3 MATERIALS AND METHODS

3.1 CHEMICALS AND MATERIALS

Chemicals were purchased from Sigma Aldrich, India, Sd-Fine chemicals, India and were used without further purification. All the solvents were purchased from Sd-Fine chemicals, India and used without further purification.

3.2 PREPARATION OF CASEIN MICROPARTICLES

Casein microparticles were prepared by steric stabilization process. The polymer dissolved in aqueous phase is dispersed in continuous organic phase containing steric agent and this emulsion is homogenized to get particles. The preparation of microparticles includes an aqueous phase containing casein solution which is dispersed in organic phase containing chloroform (50 ml). Ethyl cellulose is added as steric hindrance agent in chloroform. The emulsion is homogenized at 20,000 rpm for period of half an hour. Glutaraldehyde cross-linked particles are stirred magnetically (for 24 hours) for effective crosslinking. Acetone (above three fourth) is added to the final solution to harden the particles. The particles were separated by centrifugation at 5500 rpm for 10 min and washed repeatedly with acetone to remove excess ethylcellulose and chloroform. The casein microparticles obtained was dried and packed tightly for further usage.

Fig 3.1 Preparation of Casein Microparticles
3.3 OPTIMIZATION OF CASEIN MICROPARTICLES

The effect of casein concentration, casein volume and ethyl cellulose concentration on the size and morphology of casein microparticles were studied. Effect of various parameters is analyzed by varying their concentration. The combinations of the parameter are given in the Table 3.1. The concentrations of these parameters are selected based on smaller size and good morphology of casein microparticles. The end quality of the particles depends on the effective washing. Washing removes excess of ethyl cellulose and chloroform. The particles are washed with

1. Acetone alone for three times.
2. Chloroform and continued with acetone for two times.
3. Chloroform, then Sodium bisulfite and then with acetone.

<table>
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<th>Formulation code</th>
<th>Casein concentration</th>
<th>Ethyl cellulose concentration</th>
<th>Stirring rate</th>
<th>Glutaraldehyde concentration</th>
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<td>20,000 rpm</td>
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3.4 DRUG LOADING OF CASEIN MICROPARTICLES

Diffusion filling method and the loading of drugs while preparing the microparticles are the commonly employed method in drug loading. Due to the heavy loss of drug the diffusion loading method was not preferred and thereby the drugs were added separately to the casein solution before preparing the particles. The various drugs used were

1. Methotrexate – Anticancer drug
2. Levocetirizine dihydrochloride - Anti-Histaminic drug
3. Donepezil hydrochloride - Acetylcholinesterase inhibitor
4. Phenytoin sodium – Anti-epileptic drug
5. Glimepiride – Antidiabetic drug
6. Propranolol HCl – Anti-hypertensive drug

The drug containing casein solution is mixed thoroughly to get a homogenous solution and is used for the preparation of the particles. The remaining protocol for preparing the drug loaded casein microparticles remains the same.

3.5 MORPHOLOGICAL OBSERVATION UNDER LIGHT MICROSCOPE

Initial observation is done using light microscopy under oil immersion. The particles were also confirmed by the 1000X Microscopy.

3.6 MORPHOLOGICAL CHARACTERIZATION USING SCANNING ELECTRON MICROSCOPY (SEM)

The size of casein microparticles (plain and drug loaded) were qualitatively analyzed through Scanning Electron Microscopy (SEM). For SEM analysis particles are sprinkled on adhesive aluminum stub and then surface coated with gold to a thickness of 300A using a sputter coater. The micrograph of these samples was then taken for both loaded and unloaded casein microparticles.

3.7 ENCAPSULATION EFFICIENCY

Encapsulation efficiency is the amount of added drug (in percent) that is encapsulated in the formulation of microspheres. Encapsulation efficiency was calculated
in terms of the ratio of drug in the final formulation to the amount of added drug as described by Mohini et al 2006.

\[
\text{Encapsulation efficiency (\% )} = \frac{\text{Actual Weight (W_a)}}{\text{Theoretical Weight (W_t)}} \times 100
\]

An accurately weighted amount (10 mg) of the formulation of microspheres was dispersed in 10 ml of Phosphate Buffer Saline (PBS, pH 7.4). The sample was ultrasonicated and left to equilibrate for 24 hours at room temperature. The suspension was then centrifuged at 5500 rpm for 10 min. the supernatant was diluted approximately with PBS (pH 7.4) and analyzed for concentration of the drugs using UV-Vis Spectrophotometer. The drug content of each sample was determined in triplicate and the results were averaged.

3.8 PARTICLE SIZE DISTRIBUTION

Particle size distribution was performed using Mastersizer 2000 (Malvern India Pvt ltd). The microparticles loaded with drugs were dispersed with poly (dimethylsiloxane). The refractive index for the powder samples was set to 1.52, and the poly (dimethylsiloxane) was 1.40. Each run was measured three times with about 1000 particles being measured for each run. Data is reported in % volume distribution since it is pharmaceutically relevant.

3.9 X-RAY DIFFRACTION (XRD) STUDIES

XRPD (X-Ray Power Diffraction) can also be used for crystallographic structure refinement and determining crystallographic parameters such as: lattice constants, displacement parameters, coordinates of atoms, site occupation factors, preferred orientation etc. The XRD technique takes the sample and places the powdered sample in a holder and then the sample is illuminated with X-rays of a fixed wave-length and the intensity of the reflected radiation is recorded using a goniometer. This data is then analyzed for the reflection angle to calculate the inter-atomic spacing (D value in Angstrom units- \(10^{-8}\) cm). The intensity (I) is measured to discriminate (using I ratios) the various D spacing’s and the results are to identify possible matches. The geometry of an
X-ray diffractometers is such that the sample rotates in the path of the collimated X-ray beam at angle $\theta$ while the X-ray detector is mounted on an arm to collect the diffracted X-rays and rotates at an angle $2\theta$. The instrument used to maintain the angle and rotate the sample is termed as a goniometer. For typical powder patterns, data is collected at $2\theta$ from $-5^\circ$ to $70^\circ$, angles that are preset in the X-ray scan. The X-ray diffractograms of the microparticles and the drug loaded microparticles were obtained in a D8 Advance Model X-Ray Diffractometer (Bruker, Germany) using Ni filtered radiation ($\lambda = 15.4$ nm, 40 kV and 30 mA). The measurements were carried out using poly methyl methacrylic acid (PMMA) sample holder and lynx eye detector.

3.10 FOURIER TRANSFORM INFRA RED (FTIR) SPECTRAL ANALYSIS.

Samples for FTIR can be prepared in a variety of ways. One common method is to crush the sample with an oily mulling agent (Usually Nujol) in a marble or agate mortar, with a pestle. A thin film of the mull is smeared onto salt plates and measured. The second method is to grind a quantity of the sample with a specially purified salt (usually potassium bromide) finely (to remove scattering effects from large crystals). This powder mixture is then pressed in a mechanical press to form a translucent pellet through which the beam of the spectrometer can pass. A third technique is the “cast film” technique, which is used mainly for polymeric materials. The sample is first dissolved in a suitable, non hygroscopic solvent. A drop of this solution is deposited on surface of KBr or NaCl cell. The solution is then evaporated to dryness and the film formed on the cell is analyzed directly. Care is important to ensure that the film is not too thick otherwise light cannot pass through. This technique is suitable for qualitative analysis. The final method is to use microtomy to cut a thin (20-100 micrometres) film from a solid sample.

FTIR spectroscopic analysis of casein microparticles and drug loaded casein microparticles was studied for qualitative analysis of physical mixture for microparticles which was carried out using KBr pellets. FTIR spectra of pure compounds are generally so unique that they are like a molecular “fingerprint”. While organic compounds have rich, detailed spectra, inorganic compounds are usually much simpler.
3.11 THERMAL ANALYSIS - DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Differential Scanning Calorimetry (DSC) is a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. Both the sample and reference are maintained at nearly the same temperature throughout the experiment. Generally, the temperature program for a DSC analysis is designed such that the sample holder temperature increases linearly as a function of time. The reference sample should have a well-defined heat capacity over the range of temperatures to be scanned.

The cross-linking of polymer molecules that occurs in the curing process is exothermic, resulting in a positive peak in the DSC curve that usually appears soon after the glass transition. In the pharmaceutical industry it is necessary to have well-characterized drug compounds in order to define processing parameters. For instance, if it is necessary to deliver a drug in the amorphous form, it is desirable to process the drug at temperatures below those at which crystallization can occur. The DSC thermograms of placebo and drug loaded casein microparticles were carried out using Netzsch DSC 204. The samples were heated from 50 to 230°C at a heating rate of 10°C/min in an inert nitrogen atmosphere.

3.12 DRUG POLYMER INTERACTION

The extent of drug interaction with the polymer was determined using an equilibrium dialysis technique as given by Mohini et al 2006. For this the physical mixture has to be equilibrated with PBS in dialysis membrane and the amount of drug released at particular time (about 8 hours) was determined. The extent of drug-polymer interaction (\(\beta\)) was expressed by the ratio of Sb to St

\[
\beta = \frac{S_b}{S_t} = \frac{S_t - S_f}{S_f - S_R}
\]
where as Sb and ST are the amount of bound drug molecules inside the dialysis membrane and the total amount of drug used, respectively. St is the amount of drug inside the dialysis membrane after equilibrium is accomplished, and SR is the amount of drug in the beaker after equilibrium is accomplished. Sf is the amount of free drug in the beaker.

3.13 ANALYSIS OF SURFACE BOUND DRUG

The amount of drug in the surface of the casein microparticles can be determined by taking known amount of drug loaded microparticles (10 mg) in PBS (pH 7.4). It was mixed gently for 5 to 10 seconds and centrifuged at 5500 rpm for 10 min. The supernatant was diluted with PBS (pH 7.4) and the amount of drug was quantified by UV-Vis Spectrophotometer.

3.14 EQUILIBRIUM SWELLING STUDIES OF MICROPARTICLES

The swelling studies of casein microparticles were done as described by Latha et al 1994. A preweighed amount (100 mg) of microparticles was placed in Phosphate Buffer Saline (PBS, pH 7.4) and allowed to swell up to a constant weight. The microparticles were removed and blotted with filter paper, and their changes in weight were measured. The degree of swelling (α) was then calculated from the following formula,

\[
\alpha = \frac{W_g - W_o}{W_o}
\]

where Wo is the initial weight of the microparticles and Wg is the weight of the microparticles at equilibrium swelling in the medium.

3.15 IN VITRO DRUG RELEASE

In vitro release of drugs from casein microparticles were performed in Simulated Intestinal Fluid (SIF pH 7.4) and Simulated Gastric Fluid (SGF pH 1.2) without enzymes.

3.15.1 IN SIMULATED INTESTINAL FLUID (WITHOUT ENZYMES)

Known amount of drug loaded casein microparticles (50 mg) was taken in a dialysis tube with 0.5 ml of Simulated Intestinal Fluid (SIF, pH 7.4) is immersed in 50 ml of SIF in a beaker. The system was kept at 50 rpm at 37°C. Aliquots (0.5 ml) were
collected at pre determined time and equal volume of buffer was replaced to maintain constant volume. The amount of drug was quantified by UV-Vis Spectrophotometer.

3.15.2 IN SIMULATED GASTRIC FLUID (WITHOUT ENZYMES)

Known amount of drug loaded casein microparticles (50 mg) was taken in a dialysis tube with 0.5 ml of Simulated Gastric Fluid (SGF pH 1.2) is immersed in 50 ml of SGF in a beaker. The system was kept at 50 rpm at 37°C. Aliquots (0.5 ml) were collected at pre determined time and equal volume of buffer was replaced to maintain constant volume. The amount of drug was quantified by UV-Vis Spectrophotometer.

3.16 IN VIVO ANTI-DIABETIC STUDY

3.16.1 INDUCTION OF DIABETES IN MICE

Streptozotocin (STZ) was obtained from Sigma Chemicals. STZ was dissolved in cold 0.01 M citrate buffer, pH 4.5 and always prepared freshly for immediate use within 5 min. STZ injections were given intraperitoneally and the doses were determined according to the body weight of animals. The blood glucose concentration was measured every week from the day of STZ injection. The blood samples were collected from the tail vein once a week. Single high dose was optimized for inducing Diabetes in Albino Mice. We started with a single dose of 40mg/kg and the final dose optimized for inducing diabetes in mice was 200mg/kg.

3.16.2 EXPERIMENTAL STUDY

The animals were distributed into three experimental groups. Each group consisted of 3 mice in the beginning of the study. On the first day of experiment, all the food was removed from the cages for all the groups involved in the experiment, six hours prior to STZ treatment but water was provided as normal. The glucose levels were tested in the blood from the tail vein using the Accu-Chek Active blood glucose monitoring system just prior to STZ treatment. STZ was dissolved in freshly prepared sterile cold 0.05 M (50 mM) sodium citrate buffer, pH 4.5 in an aluminum foil covered 1.5 ml microcentrifuge tube immediately (not more than 10 min) prior to injection to a final concentration of 12 mg/lasts injections were given intraperitoneally (i.p.) using 1-ml syringes and 25-G needles at 200 mg per kg body weight. The doses were determined according to the body weight of animals but the volume injected was not more than 300
µl per animal. Mice in group I which served as a control group were injected with an equivalent volume of citrate buffer (pH 4.5) intraperitoneally. All the mice were returned to their cages and provided normal food; the mice in group II and Group III were provided 10% sucrose water instead of regular water. This was done to prevent fatal hypoglycemia and associated morbidity.

3.17. ENZYMATIC STUDIES

3.17.1 IN VITRO QUANTITATIVE DETERMINATION OF ASPARTATE AMINOTRANFERASE (AST)

This is being done by properly mixing 100 µl of Plasma and 1000 µl of AST reagent and by recording the absorbance at 60, 90 and 120 seconds respectively at 340nm. The mean absorbance change per minute was noted (Δ A/min).

Calculation:
AST activity (IU/L) = Δ A/min * kinetic factor
Δ A/min is the change in absorbance per minute
Kinetic factor K = 1768
AST activity in nkat/L = AST activity in IU/L * 16.67

3.17.2 IN VITRO QUANTITATIVE DETERMINATION OF ALANINE TRANSAMINASE (ALT)

This is performed by properly mixing 100 µl of Plasma and 1000 µl of ALT reagent and by recording the absorbance at 60, 90 and 120 seconds respectively at 340 nm. The mean absorbance change per minute was noted (Δ A/min).

Calculation:
ALT activity (IU/L) = Δ A/min * kinetic factor
Δ A/min is the change in absorbance per minute
Kinetic factor K = 1768
ALT activity in nkat/L = ALT activity in IU/L * 16.67

3.17.3 IN VITRO QUANTITATIVE DETERMINATION OF ALKALINE PHOSPHATASE (ALP)
This is done by properly mixing 20 µl of Plasma and 1000 µl of ALP reagent and by recording the absorbance at 60, 90 and 120 seconds respectively at 405 nm. The mean absorbance change per minute was noted (Δ A/min).

Calculation:
ALP activity (IU/L) = Δ A/min * kinetic factor
Δ A/min is the change in absorbance per minute
Kinetic factor K = 2712
ALP activity in nkat/L = ALP activity in IU/L * 16.67

3.18 HISTOPATHOLOGICAL STUDIES

On the experimental day 15, all the mice were fasted for six hours and then euthanized by cervical dislocation. The blood was collected by cardiac puncture from mice of all the three groups. The organs liver, kidney was isolated from all the mice. The blood from all the mice was processed further to obtain serum samples which were stored at -20°C which was used for enzymatic assays.

3.19 HEMATOLOGICAL STUDIES

Gently restrain the rabbit and place the rabbit on a towel and wrap the rabbit firmly to ensure it does not move.
Shave the ear or damp down the injection site with surgical spirit, smear Lignocaine on the vein wait at least 5 mins.
Hold the ear firmly with one hand, using a 23 gauge winged infusion needle and a (5 -10 ml) syringe insert the needle gently into the vein. The needle should be visible though the wall of the vein.
Draw the syringe plunger back “very slowly”.
Blood should appear in the tubing, continue slowly but don’t pull back the plunger too quickly or the vein will collapse.
If the vein does collapse, release the plunger and wait for the vein to refill with blood before recommencing.
Once the desired amount of blood has been collected, remove the needle and apply pressure to the vein with a tissue until the bleeding stops.
Gently expel the blood into 10ml serum tubes.
TC RBC, TC WBC, DC POLYMORPHS, DC LYMPHOCYTES, DC EOSINOPHILS, DC MONOCYTES were screened before and after the administration of microparticles in rabbit through the oral route.

3.20 PREPARATION OF CASEIN FILMS

500 mg of Propranolol hydrochloride was added to 100 ml of 10 % casein solution with glycerol and homogenized for 30 min @ 10,000 rpm. The solution was poured onto the petriplates and dried overnight at room temperature. The films produced were peeled, washed with 30 % of ethanol and air dried. The parameters such as casein concentration – Homogenization time, Homogenization rpm and Glycerol concentration were optimized and found as 10%, 30 min, 10,000 rpm and 1%.

<table>
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<th>Batch No</th>
<th>Casein volume</th>
<th>Casein conc</th>
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3.21 PHYSICAL EVALUATION OF FILMS

3.21.1. THICKNESS

The film thickness was evaluated using digital Vernier calipers and thickness uniformity was measured as standard deviation of average of five readings taken at five different sites.

3.21.2. FOLDING ENDURANCE

The folding endurance was calculated by cutting a strip of film (4x3 cm) evenly and repeatedly folding at the same place till it broke. This probability of the film to fold at the same place without breaking gives folding endurance.

3.21.3. WEIGHT VARIATION

Weight variation test was done by cutting three disks of 2*2 cm² weighing on electronic balance for. This test checks the uniformity of weight and in turn gives the batch-to-batch variation.

3.21.4. DRUG CONTENT DETERMINATION

The specified surface area of drug contained patches were cut and dissolved in (5% of methanol contained) 100ml of pH 7.4 phosphate buffer, vigorously shaked for 12hrs, and sonicated for 15minutes and centrifuged at 5000 rpm for 30 min. The drug contained polymeric solution were filtered through 42 number whatmann filter paper and 1ml of the filtrate was taken in a test tube and diluted for five times with same solvent using double beam UV-Visible spectrophotometer to determine drug content. Placebo patch was taken as a blank solution.

3.21.5. SURFACE pH

Bottenberg et al., (1991) described a method to determine Surface pH of the patches. Following the method, the patches were allowed to swell by keeping them in contact with 0.5 ml of double distilled water for 1 hour in glass tubes. The surface pH was identified by bringing a combined glass electrode near the surface of the patch and allowing it to equilibrate for 1 minute.

3.22 ENTRAPMENT EFFICIENCY OF CASEIN FILMS

By placing the films of 1 cm² in area in 50 ml of phosphate buffer saline, pH 7.4, entrapment efficiency was monitored.
3.23 IN-VITRO DRUG DIFFUSION

Dialysis membrane experiments using vertical type diffusion cell (Franz type) having receptor compartment 15ml volume with 2cm² area was done to study the drug diffusion. The receptor compartment was filled 15ml of phosphate buffer pH 7.4; the activated dialysis membrane was mounted on the flange of the diffusion cell receptor compartment. The prepared Transdermal patch with surface area 2cm² was placed on center of membrane and the donor compartment was then placed in position by clamping the two valves of the cell together. The whole assembly was kept on a magnetic stirrer and solution in the receptor compartment was constantly and continuously stirred using a magnetic bead and at 32.1°C maintained. Propranolol HCl loaded casein films were subjected to in-vitro release in Franz diffusion cell with at 100 rpm using PBS as the receptor medium. Aliquots were collected at predetermined points and an equal amount of buffer was replaced to maintain the volume. The amount of Propranolol HCl was quantified by UV-Vis Spectrophotometer at 290nm.

![Fig 3.2 Franz Diffusion Cell](image)