5.0 EXPERIMENTAL WORK

Plan of work

Experimental work

Analytical method development

Preformulation Study

Development, Characterization and Optimization of Pulsatile Drug Delivery.

ACE+DIA Combination System | ACE+LEF Combination System | Salai gugul Formulation

- Core Tablet Formulation
- Compression Coated Tablet
- pH sensitive coated system (single pulse)
- Surface eroding polymer coated system (single pulse)
- Multi-coat system (double pulse)
- Tablets in capsule formulation (TIC)

- Core Tablet Formulation
- Compression Coated Tablet
- Coated system (double pulse)

- Final Double pulse system (multi coat)

- X-ray study

- Comparative pharmaco dynamic study

- Selection of best formulation

- Pharmacokinetic study of final formulation
Formulation and Development of DDSs for Combination therapy for Arthritis and Herbal Formulation

1. Development of Aceclofanc + Diacerein Combination (ACE+DIA)
   - Development and characterization of core formulation
   - Development & characterization of Tab in Tab formulation
   - Development and characterization of pulsatile system using pH sensitive polymers and surface eroding polymers
   - For pH sensitive coatings, optimizing the ratio of Eudragit and process optimization
   - For surface eroding coating - selection of suitable polymer and pore former, their optimization and process optimization
   - Development and characterization of Tablets in capsule dosage form in pulsatile release form.
   - Development and characterization of multi pulse system
   - In vitro characterization

2. Development of Aceclofanc + Leflunomide Combination (ACE+LEF)
   - Development and characterization of core formulation
   - Development & characterization of Tab in Tab formulation
   - Development and characterization of pulsatile system using pH sensitive polymers and surface eroding polymers
   - For pH sensitive coatings, optimizing the ratio of Eudragit and process optimization
   - For surface eroding coating - selection of suitable polymer and pore former, their optimization and process optimization
   - Development and characterization of Tablets in capsule dosage form in pulsatile release form.
   - Development and characterization of multi pulse system
   - In vitro characterization

3. Development of Herbal Formulation (Salai gugul)
   - Development & characterization of Core Tablets
   - Development and characterization of double pulse system
   - In vitro and pharmacognostic evaluation of Herbal formulations
Characterization of delivery systems (Wherever applicable)

1. Preformulation study
   - Drug preformulation & characterization
   - Drug-Excipients compatibility
   - Drug - Excipients compatibility study by FTIR spectra

2. Analytical method development
   - For two combinations
   - For Herbal formulation
   - Measurement of drug concentration in plasma

3. Physico-chemical evaluation
   - Pharmacopoeial specifications (Hardness, friability, weight variation, drug content, content uniformity, DT)
   - Thickness and diameter

4. Performance evaluation
   - In vitro drug release study for pulsatile delivery
   - Effect of dissolution variables on drug release through DOE
   - pH solubility of different ratios of polymers
   - Pharmacognostic and microbial attributes for herbal formulation
   - X-ray study to evaluate the desired lag phase in-vivo.

5. Stability Testing
   - Stability testing at 40°C/75% RH condition for 6 months for final optimized formulation
   - Ageing effect on assay and dissolution of final optimized formulation.

6. Pharmacokinetics and Pharmacodynamic study
   - Comparative dynamic study for multipulse system to select the best treatment.
   - Pharmacokinetics study with final formulation to evaluate the in-vivo lag phase
Material and Equipments used in the study

Table 5.1 shows list of materials used in the research work. All other materials/excipients used are of proper grade, of pharmaceutical category and GRAS certified (Generally recognized as safe). All solvents and/or chemicals used are of analytical grade and free from any contamination. Equipments used in manufacturing process and for characterization are given in Table 5.2. All glassware and other utensils used are of pharmaceutical grade and made up of suitable material of construction.

Table 5.1: List of materials used in the research work and Source

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Material</th>
<th>Vendor/Supplier/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aceclofenac</td>
<td>Amoli organics</td>
</tr>
<tr>
<td>2</td>
<td>Leflunomide</td>
<td>Alembic</td>
</tr>
<tr>
<td>3</td>
<td>Diacerein</td>
<td>Ami life sciences</td>
</tr>
<tr>
<td>4</td>
<td>Eudragit S100 &amp; Eudragit L 100</td>
<td>Evonik</td>
</tr>
<tr>
<td>5</td>
<td>Ethyl cellulose 10cps</td>
<td>Signet Chemical (Hercules)</td>
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<tr>
<td>6</td>
<td>Hard Gelatin Capsule</td>
<td>ACG</td>
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<tr>
<td>7</td>
<td>Lactose (200m)</td>
<td>DMV</td>
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<tr>
<td>8</td>
<td>Povidone (K-30)</td>
<td>ISP Tech</td>
</tr>
<tr>
<td>9</td>
<td>HPMC (6 cps) &amp; HPC (Klucel LF)</td>
<td>Ashland</td>
</tr>
</tbody>
</table>
Table 5.2: List of Equipments used in the work

<table>
<thead>
<tr>
<th>Sr No</th>
<th>Equipment /Software (model no.)</th>
<th>Supplier/Vendor (Place)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Weigh Balance</td>
<td>Sartorius (Mumbai)</td>
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<tr>
<td>2</td>
<td>UV Spectrometer (model: 2202)</td>
<td>Systronics (Ahmedabad, India)</td>
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<tr>
<td>3</td>
<td>HPLC (LC-2010 HT)</td>
<td>Shimandzu (Kyoto, Japan)</td>
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<tr>
<td>4</td>
<td>FTIR (Nicolet™ iS™ 10 FT-IR)</td>
<td>Thermo Scientific (Massachusetts, United States)</td>
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<tr>
<td>5</td>
<td>Rapid mixer granulator (Diosna, P 100)</td>
<td>Diosna (Germany)</td>
</tr>
<tr>
<td>6</td>
<td>Rotary tablet compression machine (CMD 4)</td>
<td>Cadmach (Ahmedabad)</td>
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<tr>
<td>7</td>
<td>Erweka Hardness tester (TBH 125)</td>
<td>Erweka GmbH (Ahmedabad)</td>
</tr>
<tr>
<td>8</td>
<td>Friability tester</td>
<td>Lab India (Mumbai)</td>
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<tr>
<td>9</td>
<td>Vernier Caliper</td>
<td>Tolexo</td>
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<tr>
<td>10</td>
<td>Dissolution Apparatus (Model TDT 06-T)</td>
<td>Electrolab (Mumbai)</td>
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<tr>
<td>11</td>
<td>Disintegration test apparatus (ED 2L)</td>
<td>Electrolab (Mumbai)</td>
</tr>
<tr>
<td>12</td>
<td>Punches &amp; Dies</td>
<td>GMI (Mumbai)</td>
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<td>13</td>
<td>Neocoata® (20 M)</td>
<td>Neo machine (Mumbai)</td>
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<tr>
<td>14</td>
<td>Design Expert® software (Version 10)</td>
<td>Stat Ease® Inc. (Minneapolis, US)</td>
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<td>15</td>
<td>Stirrer</td>
<td>IKA Labs (Germany)</td>
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<tr>
<td>16</td>
<td>Colloidal mill</td>
<td>Riddhi pharma (Ahmedabad)</td>
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<td>17</td>
<td>Stability Chamber</td>
<td>Thermo lab group (Mumbai)</td>
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<td>18</td>
<td>DSC (model no.: DSC821e)</td>
<td>Mettler-toledo Inc. (Ohio, US)</td>
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<tr>
<td>19</td>
<td>HPTLC (Linomat 5)</td>
<td>Camag (Switzerland)</td>
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</table>
5.1 Analytical Method Development

Simultaneous UV method was developed for the proposed combinations. Based on the solubility of the drug common solvent was selected for the method development.

5.1.1 ACE+DIA Method Development [105-108]

First order derivative UV method was developed using acidic methanol (methanol: acetic acid 99.8:0.2 % v/v) as a common solvent. In this method solutions of DIA and ACE were prepared separately by appropriate dilution of standard stock solution and scanned in the spectrum mode from 400 nm to 200 nm. The absorption spectra thus obtained were derivatized from first to fourth order and first order derivative spectra were selected for analysis of both drugs.

Simultaneous UV method

Overlay spectra was obtained in the range of 200 – 400 nm for 0.1 M acidic methanol solution of DIA (50 µg/ml) and ACE (25 µg/ml) (Fig 5.1).

![Figure 5.1: Overlay UV spectra for Diacerein and Aceclofenac](image)

From first to fourth order derivative spectra, first order derivative spectra was selected for quantification. (Fig 5.2)
Figure 5.2: First derivative graph (overlay) for Diacerein and Aceclofenac

From the derivative spectra, following wave length was selected for quantification in case of above combination

For Diacerein – 250 nm (Zero cross for Aceclofenac)
For Aceclofenac – 258 nm (Zero cross for Diacerein)
**Calibration curve for Aceclofenac**

Standard solutions were prepared within 10-50 µg/ml by appropriate dilution and from the absorption values calibration curve was prepared. (Fig 5.3)

![Calibration curve for Aceclofenac](image)

**Figure 5.3: Calibration curve for Aceclofenac (for Diacerein + Aceclofenac Combination)**

**Calibration curve for Diacerein**

Standard solutions were prepared within 10-30 µg/ml by appropriate dilution and from the absorption values calibration curve was prepared. (Fig 5.4)

![Calibration curve for Diacerein](image)

**Figure 5.4: Calibration curve for Diacerein (for Diacerein + Aceclofenac Combination)**

**Note:** Each results indicate the average value where n=3
Good linearity was observed within the developed range with \( R^2 \) value 0.995 and 0.997 respectively. Range of linearity observed for ACE 10-60 µg/ml and 10-50 µg/ml for DIA.

### 5.1.2 ACE+LEF Method Development [109-110]

First order derivative UV method was developed using acidic methanol (methanol: acetic acid 99.8:0.2 % v/v) as a common solvent. In this method solutions of LEF and ACE were prepared separately by appropriate dilution of standard stock solution and scanned in the spectrum mode from 400 nm to 200 nm. The absorption spectra thus obtained were derivatized from first to fourth order and First order derivative spectra were selected for analysis of both drugs.

**Simultaneous UV method**

Standard Methanolic solution of ACE + LEF were prepared by appropriate dilution of standard stock solution and scanned from 400 nm to 200 nm. (Fig 5.5)

![UV overlay plot for Leflunomide + Aceclofenac](image)

**Figure 5.5: UV overlay plot for Leflunomide + Aceclofenac**

Their first order derivative spectra were selected for quantification as shown in Fig 5.6.
Figure 5.6: First order derivative spectra (overlay) for LEF and ACE

From first order spectra following wavelength was selected for quantification.

For Leflunomide – 249 nm (Zero cross for Aceclofenac)

For Aceclofenac – 259 nm (Zero cross for Leflunomide)
**Calibration curve for Aceclofenac**

Standard solutions were prepared in the range of 10-60 µg/ml and standard calibration curve was prepared (Fig 5.7 for ACE and Fig 5.8 for LEF)

Figure 5.7: Calibration curve for Aceclofenac (for ACE + LEF combination)

**Note:** Each results indicate the average value where n=3

**Calibration curve for LEF**

Figure 5.8: Calibration curve for LEF for ACE+LEF Combination

**Note:** Each results indicate the average value where n=3
Good linearity was observed within the developed range with $R^2$ value 0.995 and 0.996 respectively. Range of linearity observed is 10-70 µg/ml for both ACE and LEF.

Both repeatability (within a day precision) and reproducibility (between days precision) were determined for the developed simultaneous UV methods for the proposed combination. Mean and relative standard deviations (n=3) were calculated and used to predict the accuracy and precision of the method. The relative standard deviation (RSD) of intra-day assay for two methods developed was ranged from 3% to 5 % and for the inter-day assay was from 4.5 to 6.0%. Accurate data ranged from 98.52 to 99.97%. Further specificity of method was evaluated by studying the excipients interfering study. Placebo samples were run and compared with drug spectra, showed no interfering effects. (Placebo samples show no absorbance at the $\lambda$ of each drug sample)

**5.1.3 Analytical method for Herbal formulation containing Salai gugul [111]**

HPLC method was used for the quantitative estimation of Boswellic acid. The mobile phase consisted of acetonitrile–water (90:10, % v/v) adjusted to pH 4 with glacial acetic acid. Samples were analyzed using the following parameters: flow rate, 2.0 mL/min; injection volume, 20 µL; run time 12 min; temperature, 27 ± 2ºC; detection wavelength, 260 nm, Column: Kromasil 100 C18. Unknown concentration was measured against the standard area of known Boswellic acid concentration. (Fig 5.9)
Fig 5.9: Chromatogram of standard BSE (Boswellia Serrata Extract) solution with peak for BA (Boswellic acid) at around 7 min

Linearity range observed was 1-100 µg/ml. Linear equation observed $y = 15774x - 10558$ with $r^2$ value 0.9978. Limit of detection (LOD) was found to be 0.4 µg/ml and Limit of quantification was found to be 1 µg/ml.

5.1.4 Analytical method for plasma concentration measurement of Aceclofenac [112]

The blood collected in kinetic study was mixed with the EDTA properly and centrifuged at 5000 rpm for 25 min for separation of plasma. The separated plasma was stored at -20°C until drug analysis was carried out using high performance liquid chromatographic (HPLC) method. Aceclofenac in plasma was determined by the reported validated HPLC method. Analysis was performed on a C18 RP-HPLC column. The mobile phase consisted of acetonitrile: phosphate buffer (40:60). The mobile phase was delivered at the flow rate of 1 ml/min. Detection was performed at 282 nm in a UV detector. Injection volume was 20 µl (flow rate – 1ml/min). The concentration of unknown plasma samples was calculated from the calibration curve plotted between peak area ratios of aceclofenac to IS (diclofenac sodium) against corresponding aceclofenac concentrations (Fig 5.10 & 5.11). LOD (Limit of Detection) & LOQ (Limit of Quantification) values were 6.8 & 50 µg/ml respectively.
Good linearity was observed within the range with $R^2$ of 0.972. Linearity range observed was 2.5-15 µg/ml for ACE.
5.1.5 Analytical method for plasma concentration measurement of Leflunomide [113]

Reported Reverse-phase high-performance liquid chromatography (HPLC) methods have been used for concentration measurement. The mobile phase consisted of 10 mmol/L potassium dihydrogen phosphate and 100 mmol/L potassium chloride in aqueous 25% acetonitrile, acidified to pH 3 with o-phosphoric acid (ratio 50:50). A mobile phase flow rate of 0.7 mL/min was used, corresponding to a column pressure of about 65 bar (6,500 kPa). Peaks were detected at an absorbance wavelength of 280 nm. The concentration of unknown plasma samples was calculated from the calibration curve plotted between peak area ratios of Leflunomide to IS (4-aminopyridine) against corresponding Leflunomide concentrations (Fig 5.12 & 5.13). LOD & LOQ values were 0.05 & 0.4 µg/ml respectively.

![Calibration curve for Leflunomide](image_url)

*Figure 5.12: Calibration curve for LEF (for kinetic study)*
Figure 5.13: Typical chromatogram for Leflunomide (Rt = 3 min) and IS (Rt = 5 min 15 sec)

Good linearity was observed within the range with $R^2$ of 0.9975. Linearity was observed within 2-200 µg/ml range.