CHAPTER - 4

EXPERIMENTAL METHODOLOGY

4.1 PLAN OF THE WORK

SUMATRIPTAN SUCCINATE+RESOMER RG 502 H

PREFORMULATION

FORMULATION

METHOD OF PREPARATION

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2] SPRAY DRYING

3] NANOPRECIPITATION

METHOD OF HARVEST

ULTRACENTRIFUGATION

MEMBRANE FILTRATION

[EVAPOATION UNDER REDUCED PRESSURE/

FREEZE DRYING]

FOR ALL SIX SS: RsG RATIO
EVALUATION

PROCESS EFFICIENCY.

SS CONTENT

SS LOADING

ENTRAPMENT EFFICIENCY

IN VITRO SS RELEASE

SS DIFFUSION (EX VIVO PERMEATION STUDY)

PARTICLE MORPHOLOGY [SEM]

PARTICLE SIZE DISTRIBUTION

STABILITY STUDIES

IN SITU NASAL PERFUSION STUDY

PHARMACOKINETIC STUDY

IN VIVO PHARMACODYNAMIC EFFECT
SCHEMATIC REPRESENTATION OF PREPARATION BY MULTIPLE EMULSION (W/O/W) FOLLOWED BY SOLVENT EVAPORATION

1. SS dissolved in distilled water
2. Homogenization/Sonication in ice bath
3. Formation of water-in-oil (w/o) emulsion
4. RsG dissolved in dichloromethane and methanol (90:10)
5. PVA (0.4%) in distilled water
6. Homogenization/Sonication
7. Formation of water-in-oil-in-water (w/o/w) multiple emulsion
8. Solvent evaporation
9. Harvesting
   1) Centrifugation
   2) Membrane filtration

EVAPORATION UNDER REDUCED PRESSURE
LYOPHILISATION
## 4.2 LIST OF EQUIPMENTS, APPARATUS

<table>
<thead>
<tr>
<th>NAME</th>
<th>MAKE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IKA®–WERKE.D-79219</td>
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<td>2. Sonicator</td>
<td>Sonics –Vibra Cell Model CV 18</td>
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<td>S.No 8219.</td>
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<td>3. Spray dryer</td>
<td>SD-05, Lab Plant Ltd., and United Kingdom.</td>
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<td>4. Rotary vacuum</td>
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<td>(-86°C),ULT FREEZER.Speed Vac Lyophilizer.</td>
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<td>Make: - Savant, Farmingdale, NY.</td>
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<td>5. Freeze Dryer</td>
<td>Electrolab equipments, Chennai, India.</td>
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<td>6. Perfusion pumps</td>
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<td>Scientific equipments.</td>
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<td>7. UV-visible spectrophotometer</td>
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<td>Scientific equipments.</td>
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<td>9. Hot plate</td>
<td>Dalal and co., Chennai, India.</td>
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<td>10. Magnetic stirrer</td>
<td>Dalal and co., Chennai, India.</td>
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<td>Sankar Scientifics,Chennai,India</td>
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13. Scanning electron microscope
   Joel, Japan
14. Particle sizer
   Malvern, UK
15. Micro centrifuge
   Electrolab, Chennai, India.
16. Cyclo mixer
   M.S. Dalal, Chennai, India.

Glass wares - All glassware used were of Borosil make.

4.3 LIST OF CHEMICALS

Sumatriptan succinate
   Gift sample Orchid chemicals, Chennai
Resomer RG 502 H –
   Boehringer Ingelheim, Germany
MW12000 D Tg + 46 °C.
Dichloromethane AR
   S.D fine chemicals, Chennai, India.
Methanol AR
   S.D fine chemicals, Chennai, India.
Poly vinyl alcohol
   S.D fine chemicals, Chennai, India.
Disodium hydrogen phosphate
   S.D fine chemicals, Chennai, India.
Sodium dihydrogen phosphate
   S.D fine chemicals, Chennai, India.
Sodium hydroxide
   S.D fine chemicals, Chennai, India.
Ethylacetate
   S.D fine chemicals, Chennai, India.
Acetonitrile
   S.D fine chemicals, Chennai, India.
Sodium pentobarbital
   S.D fine chemicals, Chennai, India.
Saccharose
   S.D fine chemicals, Chennai, India.
Morphine
   Sigma Aldrich.
Water for injection
Distilled water
4.4 PROCEDURE

4.4.1 PREFORMULATION

Calibration curve for SS in distilled water.

An accurately weighed sample of 100 mg of SS was dissolved in distilled water and made up to 100 ml in a standard flask with the same. Aliquots from this stock solution were taken and dilution was done using distilled water to obtain concentrations of 10, 20, 30, 40, 50, 60, 70 µg/ml. Absorbances of these were measured at \( \lambda_{\text{max}} \) of 228 nm. The data reproduced in Table 6. Graphical representation given in Figure 4.

Linear regression analysis (LRA) was done to obtain the LRA equation for straight line. This was used for calculating the unknown concentrations of SS.

Compatibility study:

10 g of SS was mixed with 10 g of RsG, in a glass mortar. This mixture was divided into 10 parts and stored in screw capped glass vials at the following conditions.

C-I: - At ambient room temperature

C-II: - 30 +/- 2\(^0\) C and 60 +/- 5 % Relative Humidity (RH)

C-III: - 5 +/- 3\(^0\) C

The samples were stored in the above mentioned conditions for 180 days. The parameters evaluated were physical appearance and SS content, periodically on 15, 30, 60, 90, 120 and 180 day.

SS content was evaluated by UV analysis at \( \lambda_{\text{max}} \) of 228 nm. The results shown in Table 7 for physical appearance and SS content.
4.4.2 PROCEDURE FOR PREPARATION

**Multiple-emulsion-solvent evaporation method (water-oil-water (w/o/w))**\(^{113,140}\)

SS was dissolved in distilled water (20 mg/ml). Resomer RG 502 H\(^ {®}\) (RsG) was dissolved in a mixture of methylene chloride (DCM) and methanol (90:10) (25 ml). The ratio of SS to RsG was fixed as shown in Table 8. Primary water in oil (w/o), emulsion was obtained by imparting shearing force. Two different methods were adopted to effect the process, namely-homogenization and sonication.

Homogenization was done using a high pressure, high speed homogenizer (UltraTurrax.T-25 Basic. IKA\(^ {®}\)-Werke. D-79219-Steufen) at the maximum speed of 24,000 rpm for 5 min, in an ice bath.

Sonication (Vibra cell, Sonics, USA) was effected by using a probe sonicator for 20 sec ON and 10 sec OFF cycle at 25 watts, 50% amplified for 5 min in an ice bath.\(^ {167}\)

0.4% w/v solution of poly vinyl alcohol (PVA) was prepared and was added slowly while mixing the primary emulsion by sonication or homogenization, so as to obtain a multiple emulsion of water–in–oil–in–water (w/o/w) type.

This was then magnetically stirred for 1 h at room temperature (37\(^{0}\)C), to evaporate the organic solvent. Harvesting of NPs was done by three different methods.

- **Centrifugation:** The resulting NPs in suspension were then centrifuged four times for 15-minute cycles at 15 000 x g at 4\(^{0}\)C and washed with distilled water to remove the free PVA and also any free SS from the batches. The centrifuged samples were either lyophilized or evaporated
under vacuum for the final harvesting of the NPs. Lyophilization or freeze-drying of the batches was performed at 0.03 mbar and at –60°C for 24 hours. Evaporation under reduced pressure was executed by placing the NPs suspension in a rotary vacuum evaporator and subjected to evaporation under vacuum.

- Membrane filtration: The resulting NPs suspension was passed through Ultipor®, nylon filter, fitted to a Buchner funnel.

By varying the process parameters of homogenization, sonication and the three different methods of harvesting (for each method all six ratio as mentioned in Table 8 was followed) a total of thirty six (36) formulations were prepared by w/o/w multiple emulsion method.

**Spray drying**

Modification of an established method was followed for spray drying.¹⁶⁹

SS was dissolved in distilled water (100 g in 100 ml). RsG was dissolved in sufficient volume of DCM, methanol mixture (90:10) (100 g in 100 ml). The volumes were varied according to the formula for obtaining the SS to RsG concentration as per Table 8. Tween 20 was added (1 ml for every 100 ml of the final volume). The liquid was fed to the nozzle with peristaltic pump, atomized by the force of the compressed air and blown together with hot air to the chamber where the solvent in the droplets was evaporated to the dry product.

The nozzle size was 0.5 mm. Flow rate was 2 ml/ min. Inlet temperature was fixed at 35 °C and outlet temperature was 45 °C. Compressed air flow rate 30 m³/h. The collected preparation was dried for 10 min in hot air oven at 85°C. Six different formulations FSD I to FSD VI were prepared.
Nanoprecipitation method

Modified nanoprecipitation method\textsuperscript{169,170}, was followed as explained below.

RsG was dissolved in DCM: methanol (90:10). SS was dissolved in distilled water (50 mg in 1 ml). RsG solution was taken in a syringe, placed with the needle directly inside the concentrated solution of SS in water, under high speed magnetic stirring and injected slowly. RsG was water insoluble. This lead to formation of NPs of RsG entrapping SS in the polymer matrix. The freshly formed particles were centrifuged four times for 10 min cycle at 15,000 x g and washed with distilled water. Samples were lyophilized. Six different preparations were prepared as per the ratio in table 8. Six different formulations FnP I to FnP VI were prepared.
4.4.3 EVALUATION

4.4.3.1 PROCESS EFFICIENCY:

All the prepared batches were subjected to process efficiency evaluation. The following equation was followed:\textsuperscript{140}

Process efficiency (\%) = \frac{\text{Weight of recovered NPs} \times 100}{\text{Weight of SS} + \text{Weight of RsG}}

The results shown in Table 9.

4.4.3.2 SS CONTENT:

About 100 mg of the NPs from the batches selected for evaluation were taken in a clean dry glass mortar. This was triturated with 5 ml of distilled water for 5 min. The dispersion was then placed in a cyclomixer and mixed for 10 min. Then the samples were centrifuged for 2 min (1000 x g).\textsuperscript{140} The clear supernatant was aspirated using a micropipette and SS content was determined by UV analysis at $\lambda_{\text{max}}$ of 228 nm. Results shown in Table 10.1 and 10.2.

4.4.3.3 SS LOADING:\textsuperscript{140}

This was amount of drug bound per mass of polymer (mg of SS per mg of RsG). This was numerically equivalent to drug content (when the drug content has been expressed for 100 mg).

SS loading (\%) = \frac{\text{Weight of SS in NPs} \times 100}{\text{Weight of NPs}}

The results shown in Table 11.
4.4.3.4 SS ENCAPSULATION EFFICIENCY

Drug incorporation efficiency was also referred to as drug recovery and also as drug entrapment or encapsulation efficiency.\textsuperscript{140}

Encapsulation efficiency (\%) = \frac{SS loading (\%)}{Percent of the initial SS content} \times 100

The results shown in Table 11.

4.4.3.5 IN VITRO SS RELEASE STUDY:

About 100 mg of NPs of the selected formulation were weighed and taken in a magnetically stirred covered conical flask. To this, 25 ml of pH7.2 phosphate buffer was added and mixed. Stirring was continued and samples of 5 ml were taken at periodic intervals of 0, 5, 10, 15, 30, 60, 90, 120 min. The samples were centrifuged (1000 X g or 2 min). The supernatant was used for evaluation by UV analysis at \( \lambda_{\text{max}} \) of 228 nm, after suitable dilution if necessary. Results of selected formulation shown in Table 12. Graphical representation of the same shown in Figure 5. LRA equation for the absorbance values of SS in pH 7.4 buffer at concentrations between 10 and 59 mcg/ml was applied for calculating unknown concentration. LRA equation given above Table 12.

4.4.3.6 SS DIFFUSION:

Porcine nasal mucosa was used for studying the diffusion of SS from the NPs. The porcine nasal mucosa was reported be suitable for evaluating the nasal diffusion.\textsuperscript{77}

The snout was obtained from the local slaughter house from healthy pigs. The body appearance and snout appearance were observed to avoid unhealthy pigs. Within 10 min of killing the animal, the snout was cut off and placed in phosphate buffer saline, enriched with glucose in an ice box. Transport to the lab was within 30 min.
The snout was opened up to expose the conchae. The mucosa covering the ventral nasal concha (cavity mucosa) was carefully removed using forceps and a scalpel. Circular pieces were cut for the diffusion study. Modified Ussing chamber was used for diffusion study. The mucosal side faced the donor chamber and formulations were placed on this side. The receptor fluid used was pH 6.8 phosphate buffer. 100 mg of NPs was placed on the mucosal side. The receptor fluid was stirred magnetically. Periodic samples of 2 ml were withdrawn and replaced with fresh buffer. Samples were taken at 0, 5, 10, 15, 30, 60, 90, 120 min. Samples were analyzed for SS by the reverse-phase high performance liquid chromatography (RP-HPLC) method. C_{18} column was used for separation. A mixture of ammonium phosphate monobasic (0.05 M) and acetonitrile in the ratio of 84:16 v/v was used as mobile phase. Degassed mobile phase was isocratically run at a flow rate of 1 mL/min. Detector was UV and λ_max was 228 nm. The sample chromatogram for calibration with SS reference substance shown in Figure 6. The test sample chromatogram shown in Figure 7. Results of the selected batches shown in Table 13. Graphical representation of the same shown in Figure 8.

The porcine nasal mucosa was subjected to histological evaluation before and after the SS diffusion study. A cross section of the mucosa was stained with eosin and haematoxylin stain and observed before the study. Figure 9 was the nasal mucosa before the diffusion study. After the completion of diffusion, the mucosa was removed and washed in running distilled water. It was then subjected to histological evaluation. Figure 10 was the observed mucosa after the diffusion study.

After 1 h of diffusion, NPs were collected and subjected to SEM analysis. Figure 11 shows the SEM photo of NPs (F-IV only) after 1 h of diffusion.
4.4.3.7 SCANNING ELECTRON MICROSCOPY

Samples were prepared by finely spreading the freeze dried sample of F-IV, over slabs and by drying them under vacuum as a routine procedure. The samples were then coated in a cathodic evaporator with a fine gold layer using an ion sputter. Coating was provided at 20 mA for 4 min. and observed at 20 kV in SEM using a JSM-5581 scanning electron microscope (JEOL, Tokyo, Japan).

The result reproduced in Figure 12 and Figure 13.

4.4.3.8 PARTICLE SIZE ANALYSIS

Formulation F-IV was subjected to particles size analysis in a Metasizer®, Malvern instruments, UK. F-IV was dispersed in sunflower oil at a concentration which produced approximately 14 % obscuration and analysed. The result reproduced in Figure 14. Graphical representation in Figure 15.

4.4.3.9 FOURIER TRANSFORM –INFRA RED (FT-IR) study

SS, RsG and F-IV were subjected to FT-IR analysis in Bomem model FT-IR. The potassium bromide disc method was followed for recording the FT_IR spectra of SS, RsG and F-IV. The spectra reproduced in Figure 16, 17, 18 and 19.

4.4.3.10 THERMAL ANALYSIS

Thermal analysis was performed on SS, RsG and F-IV NPs. Differential Scanning Calorimetry (DSC), Thermo Gravimetric Analysis (TG) and Differential Thermal Analysis (DTA) were executed, by standard procedures. The results shown in Figure 20 (DSC), Figure 21(DTA) and Figure 22 (TG and DTA).
4.4.3.11 STABILITY STUDY

Stability study of F-IV was carried out by the following method. Twenty vials, each contain 2 g of F-IV NPs, were taken for stability studies. The storage conditions adopted for stability testing were

C-II : 30 +/- 2°C and 60 +/- 5% RH

C-III : 5 +/- 3°C

for a period of six months.\textsuperscript{165,182}

The study was carried out for a period of 180 days. At periodic interval of 30 days, sample was taken. The parameters analyzed were

- SS content
- SEM analysis for the sample after six months
- Determination of CFPT (Critical Flocculation Point)

**SS content:**

SS content was determined by UV analysis at \(\lambda_{\text{max}}\) of 228 nm. Results shown in Table 14.

**SEM:**

Procedure described under 4.4.3.7 was followed for F-IV samples stored at C-II and C-III, after 180 days. Figure 23 and 24 show the same.

**Determination of CFPT:**\textsuperscript{171}

The colloidal stability of the NPs was evaluated by the determination of CFPT. This was measured as the resistance offered by NPs to electrolyte (sodium...
sulphate) induced NPs aggregation. F –IV, was dispersed in water for injection (10 mg in 5 ml). Sodium sulphate solutions of different molar concentrations between 0-1 M (0.1 M; 0.2 M; 0.3 M------1.0 M) were prepared. 125 µl of NP dispersion was added to 1 ml of the different concentration of the sodium sulphate solution and incubated at 37 °C in a mildly shaking water bath. Multiple samples were prepared and evaluated. After 10 minutes, the turbidity of the dispersions was measured at 564 nm. The CFPT was determined. CFTP is the electrolyte concentration at which a dramatic increase in the turbidity was first observed. The graphical representation of the results shown in Figure 25.1 and 25.2.

4.4.4 **EX-VIVO STUDY:**

This study was executed under two aspects. First the nasal perfusion study, followed by brain tissue sample analysis.

4.4.4.1 **IN SITU NASAL PERFUSION:**

The study was executed after getting due approval from the 14th IAEC/CLBMCP / Proposal number 16 and 18/2005.

The animal model selected for nasal perfusion was rat. The procedure explained by Hussain 172 and Yang et al 173 was adopted.

Group I : Solvent control

Group II : Reference standard

Group III : Test – F-IV

Each group had six rats (all male, weighing 200 to 250 g), were selected and grouped randomly. All were in bred strains obtained from the animal house of C.L.Baid Metha college of Pharmacy, Chennai-97, India.
Solvent control was water for injection. Reference standard was SS dissolved in water for injection at a concentration of 20 mg per 0.1 ml. Test was prepared by dispersing NPs of F-IV equivalent to 20 mg in 0.1 ml of water for injection.

The surgical procedure detailed below was proceeded\textsuperscript{172}.

The rats were anesthetized with i.p injection of sodium pentobarbital. The animals were made to lie supine and secure in the position through out the experiment. An incision was made in the neck and the trachea was cannulated with a polythene tube of suitable diameter. Another tube was inserted through the posterior part of the nasal cavity. The passage of the nasal palantine tract was sealed to prevent drainage of drug solution from the nasal cavity into the mouth. The perfusion pump was used to perfuse the drug solution, both test and reference at the rate of 0.1 ml per minute, through the nostrils. A cannula measuring 15 mm long and a diameter of 0.9 mm was used for the perfusate inlet. This was connected to the SS reservoir. It was adjusted in such a way that the cannula was inserted to a length of approximately 15 mm from the external end of the nostril. The perfused samples were then collected via the tube attached to the posterior part of the nasal cavity. The samples were collected after 1, 5, 10, 15, 30 min of perfusion. The samples were evaluated for SS content by validated RP-HPLC method.\textsuperscript{166} Sample chromatograms shown in Figure 26.

The difference between the outlet and inlet SS concentration was deemed to be absorbed via the nasal route. Table 15 shows the results and Figure 27B shows the graphical representation of the same.
4.4.4.2 BRAIN TISSUE SAMPLE ANALYSIS:

Three rats from the perfusion study, after 15 and 30 min interval were used for brain tissue sample analysis. After perfusing for 15 min, three of the six rats from groups I, II and III were removed. The heads was severed, skull cut open and brain was harvested. Brain tissue was cleaned of surrounding blood vessels, washed; weighed. The frontal area of the brain tissue where the olfactory lobes were located was sliced. The Figure 31, shows the schematic representation of the section selected for analysis. This part was homogenized in phosphate buffer saline (pH 7.4). 1.5 ml of 2M sodium hydroxide was added to the homogenate and the mixture was vortexed for 30 sec. The sample containing SS was extracted with 4 ml ethyl acetate. The mixture was shaken for 5 min and centrifuged for 15 min at 10\(^0\) C. Extraction procedure with 4 ml ethyl acetate was repeated thrice for each sample. The extracts were mixed in a culture tube. Then it was evaporated to dryness. The residue was reconstituted with the mobile phase 0.3 ml and injected to the system. The RP-HPLC was followed for SS quantification.\(^{174}\) HPLC system with UV detector was used. The separation was achieved by using 25 cm x 4.6 mm ID, 10 micro ODS Hypersil column. Column temperature was 25\(^0\) C. The mobile phase flow rate was 1.0 ml/min and consisted of mixture of ammonium phosphate monobasic (0.04 M) - acetonitrile (78:22, v/v) pH 3.3 which was adjusted with o-phosphoric acid. The mobile phase was degassed and filtered 0.22 mm prior to use. The injection volume was 20 ml. And the detection wave length was set at 228 nm.

Figure 28 show the chromatogram of SS standard and figure 29 show the chromatogram of test sample.

The amount of SS detected shown in table 16 and graphical representation in Figure 30.

Figure 31 shows the picture of rat’s brain.
4.4.5 PHARMACOKINETIC STUDY

The study was carried out after getting approval from 14th IAEC/CLBMCP / proposal number 19/2005. Animal model used were rabbits. Male rabbits weighing between 1 to 2 kg were used. Six rabbits in each group.

Group I : - Solvent control
Group II : - Reference standard (nasal route)
Group II B : - Reference standard (i.v. route)
Group III : - Test F-IV.

The solvent used was water for injection.

The dose of SS was 0.6 mg/Kg body weight. Reference standard was SS dissolved in water for injection to obtain a concentration of 0.5 mg per 0.1 ml. The same was administered to all animals in group II B. All solutions were passed through 0.22 μm Millipore bacterial filters prior to administration. Rabbits were grouped at random and route of administration was through the nostrils. For Group II B only the drug was administered through the i.v route (as the administered dose was small, the marginal ear vein was selected for administration). All animals were in the conscious state. The rabbit was restrained in a wooden box during the whole experiment. All nasal preparations were administered with the rabbit in a fixed standing position forcing its head slightly backward. Before administration, the tube was inserted into the nostril two times to prevent the sneezing reflex of the rabbits upon insertion. The rabbits’ head was held upright and using a micropipette and an in-house device, which had a polyethylene tube PE 50 to deliver the spray. The tube was placed at about 5 mm in the nostril and sprayed. Following this, the reference, test
was sprayed in to the nostril. One spray only containing equivalent dose calculated based on the body weight of each rabbit, in any one nostril, was administered. After intranasal administration the rabbit was kept in a vertical position for one minute to prevent leakage of the solution out of the nostril.\textsuperscript{80}

Blood samples were collected via the marginal ear vein at periodic intervals of 5, 15, 30, 60, 90, 120, 240 min. The samples were analyzed for SS content by the following procedure.\textsuperscript{174} A 1 ml aliquot of rabbit plasma sample was placed in a screw capped glass tube. 1 ml of 2 m sodium hydroxide was added and the mixture was vortexed for 30 sec. To this 4 ml of ethyl acetate was added. The mixture was mixed in a cyclomixture for 5 min and then centrifuged at 10\textsuperscript{0} C for 15 min. Ethyl acetate extraction was done twice for each sample. The two extracts were taken in a culture tube and evaporated to dryness. The extraction residue was reconstituted with 0.3 ml mobile phase and injected to the system.

RP-HPLC method explained under section 4.4.4.2 was followed for determination. The chromatogram after baseline correction using blank (plain rabbit plasma treated in the same manner as for the samples but devoid of SS), was obtained. Calibration was executed using known concentrations of SS. Concentrations of SS in samples were calculated from the peak areas. Results were analyzed for pharmacokinetic parameters.

Table 17 shows the plasma concentrations of different groups; Table 18 gives the pharmacokinetic parameters.

**PHARMACOKINETIC PARAMETER CALCULATIONS**

The pharmacokinetic parameters, like $C_{\text{max}}$, $T_{\text{max}}$, AUC, AUMC, and MRT were calculated using pharmacokinetic calculation software: pK summit solutions.
The calculations reproduced in pages 154 to 176.

The bioavailability of a drug after i.n. administration may be expressed in terms of absolute nasal absorption, determined from the area under the plasma concentration curve (AUC), following i.v and i.n. dose.

\[
\text{Absolute nasal absorption} = \frac{(AUC)_{i.n.} \cdot (Dose)_{i.v}}{(AUC)_{i.v} \cdot (Dose)_{i.n.}}
\]

Relative nasal bioavailability may be determined by the following equation.

\[
\text{Relative nasal absorption} = \frac{(AUC)_{i.n. \text{ test}} \cdot (Dose)_{i.n. \text{ reference}}}{(AUC)_{i.n. \text{ reference}} \cdot (Dose)_{i.n. \text{ test}}}
\]

AUC is extrapolated to an infinite time following the administration of a single i.v or i.n. dose.

All calculated pharmacokinetic parameters shown in Table 18.

4.4.6 PHARMACODYNAMIC STUDY

The \textit{in vivo} evaluation was carried out after obtaining proper approval from 14\textsuperscript{th} IAEC /CLBMCP/ Proposal number 17/2005. The animals (mice), used were inbred strains, obtained from animal house of C.L.Baid Metha college of Pharmacy, Chennai-97, India. The method validated by Galeotti \textit{et al}\textsuperscript{175}, for evaluating anti migraine compounds was adopted.

PROCEDURE: Male albino mice (20-25 g) were used for this evaluation. Water for injection was used as solvent for all the groups.

Plain SS (reference standard), in water for injection at a concentration of 1 mg/0.1 ml was used as reference standard.
Test formulation F-IV, the freeze dried powder was reconstituted immediately before administration to the animals with water for injection by simple dispersion. NPs equivalent to 1 mg SS in 0.1 ml of the final dispersion was used as test.

The classification of group and the treatment given were:

**Group 1**: Solvent control (not exposed to morphine)

**Group 1 B**: Morphine –treated solvent control

**Group 2**: Reference standard

**Group 3**: Test formulation F-IV

**Animals**: 18, six in each group. Mice – male, weighing 20-25 g.

Morphine administration schedule: - Mice were treated by administering morphine in the following manner. A solution of morphine in 5 % saccharose solution was placed in the water bottles in increasing doses as follows:

- **Day 1 and 2**: 0.1 mg / ml
- **Day 3 and 4**: 0.2 mg / ml
- **Day 5 and 6**: 0.3 mg / ml
- **Day 7 to 15**: 0.4 mg / ml

On day 15, the morphine and saccharose solutions were replaced with tap water at a time referred to as 0 h. Four hours after the replacement with water, mice showed a significant reduction of pain threshold in the hot plate test that reached the highest intensity after 6 h (1.8 ± 0.8 s of licking latency versus 5.7 ± 1.1 s in the control group). This was evaluated by the standard procedure.177

As the test executed was to evaluate the efficacy after administration through the nasal route, the test and reference were administered 10 min before the 6 h.
The mice were held gently and volume [containing 0.6 \( \mu \text{g/kg} \) body weight of SS], was measured using a micropipette. The test, reference and solvent were administered approximately 3 mm in to the nostril using a polyethylene tube PE 10 attached to a micropipette. All solutions were passed through 0.22 \( \mu \text{m} \) Millipore bacterial filters prior to administration.

HOT PLATE TEST\(^{176}\): Mice were placed inside a stainless steel container, thermostatically set at \( 52.5 \pm 0.1^0 \text{C} \), in a precision water bath. Reaction times were measured pretest and after treatment. The latency to respond with either a paw lick, paw flick, or jump (which ever comes first) was measured to the nearest 0.1 seconds. After observing the response, the mouse was immediately removed from the hot plate and returned to its home cage. Animals were tested one at a time and were not habituated to the apparatus prior to testing. Each animal was tested only once. Those mice scoring over 8 sec, in the pretest were rejected. An arbitrary cut off time was adopted. The licking latency values were recorded at 5,10,15,30 min after the 6 h. The effect of the test was compared versus the solvent control and the reference standard. Results tabulated in table-19. Values were mean of six readings +/- standard error of mean(sem). Graphical representation of the results given in figure 32.

ABDOMINAL CONSTRICTION TEST: The groups were the same as for the morphine withdrawal method. i.n. administration was followed by the same procedure explained above. After 10 min of the i.n. administration, mice were injected intraperitonially with a 0.3\% solution of acetic acid (10 ml/kg). The number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection.

Results shown in Table 20 were mean of six values +/- standard error of mean. Graphical representation of the same in Figure 33.