6. EXPERIMENTAL

6.1 PRE-FORMULATION DRUG CHARACTERIZATION

6.1.1 Infra Red spectroscopy of drugs
The infrared spectrum of pioglitazone and rosiglitazone was obtained using FTIR spectrophotometer (FTIR 8400 Shimadzu) FTIR spectrum was taken by KBr pellet method and was compared with the reference standard IR spectrums of both the drugs.

6.1.2 Solubility studies:
The solubility of selected drugs was determined in water and pH 7.4 phosphate buffer. An excess amount of drug was placed into solvent in 20ml vial and stirred with magnetic bar at 370 C till equilibrium is achieved. Saturated solutions were then filtered using a 0.45 μm filters; drug concentration was measured by UV spectroscopy.

6.1.3 Melting Point determination:
The melting temperature of drugs was determined using capillary method and confirmed by differential scanning calorimetry (DSC)

6.1.4 Thin Layer chromatography:
Aluminum backed silica gel 60 F254 HPTLC plates (10 cm × 20 cm, layer thickness 0.2 mm, E-Merck, Darmstad, Germany) prewashed with methanol was used for the study. These plates were prewashed with methanol and resolution factor (Rf) value of drugs was determined.
6.1.5 Drug – excipients compatibility:
Sample of drug and excipients (s) are intimately mixed usually equal parts and screened by I.R. with confirmation by TLC after storage under accelerated conditions of temperature and relative humidity (40°C, 75%) for three weeks. The chromatographic study was performed using aluminum backed silica gel 60 F254 HPTLC plates (10 cm × 20 cm, layer thickness 0.2 mm, E-Merck, Darmstad, Germany) as a stationary phase. The mobile phase for Pioglitazone consisted of Toluene: ethyl acetate:Formic acid (10:3:1) and for Rosiglitazone consisted of Chloroform: ethyl acetate:25% ammonium hydroxide (5:5:0.1). The Rf values of drug and physical mixtures were determined.

6.1.6 Development of analytical method

a. Pioglitazone

i. Preparation of standard Pioglitazone solution
Pioglitazone (10mg) was weighed accurately and dissolve in 60 ml 0.1N HCL. the solution was diluted up to 100 ml with distilled water, stock solution so prepared was containing 100 microgram of drug per ml of solvent.

ii. Determination of wavelength of maximum absorbance
Standard Pioglitazone solution (1ml) was pipetted in 10 ml volumetric flask. Then the volume was adjusted to the mark with 0.1N HCL. The solution (10mg) was scanned and absorbance was measured in the range of 200-400 nm against blank on Shimadhzu 1800 UV-Visible spectrophotometer. The blank was prepared in similar manner in which volume of standard drug solution was replaced by equal volume of 0.1 N HCl.

iii Calibration Curve for pioglitazone in 0.1N HCl
Take standard Pioglitazone solution and make up dilution 0-20µg/ml in 10ml volumetric flasks and volume was adjusted to mark by 0.1N HCl. Absorbance was read at against blank solution.
iv Calibration Curve for pioglitazone in pH 7.4 phosphate buffer
Take standard Pioglitazone solution and make up dilution 0-20 µg/ml in 10ml volumetric flasks and volume was adjusted to mark by pH 7.4 phosphate buffer. Absorbance was read at against blank solution.

b. Rosiglitazone

i. Preparation of standard rosiglitazone solution
Rosiglitazone (10mg) was weighed accurately and dissolve in 60 ml 0.1N HCL. The solution was diluted up to 100 ml with distilled water, stock solution so prepared was containing 100 microgram of drug per ml of solvent.

ii. Determination of wavelength of maximum absorbance
Standard rosiglitazone solution (1ml) was pipetted in 10 ml volumetric flask. Then the volume was adjusted to the mark with 0.1N HCL. The solution (10mg) was scanned and absorbance was measured in the range of 200-400 nm against blank on Shimadzu 1800 UV-Visible spectrophotometer. The blank was prepared in similar manner in which volume of standard drug solution was replaced by equal volume of 0.1 N HCl.

iii Calibration Curve for rosiglitazone in 0.1N HCl
Take standard Rosiglitazone solution and make up dilution 5, 10, 15 up to 40µg/ml in 10 ml volumetric flasks and volume was adjusted to mark by 0.1N HCl. Absorbance was read at against blank solution.

iv Calibration Curve for Rosiglitazone in pH 7.4 phosphate buffer
Take standard Rosiglitazone solution and make up dilution 5, 10, 15 up to 40µg/ml in 10 ml volumetric flasks and volume was adjusted to mark by pH 7.4 phosphate buffer Absorbance was read at against blank solution.

6.2 PREPARATION OF SOLID DISPERSION OF DRUG WITH POLOXAMER 188 AND 407 BY USING KNEADING METHOD.
a. Pioglitazone.

i. Preparation of physical mixture
A physical mixture (PM) of PG with PXM 188 or PXM 407 in 1:1 ratio was prepared by thoroughly mixing the accurately weighed quantity of drug and carrier in by using glass mortar and pestle for 5 min. This mixture was then subsequently passed through mesh no. 40 and stored in a dessicator for 48 hrs.

ii. Preparation of Solid dispersions
The Kneading method (KM) was used for the preparation of solid dispersion. Eight different drug: Carrier ratios (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7 and 1:8) were used. PG 1 to PG 8 corresponds to preparations containing PXM 188 and PG 9 to PG 16 correspond to preparations containing PXM 407. Rosiglitazone and PXM 188 or 407 were weighed according to these weighed ratios. RG and PXM were triturated using a small volume of methanol to give a thick paste, which was kneaded for 30 minutes and then dried at 40°C in an oven. The dried mass was then pulverized, passed through sieve no. 30, stored in a vacuum desiccator (48 hrs) and passed through sieve no. 60 before packaging in an airtight container.

b. Rosiglitazone

Preparation of physical mixture
A physical mixture (PM) of RG with PXM 188 or PXM 407 in 1:1 ratio was prepared by thoroughly mixing the accurately weighed quantity of drug and carrier in by using glass mortar and pestle for 5 min. This mixture was then subsequently passed through sieve no. 40 and stored in a dessicator for 48 hrs.

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6.3 MELT SONO CRYSTALLIZATION OF DRUGS (PIOGLITAZONE AND ROSIGLITAZONE)

i. Method of Preparation
The drug (2gm) was melted in vessel in paraffin oil bath. Molten mass was poured in vessel containing 20 ml deionized water and sonicated for 20 mins using probe sonicator (Chrome Tech ultrasonic) processor at pulse on off 1 sec with 5-8 mm probe diameter and 80%. The product obtained after solidification of disperse droplet was separate by filtration and dried at room temperature.

6.4 IN-VITRO CHARACTERIZATION OF DEVELOPED FORMULATION.
(FOR PIOGLITAZONE AND ROSIGLITAZONE FOR SOLID DISPERSION WITH POLOXAMER 188 AND 407 BY USING KNEADING METHOD AND MELT SONOCRYSTALLIZATION TECHNIQUE)

6.4.1 Solubility Determination

a. Pioglitazone.
Phase solubility was performed as described by Higuchi and Connors. Excess amount of solid dispersion were added to 25 ml phosphate buffer (pH 7.4) taken in a stoppered conical flasks, and mixture were shaken for 24 hrs in a rotary flask shaker. After shaking to achieve attain equilibrium, 2 ml aliquots were withdrawn at 1 hr intervals and filtered through what man filter paper no 40. The filtrate was analyzed spectrophotometrically at 270 nm. Shaking was continued until three consecutive reading were the same.

b. Rioglitazone.
i) Phase solubility was performed as described by Higuchi and Connors. Excess amount of solid dispersion were added to 25 ml phosphate buffer (pH 7.4) taken in a stoppered conical flasks, and mixture were shaken for 24 hrs in a rotary flask shaker. After shaking to achieve attain equilibrium, 2 ml aliquots were withdrawn at 1 hr intervals and filtered through what man filter paper no 40. The filtrate was analyzed spectrophotometrically at 228 nm. Shaking was continued until three consecutive reading were the same.

6.4.2 Fourier transform infra-red spectroscopy (Pioglitazone and Rosiglitazone)

FT-IR spectra were recorded on the sample prepared in KBr disks (2 mg sample in 200 mg KBr) using Shimadzu Fourier Transform Infra-Red spectrophotometer. The scanning range was 500-4000/cm with a resolution of 4/cm.

6.4.3 Differential scanning calorimetry analysis-(Pioglitazone and Rosiglitazone)

The thermal analysis was carried out with a Mettler Tolado DSC 60 (Japan). All accurately weighed samples were placed in sealed aluminum pans and heated at a rate of 20°C in the temperature range of 20-100°C temperature range under a nitrogen flow rate of 20 ml/min

6.4.4 Powder X-ray diffraction

XRD patterns were recorded using BRUKER-axs D8- ADVANCE, model generator Powder X-ray diffraction patterns were traced for drugs, various carriers and solid dispersion. The position and intensities of diffraction peaks were considered for the identification and comparison of crystallinity of the drug or carrier and melt sono crystallized powder.

6.4.5 Scanning electron microscopy-

The external morphology of solid dispersion and melt sono crystallized powder were analyzed by using a scanning Electron Microscope (SEM). The morphology of pure drug, PM and SDs was examined under a scanning electron microscope SEM – JSM 6360 A JEOL JAPAN.
6.4.6 In vitro drug dissolution

a. Pioglitazone

Accurately weighed preparations equivalent to 15 mg of pioglitazone were added to 900 ml of dissolution media (7.4 phosphate buffer) contained in USP dissolution apparatus II (Electro lab, TDT-08L) and stirred at a speed of 50 rpm at 37 ± 0.5°C. Five milliliter aliquots were withdrawn at 5, 10, 15, 20, 30 minutes and replaced by 5 ml of fresh dissolution media (37°C). The collected samples were analyzed after suitable dilution at 270 nm using UV-visible spectrophotometer against the blank. The dissolution of pure pioglitazone was done similarly. The release profile data was analyzed for cumulative percent dissolved at different time intervals and for dissolution efficiency at 5 and 15 minutes.

b. Rosiglitazone

Accurately weighed preparations equivalent to 15 mg of Rosiglitazone were added to 900 ml of dissolution media (7.4 phosphate buffer) contained in USP dissolution apparatus II (Electro lab, TDT-08L) and stirred at a speed of 50 rpm at 37 ± 0.5°C. Five milliliter aliquots were withdrawn at 5, 10, 15, 20, 30 minutes and replaced by 5 ml of fresh dissolution media (37°C). The collected samples were analyzed after suitable dilution at 228 nm using UV-visible spectrophotometer against the blank. The dissolution of pure rosiglitazone was done similarly. The release profile data was analyzed for cumulative percent dissolved at different time intervals and for dissolution efficiency at 5 and 15 minutes.

6.4.7 Stability Studies

Stability studies were carried out for optimized formulations as per ICH guidelines by placing the formulations at 40°C ± 2°C/75% RH ± 5% RH for 3 months. The Solid dispersion batch of optimized formulation were stored in stability chamber (Remi CHM-10S, India) at 40°C and 75% RH for 3 months and observed for the drug content at 1, 2, 3 months interval. The drug content and solubility was determined at the end of study.
6.5 IN VIVO STUDIES

Introduction
Pharmacokinetic profiles of drugs that are administered to the humans and animals have received growing attention as efforts to establish individual clinical dosage plans and to standardize drug preparations have increased. Evaluation of drug concentration-time data is necessary in understanding pharmacokinetic principles and is an essential aspect of pharmacokinetic research. Bioavailability is measurement of rate and extent of drug that reaches the general circulation. Bioavailability studies play a key role in the drug development period for both new drug product and their generic equivalents. Bioavailability is an important factor in the performance and quality of a dosage form and can have a major impact on the safety and efficacy of drug product. It also helpful to understand the in vivo behavior of drug. The area under drug concentration and time curve (AUC) is useful as a measure of the total amount of unaltered drug that reaches systemic circulation. Bioavailability studies are generally single dose comparisons of test dosage form with standard dosage in normal healthy animals in fasting state. Generally these studies are perform in two way cross over fashion were all the subjects receive both the test and standard formulation on different days. Fasting should occur for at least 12 hrs prior to dosing. Blood sampling must be frequent enough to design adequately the absorptive phase of the plasma concentration time course.

Pharmacokinetic analysis is performed by non compartmental (model independent) or compartmental methods. Non compartmental pharmacokinetic analysis is highly dependent on estimation of total drug exposure. Total drug exposure is most often estimated by Area Under Curve (AUC) methods, with trapezoidal rule, the most common estimation method.

6.5.1 Pharmacokinetic Terms
1. $C_{\text{max}}$ - This is the maximum drug concentration achieved in systemic circulation following drug administration.
2. $T_{\text{max}}$ - It is the time required to achieve maximum drug concentration in systemic circulation.
3. **AUC \(_{0-t}\)** - Area under the plasma concentration curve from zero hr to last quantifiable concentration to be calculated using trapezoidal rule.

4. **AUC \(_{0-\infty}\)** - Area under the plasma concentration time curve, from zero to infinity to be calculated as the sum of AUC \(_{0-t}\) plus the ratio of the last measurable concentration to the elimination rate constant.

5. **T\(_{1/2}\)** - Elimination half life of a drug is the time necessary to reduce the drug concentration in the blood, plasma or serum to one half after equilibrium is reached.

The choice of design should be based on many factors such as background information about formulation, variability within laboratory, variability between subjects etc. Good experimental design enhances the power of study. The validity of results does not necessarily increase with the number of subjects unless statistical aspects have been carefully considered. As far as possible the bioavailability study (single dose or multi dose) should be cross over in design and suitably randomized. For cross over design each subject receives more than one formulation at different time periods. Following are the advantages of cross over design.

1. It allows a within subject comparison between formulations.
2. It removes inter subject variability from the comparison between the formulations.
3. With proper randomization of subjects to the sequence of formulation administrations, it provides the best unbiased estimate for the differences between formulations.

In practice, a cross design is often considered to be the design of choice if the number of formulations to be compared is small, say no more than three.

### 6.5.2 Latin square cross over design

In case of more than two formulations, a Latin square Design should be used. For example in a bioavailability study of 3 formulations, a group of subjects will receive formulations in the sequence shown below:

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Study Period 1</th>
<th>Study Period 2</th>
<th>Study Period 3</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>B</td>
<td>C</td>
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<tr>
<td>2</td>
<td>B</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>A</td>
<td>B</td>
</tr>
</tbody>
</table>

The next group of three subjects will receive formulations in the same sequence as shown above.
6.5.3 Study Design

In-vivo Pharmacokinetic study
Based on the in-vitro dissolution profile, an optimum solid dispersion pioglitazone: PXM 188 (1:5w/w) prepared by kneading technique was selected for comparison of in-vivo performance against plain pioglitazone

Study design
6 albino New Zealand rabbits of average weight 2.5 ± 0.3 kg were used for the study. The rabbits were divided into 2 groups of 3 rabbits each with cross over technique (n=6). All rabbits were fasted overnight with adlibitum access to water during the experiment and the animals were fed 8 hours after the oral dose. One group of animals received a single dose of PG (15 mg/2ml), formulated as a suspension containing sodium carboxy methyl cellulose (equivalent to 0.5%w/w of the drug). The second group was administered a solution containing solubility enhanced kneaded PG: PXM (1:5w/w) at the same dose. The suspensions were administered orally through a sterile pediatric feeding tube (size 8) followed by 2 ml of distilled water to wash off any drug remaining in the feeding tube and upper alimentary tract. 1mL of blood sample was collected using 22 gauge needle from the shaved marginal ear vein into heparinzed Eppendorf micro-centrifuge tubes at time intervals of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 up to 8 hrs.. Xylene was applied to the marginal ear vein before withdrawal, which causes blood vessel to dilate. The blood samples were immediately centrifuged at 6000 rpm for 10 min to separate plasma and stored at -40°C until further analysis.

6.5.3 Extraction of pioglitazone from plasma
The plasma samples were spiked with known concentrations of PG in acetonitrile so as to obtain plasma concentrations of 100ng/ml, 200ng/ml up to 600ng/ml and 10.0 μg/ml. To 0.5 ml of the spiked plasma sample taken in a polypropylene centrifuge tube, 0.5 ml of acetonitrile was added and the samples were vortexes for 30 seconds to precipitate plasma proteins. 2 ml of chloroform was added and the samples were vortexed again for
2 minutes to extract PG into the organic layer. The mixture was then centrifuged for 15 minutes at 3000 rpm. Then, 1 ml of the organic layer was transferred to a clean glass vial and evaporated in a vacuum oven whose temperature was maintained constant at 40±1˚C. The dry residue was reconstituted with 1ml of acetonitrile, diluted with 1 ml of internal standard (1.5 μg/ml Rosiglitazone in acetonitrile) and vortexes for 30 seconds. The resulting solution having a final internal standard concentration of 400ng/ml, was filtered through 0.45μ syringe filter (Millipore) and 100 μl of the sample was injected and analyzed for PG content using the HPLC method mentioned below.

6.5.4 Analysis of Pioglitazone by High Pressure Liquid Chromatography (HPLC)

The concentration of PG in the plasma samples was analyzed by a standardized reverse phase HPLC method. The system consisted of a Agilent 12:20 Binary system with UV-detector, in-line degasser and an auto-sampler programmable coupled to a personal computer. Data and system management was handled by chromatography manager software. The separation was performed at 45˚C using a C{{\text{18}}} column [250 x 4.6 mm], supplied by Agilent Technologies, USA. The mobile phase comprised of buffer: acetonitrile (55:45% v/v) and was run at a flow rate of 1.0ml/min. The aliquots were loaded in an auto sampler tray in glass vials, 20μl sample was injected and the eluting peaks were monitored at a λmax of 270 nm. The developed HPLC method was validated for linearity (100 to 600 μg/ml), repeatability, and precision.

6.5.5 Pharmacokinetic Parameters Estimation and Statistical Analysis

Results from HPLC analysis were plotted as drug concentration in plasma vs. time. Non compartment pharmacokinetic parameters including \( T_{\text{max}} \), \( C_{\text{max}} \) and AUC were estimated by kinetica 5.0 computer program. The AUC values for each curve were calculated from time zero to the last data point using the trapezoidal rule with extrapolation to infinity. The \( \text{AUC}_{0-\infty} \) values obtained from curve were used to calculate the relative bioavailability Results of in vivo experiments are reported as mean ± S.D Statistical tests of significance were performed using Graph Pad Prism 5.0 software. The variables were compared with a one way ANOVA, A \( P \)-value less than 0.05 were considered significant.