Chapter 4
Experimental
(5-Fluorouracil)

4.1 MATERIALS AND METHODS:

4.1. ***Materials:*** Table 4.1 lists the materials obtained ex gratis or purchased.

Table 4.1. List of materials

Chemical/Material	Source/Manufacturer
5-Fluorouracil	Dabur India Ltd., Sahibabad
Methotrexate	Sun Pharma, Mumbai
HPMC K4M	Colorcon Asia Ltd, Mumbai
HPMC K15M	Colorcon Asia Ltd, Mumbai
Lactose monohydrate (Tablettose-100)	Meggle, Germany
Magnesium stearate	Suvidhanath chemicals, Vadodara
Ethyl cellulose, 20 cps	Dow Chemicals, Mumbai
Gantrez MS 955	ISP Ltd, Mumbai
Gantrez S 97	ISP Ltd, Mumbai
Gantrez AN 139	ISP Ltd, Mumbai
Glycerol	SD Fine Chemicals, Mumbai
Poloxamer 407	BASF, Mumbai
Polyethylene glycol-200	SD Fine Chemicals, Mumbai
Propylene glycol	SD Fine Chemicals, Mumbai
Sodium deoxycholate	SD Fine Chemicals, Mumbai
Sodium tauroglycocholate	SD Fine Chemicals, Mumbai
Sodium dodecyl sulphate	Himedia labs, Mumbai
EDTA(Ethylenediamine tetra acetic acid)	Suvidhanath, Baroda
Ethanol, AR grade	E Merck, Mumbai
Methanol, AR grade	Spectrochem, Mumbai

Chemical/Material	Source/Manufacturer
Acetone, AR grade	SD Fine Chemicals, Mumbai
Methanol (HPLC grade)	SD Fine Chemicals, Mumbai
Ethyl acetate (HPLC grade)	E. Merck India Ltd., Mumbai
Acetonitrile (HPLC grade)	SD Fine Chemicals, Mumbai
Trichloroacetic acid	SD Fine Chemicals, Mumbai
TRIS(tris-hydroxymethyl aminomethane)	Himedia Labs, Mumbai
Sodium dihydrogen phosphate. $2H_2O$	SD Fine Chemicals, Mumbai
p-Aminoacetophenone	Loba Chemie, Mumbai
Potassium dihydrogen phosphate	SD Fine Chemicals, Mumbai
Disodium hydrogen phosphate	SD Fine Chemicals, Mumbai
Hydrochloric acid	SD fine Chemicals, Mumbai
Sodium hydroxide	SD fine Chemicals, Mumbai
^{99m}Tc	Board of Radiation and Isotope Technology, Delhi
Diazepam injection	Intact Drugs Pharmaceuticals Ltd., Panipat
Ketamine HCl Injection	Themis Medicare, Vapi
Stannous chloride	Sigma Chemicals Co. St. Louis
5-Bromouracil	Loba Chemie, Mumbai
Membrane filters, nylon (0.45µ)	Millipore, Bangalore
Synthetic cellulose membrane (mol.cut off value-12,000)	Himedia Labs, Mumbai
Sodium lauryl sulphate	Himedia Labs, Mumbai
Formaldehyde	QualigenFineChemicals,Mumbai
Agar	Himedia Labs, Mumbai

* FU is reported to be light sensitive, so proper care was taken during formulation , processing and evaluation.

4.2 *IN VITRO* PERMEATION STUDIES:

Guinea pig buccal tissue was chosen, because its non keratinized morphology is reported to be quite similar to human buccal epithelium (Deneer et. al., 2002). Buccal tissue was removed after sacrificing the animal by overdose with pentobarbitone administered by intravenous injection.

Buccal tissue was stored in Phosphate buffer (pH 6.8) and immediately transported to the experimental setup. The buccal mucosal membranes were separated by removing the underlying connective tissues using surgical scissors, making sure that the basal membrane was still present. The tissue was rinsed and then stored in ice-cold PBS until mounted in the Franz diffusion cell (within 1 h upon removal) (Attia et al, 2004). Membrane thickness ranged from 2.2 to 2.5 mm.

The isolated buccal mucosa was mounted between donor and receiver chambers of the Franz diffusion cells with a diffusion area of 3.7 cm^2 and a compartment volume of 20 ml. After an equilibrium period of 30 minutes with phosphate buffer on receptor side, 2 ml FU solution (0.5%w/v) in phosphate buffer was filled on the donor side. The diffusion studies were carried out at 37 ±1°C. Samples of 1 ml were taken from the acceptor side at 1 hour intervals for 8 hours, replaced with the same amount of fresh buffer solution, and subsequently assayed using UV spectrophotometer at 267 nm.

The cumulative amount of permeated drug (from mucosal side to serosal side) was plotted versus time, and the flux was calculated from the steady state part of the curve. The permeability coefficient (Kp) was calculated using equation 4.1.

$$Kp=(dQ/dt)/(\Delta C.A) \qquad \text{-----(4.1)}$$

dQ/dt = Slope from steady state portion of the curve

ΔC = Concentration difference across the mucosa

A= Area of diffusion

The efficacy of the different enhancers was determined by comparing the permeation rate of FU in the presence and absence of enhancers. It was defined as

the enhancement factor (EF) which was calculated using equation 4.2 (Shin et al, 2004):

Enhancement Factor,

$$EF = \frac{\text{FU permeation rate at steady state in the presence of enhancer}}{\text{FU permeation rate at steady state in the absence of enhancer}} \quad \text{---- (4.2)}$$

4.3 FORMULATION OF BIOADHESIVE PATCHES FOR 5-FLUOROURACIL (FU):

FU (200mg) was added to water (12ml) and stirred to solubilise at 50°C. After cooling, glycerine and HPMC K4M were added and stirred to yield a clear solution. In another beaker, Gantrez MS-955 was added to water and stirred so as yield a clear solution. Gantrez solution was poured in the FU containing beaker. To this solution ethanol (10 ml) was added. This was mixed for 1 hour. The final weight of the gel was adjusted to 25 g with 30% aqueous ethanol. The gel (20 g) was casted into the petri plate and allowed to dry under vacuum at room temperature for 3 days. At this point residual water was determined using Karl Fischer titration (McCarron et al, 2005). Table 4.2 gives the compositions of bioadhesive patch formulations. Optimization of formulation composition was carried out by varying the ratios of HPMC K4M and Gantrez MS 955 (Table 4.2) to get the desired characteristics (viz. release, bioadhesion and other physicochemical properties).

The mechanical properties of the bioadhesive matrices were evaluated using the film formulation without any non-adhesive backing film (McCarron et al, 2005). For the formulation of backing layer, ethyl cellulose (20 cps) was dissolved in acetone (10ml). Propylene glycol was used as plasticizer and casted on the dried 1st layer and allowed to dry for 24 hrs. After drying, the patch was removed from the casting plate (Reinhold and Merkle, 1989). Table 4.3 gives the compostions of backing layer. The fully assembled bi-laminar patch was used for bioadhesive performance and other evaluation purposes.

Table: 4.2. Compositions for mucoadhesive layer

QUANTITY / PATCH	FORMULATION CODE										
	FP1	FP2	FP3	FP4	FP5	FP6	FP7	FP8	FP9	FP10	FP11
FU (mg)	20	20	20	20	20	20	20	20	20	20	20
HPMC K4M (mg)	100	80	70	60	50	40	40	40	40	40	40
Gantrez MS 955 (mg)	40	40	40	40	40	40	50	60	70	80	100

Table: 4.3. Compositions for protective layer

Excipients	Quantity
Ethyl cellulose (mg)	25
Propylene glycol (%)	5

4.3.1 *Evaluation of bioadhesive patches*:

4.3.1.1 *Assay:*

FU content was determined by dissolving an accurately weighed quantity of formulation equivalent to 20 mg FU in 100 ml of phosphate buffer (pH 6.8). 5 ml of above solution was taken, filtered through 0.4μ filter and diluted to 100ml with phosphate buffer. Absorbance of the resulting solution was taken at 267 nm (Section 3.2).

4.3.1.2 *Bioadhesion:*

The tensile strength required to detach the bioadhesive patch from the mucosal surface is applied as a measure of the bioadhesive performance. The apparatus used (Fig.4.1) was locally assembled as per the previously described apparatus (Agrawal and Mishra, 1999).The entire study was carried out in a chamber at 37 ±1°C temperature.

A circular piece (surface area 2 cm^2) of guinea pig buccal mucosa was cut and separated from connective tissue and glued with cyanoacrylate adhesive on the ground surface of a tissue holder made of plexiglass. Similarly, the patch was glued (from backing membrane side) to another tissue holder of the same size. Thereafter, the tissue holders with mucosa and patch were put in contact with each other with uniform and constant pressure for 5 min (preload time) to facilitate adhesion bonding. The tissue holder was allowed to hang on an iron stand with the help of an aluminum wire fastened with the hook provided on the back side of the holder. A preweighed lightweight polypropylene bottle was attached to the hook on the back side of the formulation holder with aluminum wire. After a preload time of 5 min, water was added to the polypropylene bottle through an intravenous infusion set at a rate of 1 drop/sec until the patch detached from the mucosal tissue.

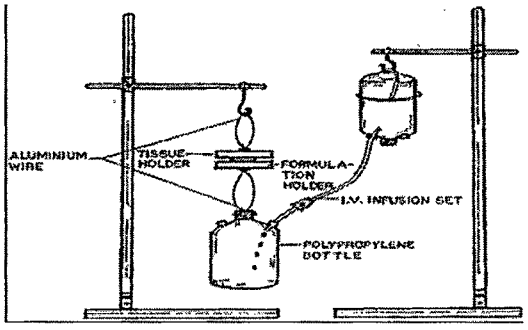

Fig 4.1 Apparatus for *in vitro* Bioadhesion test.

The water collected in the bottle was weighed and was representative of bioadhesion force (G; in grams) required for the detachment. This was then converted into dynes/cm^2 as per the reported formula given in equation 4.3 (Varma. et.al, 2004).

Force of detachment (dynes/cm^2) = G x g/A ----------(4.3)

Where,

G=Actual weight for detachment in grams;

g = the acceleration due to gravity taken as 980 cm/s^2;

A= Area of the tissue exposed

4.3.1.3 *Swelling rate*:

The swelling rate of formulations was evaluated using a 1% w/v agar gel plate (Agrawal and Mishra, 1999). Formulations (Patches) were weighed, and the average weight of was calculated (W_1). The formulations were placed with the bioadhesive surface facing the gel surface in petri dishes, which were placed in an incubator at 37 ± 0.1°C. Formulations were removed at time intervals of 2, 4, 6, and 8 hr, excess water on the surface was carefully absorbed using filter paper, and swollen formulations were weighed. The average weight (W_2) was calculated, and then the swelling index was calculated by the formula given in equation 4.4;

$$\text{Swelling Index} = (W_2 - W_1)/W_1 \qquad \text{----------(4.4)}$$

4.3.1.4 *Surface pH:*

The micro environmental pH of the formulation was measured so as to determine its effect on buccal mucosa. The formulation was first allowed to swell by adding an adequate amount of distilled water to formulation surface. The surface pH was then recorded after bringing glass electrode of pH meter (Elico, Mumbai) near the surface of the formulation and allowing it to equilibrate for 1 min (Bottenberg et al, 1991).

4.3.1.5 *Tensile strength:*

The strength required to break the patch was recorded by using tensile strength tester (Instron Universal Testing Instrument, Model 1121, Instron Ltd., Japan) equipped with microprocessor force gauge (Peh and Wong, 1999). A small strip of patch (approx. 2 x 1 cm) was pulled at a rate of about 20 mm/min till it broke.

4.3.1.6 *Folding endurance:*

Folding endurance was determined by repeatedly folding and unfolding a small strip of patch (approx. 2x 2 cm.) at the same place till it broke. Number of times the patch can be folded at the same place, without breaking gives the value of folding endurance (Patel et al., 2007).

4.3.1.7 ***Drug release studies:***

The drug release study for patches was carried out by using USP dissolution apparatus V at 37± 0.5°C temperature. The patch of circular shape was placed in a basket (50 mm diameter and 6 mm height) made from stainless steel with a sieve opening of approximately 850 mm (size No. 20) (Wong et al., 1999). The basket is designed for holding the patch system at the bottom the vessel. The basket containing the sample was submerged into the dissolution medium at approximately 10 mm from the base of the dissolution vessel (Fig 4.2). Volume of dissolution medium i.e. phosphate buffer (pH 6.8) was 500 ml and speed of the paddle was 50 rpm. The aliquots (5 ml) were withdrawn at 60, 120, 180, 240, 360, 420 and 480 minutes. The removed volume was replaced by the same volume of phosphate buffer. Analysis of the withdrawn samples was carried out by UV spectroscopy at 267 nm.

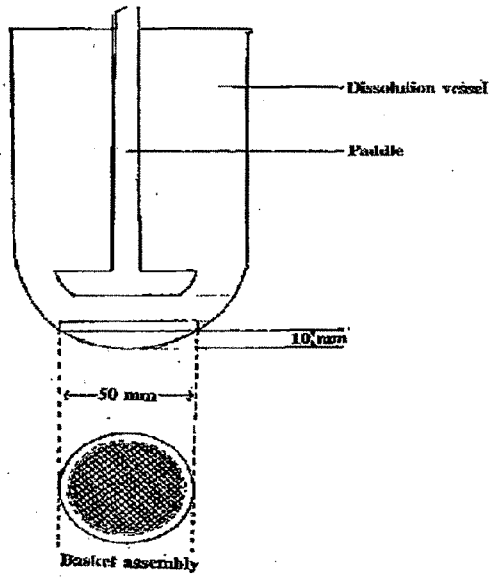

Fig. 4.2. Schematic illustration of the dissolution test apparatus.

4.3.1.8 ***Mathematical modeling of release kinetics***

Data obtained from *in vitro* release studies were fitted to various kinetic equations to understand the mechanism of drug release from formulated extended release gels.

The kinetic models used were zero order, first order, Higuchi and Peppas equation (Tas et al., 2004; Jones et al, 2004; Costa et al, 2001).

The following plots were plotted: Q_t *vs.* t (zero order kinetic model); log (Q_0-Q_t) *vs.* t (first order kinetic model,) and Q_t *vs.* square root of t (Higuchi model) and log M_t/M_∞ = nlog t + k (Peppas equation). Where Q_t is the amount of drug released at time t and Q_0 is the initial amount of drug present in gels (Korsmeyer et al, 1983). M_t/M_∞ is the fraction of drug released after time t in respect to amount of drug released at infinite time, k is the rate constant and n is the diffusional exponent which characterizes the transport mechanism (Peppas, 1985). Statistical comparisons were made using one way ANOVA by using the software PRISM (Graphpad, San Diego, CA). The level of significance was considered at $p < 0.05$.

4.3.1.9. *Compatibility studies:*

To investigate the interaction between FU and polymers, Fourier Transform Infrared (FTIR) spectrometry, X-ray diffraction (XRD) and Differential scanning calorimetry (DSC) studies were carried out.

FTIR spectra were recorded using a FTIR Shimadzu -8300 spectrophotometer (Tokyo, Japan). Samples were prepared in KBr discs by means of a hydraulic press. The scanning range was 500 to 4000 cm^{-1} and the resolution was 4 cm^{-1}.

X- Ray Diffractograms were recorded on a X-ray diffractometer (Philips PW 1710) under the following conditions: Ni-filtered Cu-Ka radiation; 40 KV voltage: 30 mA current, scan speed 2°/min in terms of 2θ angle.

DSC study was performed using a differential scanning calorimeter (DSC) (Shimadzu DSC 60, Japan) at a heating rate of 10°C/min in nitrogen atmosphere.

4.3.2 *Histology Studies:*

The histological and morphological changes in the buccal mucosa due to treatment with formulations were studied by light microscopy (using Olympus microscope). The guinea pig buccal tissue was stored in Kreb's buffer and was used within 2 hours upon removal. Most of the underlying tissues were removed from the mucosa with surgical scissors, and then the epithelium was separated from the connective

tissue making sure that basal membrane was intact. The tissue was mounted on Franz diffusion cell and incubated with the formulation (area of tissue exposed 3.14 cm^2) on one side and pH 6.8 phosphate buffer (20 ml) on the other side. For control, pH 6.8 phosphate buffer was kept on the both sides of cell. Stirring in the acceptor side was carried out using magnetic bead.

At the end of the exposure period (8 hour), small portions of tissue were fixed in 10% buffered formalin solution and dehydrated. All specimens were then embedded in paraffin wax. Sections were cut with a microtome in direction perpendicular to the epithelial surface (at Baroda Pathology Lab, Baroda). They were then stained with hematoxylin eosin (HE) and examined under light microscope (Olympus, Japan) (Aioi et al, 1993; Shin et al, 2000).

4.3.3 *Stability study of bioadhesive patch formulations:*

Patches were packed in glass Petri dishes lined with aluminum foil and kept in a stability chamber maintained at 40 ± 2 °C and 75 ± 5% RH (Accelerated) and 30 ±2°C /60±5%RH (Intermediate term) for 3 months. Changes in the appearance, release behavior and drug content of the stored bioadhesive patches were investigated after 3 months as per the procedures described previously (Nafee et al, 2003; ICH Guidlines).

4.4 FORMULATION OF BIOADHESIVE TABLETS FOR 5-FLUOROURACIL (FU):

Bilayer tablets (consisting of a backing layer and adhesive drug reservoir layer) were prepared by covering one side of the single-layer tablet with an inert ethylcellulose layer. Ethylcellulose was selected as a backing material because this hydrophobic polymer has very low water permeability thus providing an impermeable backing layer that prevents drug loss in oral cavity (Miyazaki et al, 2000).

Bilayered bioadhesive tablets were formulated using HPMC K4M, K15 M and Gantrez MS 955 as polymers as per the compositions given in table 4.4 and 4.5. Tablets were prepared by direct compression of drug blended with HPMC, Gantrez and other excipients on 8 station rotary machine containing D-Tooling (General

Machinery, Mumbai). Drug, polymers, tablettose and lubricants were mixed and then compressed using 9.5 mm flat faced punches at a lower hardness (2-3 kg/cm^2) and then backing layer made of EC (20 cps) was compressed on the drug containing layer to obtain bilayered tablets with final hardness of tablets at 4-5 kg/cm^2.

4.4.1 *Preliminary studies*

During preliminary studies, various combinations of HPMC K4M, HPMC K15M and Gantrez in different ratios were formulated to help in choosing the best possible combination for further evaluation. The formulations employed for pre-optimization investigations are enlisted in Table 4.4.

Table 4.4. Preliminary batches for FU tablets

Composition (mg)	Formulation				
	PFT1	PFT2	PFT3	PFT4	PFT5
FU	20	20	20	20	20
Tablettose 100	115	105	95	85	85
Gantrez MS-955	10	20	20	30	40
HPMC K4M	10	-	10	10	-
HPMC K15	-	10	10	10	10
Magensium stearate	2	2	2	2	2
Ethyl cellulose, 20 cps (Backing membrane)	70	70	70	70	70

4.4.2 *Experimental design*

Systematic optimization techniques are being widely practiced to alleviate inconsistencies. These techniques encompass the use of experimental designs, mathematical equations and graphic outcomes, thus depicting a complete picture of variation of the response(s) as a function of the factor(s) (Schwartz and Connors, 1996). The software DESIGN EXPERT® 7.2.2 was used for generation and evaluation of the statistical experimental design. The technique requires minimum experimentation and time, thus proving to be far more effective and cost-effective than the conventional methods of formulating dosage form (Singh et al, 2006).

A 3^2 factorial design was employed for optimization of tablet formulation in which the amount of two carrier(s) (factors) was varied at three levels as required by the design (Section 2.8). The amounts of Gantrez (X_1) and HPMC K 15M (X_2) were selected as factors, studied at 3 levels each. **Table 4.5** summarizes an account of the 9 experimental runs studied, their factor combinations, and the translation of the coded levels to the experimental units employed during the study. Bioadhesion [Y_1], release until 8 hours (rel_{8hrs}) [Y_2], time taken to release 50% of drug ($t_{50\%}$) [Y_3] and diffusion coefficient (n) [Y_4] were taken as the response variables.

Table 4.5. Factor combinations as per 3^2 factorial design

Trial No.	Coded factor levels	
	Factor 1(X_1)	**Factor 2(X_2)**
1	-1	-1
2	-1	0
3	-1	1
4	0	-1
5	0	0
6	0	1
7	1	-1
8	1	0
9	1	1

Translation of coded levels in actual units

Coded level	-1	0	+1
X_1: Gantrez MS 955 (mg)	10	20	30
X_2:HPMC K15M(mg)	4	10	16

All batches contained 20 mg FU, HPMC K4M (4mg), HPMC K15M (4-16 mg), Gantrez MS 955(10-30 mg), Magnesium stearate (2mg), and quantity sufficient of Tablettose-100.Drug containing layer's weight (155 mg); Backing layer contains Ethyl cellulose-20cps (70 mg).
Batch size = 40 tablets.

4.4.3 *Characterization of Tablets:*

4.4.3.1 *Diameter and thickness:*

The diameter and thickness of the formulated tablets were measured using Vernier Caliper (Mitutoyo, Japan).

4.4.3.2 *Hardness:*

Hardness of the tablets was tested using a Monsanto hardness tester (Campbell Electronics, Mumbai). Five tablets from each batch were used, and average values were calculated.

4.4.3.3 *Weight variation:*

To study weight variation, 20 tablets of each formulation were weighed using an electronic balance (Afcoset, USA), and the test was performed according to the method described in Indian Pharmacopoeia (Indian Pharmacopoeia, 1996).

4.4.3.4 *Friability:*

It was determined by using Roche Friabilator (Campbell Electronics, Mumbai). The tablets were carefully dedusted prior to testing. Accurately weighed 10 tablets were placed in the friabilator which was rotated for 100 revolutions, and tablets were removed. Any loose dust from the tablets was removed and tablets were accurately weighed. A maximum weight loss of not more than 1% of the weight of the tablets being tested was considered acceptable. (USP XXV, 2002).

4.4.3.5 ***Surface pH:*** Procedure same as described in section 4.3.1.4 was employed.

4.5.3.6 ***Assay:*** 10 tablets were crushed in mortar and powder equivalent 20 mg FU was taken for further analysis. The method for estimation of drug content of the tablet formulations is same as that discussed in section 4.3.1.1.

4.4.3.7 *In vitro release study:*

Tablets were stuck from backing membrane side onto the bottom of the dissolution vessel with a drop of cyanoacrylate adhesive, so that only one side of tablet was in contact with the dissolution medium (Akbari et. al, 2004). Then the prewarmed

(37±0.5 °C) dissolution medium (500 ml) was gently poured into the vessel and dissolution conducted as per United States Pharmacopoeia (USP) method II with a paddle speed of 50 rpm. Samples (5.0 ml) were withdrawn at regular intervals and replaced with an equal volume of medium. Samples were filtered using 0.4μ filter and subsequently analyzed using UV Spectrophotometer at 267 nm.

Various kinetic models like Zero order, First order, Higuchi and Peppas equation were applied to predict the mechanism of drug release as described for the patches (section 4.3.1.8).

4.4.3.8 *Optimization Data Analysis*

Various RSM (Response Surface Methodology) computations for the current optimization study were performed employing Design Expert® software (version7.1.2, Stat-Ease Inc, Minneapolis, MN). Polynomial models including interaction and quadratic terms were generated for all the response variables using multiple regression analysis (MLRA) approach (Section 2.8). The general form of MLRA model is represented as equation 4.5.

$$Y=B_0+B_1X_1+B_2X_2+B_3X_1^2+B_4X_2^2+B_5X_1X_2+B_6\ X_1^2X_2+B_7\ X_1\ X_2^2 \quad ...(4.5)$$

Where B_0 is the intercept representing the arithematic average of all quantitative outcomes of 9 runs; B_1 to B_7 are the coefficients computed from the observed experimental values of Y; and X_1 and X_2 are the coded levels of the independent variable(s). The terms X_1X_2 and X_i^2 (i=1to2) represents the interaction and quadratic terms, respectively. The main effects (X_1 and X_2) represent the average result of changing one factor at a time from its low to high value. The interaction terms (X_1X_2) show how the response changes when two factors are simultaneously changed. The polynomial terms (X_1^2 and X_2^2) are included to investigate nonlinearity. The polynomial equation was used to draw conclusions after considering the magnitude of coefficients and the mathematical sign it carries, i.e., positive or negative. A positive sign signifies a synergistic effect, whereas a negative sign stands for an antagonistic effect.

Statistical validity of the polynomials was established on the basis of ANOVA provision in the Design Expert ®software. Level of significance was considered at

$P<0.05$. The best fitting mathematical model was selected based on the comparisons of several statistical parameters including the coefficient of variation (CV), the multiple correlation coefficient (R^2), adjusted multiple correlation coefficient (adjusted R^2) , and the predicted residual sum of squares (PRESS), provided by software. Among them, PRESS indicates how well the model fits the data, and for the chosen model it should be small relative to the other models under consideration (Huang et al., 2005). Also, the 3-D response surface graphs and the 2-D contour plots were generated by the Design Expert® software. Subsequently, the desirability approach was used to generate the optimum settings for the formulations (Narendra et.al., 2005; Huang et al, 2005).

4.4.4. *Compatibility studies:*

To investigate the compatibility of matrix materials with FU, Fourier Transform Infrared (FTIR) spectrometry and Differential scanning calorimetry (DSC) studies were carried out.

FTIR spectra were recorded using a FTIR Shimadzu- 8300 spectrophotometer (Tokyo, Japan). Samples were prepared in KBr discs by means of a hydraulic press. The scanning range was 500 to 4000 cm^{-1} and the resolution was 4 cm^{-1}.

For DSC study was performed using a differential scanning calorimeter (DSC) (Shimadzu DSC 60).Approximately 10-mg sample was crimped in flat aluminum pans with an empty pan serving as the reference. The pans were ramp heated from 25 to 300°C at the rate of 10°C/min.

4.4.5 *In vivo bioadhesion study:*

The bilayered placebo tablets (without drug) were prepared using the optimized formula for tablet formulation. The backing layer was pulled off and a small hole was drilled through the matrix layer (An electric drill (Shiv Shakti Enterprises, Faridabad, India) was used to drill holes of 1.4mm diameter to a depth of 1- 1.2 mm through the centre of the tablets) for introducing radioactive solution. 1 ml of ^{99m}Tc (2 mCi/ml) was mixed with 0.1 ml of stannous chloride solution (1mg/ml) and the pH was adjusted to 7 using sodium bicarbonate solution. About 10µl of above aqueous solution of ^{99m}Tc was instilled into the drilled holes, avoiding contact with

the surface of the tablets. The solution was allowed to dry for 1 hour in a fume cupboard.

A mixture of HPMC K15M and Gantrez MS-955 in 1:1 proportion was prepared and used to seal the drilled holes so that it covered an area slightly greater than that of the hole. Then the backing layer of Ethyl cellulose was stuck over it using cyanoacrylate adhesive (Araldite®). The labelled tablets were left to dry overnight before use.

Animals (albino rabbits of either sex) were lightly anesthetized by an i.m. injection of a 1:5 mixture of Diazepam (1.9 mg/kg) and ketamine (9.3 mg/kg). Following induction of anesthesia, bioadhesive tablets were first moistened with a drop of water and inserted in the mouth and positioned in such a way that tablet surfaces were in contact with the cheek.

Gamma scintigraphy

For determination of the tablet position and the residence time in the buccal cavity, the rabbits were fixed on a board and imaging was performed after 4 hours using a Single Photon Emission Computerized Tomography gamma camera (SPECT, LC 75-005, Diacam, Siemens, USA).

4.4.6 *Histology studies:*

Histological studies were performed as per the same procedure used for patches as described in section 4.3.2.

4.4.7 *Stability study of bioadhesive tablet formulations containing FU*:

The stability study of formulations was carried out as per ICH guidelines. Tablet formulations were wrapped in aluminium foils and subjected to stability study at 40 ±2°C /75±5 %RH (Accelerated) and 30 ±2°C /60±5 %RH (Intermediate) for 3 months and then samples were observed for physical appearance analyzed for drug content.

4.5 FORMULATION OF BUCCAL BIOADHESIVE GELS CONTAINING FU:

Poloxamer 407 gels were prepared using the method described by Schmolka. (1972). Table 4.6 gives composition of various gel formulations. Required quantity of Poloxamer-407 was dissolved in 10 ml water with gentle stirring. The solution was left in a refrigerator overnight to ensure complete polymer dissolution. FU was dissolved in water and HPMC K15M and Gantrez S–97 were added. Then both the solutions were mixed and stirred at 40-45 °C for 2 hours till a gel was formed (Shin, S.C. et.al., 2004). The prepared gel formulations were filled in amber colored glass containers (Well stoppered) and stored at room temperature.

Table 4.6 Compositions of the FU Bioadhesive Gel Formulations (%w/w).

Batch No.	FU	Poloxamer - 407	HPMC K 15M	Gantrez S-97
B1	1	16	-	-
B2	1	16	1	-
B3	1	16	1.5	-
B4	1	16	2	-
B5	1	17	2	-
B6	1	17	2	2
B7	1	17	2	3
B8	1	17	2	4
B9	1	18	2	2
B10	1	18	2	3
B11	1	18	2	4

4.5.1 *Characterization*:

4.5.1.1 *Measurement of bioadhesive force*

The method is same as described in section 4.3.1.2 except few changes. Here, two circular pieces (diameter 3.6 cm) of guinea pig buccal mucosa were cut and separated from connective tissue and glued with cyanoacrylate adhesive on the

ground surface of two tissue holders made of plexiglass. Tissues were immediately used after separation. The gel (0.5 g) was applied to the mucosal tissue on one of the tissue holders. Thickness of gel layer was 1.0±0.2 mm. Thereafter, both the tissue holders with mucosa and gel formulation were allowed to come in contact with each other with uniform and constant pressure for 5 min (preload time) to facilitate adhesion bonding. Rest of the method is same as in section 4.3.1.2.

4.5.1.2 *In Vitro diffusion studies*

Prevalidated Franz diffusion cells with a receiver compartment volume of 20 ml and effective diffusion area of 3.14 cm^2 were used in this study. The synthetic cellulose membrane (Molecular cut off value: 12,000) was mounted on top of receptor compartment of diffusion cells. The receptor phase (pH 6.8 phosphate buffer) was continuously stirred by magnetic bead and kept at a temperature of 37±0.5°C during the experiments. Amount of the prepared gels equivalent to 20 mg of FU was spread on the cellulose membrane at the donor compartment; the donor cap was covered with parafilm and clamped (Tas. et al, 2004).

At appropriate times upto 8 hours, 500 µl sample was withdrawn from the receiver compartment and the same amount of fresh buffer solution was added to keep the volume constant. This dilution of the receiver content was taken into account when evaluating the data. The samples were analyzed spectrophotometrically at a wavelength of 267 nm and the concentration of FU in each sample was determined from a previously calibrated standard curve. Each data point represented the average of three determinations.

4.5.1.3 *Assay*

Method for estimation of drug content of the gel formulations is same as that discussed in section 4.3.1.1.

4.5.1.4 *pH measurements*

The pH of each gel was measured using a pH meter (Elico, Mumbai), which was calibrated before each use with buffered solutions at pH 4, 7, and 10.

4.5.1.5 ***Viscosity measurement***

Viscosity of gel was measured using Brookfield programmable rheometer (Rheocalc V 2.5, Model DV-III Ultra) using CP 40 spindle at 40 RPM (25±1°C). Effect of shear rate on shear stress and viscosity were studied.

4.5.2 ***Drug and polymer compatibility studies***

To investigate the interaction between FU and polymers, Fourier Transform Infrared (FTIR) spectrometry, X-ray diffraction (XRD) and Differential scanning calorimetry (DSC) studies were carried out with HPMC, Poloxamer, Gantrez physical mixtures and solid dispersions. Physical mixtures and solid dispersions (co precipitates) of FU with Poloxamer 407, HPMC K15 M and Gantrez® S-97 (1:4 w/w) were studied. The physical mixtures of FU and Poloxamer, HPMC K15M and Gantrez® S-97 were prepared by simple blending. The co precipitates were prepared by an evaporating method after dissolving FU and polymers in methanol and air dried at room temperature (Shin et al., 2000). Residual organic solvent was removed by placing in a vacuum dessicator.

4.5.2.1 *Fourier Transform Infrared (FTIR) Spectroscopy:* FTIR spectra were recorded using a FTIR Shimadzu -8300 spectrophotometer (Tokyo, Japan). Samples were prepared in KBr discs by means of a hydraulic press. The scanning range was 500 to 4000 cm^{-1} and the resolution was 4 cm^{-1}.

4.5.2.2 *X-Ray Diffraction:* X- Ray Diffractograms were recorded on a X-ray diffractometer (Philips PW 1710) under the following conditions: Ni-filtered Cu-Ka radiation; 40 KV voltage: 30 mA current, scan speed 2°/min in terms of 2θ angle.

4.5.2.3 *Differential scanning calorimetry (DSC):* DSC study was performed using a differential scanning calorimeter (DSC) (Shimadzu DSC 60, Japan) at a heating rate of 10°C/min in nitrogen atmosphere.

4.5.3 ***In vivo bioadhesion of gels:***

Radiolabelling of the gels and their application

1 ml of technetium-99m [^{99m}Tc] (2 mCi/ml) was mixed with 0.1 ml of stannous chloride solution (1mg/ml) and the pH was adjusted to 7 using sodium bicarbonate

solution. 10µl of above aqueous solution of ^{99m}Tc was added to approximately 2 g of gel formulation (placebo) to be tested and thoroughly mixed. The average final activity per dose and per subject, at the time of administrations ranged from 3995 to 4210 kcpm (kilocounts per minute). Rabbits were anaesthetized as described previously (section 4.4.5) and 200 mg of the gel formulation was applied with a small Teflon spatula on an area of approximately 1 cm^2 of the oral mucosa. Each animal was used only once throughout these trials.

Gamma scintigraphy

For the determination of residence time of gel formulations in the buccal cavity, the rabbits were fixed on a board and imaging was performed using a Single Photon Emission Computerized Tomography gamma camera (SPECT, LC 75-005, Diacam, Siemens, USA).

4.5.4 *Histology Studies:*

The histological and morphological changes in the buccal mucosa due to treatment with gel formulations were studied as per the method described under section 4.3.2.

4.5.5 *Stability study of bioadhesive gel formulations containing FU:*

Gels were stored in amber colored glass containers (well stoppered) for three months at room temperature (25±2°C). They were checked for physical appearance, pH, assay and release after preparation and periodically throughout this period. Physical evaluation of stability of the samples was carried out by visual inspection (Tas et al., 2004).

4.6 *IN VIVO* STUDIES OF FU CONTAINING BIOADHESIVE PATCHES, TABLETS AND GELS:

4.6.1 *Animal treatment*

All experiments and protocols described in this study were approved by the Institutional Animal Ethics Committee of MS University of Baroda and are in accordance with the Committee for Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. Albino rabbits (of either sex) weighing 2.0–2.6 kg were

housed individually over 2 weeks in a temperature-controlled environment (20–25°C). The relative humidity varied between 50 and 60%. The animals were fasted overnight prior to the experiment but had free access to water.

Animals were lightly anesthetized by an i.m. injection of a 1:5 mixture of Diazepam (1.9 mg/kg) and ketamine (9.3 mg/kg). Following induction of anesthesia, a catheter was placed in the marginal ear vein for blood sample collection. Bioadhesive tablet/patch containing FU (20 mg) were first moistened with a drop of water and inserted in the mouth and positioned in such a way that tablet/ patch surfaces were in contact with the cheek. The gel formulations were applied directly on the cheek of rabbits. 0.5 ml blood samples were collected before and at 1, 2, 3, 4, 6, 8, and 24 h after buccal administration of the mucoadhesive buccal patch/tablet/gels and at 15 min and 1, 2, 4 h after intravenous administration. The blood samples were transferred into heparinized microcentrifuge tubes.

A light plane of anesthesia was maintained by an i.m. injection of one-third of the initial dose of Diazepam and Ketamine mixture as needed. Then the samples were subjected to centrifugation on a laboratory centrifuge (Sigma, 3K30) at 10 000 rpm for 10 minutes at 0°C, and supernatant plasma was collected into another microcentrifuge tube and kept at –20°C until analysis (Alur et al., 1999).The plasma samples were analysed by HPLC as per method described in section 3.3.

4.6.2 *Pharmacokinetic data analysis*

The noncompartmental pharmacokinetic analysis was performed. Trapezoidal method was employed to calculate the AUC of plasma concentration (C) as a function of time (t). Mean residence time (MRT) was calculated as area under the first moment curve (AUMC) divided by area under the curve (AUC). AUMC was determined using a plot of plasma concentration multiplied by time (C X t) versus time and calculation of its area under the curve by the Trapezoidal method. All the pharmacokinetic parameters were calculated in MS-Excel software. The maximum plasma concentration (C_{max}) and time to reach maximum plasma concentration (t_{max}) were determined by visual inspection of the experimental data as well as the plasma concentration curve using MS Excel software. The elimination rate constant (K_{el}) was calculated by the regression analysis from the slope of the line and the

half-life ($t_{1/2}$) of the drug was obtained by $0.693/K_{el}$. The absolute bioavailability (A.B.) of FU after buccal administration per the I.V. administration was calculated as given below in equation 4.6 (Shin et al, 2000; Alur et al, 1999).

$$A.B. = \frac{(AUC)_{sample}}{(AUC)_{i.v.}} \times \frac{(Dose)_{i.v.}}{(Dose)_{sample}} \times 100 \text{ ---------- } (4.6)$$

The statistical significance of the differences between the formulations was tested by the Student's paired t-test. All the values were reported as mean ± standard error of mean (S.E.M.) of six determinations.

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