response plot for response R2- Mucoadhesive strength.

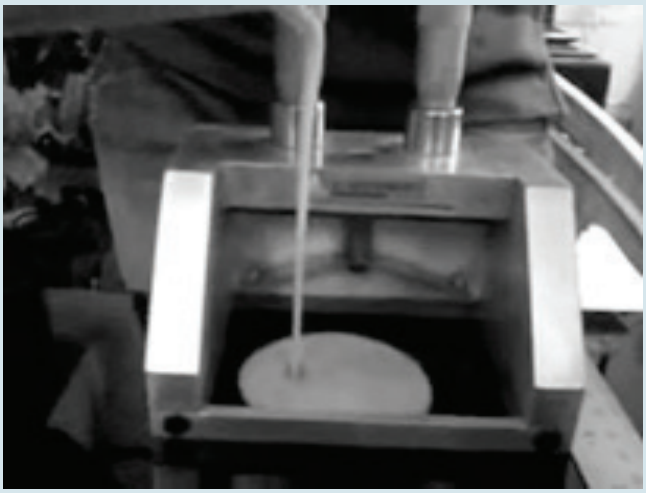

Figure 16: Film former Machine by VJ Instrument, Amravati used for preparation of final optimized batch buccal films.

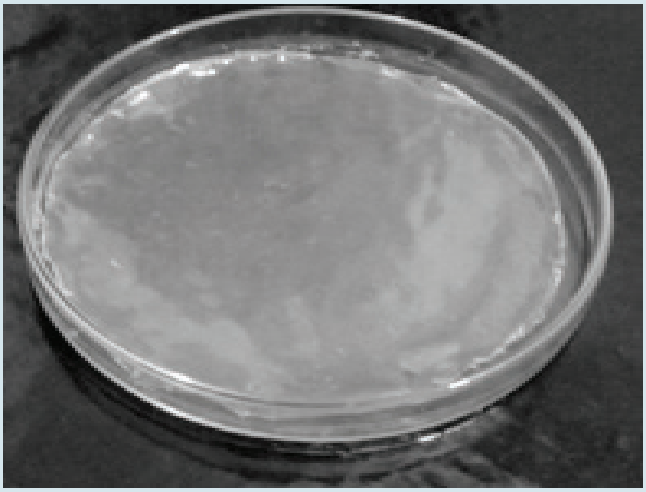

Figure 17: Film formation in petri plate.

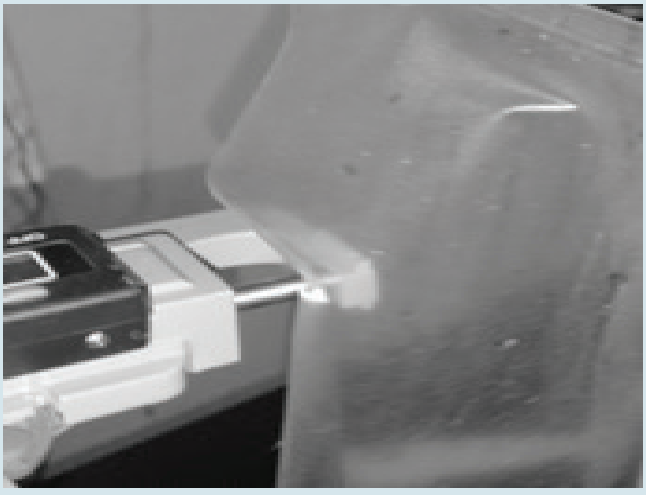

Figure 18: Digital thickness measurement of Film.

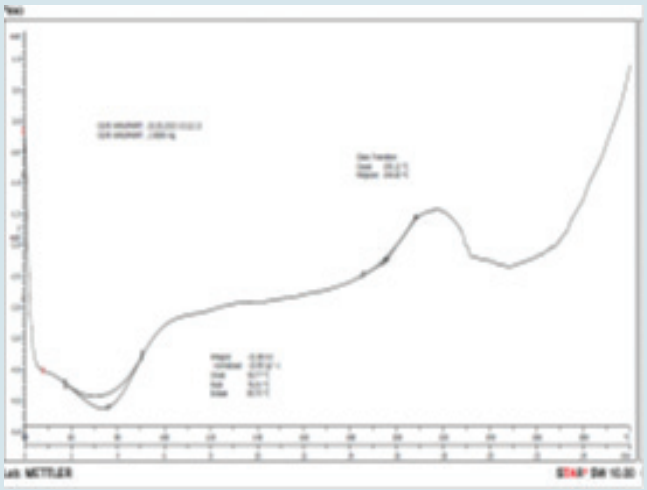

Figure 19: Graph of Differential scanning calorimetric studies.

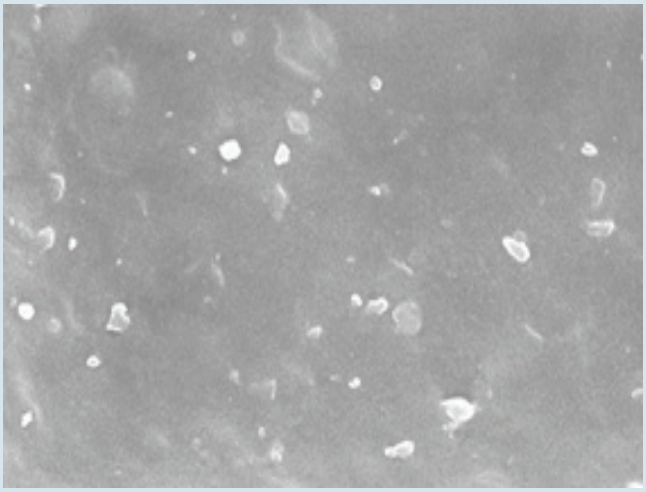

Figure 20: SEM images (×500 magnification) showing the surface morphology of films before stability studies.

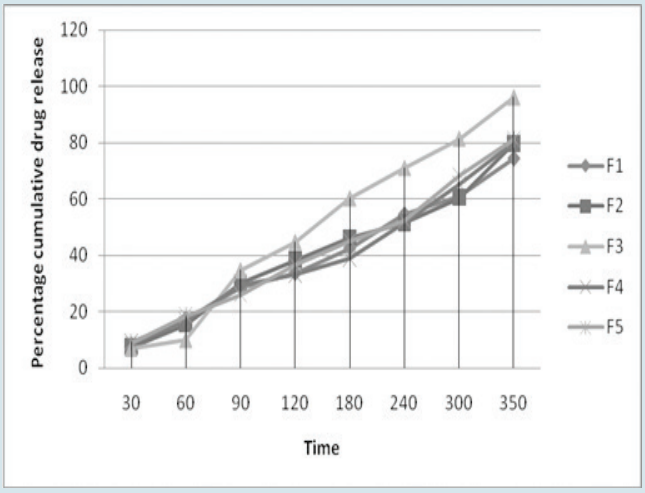

Figure 21: Percent Cumulative Drug Release.

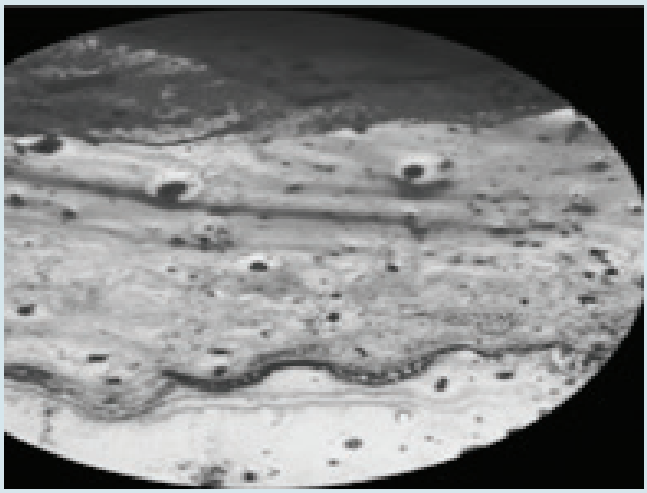

Figure 22: Buccal sheep mucosa without treatment.

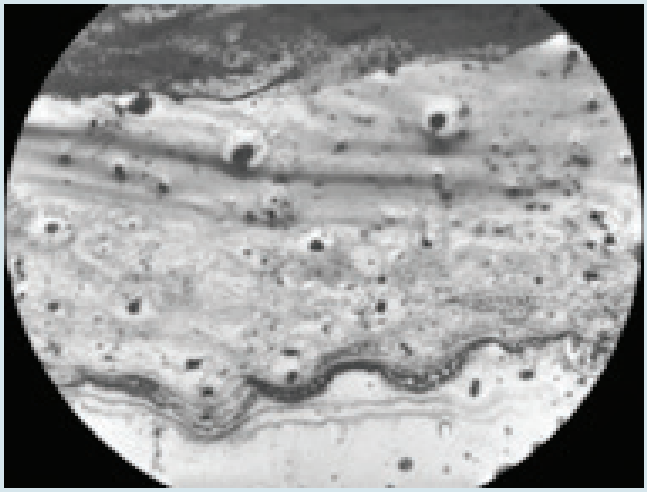

Figure 23: Buccal sheep mucosa after candesartan film treatment.

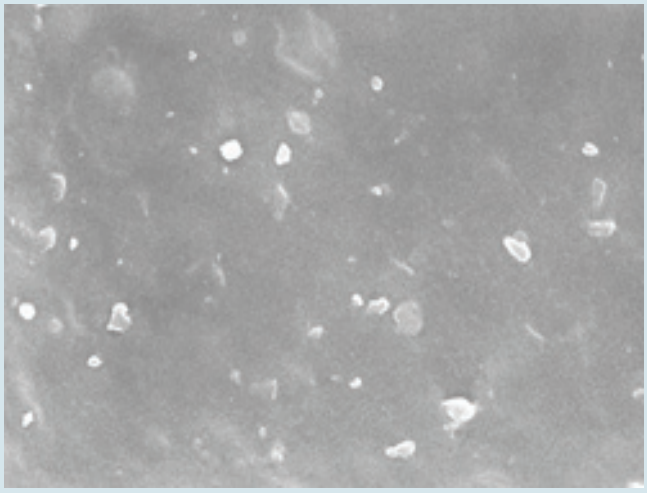

Figure 24: SEM images (×500 magnification) showing the surface morphology of films before stability studies.

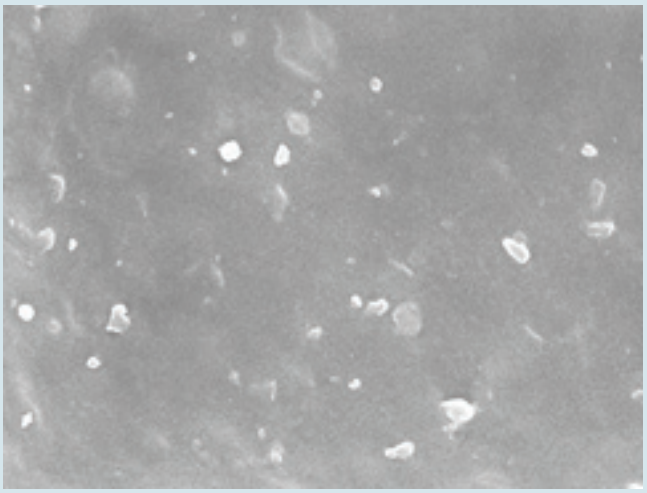

Figure 25: SEM images (×500 magnification) showing the surface morphology of films after stability studies.

physical stability by means of creaming, phase separation, or flocculation, accelerated centrifugation cycle (3000 × g for 15 min).[18]

Chemical Stability

The optimized formulations, F-3 were subjected to chemical stability by means of drug content, pH, Viscosity.

Thermodynamic stability

These studies included the exposure of prepared film to thermal (both low and high) as well as mechanical stress and observing the effect on homogeneity of film. The test were carried out in two part.[19]

Accelerated Stability Tests:

Freeze-Thaw Cycles (FTC): To access any change in stability of film they are subjected to store at 25^{0}C for 24 h and followed by 24 h at - 5^{0}C, the cycle is repeated three times and change is noted.[20]

Centrifugations test: It included centrifugation of formulations for 30min at 3500 rpm. Formulations which still remained clear and did not show any phase separation were included in freeze thaws cycles. Thermodynamic stability study was carried out in order to determine physical stability of the formulations. Repeated heating and cooling cycle lead to screening of formulations which would remain stable on long storage.[20]

RESULT AND DISCUSSION

Pre-formulation studies

Pre-formulation studies or preliminary studies to generate supportive data to understand physicochemical behavior of a drug and necessary modifications to design develop and evaluate dosage form.

The absorption maxima for *Candesartan* (10µg/ml) in P^H 6.8 were found to be 257 nm. (Figure 1) A series of external standard solutions is prepared and their absorbance is measured. A line or curve is fit to the data and the resulting equation is used to calculate concentration of unknown samples.10 µg/ml to 100 µg/ml dilutions of candesartan were prepared and absorbance of the solution was recorded at 257 nm using double beam UV spectrophotometer with water as a blank. The results are shown in Figure 2. A linear relationship (R2 = 0.993) between absorbance and *Candesartan* concentration passing through the origin, which obeys Beer-Lambert's law, was observed.[21]

To know compatibility of selected excipients with active drugs it is always preferred to study drug excipients interaction by using spectroscopy like FTIR, differential scanning colorimeter. Incompatibility is actually inactivation of active drug due to decomposition or alteration to a less effective physical or chemical form. To characterize the possible interactions between the drug and excipients, FT-IR spectroscopy was employed in the solid state on a Bruker Vector 22. Conventional KBr pellet method used to record FT-IR spectra. The spectra were scanned over a frequency range 400-4000/cm with a resolution of 4/cm.(Figure 3,4,5,6 and 7)For pure HPMC, the band at 3579.88 cm−1 is due to O–H stretching. The band at 2902.87 cm−1 represents C–H stretching of the –CH2 groups. The bands due to ring stretching of galactose and mannose appear at 1668.43 cm−1. Moreover, the bands in the region of 1350–1450 cm−1 show the symmetrical deformations of the CH2 and COH groups. The bands representing the primary alcoholic –CH2OH stretching mode and CH2 twisting vibrations appear at 1078 and 1024 cm−1, respectively. FTIR spectrum of chitosan showed characteristic signals for the polysaccharide structure at 898 cm-1and 1154 cm-1 and a strong amino characteristic peak at around 1575 cm-1.FTIR spectrum of Candesartan showed characteristic signals at 1752.70 cm−1and 1715.14 cm−1for ester –C=O stretching vibration and 1315.96 cm−1and 1241.93 cm−1for C–O stretching of aromatic esters. There is no interaction in selected polymers and drug candesartan.[22-23]

Optimisation of Buccal Film Composition

A response surface methodology experimental design was applied for the optimization of buccal film using Box-Behnken experimental design as it requires few runs with three or four variables. Here three variables at three levels were studied using total 17 runs. (Table 2) The amount of HPM (X1), Chitosan (X2) and gelatin (X3) were selected as independent variables and the dependent variable were % cumulative drug release (R1) and mucoadhesive strength (R2). The data obtained was treated using DE software (Design Expert® trial version 9.0.6; State- Ease Inc., Minneapolis, MN, USA) and analyzed statistically using analysis of variance (ANOVA).[3-5] The levels of these factors were selected on the basis of initial studies and observations. All the other formulation aspects and processing variables were kept invariant throughout the study period. Polynomial models including interaction and quadratic terms were generated for the entire response variables using multiple linear regression analysis (MLRA) approach. The general polynomial equation quadratic model is

$$Y = \beta0 + \beta1\,X1 + \beta2\,X2 + \beta3\,X3 + \beta12\,X1X2 + \beta13\,X1X3 + \beta23\,X2X3 + \beta11\,X12 + \beta22\,X22 + \beta33\,X32 + \cdots$$

Where, Y is the measured response associated with each factor level combination; β0 is constant; β1, β2, β3 are linear coefficients, β12, β13, β23 are interaction coefficients between the three factors, β11, β22, β33 are quadratic coefficients computed from the observed experimental values of Y from experimental runs and A, B and C are the coded levels of independent variables high (+), low (−) and center point (0). The result of ANOVA demonstrates that the model was significant for all dependent variables. Regression analysis was carried out to determine the regression coefficients. All the independent variables were found to be significant for all response variables. The quadratic model was found to be significant for both responses Y1 (Percentage Cumulative drug release) and Y2 (Muco adhesive strength).

Y1 (Percentage Cumulative drug release)

ANOVA results of the quadratic regression model indicate a highly significant model, as evidenced by the F value (3.83) and p value (<0.0001) in statistical analysis. The high value of R2 (0.5609) indicates a good fit of the quadratic regression model to the observed responses. The value of AdjR2 is also very high (0.4146), further demonstrating a high significance of the model. The relationships between the variables and responses could be better illustrated by the 3D plots obtained from the

predicted model using Design-Expert software (Design Expert 9.0.6, Stat-Ease Inc., Minneapolis, MN, USA). These plots were generated for the pair-wise combination of the three variables while keeping the third one at "0" level. Various statistical parameters (Table 3) models graphs (Figure 8-11) with result indicates that the factor play an important role in the formulation of film containing candesartan. The data of pure error and lack of fit can provide a mean response and an estimate of pure experimental uncertainty.[4]

Y2 (Mucoadhesive strength)

ANOVA results of the quadratic regression model indicate in a highly significant model, as evidenced by the F value (3.83) and p value (<0.0001) in statistical analysis. (Table 3) The high value of R2 (0.9159) indicates a good fit of the quadratic regression model to the observed responses. The value of AdjR2 is also very high (0.8079), further demonstrating a high significance of the model. The relationships between the variables and responses could be better illustrated by the 3D plots obtained from the predicted model using Design-Expert software (Design Expert 9.0.6, Stat-Ease Inc., Minneapolis, MN, USA). These plots were generated for the pair-wise combination of the three variables while keeping the third one at "0" level. Various statistical parameters (Table 3) models graphs (Figure 12-15) with result indicates that the factor play an important role in the formulation of film containing candesartan. The data of pure error and lack of fit can provide a mean response and an estimate of pure experimental uncertainty.

The residuals are the difference between observed and predicted values. The ANOVA for the dependent variables demonstrates that the model was significant for all response variables. The effects are like, the amount of chitosan, HPMC and gelatin were found to be significant, along with its quadratic and interaction terms for all the dependent variables.

A positive value represents an effect that favors the optimization, while a negative value indicates an inverse relationship between the factor and the response. Coefficients with higher order terms or more than one factor term in the regression equation represent quadratic relationships or interaction terms, respectively. It also shows that the relationship between responses and factors is not always linear. Used at different levels in a formulation or when more than one factors are changed simultaneously, a factor can produce different degree of response.[5]

Two-dimensional contour plots and three-dimensional response surface plots are obtained which are very useful to study the interaction effects of the factors on the responses. These types of plots show the effects of three factors on two responses at a time. The contour plot and response surface plot (Figure 8-15) revealed that a corresponding decrease in the cumulative drug release and muco adhesive strength takes place with an increase in the concentration of HPMC and chitosan upto a specific level and then decline is observed.

Developed model was further validated based on experimental versus predicted values and their corresponding residual plot. Finally, the best formulation was selected from 17 trial formulations based on achieving optimum values set for the response variables. Various response variables were adjusted and comprehensive evaluation of feasibility search alongwith exhaustive grid search was done. This led us to the formulation 5 that was found to fulfill the maximum requisite of an optimum formulation, which is given in FDS and overlay graph. (Figure 8-15) Five different batches from Box-Behnken design suggested overlay graph were selected for preparation and further details evaluation.

Development of Buccal Film

Initially placebo films without drug were prepared and those five batches exhibiting appreciable organoleptic properties like uniformity in physical appearance and non-stickiness were selected from Box-Behnken design for incorporating *Candesartan*. Solvent casting method used in following steps to prepare buccal films. Propylene Glycol (PG), Glycerine, Polyethylene glycol (PEG) used as Plasticizers. 1%v/v Acetic acid solution system for dissolve chitosan, distilled water was used as solvent system for polymer and drugs. EDTA was found penetration enhancers.[10] The weighed quantity of HPMC was gradually added to the drug solution under continuous stirring using magnetic stirrer and was left overnight at room temperature to ensure clear, bubble-free viscous solution.

Pharmaceutical Evaluation of Films

Appearance

The general appearance and elegance (shape, color, presence of an odor, taste, and surface texture) found satisfactory in each formulation. (Table 3) Candesartan-chitosan films were homogenous, clear and flexible (Figure 17). The additions of enhancers to the prepared films increased their flexibility and enhanced their moisture uptake. Glycerin in F-2 played its plasticizer role and gave a film better transparency.

Weight variation studies

Average weight was found in range of 32.4 ± 0.007 mg and 77.2 ± 0.002 mg. Drug loaded films (2 x 2 cm^2) were tested for uniformity of weight and the results are given in the Table 7. All the films were found uniform. Standard deviation of all the films ranged between 0.684 and 1.103. The optimized F-3 film was found to have thickness of 22.1 ± 1.103 mg.

Thickness and Diameter

Film thickness should be controlled within a range of 0.14 ± 0.01 mm and 0.29 ± 0.06 mm ± 5% variation of standard value.[3] All the drug-loaded films have uniform thickness throughout. The average thickness of all the films ranged between 0.110 ± 0.059 to 0.183 ± 0.066 which are in listed in Table 7. The optimized F-3 film was found to have thickness of 0.127 ± 0.078 mm.

Percent moisture absorption

Moisture interaction studies are necessary to find out the physical stability of the film at high humid conditions and integrity of the film at dry conditions.[4] The percent moisture absorption study was done over a period of 3 days and the results were found to be varied between 3.2% ± 0.51 percentage and 6.4% ± 1.82 percentage. (Table 7) The moisture absorption was found to increase with an increase in the viscosity of the polymer (HPMC K4M, gelatin) as well as with the polymer concentration. Microbial contaminations and bulkiness of the film can be reduced by presence of low moisture content but low moisture content can make film completely dried and brittle. Hence gelatin film found more brittle as compared to chitosan. Chitosan absorbs more moisture than gelatin.

Percent moisture loss

The results of percent moisture loss varied between 1.21% ± 0.42 percentage and 3.40% ± 0.41 percentage as shown in Table 7. It is found that increase in the viscosity of the polymer causes retention of moisture capacity and thus slow decline of percent moisture loss. Capacity of excipients to absorb water in vapour form decides percentage moisture absorption. Less moisture capacity is observed in F-3 and high moisture absorbing capacity in F-4. Chiotsan shows less moisture loss and gelatin shows higher moisture loss. High moisture content in films can be observed by percentage moisture loss. There is inverse relationship between percentage moisture loss and percentage moisture absorption.

Surface pH

The surface pH of the films was determined to examine the possible side effects due to acidic or alkaline pH, which can leads to irritation of buccal mucosa. The buccal film was allowed to swell by keeping in contact with 5 ml distilled water for one hour at room temperature. Acidic or

alkaline pH may cause irritation to the buccal mucosa and influence the rate of hydration of polymer.[5] The surface pH was measured by placing a pH paper on the surface of the swollen film. The surface pH of all formulations was within + 0.5 units of the neutral pH and hence no mucosal irritation were expected and ultimately achieve patient compliance. (Table 7)

Folding endurance

The average number of times that the patch could fold at the same place without breaking was the value of the folding endurance. Folding endurance of 3 films of each batch was determined by repeatedly folding one film at the same place up to 200 times till it broke or folded, which is considered satisfactory to reveal good patch properties.[6-7] Films did not show any cracks even after folding for more than 200 times. (Table 7) Hence it was taken as the end point. Folding endurance did not vary when the comparison was made between plain films and drug loaded films.

Tensile Strength

Tensile strength (TS) is the maximum stress applied to a point at which the film specimen breaks. Normal stress required to apply film in buccal mucosa must be withstand by a good pharmaceutical buccal film. The maximum tensile stress continued by the film during the strain test is called as tensile strength.[8] If maximum tensile stress occurs at either the yield point or the breaking point, it is designated tensile strength at yield or at break, respectively. Tensile strength of formulae F-1to F-5 found in range 3.96 to 9.06 MPa. 8.21, 8.10, 9.06, 4.53 and 3.96 Mpa were tensile strengths of F1 to F5 formulae respectively. (Table 7) Changing plasticizer type into PEG (F-3) showed different mechanical properties than the gelatin and glycerin.

Swelling Index

All the films hydrated very quickly and reached 80% hydration after just few minutes. (Table 7) Maximum hydration (115-120%) was obtained with formulations containing Chitosan i.e. F3. Films containing only HPMC showed a slightly lower hydration by 4-8%. Fragmentation was already evident at 60 min in all formulae. The highest losses were observed for films containing Chitosan as mucoadhesive polymer; for some of these films fragmentation was so high that it was not possible to recover and handle the film from the PBS 6.6, even immediately after the beginning of the experiment (Figure 3). This higher figure ragility of the films might be due to the larger swelling in water of this polymer with respect to gelatin. The consequence could be the formation of empty spaces within the film matrix that could make this structure less resistant to mechanical stresses uptake then chitosan films as expected. The degree of swelling of the bio adhesive polymers is an important factor affecting film bio adhesion. The faster the swelling of the polymer is the faster the initiation of drug diffusion and formation of adhesive bonds resulting in faster initiation of bio adhesion.

Drug Content uniformity

Content uniformity is determined by as per standard assay. The results of content uniformity indicated that the drug was uniformly dispersed. Recovery was possible to the tune of 88 to 97 %.

Differential Scanning Calorimetry (DSC) studies

The thermo grams of the physical mixtures of *Candesartan* with other excipients (1:1) showed the existence of the drug exothermic peak which indicated the absence of interaction between *Candesartan* and other excipients. In the optimized formulation, endothermic peak of drug was well preserved with slight changes in terms of broadening or shifting towards the lower temperature. It has been reported that the quantity of material used, especially in drug–excipients mixtures, affects the peak shape and enthalpy.[10] Thus, these minor changes in the melting endotherm of drug could be due to the mixing of drug and excipient, which lowers the purity of each component in the mixture and may not necessarily indicate potential incompatibility. Thermo grams recorded from (40-300^{O}C) for film was similar showing no thermal events. The DSC pattern of film, showed complete disappearance of the drug characteristic melting point peak at 89.70°C (Figure 19) indicating that *Candesartan* was molecularly dispersed in an amorphous form, slight downward shift of *Candesartan* endothermic peak with reduced intensity was observed at 58.77 °C and 76.01 °C respectively.

Morphological analysis by Scanning Electron Microscopy (SEM)

SEM has been used to determine particle size distribution, surface topography and texture and to examine the morphology of fractured or sectioned surface. The same generally used for generating three dimensional surface relief images derived from secondary electrons.[11] The surface of buccal film having the proportions of drug and polymer under microscopical examination can give the information of morphology and porosity of the film study. Film morphology the optimized selected formulation was characterized by scanning electron microscopy (SEM). Samples were mounted on round brass stubs (12 mm diameter) using double-backed adhesive tape.[12] The stubs were then coated with platinum to a thickness of about 200 nm under an argon atmosphere using a gold sputter module in a high vacuum evaporator. Afterwards, the stub containing the coated samples was placed in the scanning electron microscope chamber. It shows a uniform distribution of the drug within the film matrix which is highly desirable, in order to prevent potential re-crystallization and crystal growth, which could lead to instability (Figure 20).

Determination of the in- vitro bio adhesion strength

Buccal film is intended to be delivered by buccal route for either local or systemic action. In either case, it has to be hold on to the buccal mucosa for a extended period of time. [11] Therefore, it must display good mucoadhesive characteristics .Different polymeric combinations showed variations in mucoadhesive strength of films. Mucoadhesive strength also relates to drug release and permeation of drug from buccal mucosa. It was interesting to note that there was no noteworthy effect of either penetration enhancer or plasticizer in the mucoadhesive strength of films. Bio adhesion strength was found in range of 34.41 to 49.02 gm for F-1 to F-5 formulae. The maximum buccoadhesive strength has observed in the formulation F-3. The results are presented in the Table 7.

Determination of ex-vivo mucoadhesion time

Film mucoadhesion times varied from 90 to 120 min in various batches. But F-3 showed the highest adhesion time of 121 min whereas the films from F-4 showed the lowest muco adhesion time of 91 min. (Table 7) This difference depends upon several factors that affect the effectiveness of such a formulation. First of all, the use of Chitosan favors hydration and the outward diffusion of the drug from the film matrix. In fact, when using Chitosan, mucoadhesion time always resulted high, because the polymer although manifesting decisively higher swelling is less water affined and hence tends to retain its structure better than gelatin that, in turn, is better dissolved. Another important factor to be considered is the kind of film forming polymer used for the film preparation and the goodness and homogeneity of the polymer solution mixtures.[12]

In-vitro drug release studies

Varying proportions of polymeric substances showed noticeable difference in the release pattern of Candesartan in all film formulations. Reasonable release of Candesartan in most of formulations is producing at the end of 6 h. It is also observed that rate of Candesartan release is related to swelling index and bucco adhesive strength, which again

depends on properties and composition of basic matrix forming polymers in the various film formulations. Thus the rate of drug release found to be rise by raising proportions of hydrophilic polymer. Good swelling index and buccoadhesive strength of formulation F3 in proportions of chitosan and HPMC relates well with the maximum cumulative percentage release of Candesartan. Different kinetic models were used to analyze the *in- vitro* release data. *In vitro* drug release of prepared film showed that *Candesartan* was rapidly released during the first 1.5 h (30%), and the release was completed after 6 h and 30 min. Percent drug release after 6 h. was found out to be 82% for film code F-3 and for film code F1, F-2, F-4 and F-5 found to be above 65 to 74% (Table 7). It is evaluated all the data of drug released of batches and it showed that prepared buccal films follows Higuchi pattern of drug release. (Figure 21)

BIOLOGICAL EVALUATION

Ex-vivo permeation studies

The oral mucosa shows intermediate permeability characteristics between skin epidermis and the gut. It acts as a barrier to drug permeation.[12] Hence to get idea of total buccal barrier and effectiveness of buccal absorption for candesartan in the form of buccal film is determined by ex- vivo permeation studies. Permeation studies were performed for optimized formulation: F3. The percentage of the released drug was found in range of 74 - 93%. F-3 batch shown highest drug release of 96.21% as compared to other batches.

Pharmacokinetics study

To understand the mechanism of drug release from hydrophilic matrices, the *in vitro* dissolution data of each formulation were calculated with different kinetic drug release equations, namely zero order: Q=K0t; Higuchi's square rate at time: Q=KHt1/2 and Peppas: F=Kmtn, where Q is amount of drug release at time t, F is Fraction of drug release at time t, K0 is zero order kinetic drug release constant, KH is Higuchi's square root of time kinetic drug release constant, Km is constant incorporating geometric and structural characteristic of the films and n is the diffusion exponent indicative of the release mechanism. The correlation coefficient values (R) indicate the kinetic of drug release was zero order in F-1. The mechanism of drug release was by Peppas model (Table 5) indicates the non-Fickian release kinetics, evidenced with diffusion exponent values (n).

Ex-vivo muco irritation studies

Irritation is local cell damage with or without pain and inflammation. Mucosal membrane is most common site of irritation due to presence of sticky mucous secreting glands that attracts the allergens due to its nature. Buccal irritation studies must be conducted to determine the viability of this route for better administration for the selected drug before formulating a buccal drug delivery system. These studies involve methods that would examine *in vitro* and/or *in vivo* which determines possible toxicity of drug and excipients in the form of buccal patch. To evaluate the pathological changes in tissue morphology and organization during application of bucco adhesive film, histological examination was performed.[13] It is expected that buccal film should not cause any irritation, ulceration, inflammation and redness to buccal mucosa, and it be similar to controlled buccal mucosa. In present study optimized F-3 formulation taken for *Ex-vivo* muco irritation study using eosin stain. Eosin is a fluorescent acidic compound that binds to positively charged compounds like proteins, collagen, muscle fibers and stains them dark red or pink. Eosin also stains red blood cells intensely red. It is most widely used stain in histological study. *Ex-vivo* muco irritation was performed by using a fresh sheep buccal mucosa. (Figure 22) Results compared with untreated buccal mucosa. (Figure 23)

In-vitro ACE Inhibitory Activity

Angiotensin-converting enzyme inhibitors are called as ACE inhibitors which blocks alteration of angiotensin I enzyme into angiotensin II where later is a potent vasoconstrictor.[14] These class drugs are found effective more than calcium channel blockers and beta blockers. It is preferred choice in treatment of hypertension associated with chronic kidney disease state. Candesartan is an antihypertensive drug acts as angiotensin II receptor antagonist.[15] It is usually preferred in treatment of diabetic nephropathy and in patients who are not responding to ACE therapy than other drugs in the treatment of congestive heart failure, systolic dysfunction, myocardial infarction and coronary artery disease. Blockage of rennin-angiotensin system by angiotensin II receptor antagonists produces antihypertensive effects due to vasodilatation. ACE Inhibitory activity of developed film formulations were determined by in-vitro method and results calculated (Table 7) using formula:% inhibition=hippuric acid (control)–hippuric acid (sample)/hippuric acid (control) * 100[16]

Stability Studies

Saliva is the protective fluid for all tissues of the oral cavity. It protects the soft tissues from abrasion by rough materials and from chemicals. It allows for the continuous mineralization of the tooth enamel after eruption and helps in remineralisation of the enamel in the early stages of dental caries.[17] The stability study of films in natural human saliva shown acceptable change in color, shape, and physical stability. Stability is defined as the capacity of drug product to remain within specification established to ensure its identity, strength, quality and purity.[18] The purpose of this is not only to determine the rate of chemical and physical reaction but also predict a tentative expiration-dating period under ambient condition. FDA and ICH specifies the guidelines for stability testing of new drug product, the final formulation was reproduced in large scale and packed.[19-20] The packed samples were kept for stability study at 40^{0}C with 75% RH for 1 month. Sample were collected after 1 month and evaluated. The drug content and other parameters were compared with initial profile to check the effect of storage on drug release of the formulation. Stability studies parameters for optimized F-III Batch evaluated are Mucoadhesion Time 19.23, Mucoadhesive Strength 15.45 and Drug Content 93.12. (Table 6) No change in before and after stability studies sample of F-III has been observed in SEM analysis. (Figure 24 and 25)

CONCLUSION

We conclude that, chitosan with HPMC and gelatin can meet the ideal requirement for buccalmucoadhesive candesartan film, which can be good way to bypass the extensive hepatic first pass metabolism of candesartan, substantial dose reduction and increase bioavailability. Present research can be further evaluated to perform *in-vivo* drug release studies in suitable animal model. Further work is recommended to support its efficacy claims by long-term pharmacokinetic and pharmacodynamic studies in human beings. Thus clinical investigation will only decide its suitability of dosage form in the actual clinical practice.

CONFLICT OF INTERESTS

The authors declare that this paper content has no conflict of interests.

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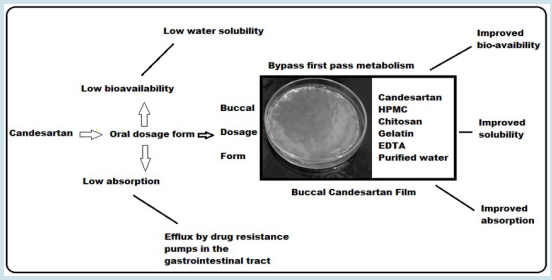
PICTORIAL ABSTRACT



SUMMARY

- In the present study, buccal films of candesartan cilexetil were prepared by solvent casting method employing polymer such as HPMC K4M, Chitosan, PEG, EDTA in different combinations. The optimized batch found transparent, uniform, flexible, and without bubbles. It showed maximum in-vitro drug release and fairly good amount of drug permeation through the membrane in 6 hrs with satisfactory physical stability. The present study indicated enormous potential of mucoadhesive buccal films containing *Candesartan cilexetil* for systemic delivery with an added advantage of circumventing hepatic first pass metabolism.

ABOUT AUTHORS

Dr. S. L Deore: Is working as Assistant Professor in Govt. College of Pharmacy, Amravati. She has published 03 books and more than 50 research papers. She has filed 02 Indian patents. She has been awarded with CAYT by AICTE. Her areas of research are intellectual property rights and traditional medicine, isolation and structural elucidation of phytochemicals, neutraceutical development, and traditional medicine screening, chromatographic and phytochemical analysis of extracts. Recently she has transferred technology of taste masking for one of popular mushroom nutraceutical.