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Permeation enhancement of furosemide by using 2 level full factorial design: An *ex vivo* study

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ABSTRACT

Context: Various targeted and modified drug delivery systems have been developed to overcome the challenges for BCS Class IV drug and to obtain a promising drug delivery system. Objective: The present work is aimed to enhance the permeability and solubility of furosemide, an antihypertensive drug by using bile salt as permeation enhancer as well as by formation of cyclodextrin inclusion complex with β- cyclodextin. Method: Furosemide multipolymericbuccoadhesive bilayer films for systemic delivery of furosemide were prepared using solvent casting method. The multipolymeric film contained Chitosan (CH), polyvinyl pyrollidone (PVP) K30, glycerol, sodium glycocholate (SGC) and inclusion complex. A 2^2 full factorial design was employed to study the effect of independent variables like concentration of CH (x_1) and SGC (x_2), which significantly influenced permeability of drug. Steady state flux (J_{SS}) was chosen as dependent variable. Result: It was found that the film having higher concentration of CH and SGC (P_2) showed high values for J_{SS}. Incorporation of inclusion complex further increased the solubility of furosemide. Conclusion: The present approach for permeation and solubility enhancement using transbuccal route can be further explored for systemic delivery of BCS Class IV drugs.

Keywords: Mucoadhesive Bilayer film, Chitosan, Sodium glycocholate, Buccal drug delivery, Steady state flux (J_{SS}), Furosemide, PVP K30, Hydroxypropyl-β-cyclodextrin, Permeation enhancement

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INTRODUCTION

BCS classification as originated by Dr. Gordon L. Amidon faces a major challenge of development of drug delivery system and achieving a target release profile for BCS Class IV drugs [1]. Since aqueous solubility and slow dissolution rate ofBCS Class IV drugs is a major challenge in the drug development and delivery processes, improving aqueous solubility and slow dissolution have been investigated extensively [2]. The other challenge for formulation scientists is the permeability aspect of drug belonging to Class IV. Attempts have been made to enhance the permeability of drugs by physical and chemical means. Among the latter two, approaches chemical permeation enhancers provide a good opportunity to enhance the permeation of such drugs.

Furosemide is a loop diuretic agent that is used orally in the treatment of edematous states associated with cardiac, renal, and hepatic failure and the treatment of hypertension. It is a model Class IV drug[3].It is incompletely absorbed after oral administration to healthy subjects and also in patients with various diseases[4]. In healthy patients, the bioavailability is approximately 50% [5]. Different ways have been studied so far to enhance the bioavailability of furosemide by employing novel approaches of drug delivery. Attempts have been made to increase the solubility of furosemide by complexation with cyclodextrin[6, 7] and by formulating solid dispersions[8-10].

Similarly FurosemideCalcium-pectinate microcapsules with self-micro emulsifying core to enhance the solubility and permeability have been prepared [11]. Transdermal delivery systems and matrix granules for furosemide have been developed [12-15].Buccal drug delivery for systemic delivery of furosemide has not been studied till date.

Oral mucosal drug delivery is an alternate method for systemic delivery of drugs and it offers several advantages over both injectable and eternal methods. It also enhances drug bioavailability because the mucosal surfaces are usually rich in blood supply and avoids first pass metabolism [16, 17]. Severalbuccal formulation have been developed to enhance systemic delivery.[18]Buccalbioadhesive formulations include buccal tablet, buccal films, buccal wafers, buccal patches, buccal gels and liquid preparations for local and systemic delivery of drugs. Buccal patches and films have been formulated for drugs like lidocaine, miconazole, acyclovir, clotrimazole , sumatriptanetc for either local or systemic delivery.[17]

The present work is aimed to develop a buccoadhesive drug delivery for furosemide with aim to enhance the permeability and solubility. An attempt has been made by way of enhancement of dissolution and permeability and also by avoiding first pass metabolism.

Multipolymericbuccoadhesive bilayer films with non permeable backing layer and a mucoadhesive layer for systemic delivery furosemide via transbuccal route were prepared. Drug was incorporated in the film as free drug and also in the form of inclusion complex with hydroxypropyl-β-cyclodextrin[19-21]. Chemical permeation enhancer, sodium glycocholate was incorporated in the formulation to enhance the permeability of drug through ovine buccal mucosa. Chitosan a natural, mucoadhesive and biodegradable polymer with film forming properties is used as base matrix and hydrophilic polymer PVP K30 was incorporated in the formulation to modify the release of drug from the base polymer matrix [22]. Glycerine was used as a plasticizer. The films were formulated by solvent casting method. The films were evaluated for permeation study through ovine buccal mucosa.

Chitosan was selected as a base matrix for buccoadhesive film as suggested by Bonferroni et al [23]. Chitosan is a biodegradable, natural polymer, non toxic and mucoadhesive, biocompatible and cationic polymer. Various researchers have worked on mucoadhesive systems using chitosan as a polymer. It is used for the formulation of mucoadhesive tablets, patches, films and gels for buccal use [24]. Also, chitosan has permeation enhancing property depending on the degree of deacetylation and molecular mass. The penetration enhancement effect of chitosan is due to prolonged mucoadhesion properties and ability to open tight junctions complexes of the mucosa [23]. The enhancement effect of chitosan in gel form for oral mucosa was investigated with transforming growth factor-β TGB-β. It showed marked permeation enhancing effect on buccal mucosa [25]. Chitosan has excellent gel forming and film forming properties; so it is a good candidate for mucoadhesive polymer.

Nowadays, researchers use a blend of polymers to enhance the mucoadhesion properties and also modify the drug release. Over hydration of chitosan may lead to slippery mucilage. So, to avoid mucoadhesion failure other polymers are added to the films [26]. The other polymer used was PVP K30 (polyvinyl pyrollidone K30). It was incorporated in the system to modify the release of drug from the base matrix. PVP K30 being a hydrophillic polymer it can be hypothetized that it would produce pores in the matrix structure and thus promote the diffusion of drug from the matrix. Various scientists have used PVP K30 as a drug release modifying polymer for buccal films and patches [22, 27-29]

Permeation enhancers are used to modify drug permeation. Various classes of chemical permeation enhancers are used like bile salts, terpenes, chitosan, cyclodextrins, surfactants, medium chain fatty acids, azone etc. But the criterion for selection of permeation enhancer was least toxic to the buccal mucosa, which have reversible type of effect on mucosa and which have GRAS status. Out of all permeation enhancers, bile salts have less effect on buccal mucosa and most widely used and mucosal damage caused by them is reversible [30]. Bile salts are investigated for transbuccal delivery of morphine sulphate, fluoresinisothiocyanate, triamcinolone acetanolide, insulin and calcitonin [30]. Sodium glycocholate was selected as a permeation enhancer as it has been explored as permeation enhancer for delivery of morphine sulphate[S31], acyclovir [32]. Ethyl cellulose has been used as backing layer extensively [29].

MATERIALS AND METHODS

Furosemide was obtained as a gift sample from Sanofi Aventis, Ankleshwar, Gujarat, India. Chitosan (CH), Polyvinyl pyrollidone K30 (PVP K30), Sodium Glycocholate (SGC), Ethyl cellulose (EC) and Hydroxypropyl–β-

cyclodextrin (HP-β-CD) were purchased from HiMedia Laboratories Pvt. Ltd, Mumbai, India. All other chemicals, excipients and solvents used were of analytical grade

Solubility study (Phase solubility study)

Phase solubility study was performed according to the method described by Higuchi and Connors [33-36] . An excess amount of furosemide (50 mg) was added to 5 mL of aqueous HP-β-CD solution in concentration ranging from 0 to 0.05 mM. The suspension was shaken at 50 rpm at 25 ± 2 ^{0} C for 24 hours until equilibrium was achieved. The samples were filtered through 0.22 µm filter membrane in a vacuum filter and drug concentrations in the filtrate were detected at 229 nm wavelength in a UV spectrophotometer. The apparent stability constant K_s was calculated from phase solubility diagram using the following equation (1):

K_S= Slope/(S_o ×(1-Slope)) (1)

Where, S_o = the solubility of furosemide in water. (Intercept)

The solubilization efficiency of cyclodextrin is an important aspect for determining the amount of cyclodextrin to be used in the pharmaceutical formulation. It can be determined as the slope of the phase solubility profile or as the ratio of complex to free cyclodextrin concentration [37]. The complexation efficiency (CE) was calculated using equation[38, 39]:CE= Slope/(1-Slope)

Preparation of inclusion complex by Co-precipitation method

The complex of furosemide HP-β-CD were prepared by co-precipitation method with slight modification as described byFarcas et al [40]. The complex was prepared in molar ratio of 1: 1.5, drug: HP-β-CD. HP-β-CD was dissolved in 50 ml of distilled water on a magnetic stirrer with heater. Furosemide (330 mg) was dissolved in 5 ml of acetone. The HP-β-CD solution was heated up to 70 ^{0} C and furosemide solution was added drop by drop to it. The mixture was stirred till precipitate appeared in the solution. The precipitates were filtered, and dried at room temperature.

Evaluation of Furosemide HP-β- CD complex

Differential scanning calorimetry

Differential scanning calorimetry (DSC) thermograms of solid product were recorded on a DSC instrument. Accurately weighed samples (2-5 mg) were placed in the pan and scanned at a heating rate of 10 ^{0} C / minute over temperature range of 25 ^{0} C to 300 ^{0} C with a nitrogen purge 20ml/ minute and an empty pan was used as a reference. [7, 38]

Fourier transformed infrared spectroscopic studies

Fourier transformed infrared (FTIR) spectra of the solid product were recorded on FTIR spectrophotometer. The samples were prepared by using the potassium bromide disk method and scanned for transmittance in the range of 4000 to 400 cm^{-1}.

X- Ray powder diffractometry

X- Ray powder diffractometry (XRD) patterns were obtained at room temperature (25 ± 2 ^{0} C) using X- ray diffractometer. The measurement conditions were range for 2θ (2 theta) from 5^0 to 60 ^{0}. LynxEyeDetector was used and X- ray generator voltage was kept 30 kV and current 10 mA. The step size was set as 0.02 and scan speed was set as 0.1 second. The XRD patterns of furosemide drug, HP-β-CD, physical mixture of drug and HP-β-CD; as well as inclusion complex were compared[7].

In vitro dissolution study of complex

In vitro dissolution was done using USP dissolution apparatus Type II at 50 rpm, to know the effect of complexation on solubility of drug. Thus three different powders containing free drug, its stoichiometric physical mixture with HP-β-CD (1:1.5) and their inclusion complex were taken. They were placed in the jar containing 900 mL of pH 7.4 phosphate buffer maintained at 37 ± 2^0 C and dissolution study was carried out for period of 60 minutes. 5 ml samples were withdrawn and analyzed for UV absorbance at 229 nm.

Preparation of multipolymeric bilayer buccoadhesive films

Preparation of Mucoadhesive layer containing drug/inclusion complex

Furosemide, films were prepared using solvent casting method [29, 41]. Initially required amount of chitosan was dissolved in 0.5% v/v of acetic acid under constant stirring till a clear solution was obtained. To this solution required amount of PVP K30 was added. Glycerine was added as a plasticizer under constant stirring. Required amount of sodium glycocholate was dissolved in 2 ml of water and added to the above solution. Required amount of furosemide [42, 43] was added to the solution with continuous stirring after dissolving in 99.6 % v/v ethanol so as to have 5 mg of drug per 1 cm^2. The mucoadhesive layer was cast on the pre formed ethyl cellulose backing layer in a Petri dish. The film was allowed to dry overnight at a temperature of 60 ± 5^{0} C in a tray dryer.

Preparation of Backing Layer

To prepare ethyl cellulose backing layer 300 mg of ethyl cellulose was dissolved in 10 ml acetone under constant stirring and to that 0.5 ml of glycerine was used as a plasticizer. The solution was poured in 70-72 mm Petri dish. The solvent was allowed to be evaporated at room temperature for 4 hours.

Experimental design

A 2^2 factorial design was employed to study the effect of 2 independent variables at 2 levels. Independent variables: concentration of permeation enhancer (SGC) and concentration of chitosan and dependent variable: steady state flux J_{SS}. **Table 1**indicated the actual values and coded/ transformed values of independent variables.

Statistical Data Analysis

And the overall effect of chitosan (CH) and permeation enhancer (SGC) on the permeation of the drug was studied. Design expert® software [trial version 8.0.4 (www.statease.com)] was used to derive a polynomial equation for the design.$Y=b_0+ b_1 x_1+ b_2 x_2+ b_3 x_1 x_2$. Here, Yis the measured response of the dependent variable and x_1, x_2 are the coded levels of independent variables. The term $x_1 x_2$ represent the interaction terms. Here b_0, b_1, b_2, b_3 are regression coefficients of the respective variables and their interaction terms computed from the observed experimental values of Y.

Each batch was evaluated for permeation enhancement and the steady state flux was calculated. The study was carried out in triplicate (n= 3). The data was analyzed by Design expert® software version 8.0.4. The effect of independent variables on the dependent variable here, steady state flux J_{SS} was evaluated by using 1 way ANOVA (analysis of variance). Statistical differences yielding $p \leq 0.05$ were considered significant.

Next the optimized batch obtained from the above experimental design was compared with the same formulation of film containing furosemide in the form of inclusion complex with HP-β-CD. Each formulation was prepared as shown in **Table 1.**

Validation of the model

An optimal J_{SS} value was predicted from the model for a formulation with chitosan concentration of 70 % and permeation enhancer, SGC concentration of 3%. A batch was prepared from it and the response was measured.

Evaluation of the multipolymeric bilayer buccoadhesive films

Physical Characterization of prepared patches

➢ Weight and Thickness of the patch

The $1cm^2$ patches were weighed on a Digital Analytical Balance and the thickness of the patch was measured using Digital Vernier Caliper; an average thickness and weight of 3 films was determined.

➢ Folding Endurance

Films of $1cm^2$ were cut and folding endurance was determined by repeatedly folding film at the same place till it broke. The number of times , the film could be folded at the same place without breaking gave the value of folding endurance.[22]

In- vitro Permeation Study

➢ Preparation of Mucosal Tissue

In vitro permeation study was done using ovine buccal mucosa (sheep, *ovis aeries*) [27]. The ovine buccal mucosa were obtained from animals sacrificed at the local slaughterhouse and were transported to the laboratory in isotonic

phosphate buffer, pH 7.4. The buccal mucosa was carefully removed from the underlying muscle and connective tissue with scissors.

➢ Permeation study

Prepared furosemide bilayer films were subjected to permeation measurement by using ovine cheek mucosa as a substrate using Franz diffusion cell. A mucosa membrane that can cover an area above 2.54 cm^2 was cut and supported on dialysis membranes, which was applied on the receptor chamber of a Franz diffusion cell. Buccoadhesive bilayer film was applied on the mucosa, in such a way that the mucoadhesive layer facing the mucosal surface. 1 ml of simulated salivary fluid, pH 6.2 [44] was filled in the donor chamber and isotonic phosphate buffer pH 7.4 was used in the receptor chamber. At regular time interval of 15, 30, 45, 60, 90 and 120 minutes and then every hour up to 8 hours 200 µL sample was withdrawn from the receptor chamber and replaced by isotonic phosphate buffer pH 7.4. The samples were assayed by the UV spectrophotometer at 229 nm.

Calculation of Steady State Flux (J_{ss})

The amount of drug present in the receptor compartment was determined and plotted as a function of time. The permeability coefficients (P) were calculated from the linear part of the curves as follows[45]:

$$P= (dQ/dt)/(A \times Cd) \quad (2)$$

Where, A= the surface area of diffusion = 1 cm^2, dQ/dt =J= steady state flux = amount of drug permeated per unit time at steady state, and Cd = drug concentration in the donor solution. Here, as furosemide is an acidic drug, having pKa value of 3.8 it remains in ionized form in the pH 7.4. So, transport of furosemide is assumed to be via paracellular path and so the steady state flux for paracellular path is considered. $J_{ss} = P * Cd$ [45].

➢ Enhancement ratio

The permeability of furosemide in presence of sodium glycocholate was evaluated by enhancement ratio. It was calculated as [32]:

$$\text{Enhancement ratio} = P_{SGC}/P_{FUR} \quad (3)$$

Where, P_{SGC} = Permeability coefficient in presence of SGC and P_{FUR} = Permeability coefficient in absence of any permeation enhancer.

Swelling Index and Surface pH

The Bilayered films were weighed (W_1) and placed separately in a Petri dish containing 25 ml of simulated salivary fluid (SSF) [1.632 gm KH_2PO_4, 2.34 gm of NaCl and 0.1257 gm of $CaCl_2$ dissolved in 1 litre of distilled water adjusted up to pH 6.2 with 0.2 M NaOH solution [44]]. The dishes were stored at room temperature. After 5, 15, 30. 45, 60 and 120 minutes time interval, the films were removed and the excess water on their surface was removed using filter paper. The swollen patches were then weighed (W_2) and percentage swelling was calculated by the following formula,

$$\text{Swelling index} = [(W_2-W_1)/W_1] \times 100 \quad (4)$$

The films used for determination of swelling index were used to determine surface pH by using pH paper [41].

Mechanical Properties

The mechanical properties were calculated using a texture analyzer using method similar to described by Mura et al [46]. Film strips of dimension 3 cm x 1 cm were cut and held between two clamps positioned at distance of 20 mm. The pulley was pulled by top clamp at a rate of 5 mm/min to a distance of 5 cm before returning to starting point. The force and elongation was measured as the films broke. The mechanical properties were calculated using following equations.

The tensile strength is defined as the resistance of the material to a force tending to tear it apart and normally identified as the maximum stress in the stress strain curve and it can be calculated as:

$$\text{Tensile strength} = (\text{Force at Failure(gm)})/(\text{Cross sectional area of the film } (mm^2)) \quad (5)$$

The elongation at break is a measurement of the maximum deformation the film can undergo before tearing apart and is calculated as:
Elongation at break= (increase in length(mm))/(initial film length(mm)) ×100 (6)

In vitro Mucoadhesive Studies

- Mucoadhesive Time

The ex-vivo mucoadhesion time was determined by a method adopted by Hassan et al. with slight modification [22, 43]. Study was done by applying the bilayer films on inert support like glass slide over freshly isolated ovine buccal mucosa fixed on slide by tieing it firmly with a thread. The bilayer film was stuck with the mucosa by applying little force with the thumb and this assembly was dipped in 100 ml of SSF (simulated salivary fluid) pH 6.2 in a beaker kept on the magnetic stirrer. The assembly was maintained at 37 ± 2^0C and kept at 50 rpm of stirring rate. The film adhesion was observed for 12 hours and the time when the film detached from mucosal surface was recorded as mucoadhesion time.

- Mucoadhesive Strength

The determination of the mucoadhesive strength was evaluated by using texture analyzer using the method described by Mura et al [46] . These thin films were cut out in 2.25 cm^2. A piece of sheep mucosa was fixed to a support. The film was fixed to the upper support and wetted with simulated salivary fluid (pH 6.2) 50 µL. The upper support was lowered at speed of 1mm/ min to contact with the tissue at a force of 1 N for a contact time of 30 seconds. It was then withdrawn at a rate of 1 mm/min upto a distance of 5 mm. The force needed for detaching the film from the tissue was used to evaluate the bioadhesive strength of the films.

In vitro drug release studies

The in vitro drug release study was carried out using Franz diffusion cell [47, 48]. The diffusion area of Franz diffusion cell was 2.54 cm^2 and the volume of the receptor chamber was 25 ml. The in vitro drug release was carried out in isotonic physiological buffer pH 7.4. Furosemide free drug or as inclusion complex containing buccoadhesive films were applied to previously hydrated dialysis membrane (molecular weight 12-16 kDa) clamped between two chambers of diffusion cell. The membrane was wetted with 0.1 ml of SSF and the donor chamber was covered with aluminum foil to avoid evaporation of fluid. The receptor chamber medium (isotonic physiological buffer pH 7.4) was continuously stirred at 600 rpm using magnetic stirrer. The cells were maintained at a temperature of 37±2 ^{0}C. At set of time intervals, 0.2 ml of samples were withdrawn and replaced by same medium. The amount of drug present was determined by measuring the absorbance of sample at 229 nm by UV spectrophotometer.

Kinetics of drug release

The kinetics of drug release was determined by fitting the best fit of the dissolution data to distinct models [49].

RESULTS

Evaluation of Inclusion Complex

The phase solubility diagram for furosemide/ HP-β-CD is represented in **Figure 1**.The increase in solubility of furosemide occurred as a linear function of HP-β-CD concentration, corresponding to A_L type profile defined by Higuchi and Connors [36, 50]. The apparent stability constant K_S and complexation efficiency CE at 25^{0}C were calculated from the parameters of solubility diagram. The apparent stability constant K_S was calculated to be 130.01 M^{-1} and CE 0.1574.

The DSC thermograms of furosemide, HP-β-CD, their physical mixture as well as their inclusion complex (1: 1.5) prepared by co precipitation method are shown(**Figure 2(A)&2(B)**). Furosemide exhibits a sharp exothermic peak at 222.57^{0}C and the endothermic peak is at 268.8^{0}C. FTIR spectra of furosemide, HP-β-CD, their physical mixture as well as their inclusion complex (1: 1.5) are shown in **Figure 3**.X-Raydiffractograms are shown in **Figure 4**. In vitro drug release data are presented in the graph format. **Figure 5** shows in vitro drug release data for furosemide, physical mixture of furosemide with HP-β-CD (1:1.5) and inclusion complex of furosemide with HP-β-CD (1:1.5). It was found that after time period of 60 minutes pure drug furosemide showed drug release of only 30 % and physical mixture showed around 50 % drug release where as the inclusion complex of furosemide with HP-β-CD showed drug release of about 85%.

Table 1: Levels of Independent Variables for 2^2Facotrial Design for Optimization for Formulation

Independent Variables	Levels			
	Coded Values		Actual Values (%)	
Concentration of CH (%)* X_1	-1	1	55	85
Concentration of SGC (%) X_2**	-1	1	1	5
Dependent Variables	J_{ss}			
Batch code	**P_1**	**P_2**	**P_3**	**P_4**
Concentration of CH (% w/w) (x_1)	**85**	**85**	**55**	**55**
Concentration of SGC (% w/w) (x_2)	**1**	**5**	**1**	**5**

***The concentration of chitosan constitutes as % of total polymer weight = 600 mg.*
***The concentration of Sodium glycocholate is taken as % of total polymer weight.*
Rest all parameters of formulation were kept constant. Total weight of polymer = 600 mg; volume of 0.5 % acetic acid = 25 ml; volume of glycerine= 0.5 ml; volume of ethanol= 15 ml; stirring speed of propellor stirrer= 750 rpm; diameter of petry dish= 70-72 mm

Table 2: Evaluation of multipolymeric bilayer buccoadhesive films

Batch code	Thickness of mucoadhesive layer (mm)	Thickness of bilayer (mm)	Folding endurance	J_{SS} (steady state flux) (µg/cm^2/hr)	Permeability Coefficient (cm/hr)	Enhancement ratio
P_1	0.373 ± 0.01	0.440 ± 0.01	>100	164.63 ± 11.85	0.032 ± 0.002	0.998
P_2	0.320 ±0.03	0.526 ± 0.04	>100	199.03 ± 13.79	0.039 ± 0.002	1.209
P_3	0.246 ± 0.01	0.650 ± 0.04	>100	138.73 ± 10.61	0.027 ± 0.002	0.842
P_4	0.210 ± 0.01	0.330 ± 0.02	>100	143.13 ± 10.01	0.029 ± 0.001	0.885
P_5	0.330± 0.01	0.514 ± 0.04	>100	206.5± 10.09	0.0413± 0.002	1.255

Table 3: Response: JSS - Analysis of variance for selected factorial model

Coefficient	Numerical value	p- value
b_0	161.38	0.0008
b_1	20.45	0.0003
b_2	9.70	0.0204
b_3	7.50	0.0567
Regression		**0.8625**

** Model generated, $J_{SS} = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_1x_2$ .Values of "p- value" less than 0.0500 indicate model terms are significant. In this case x_1 and x_2 are significant model terms. Polynomial equation for the model, $J_{SS}=161.38+20.45 x_1+ 9.70 x_2+ 7.50 x_1x_2$*

Table 4: In vitro dissolution profile of films containing furosemide P_2 and furosemide- HP-β-CD complex P_5

Time in hours	% Drug release	
	P_2	**P_5**
0.25	7.04 ± 0.18	2.24 ± 0.90
0.50	12.37± 0.36	4.90 ± 0.55
0.75	15.82 ± 0.22	8.24 ± 0.60
1	35.69 ± 0.22	17.20 ± 1.37
2	38.93 ± 0.38	21.76 ± 0.10
3	42.27 ± 0.16	28.05 ± 0.91
4	54.75 ± 0.16	44.51 ±0.62
5	68.08 ± 0.61	64.53 ± 0.53
6	68.94 ± 0.86	66.34 ± 0.64
7	69.19 ± 0.37	79.53 ± 0.48
8	69.79 ± 0.80	90.95 ± 0.55

Table 5: Correlation coefficients for different models for batch P_2 and P_5

Model	R^2 value (furosemide) P_2	R^2 value (inclusion complex) P_5
Zero order release model	0.8777	0.9870
First order release model	0.7063	0.7536
Hixson Crowell model	0.9132	0.9605
Higuchi's model	0.9450	0.9450
Korsmeyer'sPeppas model	0.9370	0.9604

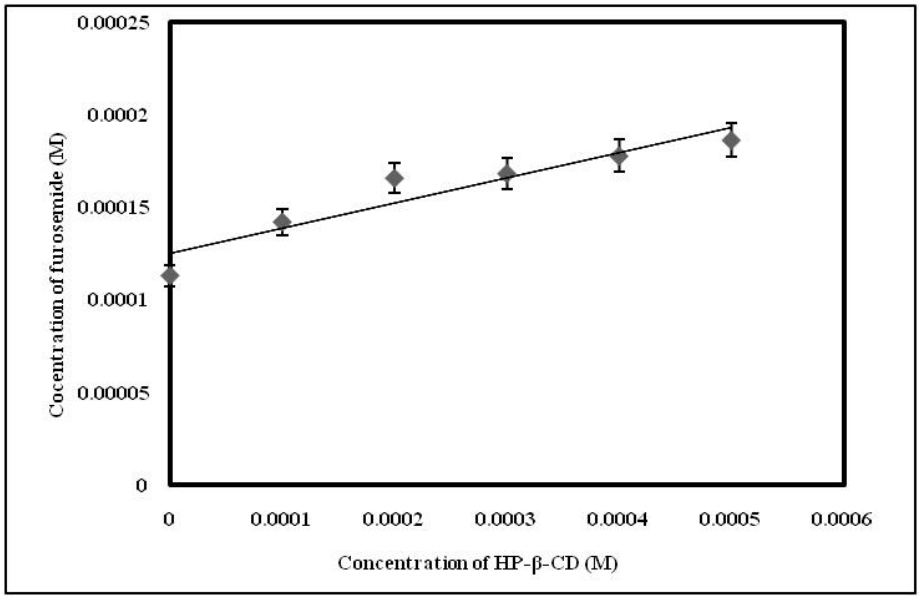

Figure 1: Phase solubility curve (Concentration of furosemide (M) Vs Concentration of HP-β-CD (M))

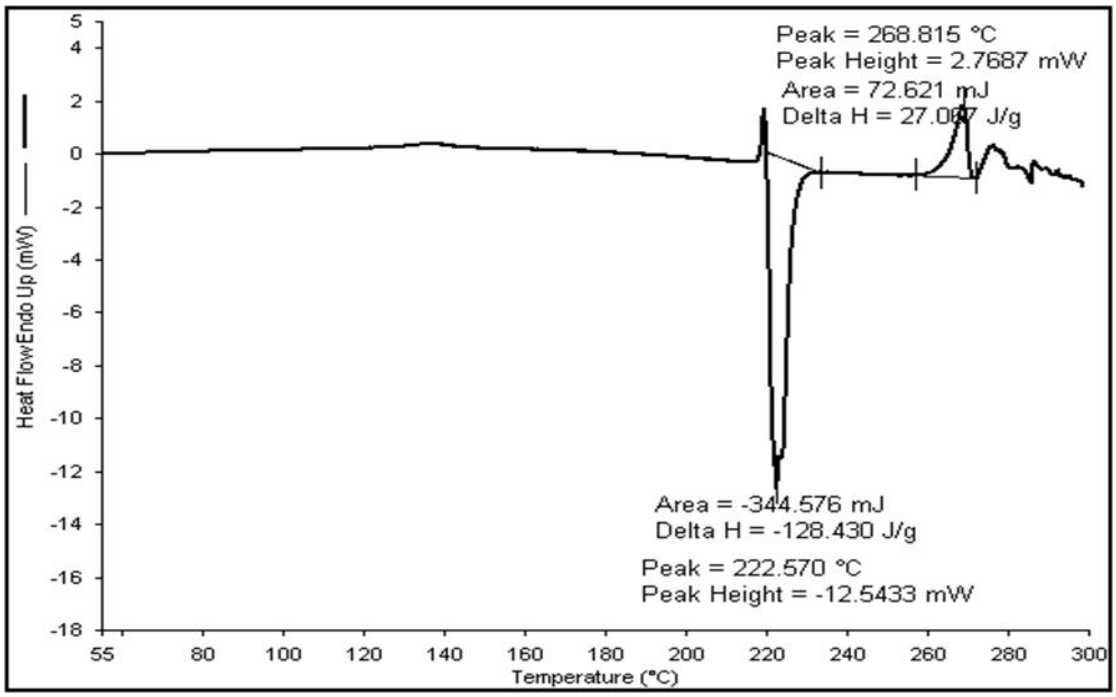

Figure 2(A) DSC thermograph of furosemide

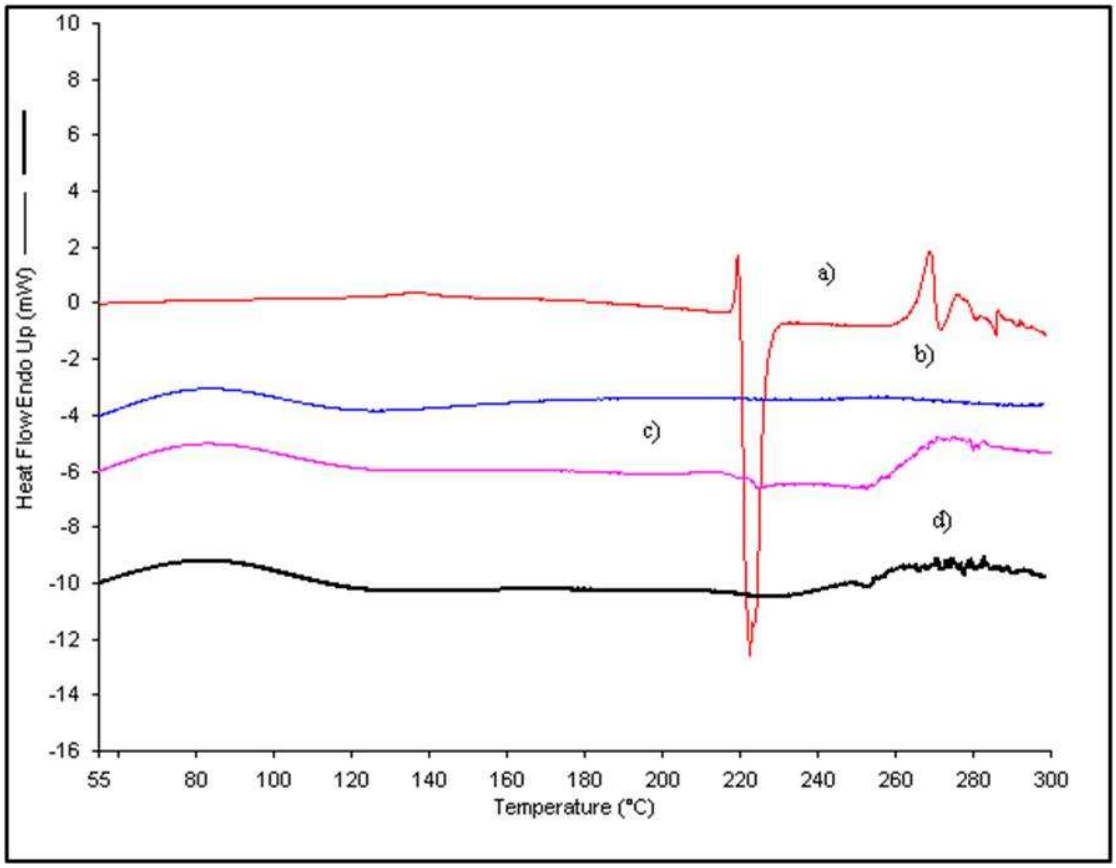

Figure 2(B) Overlay DSC thermographs of a) Furosemide; b) HP-β-CD; c) physical mixture of furosemide: HP-β-CD (1: 1.5) d) furosemide: HP-β-CD complex (1:1.5)

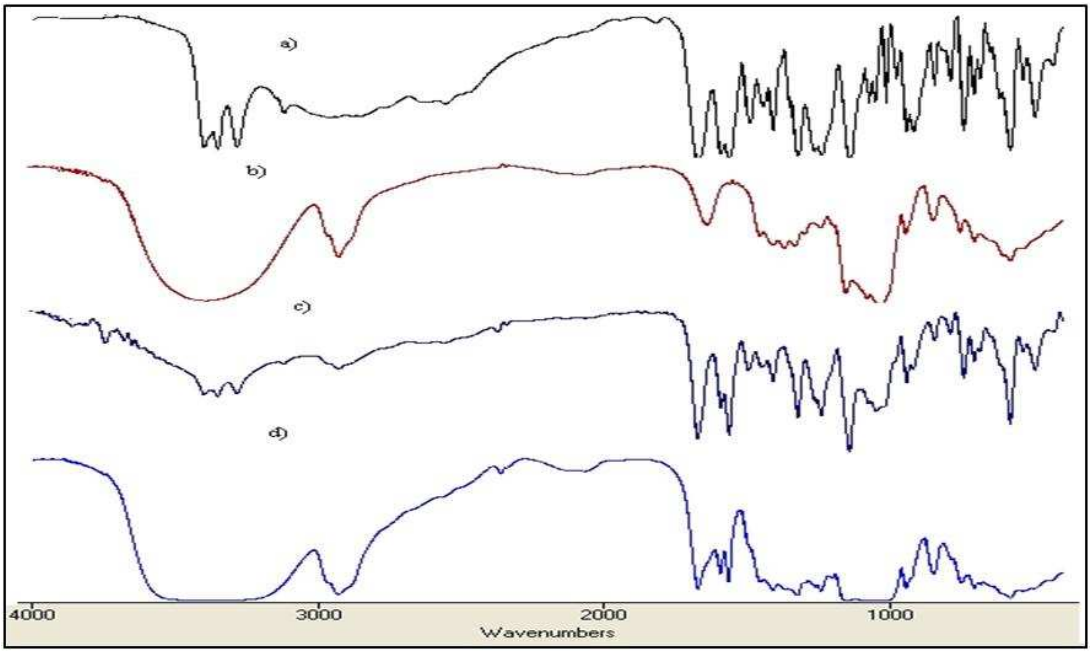

Figure 3: FTIR spectra for (a) furosemide; (b) HP-β-CD; (c) physical mixture of furosemide: HP-β-CD (1:1.5); (d) inclusion complex furosemide: HP-β-CD (1:1.5)

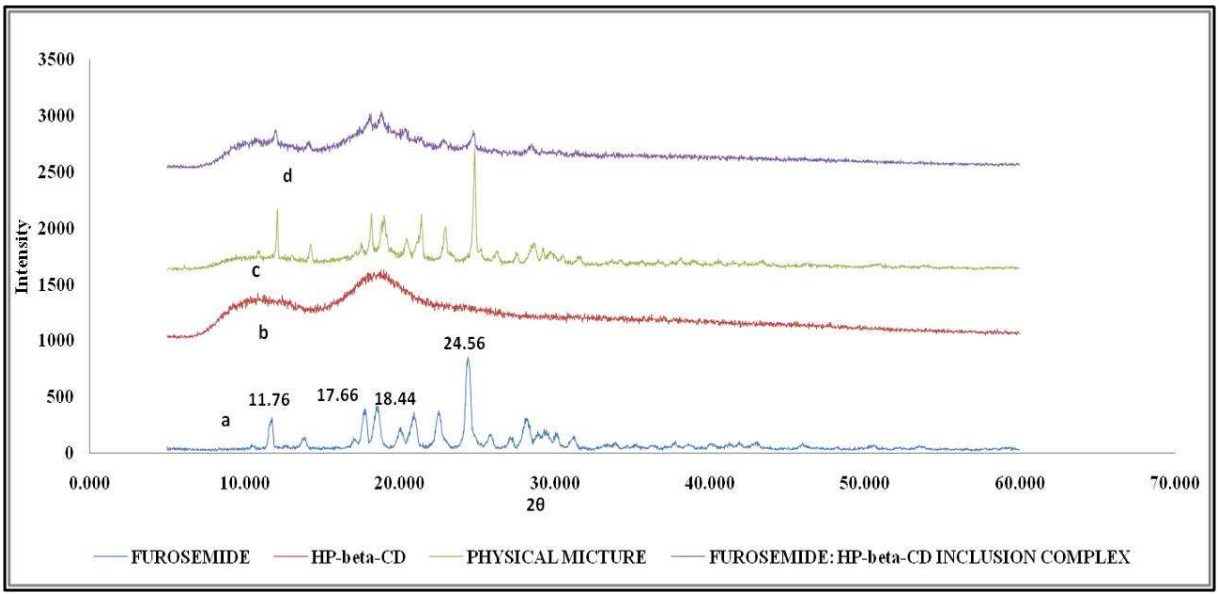

Figure 4: X ray diffractograms of (a) furosemide; (b) HP-β-CD; (c) their physical mixture (1:1.5; drug: HP-β-CD) and (d) inclusion complex (1:1.5; drug: HP-β-CD)

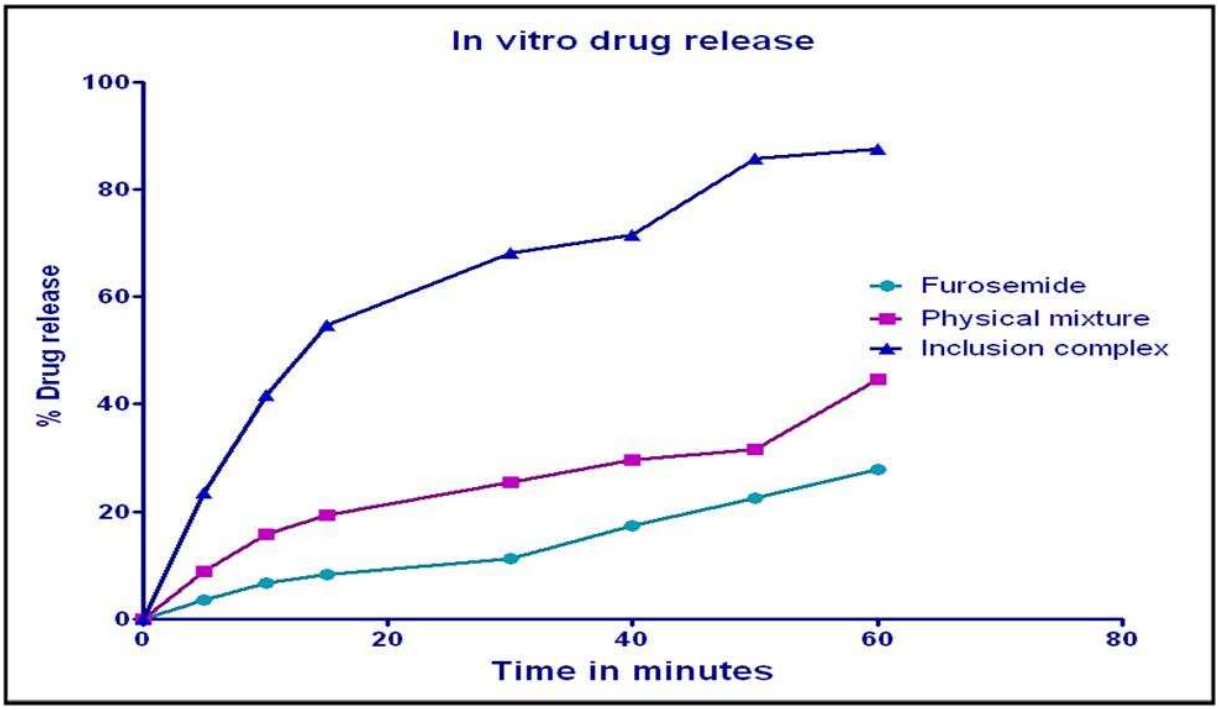

Figure 5: In vitro drug release data for furosemide, physical mixture of furosemide with HP-β-CD (1:1.5) and inclusion complex of furosemide with HP-β-CD (1:1.5)

Evaluation of multipolymeric bilayer buccoadhesive films

The films obtained after employing 2^2 factorial design were evaluated for general appearance. Films of all batches showed good appearance with least amount of air bubbles. The batches were prepared in triplicate and batches were evaluated for permeation study and Jss was calculated. The batches were also evaluated for physical appearance and thickness and folding endurance. **Table 2** shows results of thickness, folding endurance for the batches of factorial design.

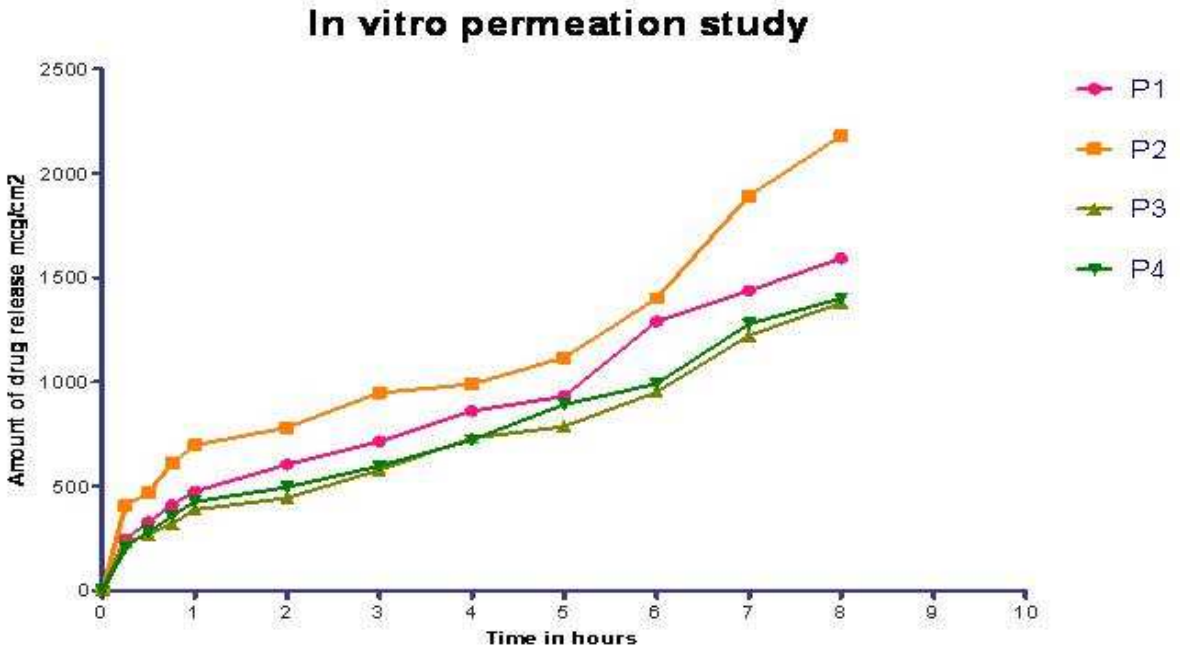

Figure 6: Amount of drug permeated Vs time for batches P_1 – P_4

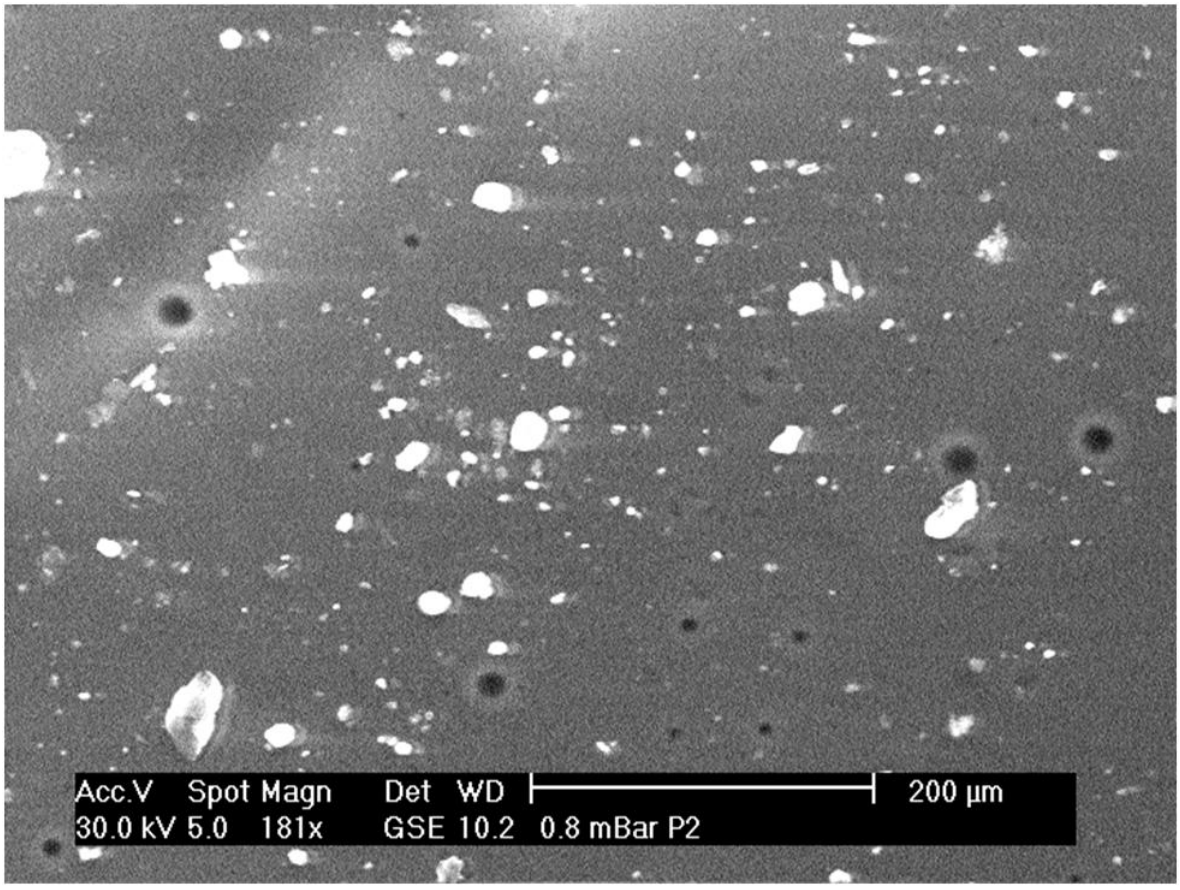

Figure 7: SEM image of mucoadhesive layer of film

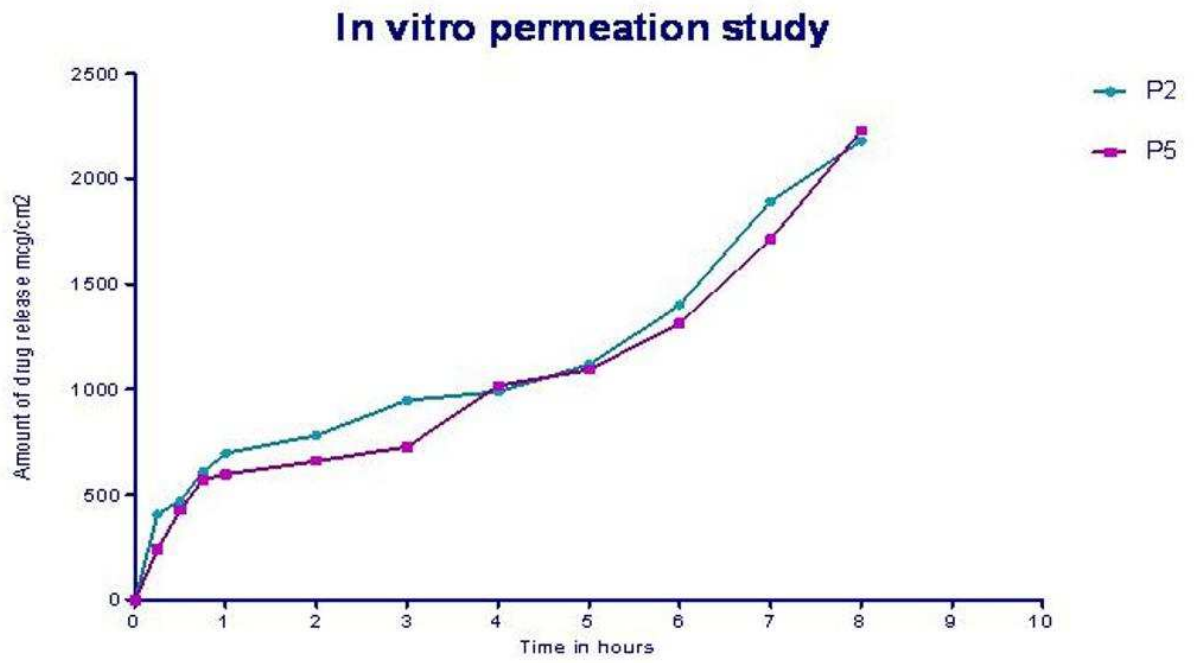

Figure 8: Comparison of amount of drug permeated versus time for films containing furosemide (P_2) and furosemide complex (P_5)

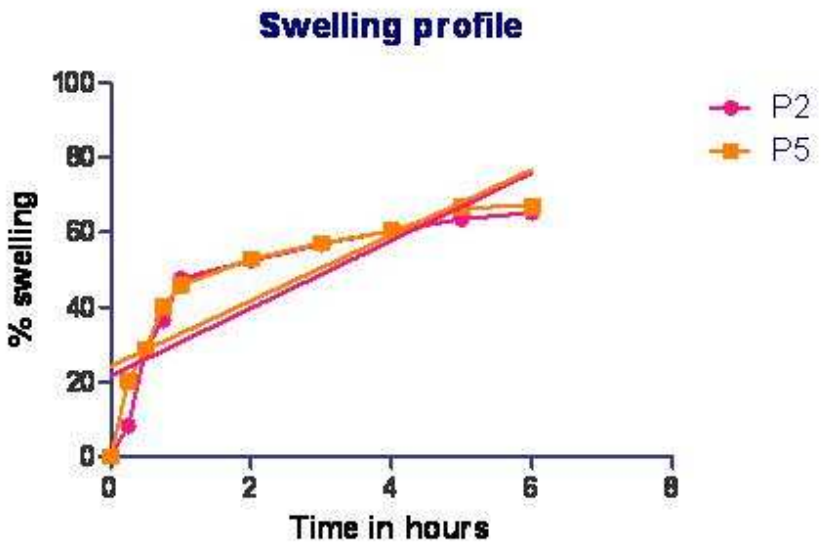

Figure 9: Swelling study of batch P_2 and P_5

Optimization of formulation for multipolymeric bilayer buccoadhesive film was done using a 2^2 full factorial design. The response measured was J_{SS} (steady state flux). The optimized batch was selected (P_2) on the basis of higher J_{SS}. The steady state flux (J_{SS}) was calculated by plotting a graph of amount of drug (µg) permeated per cm^2 verses time (hours); the straight line of the graph gives us the value of J_{SS} and Permeability coefficient [45] The results of J_{SS} were analyzed using Design Expert software. The response J_{SS} are presented in **Table 2**. **Figure 6** shows permeation study results of all four batches graphically. The enhancement ratio for both concentrations of SGC was calculated. These observations depict that with the increase in concentration of SGC the permeation increases.

SEM (scanning electron microscopy) was done for the film of best batch P_2 and it was found that furosemide was evenly distributed in the matrix of the polymers. Also, cavitization due to evaporation of ethanol was observed.

There was a phase separation and precipitation of drug in the matrix. **Figure 7** shows SEM image of mucoadhesive layer film.

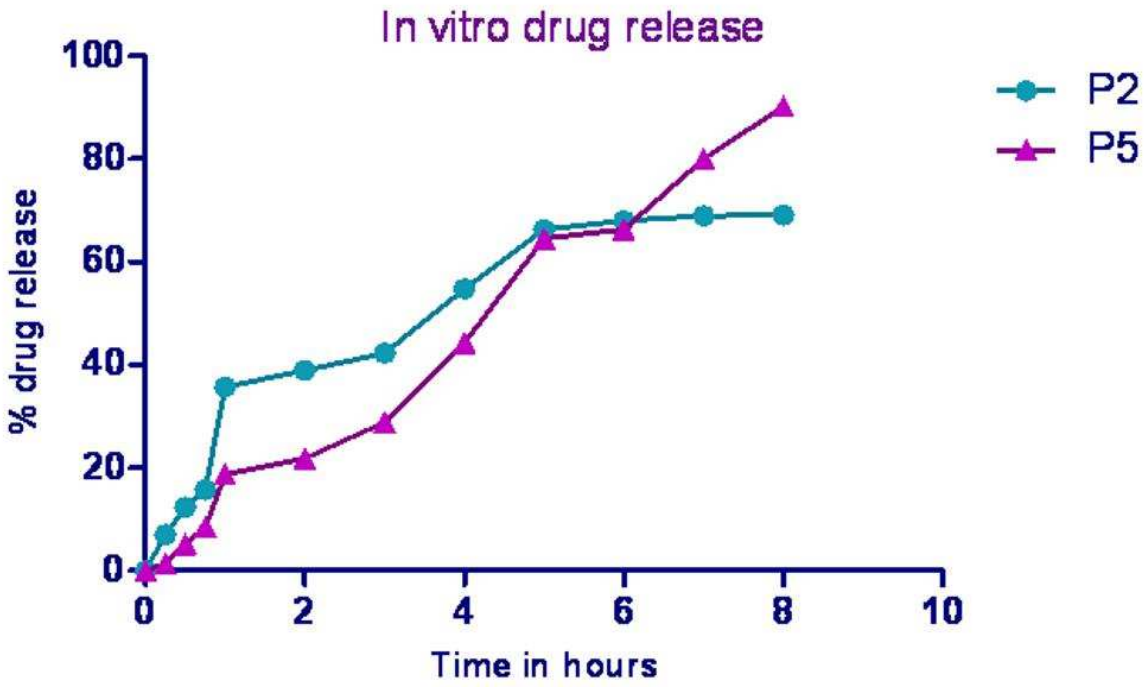

Figure 10: In vitro drug release profiles for films containing furosemide and furosemide-HP-β-CD complex

A batch was prepared which contained the same formulation of P_2 but furosemide was incorporated in the form of inclusion complex with HP-β- CD, this batch was coded as P_5. The thickness and folding endurance of the films were in acceptable range. The permeation study was done for complex containing buccoadhesive film and the obtained value of J_{SS} was compared with that of batch P_2. The value of J_{SS} and permeability coefficient and enhancement ratio for Batch P_5 are given in **Table 2**.The statistical analysis data through regression model is presented in **Table 3.**Comparison of amount of drug permeated versus time for films containing furosemide (P2) and furosemide complex (P5) is shown in **Figure 8.**

The swelling index of the film after a period of 6 hours was recorded. After 6 hours it was observed that the film due to over hydration disintegrated to particles.**Figure9** shows % swelling curves for two formulations P_2 and P_5. Surface pH evaluation of films is an important study to investigate possibility of irritation to the buccal mucosa. Acidic and alkaline pH causes irritation to the buccal mucosa. The surface pH was found to be in the range of 6 to 7 for both the films containing furosemide alone and furosemide inclusion complex [22, 51].

The mechanical properties of films describe the ability of film to with stand the damage during mouth activities. The mechanical properties of the films were evaluated using Texture analyzer. Here two parameters were measured, tensile strength and elongation at break which were obtained from load versus distance graph. Both the BatchesP_2 and P_5were found to have brittle nature. The film strength was not good enough. Due to less elasticity the films were broken easily. Here, the mucoadhesion time was determined for the bilayer film. It was observed that the backing layer detached earlier and got separated from the mucoadhesive layer. After a time of 90 minutes the backing layer detached in both the filmsP_2 and P_5. Also mucoadhesive time for both the batches was found to higher enough up to 300 minutes and above.

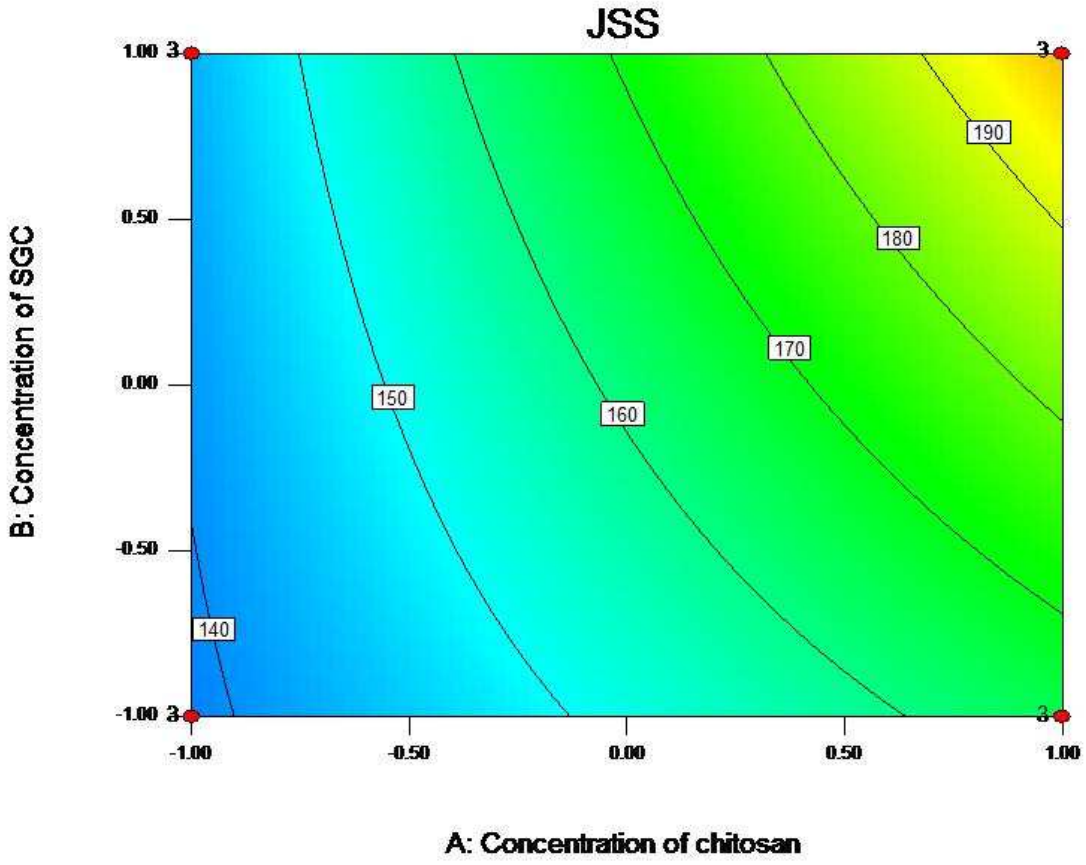

Figure 11(a) Contour plot

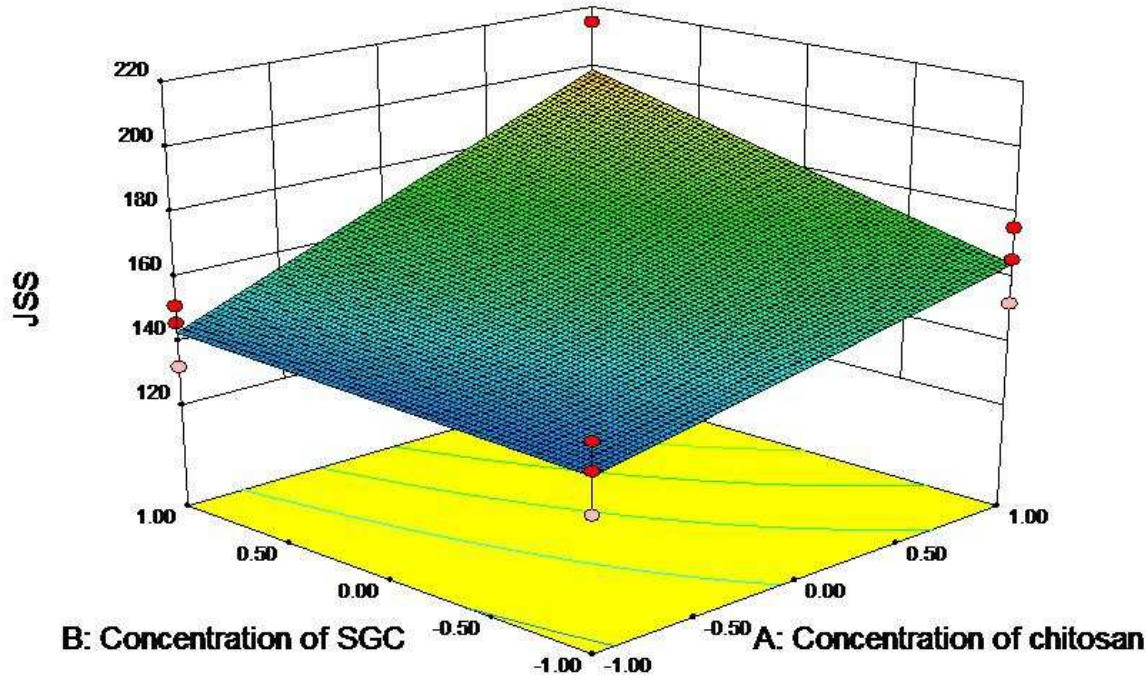

Figure 11 (b)Response surface plot for the factorial design

Mucoadhesion is considered to occur in different stages, namely wetting, interpenetration and mechanical interlocking [22, 43]. The mucoadhesion is dependent on factors like hydration of polymer, contact time with mucus, degree of swelling of polymer, mucous surface used etc. The mucoadhesive strength was determined as the force of detachment or force of adhesion. Here, adhesiveness was measured. The force of adhesiveness was measured and it was found to be 45 gm and 66 gm for films containing furosemide (P_2) and inclusion complex (P_5) respectively. Both the films do not show significant difference in mucoadhesive force p= 0.1190 (p> 0.05).

Table 4&**Table 5**shows dissolution profile of films containing furosemide P_2 and furosemide- HP-β-CD complex P_5 and Correlation coefficients for different models for batch P2 and P5respectively. **Figure 10** shows in vitro drug release profile comparison for batch P2 and P5 respectively.

DISCUSSION

The value of K_S from the phase solubility study indicates fair affinity of furosemide for complexation with HP-β-CD. Thus, HP-β-CD is a good complexing agent for furosemide[52] showed 11 fold increase in solubility of furosemide with HP-β-CD. The value of CE defines the number of molecules of HP-β-CD which take part in the complexation process and forms complex with the drug. The value 0.1 of CE indicates that 1 out of 11 cyclodextrin molecules may take part in complexation with drug. Researchers have calculated CE for 28 different drugs and found that CE value on an average was 0.3 and concluded that one out of four cyclodextrin molecules takes part in complexation with drug[37]. Here the results indicate that more HP-β-CD molecules are in free form in the system and concentration of HP-β-CD in complex form is less, thus indicating fair affinity of furosemide with HP-β-CD for complexation. The solubility of furosemide in water is 37.25 µg/ mL thus less amount of furosemide is in dissolved form in the system for inclusion complex formation.

The FTIR spectra of physical mixture shows peaks representing presence of furosemide and HP-β-CD and no peaks of interaction product, so it indicates there is no interaction between drug and HP-β-CD. In the spectra of furosemide: HP-β-CD inclusion complex 1:1.5 the peaks of furosemide due to NH stretch near 3398 cm^{-1} due to C-NH_2 have disappeared. Also, the characteristic peaks of HP-β-CD appear in the inclusion complex spectra. These spectral changes confirm formation of inclusion complex between furosemide and HP-β-CD.

The disappearance of the endo or exothermic peaks in DSC thermographsof drugs is usually indication of formation of inclusion complex[7]. The absence of peak of furosemide at 222.57^0C in the **Figure 2(B)** is strong evidence of formation of inclusion complex.

The X-Ray diffraction pattern of furosemide exhibited sharp, highly intense and less diffused peaks indicating the crystalline nature of drug, as shown in **Figure 4(a)**. However the x-ray diffraction patterns of the HP-β-CD have amorphous structures **Figure 4(b)**. The physical mixture of furosemide: HP-β-CD and were simply a superimposition of each component with respect to the peaks of furosemide **Figure 4(c)**. The inclusion complexes of furosemide with HP-β-CD physical mixture (1:1.5) show undefined, broad, diffuse peaks of low intensities **Figure 4(d)**. The inclusion complexes of furosemide with HP-β-CD prepared by co-precipitation method (1:1.5) shows a broad large background and the crystalline peaks of furosemide have diminished. The peak corresponding to 24.56° (2θ) in furosemide is decreased in intensity in the case of inclusion complex and this is a proof for the intimate changes at the lattice level during interpenetrations of the two substances. This feature indicates the formation of a significant amount of amorphous material. The diffractogram of furosemide, HP-β-CD, their physical mixture differs from that of inclusion complex. Similar results were found by Spamer et al. for complexation of furosemide with HP-β-CD [7].

In vitro drug release of complex showed marked increase in drug release after complex formation. Thus solubility of drug increases by formation of inclusion complex with HP-β-CD. Thus solubility and hence the dissolution of complex increases by formation of inclusion complex.

Multipolymericbilayer buccoadhesive films

Polynomial equation for 2^2 full factorial design involving the main effect and interaction factors were determined based on estimation of statistical parameters such as multiple correlation coefficient, adjusted multiple correlation coefficient, and the predicted residual sum of squares generated by Design-Expert® software. A mathematical relationship was generated between dependent and independent variables using the Design expert software. The

response polynomial coefficients were determined in order to evaluate the response. Each response coefficient was studied for its statistical significance by p- value. Thus, non-significant response coefficients were deleted, and the significant polynomial response equation for J_{SS} was generated. Response surface analyses plotted in three-dimensional model graph for depicting the effects of the predetermined factors on the response J_{SS} was generated. The qualitative effect of each variable on the response parameter could be visualized by response surface plot.

Reduced model generated which represents the quantitative effect of formulation parameters on J_{SS} (steady state flux),

$$J_{SS} = 161.38 + 20.45\ x_1 + 9.70\ x_2 \quad (7)$$

The contour plot and surface response plot of the model are presented in **Figure 11(A) and 11 (B)** respectively.

It shows that CH concentration and concentration of SGC has a significant positive effect on the response J_{SS} ($p < 0.05$). This observation depicts that increase in concentration of chitosan as well as SGC increases the J_{SS}. Similar results have been reported in prior work[53-58] ;[59]. The enhancement effect of chitosan in gel form for oral mucosa was investigated with a large bioactive peptide, transforming growth factor-² (TGF-²) and it was found that chitosan has a marked permeabilizing effect on buccal mucosa for peptide drug [25]. The permeation enhancement properties of chitosan is favored because of higher mucosal adhesivity[23]. Permeation enhancement property is due to its mechanism of opening tight junction complexes and partial alteration of cytoskeleton. The structural properties i.e. degree of deacetylation and molecular mass determines the absorption enhancing properties [23].Buccal mucosa is lined by non keratinized squamous epithelium supported by connective tissue. The permeability barrier is due to intercellular materials derived from membrane coating granules found in both keratinized and non keratinized epithelia. These intercellular spaces contain neutral lipids and glycolipids. The permeation enhancement effect seems due to repacking of epithelial cells up to basal membrane and disarrangement of the desmosomes. The intercellular spaces between contiguous cells enlarged folding contact with polymer, due to drainage of fluids from basal layers. This behaviour modifies or disrupts the lipid lamella that represents the principal barrier, causing a permeabilizing effect. Chitosan is supposed to have the similar effect on the lipid lamella [23]. The permeability enhancing effect is also partially attributed to higher mucoadhesion of chitosan compared to other polymers[60].

Chitosan increases permeation via other routes also i.e., transdermal, intestinal, nasal, and vaginal.[53, 56, 57, 61].

Also, the concentration of SGC has a significant effect on permeation enhancement and J_{SS} increases with the increase in concentration of SGC. Similar findings have been reported priorly[62-66]. The effect of SGC concentration on transbuccal permeation of morphine sulphateshowed that increase in concentration increased of SGC increased the permeation of morphine sulphate[31]. The concentration effects of bile salts on permeability of molecules have been studied widely. In general the concentration of bile salts above CMC (critical micellar concentration), is the basic mechanism of permeation enhancement [31]. Also presence of sodium glycocholate on permeation of acyclovir via buccal route and found that it increase permeability 2 to 9 times [32].

It can be concluded that concentration of CH and SGC has a significant effect on the permeation enhancement. From the polynomial equation it can be presented that the value of the coefficient of x_1 is higher than coefficient of x_2, thus chitosan concentration has higher effect than SGC concentration. The permeation study was done for complex containing buccoadhesive film batch P_5 and the obtained value of J_{SS} was compared with that of batch P_2. The difference in the J_{SS} value of inclusion complex and free drug containing film was found to be significant. The p-value for t test was 0.0117 ($p < 0.05$) compared to J_{SS} of batch P_2.

The reason for enhancement of the permeation can be the increase in solubility of drug by incorporation in the form of inclusion complex. Also, cyclodextrins act as permeation enhancers for transbuccal route [30]. The effect of cyclodextrins on enhancement of permeability via buccal route for omeprazole has been studied[30]. HP-β- CD also increased the amount of drug permeation of Carvediol[59]. The mechanism of permeation enhancement can be inclusion of membrane compounds by the cyclodextrins[59, 67].

PVP K30 aids to the process of hydration for swelling of the film. P_2 and P_5 batches contain 15% w/w of PVP K 30. PVP K 30 is supposed to increase the surface wettability and consequently water penetration within the matrix. Also, film hydration properties are supposed to increase with increase in concentration of PVP K30. The swelling

index was found to be dependent on concentration of PVP K 30 and increased at higher concentration of PVP K30 [22, 27]. It was also observed the profile for water hydration stops after certain time; this is because of poor solubility of chitosan in water thus liquid uptake stops after certain limit. Similar results were found by Rossi et al [22, 68]. As similar results were found for buccoadhesive films containing drug in the form of inclusion complex, it can be presented that cyclodextrin do not have a significant influence swelling study. Contrary results have been reported where the swelling behaviour of the film is decreased due to presence of cyclodextrins[48].

The mechanical properties of films describe the ability of film to with stand the damage during mouth activities. Here two parameters were measured, tensile strength and elongation at break which were obtained from load versus distance graph. Both the formulation was found to have brittle nature. Due to less elasticity the films were broken easily. High strain value, moderate tensile strength and low elastic modulus are indicative of strong soft and elastic film [46]. The tensile strength of films containing drug and inclusion complex had values of 0.19 and 0.21 g/mm^2. The tensile strength is higher in the films containing inclusion complex and the difference is significant (p= 0.03). The concentration of polymers was same in both the films. So, this indicates some interaction of the polymer and the complex present in the film renders change in tensile property. The concentration of chitosan 85% w/w and drug as free form gives elongation at break of 31.2 % where as the films contain inclusion complex has 16.6 %. Elongation at break is decreasing with inclusion complex incorporation. But the difference between the values of elongation was not significant as p= 0.1887 (p> 0.05). The graph of load versus distance shows, increase in the length of film due to stretching of the film between the probes, the sudden break point in the film is indicated by a sudden decline of the graph. Force at break for films containing furosemide and furosemide inclusion complex were, 38 g and 41 g respectively. And increase in length for films containing furosemide and furosemide inclusion complex were, 6.64 mm and 3.31 mm respectively. The low tensile properties may be attributed to lower concentration of glycerin. Similar result were reported, CMC films were studied for tensile properties with different concentration of plasticizer, glycerol and it was found that with increase in concentration of glycerol tensile strength decreased, but percentage strain at break increased [69]. Studies also show that the tensile strength increases with addition of chitosan to polymeric composition of film [26, 51]

The higher mucoadhesion time can be attributed to large volume of hydrating fluid present to hydrate the film and also, the force applied by thumb to stick the film to the mucosal surface and to presence of PVP K30 in the matrix. Both the films do not show significant difference in mucoadhesive force p= 0.1190 (p> 0.05).

Chitosan was presented to have good mucoadhesive properties due to cationic nature which allows interaction with mucosal surface and hence attachment [26]. Contrary chitosandid not show good mucoadhesive properties and the adhesion forces had very lower values. This can be due to hydrophobic nature of chitosan, that it does not interact with mucosal surface in short time period, and also, hydration of films was not adequate to show adhesiveness to mucosal surface. Similar observation has been reported[46]. Also glycerin content of 5% or higher show good bioadhesive properties [46]. But the amount of glycerin used in the formulation was less than 2% v/v thus it can be assumed that lower amount of glycerin could be the reason for less mucoadhesive properties. So, the batches containing furosemide and inclusion complex showed poor mucoadhesive properties.

The drug release pattern of the buccoadhesive film showed a sustained release pattern, for batch P_2 and P_5. The release of drug from the film showed a burst effect in initial hours and then it showed a sustained effect for film P_2[46]. Also higher drug release can be attributed to presence of hydrophillic polymer PVP K30 which imparts good swelling properties to the film. PVP K30 dissolves creating pores in the film structure for drug to diffuse but due to higher concentration of chitosan the gelling barrier is more so, drug release is incomplete [29].

Films containing furosemide in the form of inclusion complex with HP-β-CD showed higher drug release. The burst release pattern was not found and it showed a sustained release of drug. This result indicates the increases in solubility of furosemide in the form of complex hence increase in dissolution and thus increase in the improved dug release.

Similar results were observed for the release kinetic of atenolol and its complex and flufenamic acid which followed the higuchi's model [46, 48]. It was found that swellable type of systems follow this type of mechanism where drug release is affected by rate of penetration of liquid and relaxation rate of polymeric chains [46].

The higuchi's model represents two limit cases in the transport and drug release phenomenon. In the drug release phenomenon of the film, the drug molecules have to diffuse across unstirred aqueous layer on the membrane surface in the donor compartment followed by diffusion across semi permeable membrane to the receptor compartment. The observed release kinetics indicates that the drug across the membrane surface is a rate limiting step-in overall drug release process [38]. Also the swelling of polymer in the presence of simulated salivary fluid, the extent of swelling depends on the type of polymer, thus it also creates an additional diffusional pathway for the drug molecules.

The complex containing film follows Zero order kinetics. Complex formation facilitated the diffusion of drug across the membrane and hence increased the dissolution of drug. The drug can pass through semi permeable membrane and also the complex as the pore size of membrane was 12-14 kDa. The drug release thus increased with formation of complex of furosemide with HP-β-CD. There is higher concentration of furosemide available due to increase in solubility after complexation; hence more amount of drug diffuses out of the polymeric matrix.

Also due to good swelling properties, of the polymeric films, the drug release is facilitated. Due to the presence of hydrophilic polymer PVP K30, the hydration of films higher and formation of pores and channels in the matrix structure increases the amount of drug release.

CONCLUSION

It can be concluded that the buccoadhesive films of FUR can be prepared and permeability of furosemide can be enhanced by using permeation enhancer. CH and SGC in highest concentration enhance the permeability of FUR via transbuccal route. Also incorporation of drug in the form of complex increases the drug release. Thus simultaneous enhancement of solubility and permeability can be carried out by this approach. The major challenge for BCS Class IV drug can be thus addressed. In addition to that swelling time, mucoadhesive time, mucoadhesive strength and film properties have impact on buccoadhesive drug delivery. Thus, the strategy of incorporation of permeation enhancer and drug inclusion complex in buccal films can be upcoming new technology for buccal drug delivery.

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