FORMULATION AND EVALUATION OF ORAL DELIVERY SYSTEMS FOR POORLY ABSORBED DRUGS USED IN OSTEOPOROSIS

A Thesis submitted to Gujarat Technological University

for the Award of

Doctor of Philosophy

in

PHARMACY

by

MILIND MADHAV THOSAR
Enrolment No.119997290025

GUJARAT TECHNOLOGICAL UNIVERSITY
AHMEDABAD
JULY 2017

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under supervision of

Dr. S. S. PANCHOLI

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ABSTRACT

Bisphosphonates (BPs) are drugs found useful in physiological regulation of calcification, bone resorption and currently have become drug of choice for the management of osteoporosis. The major drawback of the clinically utilized BPs is their poor oral absorption from the GI tract, typically less than 1% is absorbed which is due to their poor permeability. In addition, the BPs have been associated with undesirable gastrointestinal effects. Risedronate sodium (RIS) is a bisphosphonates popularly indicated for osteoporosis that belongs to BCS Class III and has poor oral bioavailability due to less permeability. The present work attempted to improve poor permeability of RIS through dual approaches, multiple emulsion and sublingual spray formulations which were found promising. In first approach Initially RIS w/o/w multiple emulsion formulations were prepared and evaluated for determining significant causative factors and their effect on formulation properties. Consequently determined causative factors were included in central composite design and total 18 formulations were prepared and evaluated for appearance, pH, globule size, viscosity, % creaming, pourability, drug entrapment efficiency, *in vitro* drug release, *ex-vivo* permeation study. Numerically optimized F19 formulation was additionally subjected for zeta potential and stability test. Stability of F19 was improved by including surfactant blend, phase volume ratio and hydrophilic polymer in outer water phase and stabilized F19B was evaluated for *in vivo* absorption study. The second approach involved formulation of propellant free RIS sublingual spray using face centered central composite design in which independent variables considered were concentration of drug, co-solvent and spreading agent while responses as spray pattern, spray angle and drug permeation and optimized by numerical method to get optimized sublingual spray FO. Total 16 formulations of RIS sublingual spray were prepared. All formulations were evaluated for spray pattern, spray angle, leak test, prime test, drug delivery uniformity, drug content per spray, *in-vitro* drug release and *ex-vivo* drug permeation study data. It was concluded from the study that independent variables had not caused any significant effect on delivery characteristics but influenced the permeation. Higher level of independent variables showed higher drug permeation. The study concluded with satisfactory performance of the device and feasibility of the sublingual formulation of RIS. *In vivo* absorption study was aimed to know the enhancement in permeability of optimized and stabilized RIS formulations FO and F19B after oral administration in rats and the extent of absorption was determined. Rats were divided in different four groups and blood samples

were collected at regular interval for 8 hours. Various pharmacokinetic parameters like C_{max}, T_{max} and AUC were found 1300.10 ± 50 ng/ml, 4 hr and 4425 ± 0.20 ng.hr/ml for optimized multiple emulsion formulation F19B and 920 ± 0.045 ng/ml, 5 hr and 3710 ± 0.18 ng.hr/ml for sublingual formulations FO. The enhancement in permeability of RIS was evident from pharmacokinetic data when compared with drug solution and conventional preparation which were 420 ± 20 ng/ml, 8 hr, 1630 ± 80 ng.hr/ml and 460 ± 23 ng/ml, 6 hr, 1855 ± 0.10 ng.hr/ml accordingly for C_{max}, T_{max} and $AUC_{0-8.}$ Relative bioavability of RIS multiple emulsion formulation was 3 fold and sublingual formulation was 2 fold than the plain drug solution and conventional preparation. The study suggested sublingual spray and multiple emulsion formulation may be an alternative way of administration of RIS, providing enhanced bioavailability and also opened future scope for incorporation of other bisphosphonates.

Acknowledgement

Table of Content

List of abbreviations

Abbreviations	Original phrase
ΔA	Absorbance difference
μg	Microgram
API	Active pharmaceutical ingredient
ATP	Adenosine triphosphate
AUC	Area under curve
BCS	Biopharmaceutical classification system
BPs	Bisphosphonates
BSA	Bovine serum albumin
D_{max}	Maximum diameter
D_{min}	Minimum diameter
EDTA	Ethylene diamine tetra acetic acid
EE	Entrapment efficiency
F	relative bioavailability
FT IR	Fourier transform infrared spectroscopy
GIT	Gastro intestinal tract
GRAS	Generally regarded as safe
HLB	Hydrophilic lipophilic balance
ICH	International conference on harmonization
IOD	Intraoral dosage form
IPM	Isopropyl myristate
KBr	Potassium bromide
LOD	Limit of detection

LOQ	Limit of quantification
ME	Multiple emulsion
Mins	Minutes
O/W	Oil-in-water
OC	oral cavity
OMD	Oral mucosal delivery
OTD	Oral transmucosal delivery
PEG	Polyethylene glycol
q.s	Quantity sufficient
RH	Relative humidity
RIS	Risedronate
RP-HPLC	Reverse phase high performance liquid chromatography
RPM	Rotation per minute
SDEDDS	Self-double-emulsifying drug delivery systems
UV	Ultra violet
VCM	Vancomycin
W/O/W	Water-oil-water
λ_{max}	Wave length of maximum absorption

List of Symbols

Symbol	Description
pH	Concentration of hydronium ion
λ_{max}	Wave length of maximum absorption
mg	Milligram
µg	Microgram
ml	Millilitre
ng	Nanogram
nm	Nanometer
Δ	Difference
%	Percentage
θ	Angle in degree
J_{ss}	Flux
t	Time

List of Figures

List of Tables

List of appendices

Appendix A: IAEC approval certificate

CHAPTER -1

Introduction

1.1 Background of the study

Osteoporosis is characterized by low bone density with slow deterioration of bone tissue resulting in fragile and porous bone, thus increasing the susceptibility to fracture in patients having age more than 50 years. Based on 2001 census, around 163 million Indians were above the age of 50; this number would have expected to increase to 230 million by 2015. Even conservative estimates suggest that of these, 20 per cent of women and about 10-15 per cent of men would be osteoporotic. The total affected population would, therefore, be around 25 million. The goal of treatment of osteoporosis is to prevent bone fractures by early detection simultaneous adopting universal health measures and timely treatment of osteoporosis can substantially decrease the risk of future fractures and patient can return to their routine life. However therapeutic intervention in this disease is costly and often found to be discontinued due to drug associated unwanted effects. Bisphosphonates, selective estrogen receptor modulator, Calcitonin, Parathyroid hormone and Denosumab are currently used medicines in osteoporosis. Bisphosphonates amongst are primary agents in the current pharmacological arsenal in treatment of osteoporosis and other bone diseases [1] [2].

1.1.1 Bisphosphonates and risedronate approval history

Bisphosphonates (BPs) were first used in the early 1960s as a potential therapy for bone diseases and they are current arsenal for the treatment of osteoporosis and other bone diseases. BPs intervene osteoclast mediated bone resorption and increase bone density and reduce fracture incidents. BPs approved as drug therapy in osteoporosis includes alendronate, ibandronate, zoledronic acid and risedronate (Actonel, Actonel with Calcium,

and Atelvia; Warner Chilcott). Globally actonel is approved in more than 90 countries andIts first approval for corticosteroid-induced osteoporosis was came in October 1999 and osteoporosis in men July 2006. Actonel is prescribed to huge number of patients every year in the US and throughout the world [3].
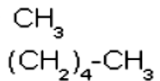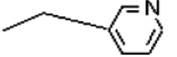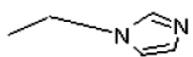
1.1.2 Structure Activity Relationship of bisphosphonates

The activity of bisphosphonates differs from one to another due to the length and substitution of the aliphatic carbon atom [4]. The potency of inhibiting bone resorption varies between different bisphosphonates by up to 5000-fold to 1. The antiresorptive activity increases in order, as etidronate < tiludronate < clodronate < pamidronate < alendronate <Risedronate [5]. The biological activity of the bisphosphonates can be modified by altering the structure of the two side chains on the carbon atom. The binding to bone mineral depends upon the P–C–P structure and is enhanced by including a hydroxyl group at R1. The structure and three-dimensional configuration of the R2 side chain determines the cellular effects of bisphosphonates, and their relative efficacies as inhibitors of bone resorption. Each bisphosphonate has its own profile of activity, determined by its unique side chain and bisphosphonates side chains in Fig. 1.1 and 1.2.
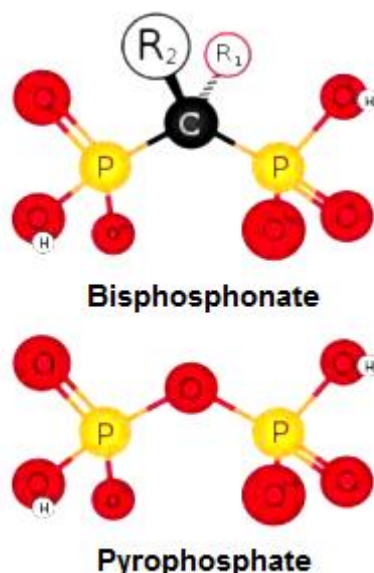
(Source *https://en.wikipedia.org/wiki/Bisphosphonate*)

FIGURE 1.1 Chemical structure of Bisphosphonates

Agent	R_1 side chain	R_2 side chain
Etidronate	-OH	$-CH_3$
Clodronate	-Cl	-Cl
Tiludronate	-H	$-S-\langle\bigcirc\rangle-Cl$
Pamidronate	-OH	$-CH_2-CH_2-NH_2$
Neridronate	-OH	$-(CH_2)_5-NH_2$
Olpadronate	-OH	$-(CH_2)_2N(CH_3)_2$
Alendronate	-OH	$-(CH_2)_3-NH_2$
Ibandronate	-OH	$-CH_2-CH_2N \big\langle \begin{array}{l} CH_3 \\ (CH_2)_4-CH_3 \end{array}$
Risedronate	-OH	(pyridine structure)
Zoledronate	-OH	(imidazole structure)

(Source *https://en.wikipedia.org/wiki/Bisphosphonate*)

FIGURE 1.2 Bisphosphonates side chains

After the promise shown in the early clinical use of etidronate and clodronate, newer bisphosphonates were synthesized, containing a primary nitrogen atom in an alkyl chain (pamidronate, alendronate). This increased the antiresorptive potency by up to one hundred times. Later modifications of the R2 side chain to produce compounds containing tertiary nitrogen groups, such as ibandronate and olpadronate, further increased potency. The most potent bisphosphonates to date, risedronate and zoledronate, contain a nitrogen atom within a heterocyclic ring. They are up to 10 000 times more potent than etidronate in some experimental [6].

1.2 Oral Bisphosphonates (BPs)

Oral bisphosphonates are drugs that belong to biopharmaceutical classification system (BCS) III, and available in conventional tablet and capsule dosage forms. These agents are administered in the morning in the fasting state and patients must remain without food or drink for 30 – 60 minutes after administration. Being BCS class III drugs these drugs have low permeability and an often interaction of these compounds with food present in the digestive medium causes poor absorption and lead to extent of 3% bioavability only. Poor bioavailability of oral bisphosphonates has necessitated the administration of higher than normally required oral doses which often leads to economic wastages, risk of toxicity,

erratic and unpredictable responses. However, the clinical efficiency shown by the bisphosphonates is sufficient to justify the use of the oral route. Further, to obtain an effective antiresorptive effect in tumor osteolysis, a relatively high dose of drug must be given parenteral [7] [8].

1.3 Recent work attempted on BPs (for oral absorption enhancement)

Various approaches undertaken to improve the oral absorption of bisphosphonates were based on either to modify the parent drug structure or to deliver the drug in a new formulation.

1.3.1 Molecular modification

Molecular modification approach entails the structural alteration of a known and previously characterized lead compound. The starting molecule is called a prototype, whilst the derivatives obtained from the prototype are classified as either analogues or prodrugs, depending on the type of molecular modification executed. It was only from the 1970s onwards that the concept emerged of designing prodrugs to modify the non-ideal physicochemical properties of certain compounds, thus making them more efficient. Nevertheless, a prodrug is indeed a new molecular entity, thus its safety and efficacy must be considered when applying this approach [9].

1.3.1.1 Non specific pro-drug approach (bio precursors)

Unlike most lipophilic agents, hydrophilic molecules are generally not passively absorbed across intestinal epithelia, largely due to restricted permeation across the brush border and the basolateral membranes. To improve oral absorption, a classic prodrug approach with greater permeability across the intestinal epithelium than the active can be adopted to enhance drug lipophilicity and passive diffusion. Using the prodrug approach, oral absorption can be increased by masking one or more ionisable groups in drug compound [10]. Niemi et al. (1999) synthesized Prodrug of clodronic acid which was tetra-, tri-, and P,P'- dipivaloyloxymethyl esters of clodronic acid and evaluated in vitro. All pivaloyloxymethyl esters were significantly more lipophilic (log P_{app} ranged from -2.1 to 7.4) than clodronate (log $P_{app} \leq -5.4$), which suggests that it may be possible to change the intestinal absorption mechanism of clodronate from a paracellular to a transcellular pathway by a prodrug approach. Intermediate degradation products were further degraded,

and clodronic acid was released in quantitative amounts mostly due to the chemical hydrolysis[11]. Marko et al. (1999) , in a study synthesized P,P'-Diacetyl, P,P'-dibutyroyl, P,P'-dipivaloyl, and P,P'-dibenzoyl (dichloromethylene) bisphosphonic acid dianhydride disodium salts as novel bioreversible prodrugs of clodronate. The dianhydrides alone were more lipophilic than the parent clodronate, as determined by drug partitioning between 1-octanol and phosphate buffer at pH 7.4. The aqueous solubility of clodronate decreased considerably in the presence of Ca_{2+} ions. This is most probably due to formation of poorly water-soluble chelates, which may also hinder the oral absorption of clodronate. However, Ca_{2+} ions did not have an effect on the aqueous solubility of clodronic acid dianhydrides, and therefore, these prodrugs may improve oral absorption of the parent drug. In conclusion, these novel dianhydride derivatives may be potentially useful prodrugs of clodronate which, due to their lipophilicity and lack of Ca_{2+} chelating, increase its bioavailability after   oral   administration [12].

Erez et al., (2008) used a self immolative linker to attach tryptophan to a bisphosphonate component through a carbonate-labile linker, 4-hydroxy-3, 5-dimethoxybenzyl alcohol, which was further attached through a stable carbamate linkage to the amine group of tryptophan. The carbonate linkage hydrolyzed with a half-life of 90 h, but it can be modulated through the nature of the substituent on the aromatic ring of the self-immolative linker. Prodrug showed more liphophilicity causing passive transport across intestinal membrane with more oral absorption than the parent polar, hydrophilic bisphosphonates. Prodrug was stable in gastric fluid .Prodrugs were having significant binding capability to hydroxyapatite, the major component of bone, and were hydrolytically activated under physiological conditions [13].

1.3.1.2 Pro-drug carrier-mediated transport (classical prodrugs)

Many organic solutes such as nutrients (i.e. amino acids, sugars, vitamins and bile acids) and neurotransmitters are transferred across cell membranes by means of specialized transporters. These carrier systems comprise integral membrane proteins that are capable of transferring substrates across cell membranes by means of a passive process (i.e. through channels or facilitated transporters) or an active process (i.e. with carriers). Carrier-mediated active transport requires energy obtained by adenosine triphosphate (ATP) hydrolysis or by coupling to the cotransport of a counterion down its electrochemical gradient (e.g. Na+,H+, Cl–). Several drugs and pro-drugs share this

transport pathway with nutrients and it has been shown that targeting drugs to these transporter carriers can influence their bioavailability as well as their distribution. Targeting drug delivery to intestinal nutrient transporters has emerged as an important strategy to improve oral bioavailability of poorly permeating therapeutic agents. A relatively novel method to increase membrane permeability is by utilizing the carrier systems of the brush-border membrane of intestinal mucosa which are active transporter for di- and tripeptides (hPEPT1). Gershon Golomb et al.,(2000) coupled 14C-Labeled pamidronate and alendronate with dipeptide, proline-phenylalanine to produce peptidylbisphosphonates,Pro-[3H]Phe-[14C]pamidronate, and Pro-[3H]Phe-[14C]alendronate known to be actively transported by the peptide carrier. Prodrug transport in the Caco-2 cell line was significantly better than that of the parent drugs, and the prodrugs exhibited high affinity to the intestinal tissue. Oral administration of the dipeptidyl prodrugs resulted in a three-fold increase in drug absorption following oral administration in rats, and the bioavailability of Pro-Phealendronate was higher than that of the parent drug [14].

1.3.1.3 Co-crystal compositions

The application of co-crystal technologies has just an emerging way to alter physiochemical properties of API. Co-crystallization involves combination of an API with a pharmaceutically acceptable agent, which is bound together by hydrogen bonding and yield a uniform crystalline material. Josef et al. (2010), manufactured various cocrystals of ibandronate and excipients (more hydrophobic adducts) in different ratios and under various conditions. Various pharmaceutically acceptable agents were evaluated as potential counter ions: α-D-glucose, α-D-mannose, α-Dgalactose, methyl-α-D-glucopyranoside,methyl-β-D-galactopyranoside,methyl-α-Dmannopyranoside, phenyl-β-D-glucopyranoside,and phenyl-β-D-galactopyranoside. Out of the evaluated co-crystals of ibandronate, phenyl-β-D-galactopyranoside showed similar or relatively low absorption related to permeability of ibandronate API [15].

1.3.1.4 Molecular complexes

An analogous strategy to a covalent prodrug approach is an ion-pairing approach, wherein a highly charged, polar molecule with poor membrane permeability is coupled with a lipophilic counterion of equal and opposite charge to form an ion-pair in solution that is

able to passively permeate cell membranes. The approach is simple in principle and eliminates the need for prodrug uptake by transports and activation by specific enzymes. The ion-pair in solution may be absorbed and then readily dissociate after absorption via dilution in the bloodstream. Moreover, the approach does not rely on disrupting membrane integrity to facilitate absorption. A disadvantage of the ion-pairing approach is that the ionic bonding and other non-covalent interactions (i.e. hydrogen bonding) may be too weak in solution to facilitate membrane permeation [16]. So Hee Nam et al.,2011 verified the enhancing effect of the RIS ion-pair solutions of Risedronate (RIS) with l-arginine (ARG),l-lysine (LYS), and diethylenetriamine (DETA) in different molar ratios by performing in vitro penetration tests on the skin of hairless mice. In vitro permeation tests were carried out on mice skin, and data showed that ion-paired RIS could permeate 36 times more effectively than RIS alone. The cumulative amount permeated after 24 h resulted in 475.18 ± 94.19 µg/cm^{2}2 and 511.21 ± 106.52 µg/cm^2 at molar ratio of 1:2 and 1:1of ion paired RIS while RIS alone was only 14.13 ± 5.49 µg/cm^2. In this study, it was found that RIS could be delivered transdermally, and the ion-paired system in an aqueous solution showed an enhanced flux from the skin barrier [17].

1.3.2 Formulation techniques

Formulation approaches involve compounding the hydrophilic drug in a way to increase the lipophilicity around it there by reducing the resistance offered to cross the membrane via the process of passive diffusion. Formulation approach is the safest way to increase the permeability of the poorly permeable drug since the parent drug moiety is not altered simultaneously the drug is landed in a condition to enhance absorption.

1.3.2.1 Gastro retentive drug delivery system

Gastro retentive drug delivery systems are designed to keep dosage form for those drugs having the absorption window in upper intestinal tract specifically in stomach or are stable in acidic environment. These systems work on mainly three mechanisms: floatation, size expansion and mucoadhesion. Floating systems or hydro dynamically controlled systems are low-density systems that have sufficient buoyancy to float over the gastric contents and remain buoyant in the stomach without affecting the gastric emptying rate for a prolonged period of time. While the system is floating on the gastric contents, the drug is released slowly at the desired rate from the system. After release of drug, the residual system is

emptied from the stomach . Size expansion method involves swellable cellulose type hydrocolloids, polysaccharides and matrix-forming polymers like polycarbonate, polyacrylate, polymethacrylate and polystyrene. After oral administration this dosage form swells in contact with gastric fluids and attains a bulk density less than one. The air entrapped within the swollen matrix imparts buoyancy to the dosage form. The so formed swollen gel-like structure acts as a reservoir and allows sustained release of drug through the gelatinous mass. Mucoadhesive polymeric systems can provide an intimate contact with the mucosa at the site of drug uptake by increasing the residence time of the delivery system at the site of drug absorption [18]. Chauhan et al., (2004) prepared floating matrices of risedronate sodium with an objective to avoid gastric irritancy by formulating it in lipidic material and achieve sustained release . The matrix systems were prepared by melting Gelucire® 39/01 derived from the mixtures of mono-,di- and triglycerides with polyethylene glycol (PEG) esters of fatty acids and Caprol PGE 860 at10 °C above the melting point of Gelucire®39/01. Results confirmed the use of Gelucire® 39/01as sustained release carrier in gastro retentive drug delivery system [19].

Ochiuz et al., (2008) formulated a controlled release tablet of alendronate with various grades of hydrophilic Carbapol polymer and other excipients. The tablets were intended to release the alendronate in gastric region since absorption is favoured in this region. Controlled release of alendronate over long period caused enhancement in bioavability. Three grades of Carbapol 974 P NF, Carbapol 971 P NF, Carbapol 71 G NF mixed in various ratios and the tablets were pressed. Penetration enhancers are chemical substances which alter the membrane function reversibly and increase the permeation of low permeable drugs. They act generally by disrupting the lipid structure of the membrane, open the tight junctions or extract lipid from the membrane. Raiman et al.**,** (2003)in their study assessed the effects of four different absorption enhancers—palmitoyl carnitine chloride (PCC), N-trimethyl chitosan chloride (TMC), sodium caprate (C10), and Some blends displayed the best swelling and erosion characteristics, being recommended for the preparation of the extended release alendronate tablets [20].

1.3.2.2 Micro emulsions

Micro emulsions are homogeneous, transparent, thermodynamically stable dispersions of water and oil, stabilized by a surfactant, usually in combination with a co surfactant (typically a short-chain alcohol). Micro emulsion seems to improve permeability of

hydrophobic, and hydrophilic drugs across the intestinal mucosa and thus enhance their oral bioavailability. One of the proposed mechanisms is based on enhancer-induced structural and fluidity changes in the mucosal membrane. However high amount of surfactant and viscous nature pauses some patient compliance problems [21]. Fulya and Nevin et al.,(2008) in their study formulated water in oil micro emulsion of alendronate with Captex 200®, lecithin, propylene glycol and bi distilled water. Rheological behavior, phase stability and type of the micro emulsion formulation were investigated by Brookfield viscometer, centrifugation test and dye method, consequently. Phase behavior of the formulation was found to be Newtonian. No precipitation was observed in the stressed conditions and formulation was W/O. The physical characterization of the formulation (physical appearance, viscosity, refractive index, conductivity, density and turbidity) was investigated at 4°C and 25°C during 6 months while droplet size was investigated for three months [22].

Meng et al. (2011) and his co-workers prepared positively charged micro emulsion of alendronate for bioavability improvement. The bioavailability of alendronate from the microemulsion was compared with the commercially available tablet (Fosmax) for beagle dogs. The permeability enhancement was parallel to the reduction in transendothelial electrical resistance, which indicated the micro emulsion modulated the tight junctions and widened the paracellular pathway [23].

1.3.2.3 Micro particulate drug delivery

Cruza et al. (2010) encapsulated alendronate sodium in blended micro particles composed of Eudragit® S100 and Methocel® K15M with an objective to prepare gastro protective drug delivery and sustained release system. In vivo experiments carried out in ovariectomized rats showed bone mineral density significantly higher for the sodium alendronate-loaded micro particles than for the negative control groups. Furthermore, the microencapsulation of the drug showed a significant reduction in the ulcerative lesion index. The blended micro particles were excellent oral carriers for sodium alendronate since they were able to maintain the drug antiresorptive effect and to reduce the gastrointestinal drug toxicity [24].

Dissettea et al. (2010) prepared particulate adducts of sodium risedronate and titanium dioxide for the oral bioavailability enhancement. Nanocrystalline and colloidal TiO2, was used to obtain the adducts In vivo studies indicate that after oral administration to male

wistar rats, the micro particles of adduct were able to prolong the presence of risedronate in the bloodstream during an 8 h period, resulting in a relative bioavailability almost doubled with respect to the free drug. This behavior allows envisioning an improvement of the risedronate therapeutic effects and/or a reduction of its frequency of administration with consequent reduction of gastro-oesophagal injuries typically induced by oral administration of bisphosphonates [25].

1.3.2.4 Vesicular delivery systems

Among the different formulation strategies, lipid-based formulations such as liposomes are considered potential delivery systems because of their low melt viscosity, biodegradability and biocompatibility. However disadvantages associated with liposomes, such as chemical instability, variable purity of phospholipids, and high cost limits its commercial viability. Hyo-Kyung Han et al. (2014) incorporated risedronate sodium in chitosan-coated liposomes to improve the bioavailability of orally administered risedronate. Anionic liposomes of risedronate were prepared with 1,2-distearoryl-sn-glycero-3-phosphocholine (DSPC)/1,2-distearoryl-Sn-glycero-3-[phosphoric-(1-glycerol)] sodium salt (DSPG) and then coated with chitosan. When compared for permeation study with the untreated drug, chitosan-coated liposomes showed enhanced the cellular uptake of risedronate, resulting in around 2.1–2.6-fold increase in Caco-2 cells. It was concluded that chitosan-coated liposomes containing risedronate would be effective for improving the bioavailability of risedronate [26]. Dhrubojyoti Mukherjee et al (2014) prepared Risedronate sodium mucoadhesive films composing different polymers and characterized. Mucoadhesive films showed prolonged drug release 95.274± 5.752% for a period of 3h with residence time[27].

1.3.3 Patents and technology for BPs oral delivery

Lulla et al. (2004) received an US patent for an enteric coated formulation for bisphosphonic acids and salts thereof. The pharmaceutical composition comprises an inert core surrounded by an active coating containing one or more bisphosphonic acids or salts thereof, a seal coating surrounding the active coating and an enteric coating surrounding the seal coating. Alendronic acid and alendronate sodium trihydrate are the preferred active ingredients. The composition can be provided in the form of pellets in a capsule or Peltabs. The composition includes one of the bisphosphonic acids and salts thereof, typical enteric coatings for use include one or more of hydroxypropyl methylcellulose phthalate,

hydroxypropyl cellulose acetyl succinate, cellulose acetate phthalate, polyvinyl acetate phthalate, and methacrylic acid-methyl methacrylate copolymers. The compositions are preferentially released in the lower gastrointestinal tract upon ingestion and thus avoid oesophageal discomfort and ulceritis for the prior art [28].

Khandelwal et al., (2004) received an US for Pharmaceutical preparations containing alendronate sodium. An oral composition in tablet form containing therapeutic amounts of alendronate sodium for release of the alendronate sodium in the stomach and by passing the esophagus, comprising a compacted granulated core with the alendronate sodium embedded in an inert fiber matrix, lined with a moisture barrier film and enclosed in a sugar based inert fiber matrix shell. The drug is released directly into the stomach (the site of absorption) and the swallowing of the tablets is made much simpler and no postural restrictions are required any longer. The formulation is modified to release the drug at the site of absorption. The hydrophilic matrix and the sugar inert fiber shell used impart the above characteristics [29].

Tomaz et al., (2004) patented pharmaceutical technology where a pharmaceutical composition for oral administration of alendronic acid, pharmaceutically acceptable salts or esters thereof, which reduces the potential for irritation and erosion of the mucosal tissue and pain in the upper gastrointestinal tract and provides relatively good absorption of the active substance in the gastrointestinal tract [30].

Boyd et al (2007) received an US patent on Compositions for delivering bisphosphonates and methods of preparation, administration and treatment are provided as well. The present disclosure related to compositions comprising at least one of the delivery agent compounds depicted as carboxylic acids may be in the form of the carboxylic acid or salts thereof, amines may be in the form of the free amine or salts thereof and at least one bisphosphonate. These compositions facilitate the delivery of the bisphosphonate to selected biological systems and increase or improve the bioavailability of bisphosphonate compared to administration without the delivery agent compound. The administration compositions can be in the form of a liquid. The solution medium may be water. Dosing solutions may be prepared by mixing a solution of the delivery agent compound with a solution of the bisphosphonate, just prior to administration. Alternately, a solution of the delivery agent compound (or bisphosphonate) may be mixed with the solid form of the bisphosphonate (or delivery agent compound). The delivery agent compound and the bisphosphonate can also be mixed as dry powders. The administration compositions can alternately be in the form of a solid, such as a tablet, capsule or particle, such as a powder

or sachet. Solid dosage forms may be prepared by mixing the solid form of the compound with the solid form of the bisphosphonate. Alternately, a solid can be obtained from a solution of delivery agent compound and bisphosphonate by methods known in the art, such as freeze-drying (lyophilization), precipitation, crystallization and solid dispersion. The administration compositions of the present invention can also include one or more enzyme inhibitors. Such enzyme inhibitors include, but are not limited to, compounds such as actinonin or epiactinonin and derivatives thereof. Other enzyme inhibitors include, but are not limited to, aprotinin (Trasylol) and Bowman-Birk inhibitor [31].Gastrointestinal Permeation Enhancement Technology (GIPET®) formulations (Merrion Pharmaceuticals, Dublin, Ireland) are a group of oral solid dosage forms designed to promote absorption of poorly permeable drugs. GIPET is based primarily on promoting drug absorption through the use of medium-chain fatty acids, medium-chain fatty acid derivatives and micro emulsion systems based on medium-chain fatty acid glycerides formulated in enteric-coated tablets or capsules. The typical GIPET®-I preparation contains a poorly-absorbed drug with Sodium caprate C10 as the promoter in an enteric-coated tablet. In a Phase I study, GIPET® also improved the oral F of the bisphosphonate, alendronate, 12-fold compared to alendronate sodium tablets (Fosamax®, Merck), to yield an oral F of 7.2 % based on urinary excretion data of the unchanged molecule [26].

1.4 Limitations of research undertaken for improvement of BPs

Reported research work for the improvement of absorption of bisphosphonates was based on structural modification of parent drug or formulation. Chemical modification of the parent hydrophilic drug by classical prodrug approach, prodrug for transport systems, co-crystals and ion pair complex renders new drug entity with more lipophilic nature which in turn requires consideration for safety, efficacy and economic issues. Further after delivery to the site of absorption it should overcome the problems encountered by parent drug and in addition when it enters in systemic circulation it should elicit derided therapeutic response with enhanced benefit to risk ratio. All these require enormous efforts in terms of investment and labor. However in certain cases chemical modification is prove to be effective to that parent drug. Formulation approaches based on multiparticulate drug delivery often undergo gastric emptying leading to insufficient absorption. Micro emulsion based delivery involves utilization of high amount of surfactant which works by reversibly altering the barrier structure of the membrane and in some cases it may cause irritation to

gastric mucosa where the drug inherently having similar effect. Formulations designed to be retained in stomach for prolonged time necessitates administration with large quantity of fluid or avoidance of food intake which otherwise often interfere with drug absorption. Lipid based drug delivery such as liposomal delivery requires expertise persons, sophisticated instruments for manufacture and evaluation, establishment of stability in storage and integrity. Most of these various approaches have been successfully employed to improve oral drug delivery which has often translated in enhanced drug absorption. The challenges of poor oral bioavailability are still prominent despite these breakthroughs. All together it appears that formulation approach is more suitable and practical than chemically modifying the parent drug. In this respect w/o/w multiple emulsion and sublingual delivery of the BPs drugs would give a viable solution to improve absorption.

1.5 Multiple emulsions or Double emulsions

Multiple emulsions are also known as double emulsions and are multicompartment systems, termed "emulsions of emulsions", i.e. the globules of the discontinuous phase contain even tiny dispersed globules themselves. Each dispersed globule in the multiple emulsion forms an enclosed structure with single or multiple aqueous compartments separated from the external aqueous phase by a thin layer of oil phase compartments [32]. The two major types of multiple emulsions are the water-oil-water (w/o/w) and oil-water-oil (o/w/o) double emulsions. water-oil-water (w/o/w) multiple emulsion is one where external phase is aqueous which makes it easy to administered by oral route as well by parenteral route. BCS class III drugs i.e., Hydrophilic drugs having poor permeability can be incorporated in inner aqueous phase. When this emulsion is administered orally it prevents immediate release of the hydrophilic drug from inner aqueous phase due to oil film surrounded to it. Further this oil phase impart lipophilicity, protects gastrointestinal tract mucosa from direct contact of drug, release drug in controlled manner and enhances transcellular diffusion. Further it has been reported that these oil globules containing tiny aqueous drops undergo lymphatic uptake [33]. Overall the absorption of the hydrophilic drug having poor permeability can be increased by formulating in water-oil-water multiple emulsions.

1.5.1 Applications of w/o/w multiple emulsions

1. The potential pharmaceutical applications include delivery of hydrophilic drugs having poor permeability, red blood cell substitutes, immobilization of enzymes, carriers for

sustained release, enhancement of enteral absorption.

2. Drug delivery for preferential lymphatic uptake of drugs

3. In Cosmetics, food and separation technology

4. Use as adjuvant vaccines, as sorbent reservoirs in drug over dosage treatments

5. Taste masking of bitter drugs

6. A means of site-specific delivery; for example, delivering drugs to phagocyte cells of the reticuloendothelial system for treatment of a variety of parasitic and infectious diseases [34]

1.5.2 Mechanism of absorption

After oral administration the basic pathway for absorption of drug from multiple emulsions occurs through the intestinal lymphatic system. The multiple emulsion system can be absorbed directly through the intestinal macrophages or peyer's patch or from mesenteric lymph ducts in the form of chylomicrons or lipoproteins from where they are drained into circulation through thoracic lymph duct. By this way they carry therapeutic agents within them avoiding degradation in intestine as well as liver. Another theory suggests that presence of exogenous surfactants over emulsion reduce surface tension and thereby contribute to the increased surface area of the emulsified lipid. Exogenous surfactants may increase its penetration through the aqueous boundary layer [35]. Mechanism of absorption is shown in Fig. 1.3 (a) and (b).
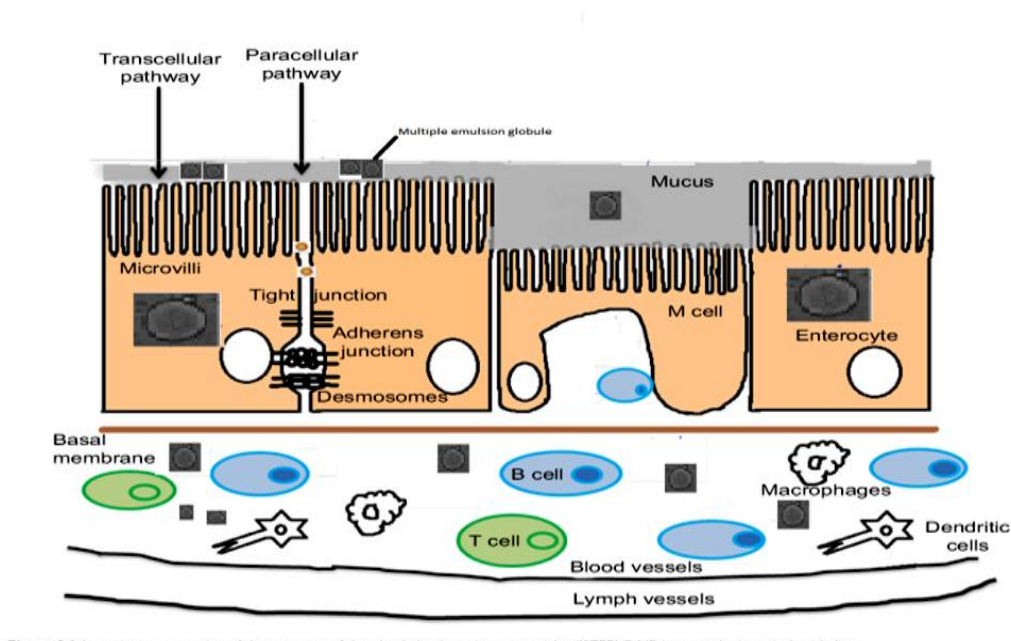
FIGURE 1.3 (a) Schematic presentation of drug loaded multiple emulsions across intestinal epithelium
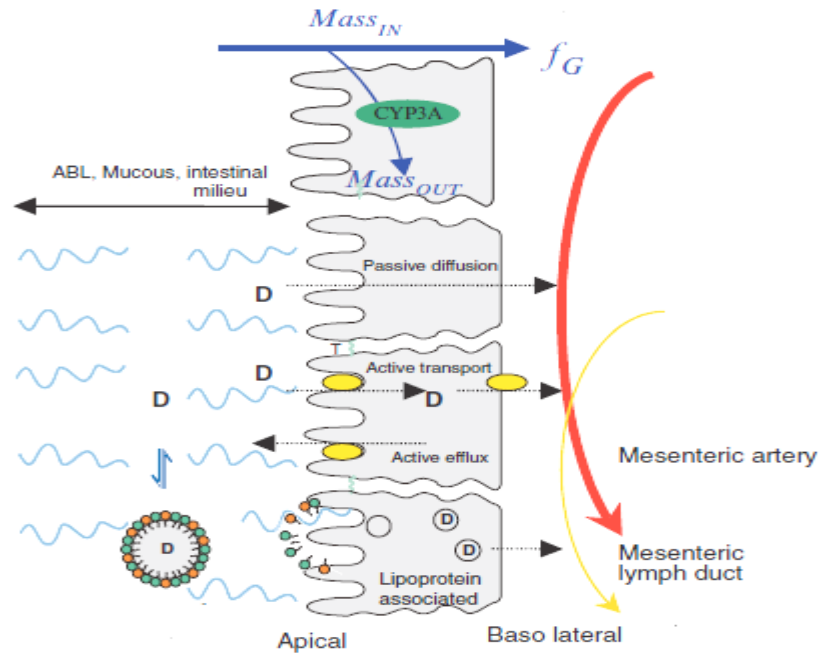
(Source : Models of the Small Intestine Brendan Griffin and Caitriona O'Driscoll, Drug Absorption Studies, Carsten Ehrhardt, Springer Science Business Media, LLC, 233 Spring Street, New York, NY 10013, USA)

FIGURE 1.3 (b) Schematic presentation of drug loaded multiple emulsions across intestinal epithelium
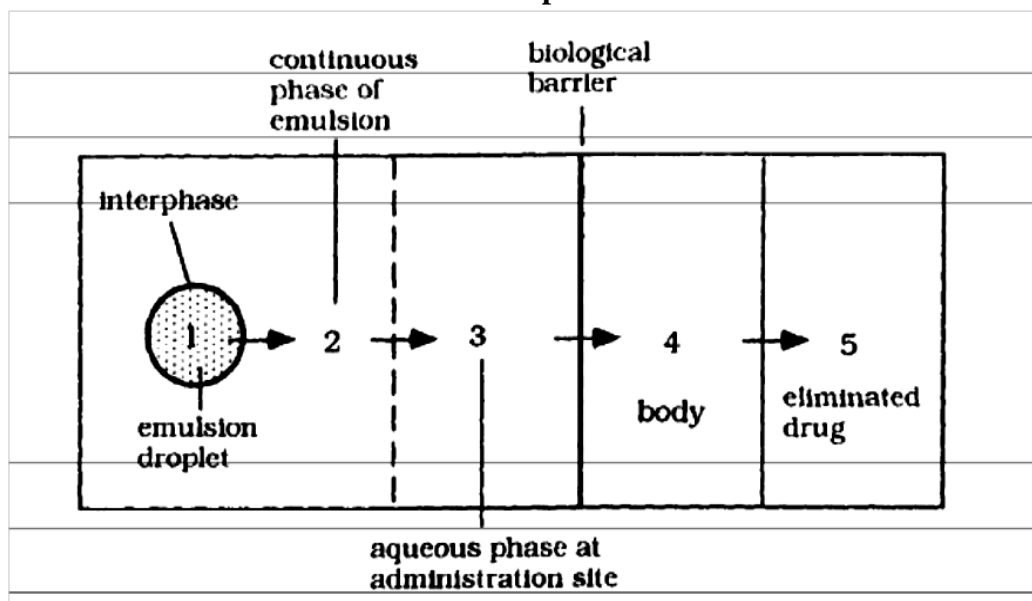
(Source: Pharmaceutical Dosage Forms: Disperse Systems, Second Edition, Volume2 2nd Edition by Herbert Lieberman)

FIGURE 1.4 Schematic diagram depicting the sequence of events governing drug absorption from an emulsion-based drug delivery system: phase I is the emulsion droplet, phase 2 is the continuous phase of the emulsion, phase 3 is the aqueous phase at the site of administration, phase 4 is composed of body fluids and tissues, and phase 5 consists of drug eliminated from the body either unchanged, in the excreta, or as metabolites. The dashed line between phase 2 and phase 3 indicates the presence of an interface when the continuous phase of the emulsion is immiscible with the aqueous phase at the administration site.

1.5.3 Formulation of multiple emulsions

Selection of formulation components for multiple emulsions requires detailed and thoughtful consideration. Despite of the inertness of these components they may affect the product performance, stability and therapeutic efficacy. A wide variety of natural and synthetic ingredients have been recommended for emulsion manufacture.

1.5.3.1 Potential drug candidates

The oral absorption of a drug largely depends on solubility of the drug and its membrane permeability across intestinal tract. Passive diffusion, endocytosis, intake via transporters, etc. are absorption pathways through which drug gets absorbed in the intestinal tract. BCS class III drugs are those candidates having sufficient solubility but lacks membrane permeability due to which they are poorly absorbed. These drugs are potential candidates to be delivered by w/o/w emulsion and can be encapsulated in inner water phase of water-in-oil-in-water (w/o/w) emulsion. Since drug is coated with oil it increases the lipophilicity and if the drug is absorbed directly as an oil droplet from the intestinal tract as mentioned earlier, the absorption of the drug will increase markedly [35][36].

1.5.3.2 Immiscible phase

The characteristics of the oil can distinctly affect the performance of the system, although nearly all edible oils will emulsify in a multiple emulsions if manufacturing conditions are properly set. For pharmaceutical uses, generally regarded as safe (GRAS) nonpolar ingredients are selected as immiscible phase such as refined hydrocarbon oils and esters of long-chain fatty acids for example, ethyl oleate, isopropyl palmitate and isopropyl myristate. Other vegetable oils such as olive oil, arachis oil, sunflower oil, castor oil and sesame oil may also be used. Apart from these polar ingredients like polyols, non polar ingredient like fatty alcohol, fatty acids, hydrocarbons, wax, and silicon fluids etc. may be included. Polar immiscible phase may be water or polyols [34].

1.5.3.3 Emulsifiers

Emulsifiers are surfactants which act by reducing interfacial tension between two immiscible phases and provide stability to disperse phase. In the absence of the emulsifier the disperse phase come together to form larger globule or flocculate. Selection of an emulsifier depends on the HLB of the oil phase used. In a w/o/w multiple emulsion

lipophilic emulsifier having HLB value less than 9 is selected to form primary emulsion (w/o) while for secondary emulsion (w/o/w) HLB value of a hydrophilic emulsifier should be more than 8. Anionic surfactants include alkyl sulphates, Soaps, Sulfosuccinates type while cationin Quaternary ammonium compounds. Non- ionic surfactants such as lanolin, poloxamers, polysorbates and Sorbitan esters are preferred for pharmaceutical emulsions because of their comparatively non toxic nature [37].

1.5.3.4 Emulsion stabilizers

After emulsification, emulsion stabilization can be achieved by attaining equal phase densities, increasing viscosity of disperse medium or adsorbing stabilizer at interface of two immiscible phases. To equalize the phase density difference between oil and water phase propylene glycol or glycerine is added in water phase while brominated vegetable oils or perfluoropolyethers are added in an oil phase. The constraint to density matching as a tool of stabilizing emulsion systems is the fluctuation in density with respect to temperature. Many hydrocolloids have been used primarily to increase the viscosity of water phase however they also get adsorb at interface between oil phase and water phase to avoid droplet coalescence. Examples of hydrocolloids include hydrophilic gums, proteins, cellulose ethers and carbomer resins. Finely divided solids such as clays, microcrystalline cellulose, oxides and hydroxides are also recommended in several cases as adsorbent [37].

1.5.3.5 Preservatives and anti-oxidants

Generally emulsions are prone to microbial contamination and degradation which can be avoided by addition of suitable preservative in outer phase. Selection of preservative depends on several factors and requires detail consideration. Alcohols (ethanol, benzyl alcohol, propylene glycol, chlorobutanol, phenylethyl alcohol), qauaternary amines (quaternium 5,benzalkoniumchloride, cetrimide), acids (sorbic acid,benzoic acid), parabens (methyl and propyl parabens), phenols (phenol, chlorocresol) etc. can be used as preservative. Antioxidants are necessary when ever unsaturated lipids are involved in formulation of emulsion to protect it from oxidation. Chelating agents (EDTA, Citric acid, Phenylalanine etc.), preferentially oxidized compounds (Ascorbic acid, Sodium bisulphite, Sodium sulphite) and chain terminators (water soluble and lipid soluble) are used as antioxidants in specified limits [37].

1.5.4 Nomenclature

Multiple emulsions are a complex system and can be defined by subscript notations suggested earlier. For example to define W/O/W multiple emulsion, W_1 water phase of the primary emulsion dispersed in oil phase O. Primary emulsion W_1O is reemulsified in external continuous water phase W_2. Thus the system can be notated as $W_1/O/W_2$. Equally oil/water/oil system can be notated as $O_1/W/O_2$ [37].

1.5.5 Manufacture

Multiple emulsions can be made in the laboratory by the re-emulsification of a primary emulsion. A two-stage emulsification procedure is adopted where in first stage primary emulsion is made which, in the preparation of a w/o/w emulsion, is a w/o emulsion. In the second step, the primary emulsion is re-emulsified in water to form the w/o/w multiple emulsion. In the formation of primary emulsion surfactant having HLB value less than 9 is mixed with oil phase and emulsified with water phase by ultrasonication or high shear mixer. Thus formed w/o emulsion is re-emulsified with low shear mixer by addition of primary emulsion in the external water phase containing surfactant having HLB value more than 9 to form w/o/w emulsion [34].

1.5.6 Evaluation tests

Following listed tests are conducted to evaluate the multiple emulsion formulation [35]

1. Visual appearance
2. Identification
3. Viscosity determination
4. pH determination
5. % creaming
7. Zeta potential
8. *In vitro* release study, *ex vivo* permeation study
9. *In vivo* absorption study

1.6 Sublingual drug delivery

The oral cavity (OC) and its highly permeable mucosal tissues have been taken advantage of for decades as a site of absorption for delivery of drugs to the systemic circulation (oral

transmucosal delivery, OTD), and for local delivery to the subjacent tissues (oral mucosal delivery, OMD). Administration of an active agent in a dosage form intended to release the drug in the oral cavity is referred as an intraoral delivery system or intraoral dosage form (IOD). Amongst the intraoral delivery system, sublingual drug administration has been proven the alternate route for drugs which are poor permeable, having food interaction and unpredictable absorption. Further sublingual delivery offers 3-10 times greater absorption than oral route, ease of administration to patients, relatively rapid onset of action and large contact surface contributes to rapid and extensive drug absorption[38]. Different formulations such as tablets, films and spray are useful for sublingual administration of drug [39]. Amongst these oral spray releases medicament rapidly in the form of micro sized droplets in intra oral cavity to be absorbed by oral mucosa, a direct and rapid dispersion of a solution of the active agent over large surface of the oral mucosa, which absorbs the active agent as shown in Fig. 1.5. In this way, a large area would be reached, thereby accelerating absorption of the active agent. Since the release medicament is in small droplet form, water is not required during administration.

1.6.1 Advantages of sublingual spray

1. The intraoral or sublingual spray method of delivery is also very helpful for individuals who have difficulty swallowing pills or capsules and, since a lower dosage is required, it is cost effective.

2. Potential faster absorption could translate into faster onset of action.

3. Patient's compliance for disabled bedridden patients and for travelling and busy people who do not have ready access to water.

4. Pre gastric absorption can result in improved bioavailability, reduced dose and improved clinical performance by reducing side effects

5. Intra oral delivery of drug avoids gastric intolerance and food interaction.

6. Sprays do not contain fillers or binders, contrary to the make-up of pills, providing exclusion of additional excipients.

1.6.2 Mechanism of drug absorption

Most studies of intra oral absorption indicate that the predominant mechanism is passive diffusion across lipid membranes via either the paracellular or transcellular pathways. The

hydrophilic nature of the paracellular spaces and cytoplasm provides a permeability barrier to lipophilic drugs but can be favourable for hydrophilic drugs.
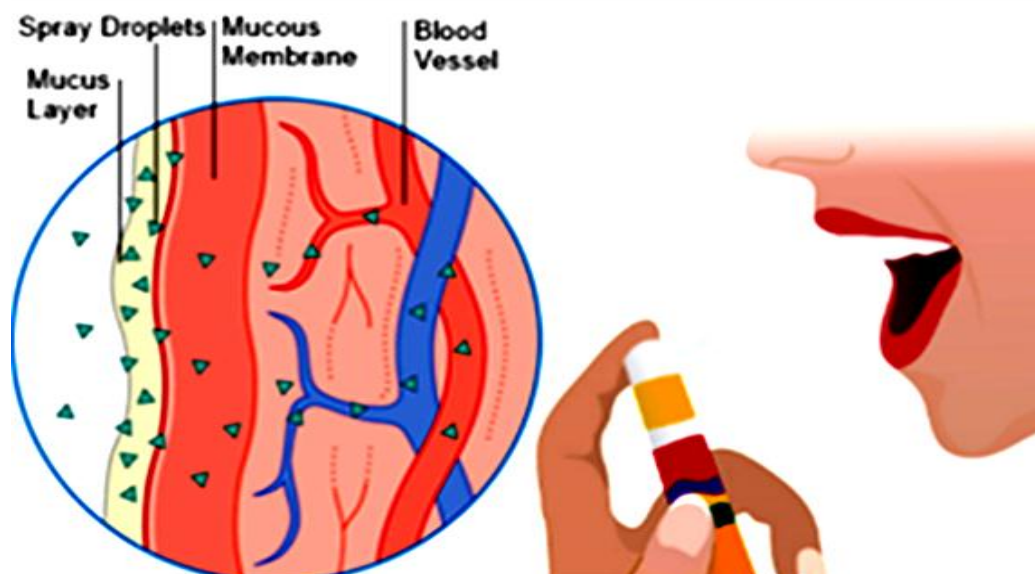
(Source: superhealthsprays.com/why-spray/)

FIGURE 1.5 Droplets formed at mucus layer

1.6.3 Formulation aspects of sublingual sprays

The permeation of drugs across mucosal membranes also depends to an extent on the formulation factors. These will determine the amount and rate of drug released from the formulation, its solubility in saliva, and thus the concentration of drug in the tissues. In addition, the formulation can also influence the time the drug remains in contact with the mucosal membrane. After release from the formulation, the drug dissolves in the surrounding saliva, and then partitions into the membrane, thus the flux of drug permeation through the oral mucosa will depend on the concentration of the drug present in the saliva. Propellant free sublingual spray is made up of the product concentrate and is described as below. In general formulation ingredient of spray formulations are put in Table 1.1.

1.6.3.1 Product concentrate

The product concentrate consist of active ingredients, or a mixture of active ingredients and other necessary agents such as penetration enhancers, solvents, antioxidants, flavouring agents, sweeteners, hydrophilic polymers, preservatives, acidifying agents, co solvent [41]. Product concentrate is filled in suitable meter dose pump spray device.

TABLE 1.1 Represents the ingredients of spray formulation [41].

Ingredients	Examples
Active ingredients	Buprenorphine, Naloxone, Scopolamine etc.
solvents	Purified water, ethanol
Antioxidants	Ascorbic acid, Amino acids
Flavouring agent	Artificial fruits flavours
Sweeteners	Neotame, Aspartame, Mannitol, Sodium Saccharin
Preseratives	Phenol, Benzoic acid, M-cresol, Methylparaben, Propylparaben, Sodium benzoate, Cetylpyridinium Chloride
Co-solvents	Propylene glycol, Ethyl alcohol , Glycerine, PEG, Soya oil, PEG-60 Hydrogenated castor oil
Hydrophilic polymer	Xanthan Gum, Sodium Carboxymethylcellulose

1.6.3.2 Evaluation tests for sublingual spray

Several tests specified for the evaluation of sublingual spray are as given under [42].

1. Spray pattern
2. Prime test
3. Average weight per meter dose
4. Content per spray
5. Drug content per spray
6. Net content
7. Density
8. Spray profiling (Delivered dose uniformity)
9. Spray angle
10. Stability study

1.7 Rationale and Motivation for this Study

The ability of orally administered drug to show the desired therapeutic effect depends on its absorption and subsequent bioavability. Therefore drug absorption (bioavailability) through GIT is crucial factor that affects the plasma concentrations of orally administered drug. Poor absorption of the drug is attributed to poor permeability and solubility in the

body fluid. Low bioavability due to poor absorption is considerable in several cases such that oral delivery becomes inefficient [43]. This issue has been addressed by numerous ways. Molecular modification of parent drug moiety is exhaustive, expensive and need establishment of safety issues. Considerable success has been attained with this approach despite that it is however short-lived in many cases as product development phase poses unexpected issues with dosage form delivery, the same challenges with older drugs that driven researches.

The advancement in novel drug delivery systems demonstrated by recent efforts in drug development has further supported the belief that new drug moieties, many of which come across with similar delivery issues might not give rise to desire remedy to the issues posed with the old ones. It is therefore much attention is focused on improvement of the delivery challenges with existing drug molecules [44] [45].

In perspective of bisphosphonates poor permeability, food interaction and variable absorption cause low bioavability up to 3%. Poor bioavailability of oral bisphosphonates has necessitated the administration of higher than normally required oral doses which often leads to economic wastages, risk of toxicity, erratic and unpredictable responses. Risedroante sodium (RIS) is a bisphopshonate and widely prescribed for the osteoporosis and has poor bioavability i.e., around 1% only. This has prompted to select the drug candidate for improvement of permeability and enhancement absorption. Thus, if increased absorption could be attained, oral administration would be a viable delivery system in situations where higher doses are required. In addition bisphosphonates therapy in osteoporosis would improve significantly with minimum occurrence of GIT side effects.

Investigations in this area have shown potential for improvement of absorption of RIS as described earlier. However amongst various approaches w/o/w multiple emulsion and sublingual spray formulation approaches are found promising for the oral delivery of RIS. Therefore novelty of this study is to formulate the RS in these oral drug delivery systems and providing a viable solution to poorly absorbed drug used in osteoporosis.

1.8 Objectives of the study

The overall aim of this study is to formulate oral drug delivery systems for poorly absorbed drug RIS in order to increase permeation across mucous membrane there by improve the oral bioavailability and opening scope for other bisphosphonates. This study considers the possibility of designing a commercializable formulation and delivery system that will

deliver drug to the systemic circulation with increased bioavailability. In order to achieve this aim, objective was to go with two approaches of oral drug delivery systems

1.8.1 W/O/W Multiple emulsion

- To formulate and evaluate RIS w/o/w multiple emulsion to select components and operation parameters
- To optimize and evaluate RIS multiple emulsion
- To carry out *ex vivo* permeation study
- To improve stability of optimized RIS multiple emulsion
- To investigate *in vivo* absorption study of stabilized formulation and compare with conventional formulation

1.8.2 Sublingual spray formulation

- To formulate sublingual formulation of RIS
- To evaluate and optimize sublingual spray formulation
- To carry out stability study of the formulation
- To carry out *ex vivo* permeation study
- To improve stability of optimized RIS sublingual spray formulation
- To investigate *in-vivo* absorption study of optimized formulation and compare with conventional formulation

Overall objective is to increase the permeability of selected drug RIS and to increase patient compliance.

1.9 Scope of the study

Attaining the objectives of the study may make feasibility to present the other members of bisphosphonates drug used in osteoporosis in selected formulation to increase oral permeability thereby increasing absorption, reducing GIT related problems and achieving patient compliance.

2.0 Original contribution in this study

One of the approach selected to enhance the permeation of RIS through the GIT was w/o/w multiple emulsion system. Enclosing the drug in inner oil phase may reduce direct contact

of RIS with gastric lumen specifically stomach mucous membrane and increase lipophilicity to increase its permeability across mucous membrane may undergo lymphatic uptake. Because of the possible lower incidence of gastrointestinal problems associated with this dosage form, it is more advantageous than dosage forms such as tablets and capsules. In addition, w/o/w multiple emulsions possess many advantages as a low viscosity due to the aqueous external phase, which makes them more convenient to handle and use, especially for oral.

Sublingual route of drug administration has been proven the alternate route for drugs which are poor permeable, having food interaction and unpredictable absorption. Further sublingual route offers ease of administration to patients, relatively rapid onset of action and large contact surface contributes to rapid and extensive drug absorption. Present work describes the formulation of Risedronate sodium sublingual spray without using any propellant.

2.1 Experimental design for formulation and optimization

Effects of the causative factors over responses were studied through designing formulation batches and optimization was carried out by analyzing obtained data from experiments by Minitab 16.0 and design expert 7.0 software.

CHAPTER -2

Literature Review

2.1 Introduction

In most of the cases, oral drug delivery remains the popular way of drug administration. However, many drug candidates do not have the required physicochemical and pharmacokinetic properties that make them unfavourable for oral administration leading to poor absorption and bioavailability. Poor absorption has caused dose dumping which often result in to economic loss, more concern for safety, variable and unpredictable effects. This may cause diversion to alternate mode of administration, which may not be comfortable and convenient to the patient for long term treatment, which may result in noncompliance. Because of this, efforts are directed to devise new formulation techniques that will make favourable administration of most drugs by oral route in parent structure and yet maintaining a therapeutic effect. This will be a huge success since most of drugs possess oral bioavailability constraints.

There is a considerable work carried out in the field of multiple emulsion and sublingual delivery for the administration of drugs having poor absorption due to poor permeability and solubility. In this chapter therefore, an attempt is made to survey the literature on research work carried on permeability enhancement of hydrophilic and poorly absorbed drugs through W/O/W multiple emulsion and sublingual formulations. This review sought to consider methods aimed at enhancing the oral absorption of drugs in terms of the objectives, scientific framework, excipients selection, commercial available dosage forms etc. with a brief drug profile.

2.2 Multiple emulsions delivery system to improve absorption of hydrophilic drugs

Multiple emulsions were first observed by Seifriz in 1925, but since two decades that they have been explored in drug delivery [43]. W/O/W multiple emulsions have been used as a

delivery system for incorporating hydrophilic drugs which have poor absorption due to poor permeability.

J.A. Omotosho et al., (1990) investigated the extent of absorption of 5 fluorouracil from the w/o/w multiple emulsion after oral administration to rats. 5 fluorouracil is an antineoplastic agent belongs to BCS class III. Its absorption from GIT varies from 28 to 80 % with severe risk of toxicity. In order to decrease the dose dependent toxicity and increase the absorption, 5 fluorouracil was incorporated in inner aqueous phase of multiple emulsion. A two-stage emulsification procedure was used to prepare emulsions. Primary emulsion w/o was prepared by addition of the aqueous phase containing 0.2% BSA, unlabeled 5-FU (1 mg/ml) and 5-fluoro [3H]uracil in normal saline to equal weight of each of isopropyl myristate and octane containing 2.5% Span 80 in a 25 ml glass container. Re-emulsification was carried using a small vortex mixer by adding w/o emulsion with an equal volume of water containing Tween 80 (1% w/v). 1 ml emulsion containing 5-fluoro [3H]uracil was given to rats and samples were collected. From the analysis it was found that isopropyl myristate composed multiple emulsion enhanced the oral absorption compare to octane composed. It was assumed that increase in absorption observed with isopropyl myristate emulsion could be attributed to the inhibitory effect of the oil and/or metabolite (myristic acid) on the gastric emptying processes. Radioactivity value of the 5-fluoro [3H] uracil following oral administration of aqueous solution and w/o/w emulsions prepared with isopropyl myristate were 0.2 (% dose /g of lymph nodes hr $^{-1}$) and 0.12 (% dose /g of lymph nodes hr $^{-1}$). Presence of the 5-fluoro [3H] uracil in lymph nodes made to assume that encapsulation of the drug by the oil phase could place it in a more lipophilic environment and would allow it, therefore, to be selectively absorbed into the lymphatic vessels rather than into the portal circulation [29].

CO Onyeji et al., (1991) in a study measured the absorption of griseofulvin from oil-in-water (O/W), water-in-oil-in-water (w/o/w) emulsions and tablet dosage forms (500 mg strength) after oral administration in 8 healthy volunteers. The absorption rate constant was measured by the urinary excretion of the major metabolite of griseofulvin (6-desmethylgriseosulvin). From these results, it was suggested that the administration of griseofulvin in the W/O/W emulsion might enhance therapeutic efficacy of the drug in man [44].

L.A.M. Ferreira et al., 1994 studied the influence of vehicle on in vitro release of metronidazole and role of w/o/w multiple emulsion. Study was designed to prepare o/w, w/o/w and w/o emulsion of hydrosoluble drug metronidazole. W/O/W emulsions were

prepared by two step emulsification method. The microscopic aspect, conductivity values and rheological parameters confirmed that three emulsion types were obtained. Metronidazole release was studied on synthetic membranes and on rat skin biopsies. In vitro release study revealed that the cellulose membrane offered negligible resistance to diffusion of the drug in which release rate of metronidazole from the w/o/w emulsion was slightly slower compared to o/w emulsion, while much slower release was observed with the w/o emulsion. On the other hand diffusion study conducted on excised skin, diffusion flux values 2.409, 2.112, 1.168J ($\mu g/cm^2$ per h) and permeability coefficient values 4.907, 3.826, 2.246 (cm/h) ($x10^{-4}$) for w/o/w, o/w and w/o emulsion were obtained correspondingly. Values suggested that the permeation of hydrosoluble drug metronidazole from w/o/w was higher than o/w and w/o emulsions which could be due to increase lipophilicity of the drug [45].

Chong-Kook Kim et al., (1995) had studied the encapsulation efficiency and release pattern of cytarabin loaded w/o/w multiple emulsion. It was assumed that cytarabin loaded emulsion would accumulate in lymph nodes in higher amount necessary for effective in lymphatic cancer. Emulsions were prepared by two step emulsification method including span 80 and tween 80 as internal and external emulsifier. Entrapment efficiency was found 72%-80% and globule size in 1-4 micron. Stability of the emulsion was improved with surfactant blend in external emulsifier to match HLB value of liquid paraffin [46].

A.J.Khopde et al., (1996) attempted to develop multiple emulsion of rifampicin for the treatment of meningitis. Study involved manufacture of rifampicin multiple emulsion by two step emulsification process where 20mg/ml rifampicin was dissolved in inner water phase. This phase was emulsified with liquid paraffin using span 80 to get w/o emulsion. In second step w/o emulsion was reemulsified with water containing tween 80 to get w/o/w emulsion. Prepared rifampicin emulsion was administered through nasal and oral route in rats. The concentration of drug at the end of 4 hours in brain was 15.68 µg/ml and 30.30 µg/m for oral and nasal delivery accordingly. Over all the drug concentration in brain was achieved higher through nasal delivery, the direct pathway to brain than oral. However, when plain drug solution was administered orally, 7.16 µg/ml drug concentration was noted in brain lower than orally administered multiple emulsion. Study concluded that nasal route could be a superior for administration of rifampicin for the said disease [47].

A. Silva-Cunha et al., (1997) developed porcine sodium insulin loaded multiple emulsion containing (soybean oil or medium-chain triglycerides -- MCT). Aim of the study was to enhance the bioavability of insulin and for that w/o/w formulation was composed of a

protease inhibitor aprotinin (AP) and an absorption enhancer sodium taurocholate (TC) in inner water phase. W/O/W multiple emulsions were prepared by two step emulsification method where Abil EM-90 ® and tween 80 were internal and external emulsifier. In vitro degradation study reported that ME1 and ME2 showed no degradation of insulin supporting effectiveness of aprotinin. Study ended with further requirement of biological effects of the developed formulation [48].

A.J.Khopde et al., (1998) prepared multiple emulsions with an oily liquid membrane (w/o/w) containing isoniazid by two step emulsification method. Microcrystalline cellulose (MCC) was used as stabilizer in external as well as internal aqueous phases, interfaces of the liquid membrane. The emulsions were characterized for droplet size, percent formation of multiple emulsion, release rate, effect of Tween-80 in external phase, phase volume ratio on release, and stability during aging at various storage conditions. In the study, results indicated formation of small droplet with fairly good yield. MCC had positive effect on droplet size. Study concluded that multiple emulsion delivery would be promising in tuberculosis therapy [49].

Masako Kajita et al., (2000) evaluated the ability of unsaturated fatty acid or docosahexaenoic acid (DHA) included in oil phase of w/o/w multiple emulsion to enhance absorption of poorly absorbed drug vancomycin (VCM) through intestinal tract. Emulsion was prepared by two step emulsification method where span 80 was used as emulsifier and triolein as oil phase. VCM containing inner water phase was emulsified to get w/o emulsion, which was reemulsified with outer water phase containing 3% tween 80 as external emulsifier. Prepared emulsion and control containing 5mg/ml VCM was administered in different rats group by making an ascending colon loop and plasma drug concentration was determined. Emulsion containing 2% linoleic, linolenic aicid and DHA in separately made emulsion as absorption enhancers showed enhancement in bioavability by 40% -50% compared to emulsion without mentioned fatty acids. In addition when the absorption enhancement ability was measured by incorporating 15% unsaturated fatty acid corresponding enchantment in bioavability was not observed. Transmembrane electrical resistance study was conducted to determine the effect of absorption enhancers on membrane resistance (R_m) and no remarkable change in membrane resistance (R_m) suggested that the VCM could have transported by transcellular way rather than paracellular uptake [50].

Yoshinori Onuki et al., (2004) studied the intestinal absorption of insulin loaded multiple emulsion in rats. He adopted statistical design to determine the causative factors that could

affect the formulation performance. In his study he utilized oleic acid as oil phase and formulated w/o/w multiple emulsion containing 200IU insulin in inner water phase. Absorption enhancement was inferred from the pharmacological effect of insulin observed with increase in oleic acid proportion upto 2% in multiple emulsion. It was also noted that the outer water phase volume ratio markedly affected the inner globule size which was increased with decreasing volume ration [51].

Kenjiro Koga et al., (2010) carried a study to enhance the intestinal absorption of BCS class III drug calcein in rats by incorporating in w/o/w emulsion. Initially, w/o emulsions were made with calcein containing inner buffer phase, condensed ricinoleic acid tetraglycerin ester as a lipophilic emulsifier and soyabin oil as oil phase. Then water-in-oil-in-water (w/o/w) emulsions were prepared with stearic acid hexaglycerin esters (HS-11) and Gelucire 44/14 as hydrophilic emulsifiers. Investigation of intestinal calcein absorption from w/o/w emulsions in rats showed that calcein bioavailability after intraduodenal (i.d.) administration was significantly higher than that of the calcein control. Analysis of samples showed C_{max} of calcein 118 ± 47 ng/ml and 13.7 ± 5.4 ng/ml after 90 mins for multiple emulsion and control formulation respectively suggesting enhanced absorption of calcein[52].

Xiaole Qi et al., (2011) prepared pidotimod novel formulation, self-double-emulsifying drug delivery systems (SDEDDS). SDEDDS act by spontaneously forming w/o/w multiple emulsion in stomach in presence of body fluid. SDEDDS was used to improve the oral absorption of pidotimod, a peptide-like drug with high solubility and low permeability. SDEDDS was formed spontaneously by formulating mixtures of hydrophilic surfactants and drug containing water-in-oil (w/o) emulsions. In stability study the optimized pidotimod-SDEDDS were found stable up to 6 months at 25 °C. Pharmacokinetic studies of pidotimod-SDEDDS in rats showed 2.56-fold ($p < 0.05$) increase compared to the pidotimod solution. Histopathologic studies supported that SDEDDS had moderately affected mucous membrane with enhanced absorption. It was concluded from study that SDEDDS could be a promising delivery for oral administration of peptide drugs [53].

Qi X et al., (2011) developed fine pidotimod-containing water-in-oil-in-water (w/o/w) double emulsions as a delivery system for promoting the oral bioavailability. A two-step emulsification was used to manufacture the multiple emulsions using medium-chain triglyceride as the oil phase, tween 80 as the hydrophilic emulsifier, and span 80 alone or in combination with phospholipids as the lipophilic emulsifiers. Optimized fine w/o/w emulsion had 82 ± 3.4% encapsulation efficiency, 3.93 ± 0.25 μm mean oil-droplet

diameter, and 36.4 ± 0.93 mPa · s at 25 °C and 300 s(-1) viscosity, which was stable for 1 month at 4 °C. In addition, the oral bioavailability of pidotimod in rats was significantly higher than that of pidotimod control solution after intragastric administration of w/o/w emulsions. Increased uptake time suggested an extra absorption pathway for w/o/w emulsions i.e., a lymphatic circulation pathway. Study results suggested that w/o/w emulsions could be a potential formulation for oral bioavailability improvement of poorly absorbed drugs and an important advancement in technology for the oral administration of peptide drugs [54].

Liang-Zhong Lv et al., (2012) prepared self double emulsifying drug delivery system (SDEDDS) of Hydroxysafflor yellow A (HSYA), the main active ingredient of the safflower plant used to treat several dysemia diseases. W/O emulsions were prepared by emulsification of HSYA (48 mg) 0.5% gelatin solution as the inner water phase and oil phase contained bean phospholipids, medium chain triglycerides, tween 80, oleic acid, and labrasol (20/65/7.4/2.5/0.1, in wt%). This w/o emulsion forms w/o/w emulsion immediately after contacts to stomach fluid. Results of the HSYA uptake and transport experiments showed that the membrane permeability of HSYA was very low. In vitro permeability test performed in Caco-2 cells showed apparent permeability (Papp) $(3.52 \pm 1.41) \times 10^{-6}$ cm/s and $(6.62 \pm 2.61) \times 10^{-6}$ cm/s for HYSA control solution and HSYA-SDEDDS correspondingly. In order to know the mechanism of absorption, endocytosis inhibitors were added in cells and there after conducted uptake study showed 4.06 ± 1.19, 3.14 ± 1.39, 2.97 ± 0.37, and 1.38 ± 0.16 µg/mg protein compared to control suggesting no effect of inhibitors on absorption. An enhanced relative bioavability 217% was observed for HSYA-SDEDDS compare to control in intra gastric absorption study in rats. Finally the safety of absorption enhancers was determined by histopathologic study and observed to cause damage intestinal mucosa. Overall study suggested possible delivery of BCS class III drug HSYA through SDEDDS to improve absorption [55].

Paul S et la., (2013) investigated design and development of acyclovir water-in-oil-in-water multiple emulsions (w/o/w emulsions) for oral bioavailability improvement. Span-80 and Span-83 as lipophilic surfactant while Brij-35 as hydrophilic surfactant was used to prepare and optimize Multiple emulsions (MEs). Stable w/o/w emulsions with an average globule size of 33.098 ± 2.985 µm and $85.25 \pm 4.865\%$ entrapment efficiency were obtained. Stability of multiple emulsions was influenced by the concentration of lipophilic surfactant. In vitro drug release study showed initially faster and there after slower release of acyclovir. All together study proved to be promising for delivery of acyclovir [56].

Mobashshera Tariq et al., (2016) investigated the antibiotic activity of amoxicillin and cefta zidime against urophathogens. These drugs are highly hygroscopic and water soluble which are poorly absorbed from GIT. Drugs were dissolved in inner water phase taking Caproyl 90 as an oil phase containing internal emulsifier transcutol. Formed w/o emulsions were reemulsified with outer water phase containing external emulsifier Solutol HS 15. Similar emulsions were prepared containing glyceryl dilaurate (GDL) in primary emulsion. Prepared w/o/w emulsions were subjected for antibiotic activity through agar well diffusion method. Amoxycillin emulsion containing GDL showed zone of inhibition (10.52 to 12.76 mm) against test organisms and without GDL did not cause inhibition suggesting antibiotic property of GDL had additive effect against organisms. Ceftazidime emulsions with and without GDL showed zone of inhibition (11.33 to 38.84 mm) against test organisms. Study concluded that protective layer could have provided by multiple emulsion to antibiotics against β-lactamase producing organisms keeping their activity [57].

B. Mishra et al., (2011) investigated the w/o/w multiple emulsion as delivery system for lamotrigine an anticonvulsant drug. Study included preparation of four lamotrigine Four, namely aqueous and oily drug suspension, simple emulsions (w/o and o/w) and multiple w/o/w emulsions made up of liquid paraffin as the oil phase. Results of the study showed entrapment efficiency varied from 84.37%–98.11% for different formulations. Zeta potential values and polydispersity indices of the emulsions were ranging from +23.46 to +28.07 and 0.256 to 0.365 which indicated a stable formulation with low droplet size distribution. In stability study the multiple emulsions found to stable since sedimentation ration was 0.88 to 1.0 as well as multiple droplet morphology was unchanged which supported the reported work where span and tween were included as emulsifier. In vitro drug release study showed sustained release pattern and particularly w/o/w followed zero order drug release. When the different formulations were administered orally to test animals for anticonvulsant activity against maximal electroshock and strychnine induced seizures, drug loaded w/o/w which emulsions were prepared by effective hydrophilic lipophilic balance provided prolonged protection compared to standard formulation [58].

2.2.1 Stability improvement of w/o/w multiple emulsion

Water-in-oil-in-water (w/o/w) multiple emulsions are complex systems often found difficult to stabilize. Some of the in stability markers in the emulsion are coalescence of

multiple droplets with each other, coalescence of internal dispersed phase, expulsion of internal droplets and shrinkage of inner dispersed phase globules due to passage into external phase. All this phenomena may results in creaming, coalescence or cracking of emulsion. The reason behind this is generally attributed to migration/extraction of internal emulsifier, osmotic pressure difference, interfacial tension, density difference between dispersed and dispersed medium, size of globules and viscosity of dispersed phase. However, the inherent instability associated with these systems has prompted many researchers to overcome this problem.

Interfacial complexation is found to stabilize the w/o/w multiple emulsion by reducing the chances of coalescence of inner aqueous globules. Some macromolecules such bovine serum albumin, gelatine, natural gums form a complex primary w/o interface membrane [58]. T.k. law et al., 1985 in his study investigated the interfacial complexation effect of bovine serum albumin (BSA) over stability of w/o/w emulsion included in inner aqueous phase of primary w/o emulsion. Initially w/o emulsion was formed by emulsifying the aqueous phase containing 0.1% sulphane blue as hydrophilic marker and 0.2% BSA with isopropyl myristate containing 5% poloxamer 331 a lipophilic surfactant. W/O emulsion was reemulsified with aqueous phase containing 0.8% poloxamer 403 a hydrophilic emulsifier. Prepared w/o/w emulsion was stable over 24 hrs at 37^0 c with average globule size of 25 μ. In the in vitro release study 40% sulphane blue was released over period of 6 hours indicated slow release pattern [59]. A viscosity enhancer (such as gum arabic, hydroxypropylmethyl cellulose, acacia, gelatine, xanthun gum) can be added in external aqueous phase of w/o/w system to avoid creaming and coalescence of disperse globules [60].

M. Kanouni et la., (2002) studied the role of interfacial films over stability of multiple emulsions. In the study a w/o emulsion was prepared by emulsifying an aqueous phase (W_1) in soyabin oil containing a low HLB emulsifier Abil EM90 using an Ultra-Turrax mixer 10 min. Next, w/o emulsion was added drop-wise to the aqueous phase (W_2) containing a high HLB emulsifier betaine and a thickener xanthun gum. The study data showed presence of medium size globules with no phase separation. It was concluded that presence of a thickener in the outer aqueous phase W_2, was an essential component to achieve viscosity ratio near to 1 between W_1/O and W_2 allowing dispersion of primary emulsion W_1/O to W_2 [61].

Density difference between aqueous phase and oil phase can be adjusted to reduce creaming phenomenon. Hideaki okochi et al., (1995) prepared vancomycin w/o/w multiple

emulsion composed of mixture of oils to balance the density difference between aqueous and oil phase. Oil phase was composed of lipiodol and isopropyl myristate in 4.5:5.5 ratio along with 5%w/v HCO-40 as an internal emulsifier which was emulsified with aqueous phase containing vancomycin hydrochloride to form w/o emulsion. W/O emulsion was reemulsified with external aqueous phase containing pluronic F68 an external emulsifier to get w/o/w emulsion. Prepared emulsion was stable over 12 h and there was no sign of phase separation. The mean particle diameter was in range of 10μ and more than 95% drug was entrapped. Photographs of the samples revealed that multiple structure of system was intact and the Stability of the emulsion was attributed to lipiodol and isopropyl myristate composition whose density was near to aqueous phase i.e., 1[62].

Amount of the lipophilic emulsifier and phase volume ratio of w/o to w/o/w influences the stability of the multiple emulsions. I. Csoka et al., (1997) carried an experiment showing the effect of lipophilic emulsifier span 80 and phase volume ration of the primary emulsion w/o over emulsion stability. Ephedrine hydrochloride was used as marker in inner aqueous phase and its presence in outer aqueous phase was measured by a chloride selective membrane electrode (Radellkis OP-208:1-OP-Cl-07 11P). An exponential equation was used to derive rate constant which explained the change in number of droplets during the storage period. Result showed that increase in lipophilic emulsifier the stability of w/o/w emulsion was increased and 0.4 gm w/o in w/o/w ration was effective. Study concluded that the knowledge of the rate constant enables to select the most suitable emulsifier for adequate stabilization [63].

2.3 Sublingual spray

Drug delivery via the oral mucous membrane is considered to be a promising alternative and a useful route when rapid onset action with better patient compliance than orally ingested tablets is offered. In terms of permeability, the sublingual area of the oral cavity is more permeable than the buccal area, which in turn is more permeable than the palatal area. Amongst new sublingual technologies sublingual sprays address many pharmaceutical and patient needs, ranging from better absorption to convenient dosing for pediatric, geriatric, and psychiatric patients with dysphagia. It also provides the opportunity to directly modify tissue permeability, inhibit protease activity, or decrease immunogenic responses to drugs. Therefore, sublingual spray has emerged as one of the delivery system for administration of drugs, particularly for those drugs that undergo first

pass metabolism, poor gastric absorption and require rapid onset of action [64][65]. Various drugs have been formulated and evaluated for pharmacokinetic parameters. The following are briefs of some of these studies.

2.3.1 Research carried on sublingual spray delivery

A pharmacokinetic study was conducted to know the plasma profile of a clemastine fumarate oral tablet (2.68 mg) with two activations of a lingual spray delivering 1.34 mg/dose. The lingual spray delivered significant amounts of the drug within 5–10 min and the peak concentration was reached within 63 min whereas the tablet only started to deliver clemastine to the systemic circulation sometime between 20 and 30 min after administration. Study values for sublingual spray and tablets of AUC, T_{max} and C_{max} were 20.99, 7.00, 0.81 and 7.57, 7.00, 0.35 correspondingly. Studies demonstrated that lingual sprays were distinctive in their ability to deliver the drug substance through the oral mucosa efficiently in therapeutically meaningful amounts [66].

Sam S et al. (2015) investigated pharmacokinetics of artemether sublingual spray (ArTiMist) formulation in african children with severe malaria or in whom gastrointestinal symptoms prevented the administration of artemether-lumefantrine tablets. Sublingual artemether spray formulation was administered in children suffering from malaria at a dose of 3.0 mg/kg of body weight to the nearest 3.0 mg using the most appropriate combination of 3.0-mg/actuation and 6-mg/actuation delivery devices. Plasma artemether and Dihydroartemisinin (active metabolite) were determined by LC –MS. Study data were simulated with reported data and bioavailability of sublingual artemether was at least equivalent to that after conventional oral artemether-lumefantrine median [interquartile range] areas under the concentration-time curve for artemether, 3,403 [2,471 to 4,771] versus 3,063 [2,358 to 4,514] µg·h/ltr, respectively; and for DHA, 2,958 [2,146 to 4,278] versus 2,839 [1,812 to 3,488] µg·h/ltr, respectively; $P > 0.42$). These findings suggested that sublingual artemether could be used as preferential treatment for sick children with less incidents of discomfort associated with oral therapy and could be transferred to oral therapy after attaining effective blood concentration of drug [67].

Peter A. Crooks et al., (2007) developed a sublingual spray formulation of scopolamine hydrobromide (L-(-)-hyoscine hydrobromide) and determined the absolute bioavailability drug following sublingual delivery. Study also included investigating the effect of a bioadhesive polymer on the pharmacokinetic parameters of this drug in a rabbit model.

Rabbits were administered a single scopolamine base equivalent sublingual dose of 100 µg/kg and this was compared to intravenous administration of the drug and the bioavailability of sublingual scopolamine was determined. Following delivery of the sublingual spray dose, the average C_{max} was 1024.4 ± 177 ng/mL, the AUC value was 61067.6 ± 9605 ng.min/mL and bioavailability was 79.8%. The addition of 2% chitosan, a bio-adhesive material in formulation caused longer residence of sprayed droplets and showed a significant improvement in scopolamine sublingual absorption ($p < 0.05$) was observed. Study concluded that the sublingual route of administration using a spray delivery dosage form would be a potential alternative mode for the prevention of nausea and vomiting associated with motion sickness [68].

Kalliopi Dodou (2012) in his review pointed the development of Ora-Lyn insulin preparation by Generax, a liquid formulation of human insulin combined with absorption enhancers that is sprayed in the buccal cavity, providing an alternative to injectable insulin formulations for the management of type 1 and type 2 diabetes. Oral spray formulations having sumatriptan for the treatment of migraine, ondasentron hydrochloride (Zensana) forchemotherapy- or radiotherapy-induced nausea and vomiting and, sildenafil (Duromist), for erectile dysfunction, are currently at preclinical stage or at phase I or II clinical trials [69].

Fiona McInnes et la., (2008) evaluated the Clearance of a Sublingual Buprenorphine Spray in the beagle dog using gamma scintigraphy. A spray formulation containing radio labeled buprenorphine (400 µg/100 µl in 30% ethanol) was administered sublingually to four beagle dogs, and the stay time in the oral cavity was determined using gamma scintigraphy. Scintigraphic imaging showed formulation clearance from the oral cavity was rapid; with a mean $T_{50\%}$ clearance of 0.86 ± 0.46 min, and $T_{80\%}$ clearance of 2.75 ± 1.52 min. T_{max} of 0.56 ± 0.13 h. indicated slow absorption of buprenorphine. Study demonstrated similar results previously reported in man with respect to $T_{50\%}$, providing useful dog model for further study [70].

Neha Parikh et al., (2013) carried out pharmacokinetic study of fentanyl sublingual spray (FSS) and oral transmucosal fentanyl citrate (OTFC) which was compared for rate of absorption and systemic bioavailability. In study, healthy volunteers received single doses of FSS (400 µg), OTFC (400 µg), and intravenous fentanyl citrate (100 µg) separated by washout periods of ≥7 days. Oral naltrexone was given to minimize potential adverse effects of fentanyl. Pharmacokinetic parameter mean C_{max} value of fentanyl was higher with FSS versus OTFC (0.81 ng/mL vs. 0.61 ng/mL) and was attained more quickly; the

median T_{max} was 1.5 hours with FSS and 2.0 hours with OTFC ($P < 0.05$). Systemic bioavailability was also greater with FSS than with OTFC (approximately 76% vs. 51%). Research came to conclusion bioavailability of fentanyl was greater with FSS than with OTFC. These findings suggested that FSS could be appropriate for the treatment of breakthrough cancer pain [72].

A N Bartoli et al., (2012) investigated melatonin oral spray formulation bioavability and compared with oral tablets. 5 mg of oral spray or oral melatonin (tablet) was administered randomly in eight subjects. After following test schedule and determining the samples for melatonin concentration in plasma, C_{max} of 17.2 ng/ml, AUC $_{0-\infty}$ 1719.32 ng.min/ml for oral spray and C_{max} 12.6 ng/ml, AUC $_{0-\infty}$ 1179.23 ng.min/ml for tablet formulation were noted. Data showed that the extent of melatonin absorption after oral spray delivery was 1.8 fold and the peak concentration was 1.5 fold of the standard oral tablet. Study indicated that the new formulation Melatonin in spray improved melatonin absorption, ensuring higher concentrations after administration, compared to the standard oral tablet [71].

2.3.2 Some patented technologies of sublingual spray

Several patents have been applied and publish for sublingual spray formulations from different persons are presented here in Table no. 1.

TABLE 2.1 Patented technologies of sublingual spray

Patent number	Title	Applicant	Publication date/Application date	Ref
US2016/0199294 A1	Sublingual naloxone spray	Insys Development Company	14.7. 2016	73
US 2016/0193142 A1	Sublingual fentanyl spray	Insys Development Company	7 .7. 2016	74
US 2016/0045430 A1	Sublingual buprenorphine spray	Insys Development Company	18 .2. 2016	75
US 2016/0022629 A1	Sublingual spray formulation comprising dihydro artemesinin	Londonpharma Limited patents	28.1. 2016	76
US 2016/0008306 A1	Diclofenac sublingual spray	Insys Pharma, Inc.	14.1. 2016	77
US 2015/0133517 A1	Ondansetron sublingual spray formulation	Insys Pharma, Inc.	14.1. 2105	78

The information provided in these patents is very useful for formulation and development of the undertaken research study. Nearly all work presented in patents represents propellant free pump spray formulations and the formulation components included in these patents hints for the selection of excipients.

2.3.3 Marketed/ Developing Spray formulations [79-85]

Enlisted products are available on prescription and used in various conditions. Marketed products reveal that the different molecules having different physiochemical property can be included in sublingual spray formulation with suitable excipients. They also help in fixing the amount to be delivered through spray.

TABLE 2.2 Marketed sublingual spray formulations

Generic name	Commercial name	company	Indication
Glyceryl Trinitrate	Glytrin Spray®	Sano.-aventis, Surry,UK	Prevention and relief Of angina attacks
Insulin	Oral-lyn™ spray	Generex Biotechnology Corporation	Treatment of Type I and Type II diabet
Nicotine	Nicorette® Quickmist®	Johnson & Johnson Inc.	Smoking secation
Zolpidem	Zolpimist	NovaDel	short-term treatment of insomnia
Isosorbide dinitrate	ISOKET® Spray	Schwarz Pharma Ltd., Germany	vasodilators
Sumatriptan	SUD-001	SUDA Ltd	Anti migraine
Fentanyl	SUBSYS	Insys	Analgesic
Sumatriptan	pipeline	Novadel	Anti migraine
Tizanidine	pipeline	Novadel	In neurological disorders

2.4 Drug profile of risedronate sodium

2.4.1 Risedronate sodium [86]

Risedronate sodium is sodium salt of (1-hydroxy-1-phosphono-2-pyridin-3-ylethyl) phosphonic acid. Molecular structure is shown in Fig.2.1.

2.4.1.1 Chemistry [87]

Molecular formula   - $C_7H_{11}NO_7P_2$

Molecular weight   -   283.112264 g/mol

Melting point   - 255^0c

Solubility   -   10.4 mg/ml (water)

Partition coefficient -   -3.6

Permeability   -   Not available

Taste   -   Not available

Chemical structure   -

(Source: https://pubchem.ncbi.nlm.nih.gov/image/imgsrv.fcgi?cid=4194514&t=l)
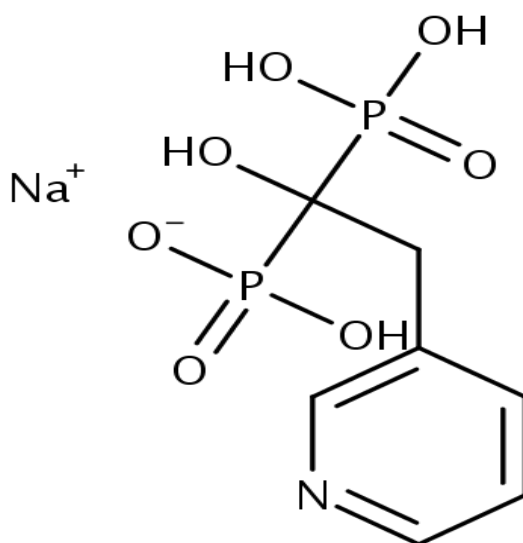
FIGURE 2.1 Risedronate sodium

2.4.1.2 Mechanism of action

Risedronate is an example of pyridinyl bisphosphonate that binds to hydroxyapatite in bone tissue and inhibits osteoclast mediated activity.

2.4.1.3 Pharmacokinetics

Absorption of the Risedronate sodium in healthy men is not site specific along the gastrointestinal tract and In healthy volunteers the bioavailability of risedronate is 0.63%. Risedronate tablet should be taken half an hour before breakfast or 2 hours after dinner for better absorption while presence of high fat content decreases the extent of absorption by 55% [87, 88].

2.4.1.4 Indications

Risedronate sodium is found useful for the prevention and treatment of postmenopausal osteoporosis, glucocorticoid induced osteoporosis and the treatment of Paget's disease of bone [89].

2.4.1.5 Available dosage forms

Risedronate sodium is marketed by Warner Chilcott Canada Co as actonel all over the world and available in 5, 30, 75 and 150 mg uncoated and film coated tablets. Its generic form Apo-risedronate and Risedronate sodium is also available in similar strength [87].

2.5 Summary of literature review

Research work carried out by different researchers in the area of multiple emulsions and sublingual spray is quite impressive and definitely has become the source of information for selection of excipients and conditions for formulation and development of the RIS w/o/w multiple emulsion and sublingual spray. Methods and experimental designs also found useful for undertaken present research work. Finally it can be said that w/o/w multiple emulsions and sublingual spray are exciting approaches towards the enhancement of uptake and transport of orally administered molecules having poor permeability with good solubility, which may lead to the requirement of relatively lower doses and decreasing incidences of adverse effects.

CHAPTER 3

Experimental Work

3.1. Materials and Equipments used

The materials and equipments, utilized in the present study are mentioned in Table no. 3.1, 3.2 and 3.3 respectively.

TABLE NO 3.1 List of materials

Sr.No.	Component Name	Category	Manufacturer
1.	Risedronate sodium	API	Vaikunth Chemicals Pvt Ltd. Ankaleshwar
2.	Isopropyl Myristate	Excipient	Chemdyes Corporation, Baroda
3.	Sunflower oil	Excipient	Chemdyes Corporation, Baroda
4.	Olive oil	Excipient	Balaji Chemicals Ltd.
5.	Arachis oil	Excipient	Chemdyes Corporation, Baroda
6.	Tween 80	Excipient	Chemdyes Corporation, Baroda
7.	Span 80	Excipient	Chemdyes Corporation, Baroda
8.	Propylene Glycol	Excipient	Suvidhanath Laboratories, Baroda
9.	Ethanol	solvent	Shree Chalthan Vibhag Khand Udyog Sahakari Mandali ltd.
10.	HPLC Grade water	solvent	Suvidhanath Laboratories, Baroda
11.	Xanthun gum	Excipient	Vaikunth Chemicals Pvt Ltd. Ankaleshwar
13.	pH 7.4 Phosphate Buffer	In-house	BIP, Baroda
14.	pH 5.5 Acetate Buffer	In house	BIP, Baroda
15	Methanol	solvent	Astrone, Ahmedabad

TABLE NO 3.1 List of materials (continued)

Sr.No.	Component Name	Category	Manufacturer
16	Sodium phosphate	buffer	Astrone, Ahmedabad
17	EDTA-2Na	Chelating agent	Astrone Ahmedabad
18	Etidronate	Internal standard	Astrone Ahmedabad
19	Tetra butyl ammonium bromide	Complexing agent	Astrone Ahmedabad
20	Poloxamer 180	polymer	Astrone Ahmedabad

TABLE NO 3.2 List of equipments

Sr. No.	Instrument	Manufacturer/Make
1.	AX200 Electronic Weighing Balance	Shimadzu Corporation japan
2.	Digital pH Meter	Equiptronics Co. Mumbai
3.	Digital Vernier Callipers	Aerospace Co.India
4.	Trinocular Microscope	Zeiss Instruments Germany
5.	High Speed Homogenizer	System Anatech Co. India
6.	Franz diffusion cell	Durga scientific, Baroda
7.	Fourier Transform Infrared Spectrophotometer	Bruker (Iindia)Pvt. Ltd. India
8.	Double Beam UV – Visible Spectrophotometer	Analytikjena Co. Germany
9.	1220 Infinity LC HPLC Instrument	Agilent Technologies USA
10.	Stability Chamber	Sun Instruments Pvt. Ltd. Ahmedabad
11.	Magnetic Stirrer	Janki Impex Pvt. Ltd. Ahmedabad

TABLE NO 3.2 List of equipments (continued)

Sr. No.	Instrument	Manufacturer/Make
12.	Melting Point Test Apparatus	Dolphin Analytical Ltd. Mumbai
13	Brookfield viscometer (DV II + Pro)	BRK Instruments India LLP
14	Vortex mixer	REMI Ahemdavad
15	Orbital shaker	Durga scientific Baroda
16	Conductivity meter	Equiptronics Co. Mumbai

TABLE 3.3 Apparatus used

1	Thermometer	Durga scientific Baroda
2	Separator funnel	Durga scientific Baroda

TABLE NO 3.4 Animals used for in-vivo studies*

Species Name	Gender	Number of Animals
Wister Rats	Male	12

*Institutional Animal Ethics Committee certificate with reference protocol number: PhD/13-14/22, is attached in appendix –

3.2. Preformulation Studies

Preformulation studies are an essential component of research wherein it supports development of formulations. Activities done prior to formulation development are called as preformulation studies. It provides the scientific basis for formulation development.

3.2.1 Identification of RIS

3.2.1.1 Appearance

Received sample of the drug was evaluated for colour, odour and texture. Taste of the drug was not determined since this requires permission from human ethical committee. Reported data for taste of the drug was utilized [91].

3.2.1.2 Melting point

The substance under test was reduced to a very fine powder. Substance was filled in glass capillary tube (closed at one end) and tied to thermometer such that closed end of the capillary was near the middle of the thermometer bulb, and remaining procedure was followed as mention in Method I for Melting Range or Temperature in IP 1996 [92].

3.2.1.3 FT-IR analysis

Identification of the drug was carried out by potassium bromide (KBr) pellet method. 1 mg of RIS was triturated with 300 mg of dry, finely powdered potassium bromide IR and compressed to get disc and mounted the resultant disc in a holder in the spectrophotometer for observation of absorbance bands in finger print region [93].

3.2.1.4 UV visible spectrophotometry determination of absorption maximum (λmax)

Stock solution of RIS (0.5 mg/ml) was prepared in distilled water and 50 μg/ml solution was scanned between 190 to 400 nm to determine λmax of RIS. [94].

3.2.2 Analytical method for quantitative determination of RIS

For the analysis of RIS in pure and in the formulation, UV spectrophotometry and HPLC method was used after validation [94].

3.2.2.1 UV Spectrophotometry method for estimation of RIS

A direct and simple spectrophotometry difference in absorbance (ΔA) method for the determination of RIS was used and validated [94].

3.2.2.1a Stock solution of RIS

Stock solution of RIS (1.5 mg/ml) was prepared by dissolving RIS in required quantity of distilled water.

3.2.2.1b Sample Procedure

Different aliquots of standard solution equivalent to 0.15—1.5 mg of RIS were transferred into two series of 10 ml volumetric flasks. The first series was completed with 0.01 moll^{-1} hydrochloric acid and the second series with 0.01 moll^{-1} sodium hydroxide. The absorbance difference (ΔA) was measured at 262 nm in 0.01 moll^{-1} hydrochloric acid and 0.01 moll^{-1}sodium hydroxide as a blank. Method was validated as per ICH guideline Q2 (R1).

3.2.2.1c Linearity

The absorbance difference (ΔA) was measured at 262 nm as described in above and calibration plots of RIS were obtained and regression equations for respective plots were calculated for the estimation of linearity.

3.2.2.1d Precision (repeatability)

INTRADAY: Three solutions (15 μg/ ml, 100 μg/ ml, 150 μg/ ml) were prepared as described in procedure and six replicate readings of each were taken by UV spectrophotometer on the same day. The repeatability was expressed in terms of % relative standard deviation.

INTERDAY: Three solutions (15 μg/ ml, 100 μg/ ml, 150 μg/ ml) were prepared as described in procedure and six replicate readings of each were taken by UV spectrophotometer on three consecutive days. The repeatability was expressed in terms of % relative standard deviation.

3.2.2.1e LOD and LOQ

Regression equations for linearity plot were derived in triplicates and standard deviation of the intercepts was calculated.

LOD and LOQ were calculated as below

$$\textbf{LOD=3.3} \times \textbf{SD / mean of slope of regression equations in triplicates} \qquad (3.1)$$

$$\textbf{LOQ=10} \times \textbf{SD / mean of slope of regression equations in triplicates} \qquad (3.2)$$

Standard Deviation = standard deviation of intercept of regression equations in triplicates

3.2.2.1f Accuracy of method

The accuracy of the method was determined by calculating the recoveries of RIS by the standard addition method. Known amounts of standard solutions of RIS were added at 50, 100 and 150 % level to prequantified sample solutions of RIS (15 μg/ ml). Concentration of RIS was estimated by applying obtained values to the respective regression line equations.

3.2.2.2 Bio analytical method of RP-HPLC for estimation of RIS

HPLC system consisted of a Series I binary gradient system, a model 525 Dual-wavelength UV detector, Hypersil C18 reverse phase column (i.d. 5mm, 4.6mm×250 mm, ODS-2) and a Hypersil guard column (5 μ, 4.6mm×10 mm) at room temperature. The mobile phase for separation of Risedronate sodium in samples consisted of buffer (5mMTBAB ion-pair reagent, 1mM etidronate,11mM sodium phosphate and 1.5mM EDTA-2Na) − methanol (88:12,v/v), adjusted to pH 6.75with 0.2 M NaOH and was pumped at a flow rate of 1.0 ml min^{-1}. The injection volume was 10 μl and the detection wavelength was 262 nm. Peak areas were used for quantitative analyses [95].

3.2.2.2a Preparation of standards

RIS Stock solutions of 1mg/ml concentration in deionised water was used to prepare working standards of 0.1, 0.5, 1.0, 2.5, and 5.0 μg/ml of RIS, after serial dilutions with water and stored under ambient laboratory conditions. Drug-free plasma samples were spiked with stock solutions prior to protein denaturation; method was validated using plasma samples from Wistar rats.

3.2.2.2b Extraction procedure of RIS from blood sample

5 μl of each working standard was spiked with 150 μl of control rat plasma to prepare standards. These standards were used to plot linearity range from 10 to 500 ng ml^{-1} for the quantization of RIS in plasma. Following procedure was adopted to extract the RIS from the plasma as represented by flow chart Fig. 3.1

Step I | 150 μl of plasma + RIS, deproteinized with 450 μl of 10% TCA

50 μl of 1.25M cacl$_2$ + 50 μl of 30% w/v of NaOH

Step II | Co-precipitation with calcium

400 μl of 1M HCL

Step III | Hydrolysis at 90^{0}c for 30 min

25 μl of 1.25M cacl$_2$ + 2.0 ml water and 90 μl of 30% w/v of NaOH

Step IV | Co-precipitation with calcium

50 μl of 1M HCL + 2.0 ml water and 40 μl of 30% w/v of NaOH

Step V | Wash the pellets with water

50 μl of 0.025M EDTA-2Na +920 μl of mobile phase

Vortex and mix for 10 s and inject

FIGURE 3.1 Sample preparation procedure

3.2.3 Physiochemical test

3.2.3.1 Solubility

Solubility was determined by common batch agitation method where excess amount of the RIS was dissolved in distilled water to form saturated solution and agitated for 48 hours. Solution was filtered and appropriate dilutions were made to analyze RIS concentration to determine solubility by spectroscopic method [91] [94] [96].

3.2.3.2 Octanol/Water coefficient (Ko/w)

Excess RIS drug sample (500 mg) was added in octanol/water mixture containing each 25 ml kept in separating funnel and shaken for sufficient time to saturate each layers. After shaking it for 24 hr an aliquot was taken from each layer and analyzed using UV absorption. Concentration of RIS in two layers was used to find Ko/w [91] [94]

$$\text{Ko/w = Concen. of drug in Octanol/Concen. of drug in water} \qquad (3.3)$$

3.2.3.3 pH of the RIS solution

1%w/v solution of RIS was prepared in distilled water and the pH of the solution was measured by pH meter [91].

3.2.3.4 Initial drug stability studies

1% w/v solution of RIS was stored for 1 month at normal condition. After, one month solution was analyzed to determine amount of RIS by UV spectroscopy [94] [95].

3.3 Formulation of RIS w/o/w multiple emulsion

3.3.1 Preliminary batches of RIS w/o/w multiple emulsions

W/O/W multiple emulsions were prepared by two step emulsification method [34]. In first step w/o emulsion i.e., primary emulsion was prepared. Different oils like Arachis oil, olive oil, sunflower oil and Isopropyl myristate (IPM) were chosen as oil phase while span 80 was selected as lipophilic emulsifier. Internal aqueous phase was made up of distilled water containing 25 mg RIS. Oil phase was poured in a glass beaker and Span 80 was added and stirred at moderate speed to make homogeneous mass. Internal aqueous phase containing drug was slowly added in this beaker with stirring at specified speed to get w/o emulsion i.e., primary emulsion. In second step primary emulsion was added in external water phase at low speed to form secondary emulsion i.e., w/o/w multiple emulsion as shown in Fig. 3.2. Minitab 16 software was utilized to design, generate and study main effects of formulation factors over the responses of RIS multiple emulsion formulation. The Placket-Barman design was selected to screen the significant factors that can affect the responses such as globule size, creaming entrapment efficiency and ex-vivo permeation rate. General formulation composition of multiple emulsions is presented in Table 3.5.

Independent factors and responses to them are presented in Table 3.6. Total 12 formulation batches were generated for each oil at two levels of five independent factors and are presented in Table 3.7. Formulations were also evaluated for other parameters like appearance, type of emulsion, viscosity and pour ability.
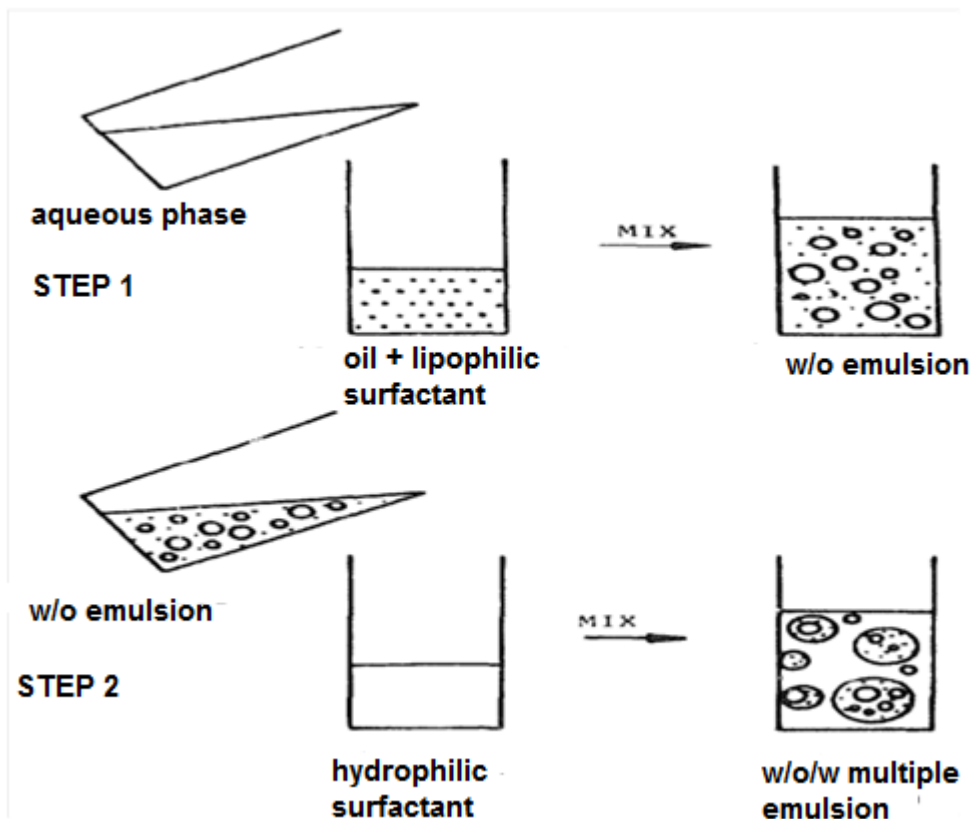
FIGURE 3.2 Two-stage preparation of w/o/w multiple emulsion.

TABLE 3.5 Preliminary formulations of multiple emulsions (w/o/w)

Formulation Components	Quantity
Primary emulsion w/o	
RIS	25 mg
Oil phase	5 ml
Span 80	5/ 10%
Internal aqueous phase	5 ml
Secondary emulsion w/o/w	
Primary emulsion w/o	10 ml
Tween 80	1 / 3%
External aqueous phase q.s to	10 ml
Total	20 ml

TABLE 3.6 Operational parameters

Causative factor	Code	Unit	Low level (-)	High level (+)	Responses
Concentration of Span 80	A	%w/w	5	10	
Homogenization speed for primary emulsion W/O	B	rpm	1000	3000	
Time of homogenization for primary emulsion W/O	C	mins	10	20	R1 Globule size(μ) R2 Creaming (%) R3 Encapsulation efficiency (%)
Tween 80	D	%w/w	1	3	
Homogenization speed for secondary emulsion	E	rpm	500	1000	

TABLE 3.7 Design Table (randomized)

Formulation	BLK	A	B	C	D	E
1	1	+	+	-	+	-
2	1	-	+	-	-	-
3	1	+	-	+	+	-
4	1	-	+	+	+	-
5	1	+	-	-	-	+
6	1	-	+	+	-	+
7	1	-	-	+	+	+
8	1	+	+	+	-	+
9	1	-	-	-	+	+
10	1	+	-	+	-	-
11	1	-	-	-	-	-
12	1	+	+	-	+	+

3.3.2 Formulation of w/o/w RIS multiple emulsion

RIS w/o/w multiple emulsion formulations were prepared according to the formula generated using Design Expert 7 Trail version. A face centered central composite experimental design was applied. Formulations were prepared considering three causal factors and dependent responses were globule size, entrapment efficiency, rate of creaming as tabulated in Table 3.8, 3.9 and 3.10. W/O/W multiple emulsions were prepared by two stage emulsification procedure. Drug and other excipients were first dissolved in purified water and were emulsified with oil phase with span 80 to form primary emulsion w/o. This primary emulsion w/o was further emulsified using tween 80 as emulsifying agent by dispersing it in aqueous phase to form w/o/w multiple emulsion. Total 18 formulation batches of multiple emulsions were prepared as presented Table 3.11and 3.12 for evaluation.

TABLE 3.8 Formulation of multiple emulsions (w/o/w)

Formulation Components	Quantity
Primary emulsion w/o	
RIS	25 mg
IPM	5 ml
Span 80	5 to 10%
Internal aqueous phase	5 ml
Secondary emulsion w/o/w	
Primary emulsion w/o	10 ml
Tween 80	1 %
External aqueous phase q.s to	10 ml
Total	20 ml

TABLE 3.9 Selected independent variables and their levels

Causative factor	Code	Unit	Low level -	High level (+)	Alpha -	Alpha +	Responses
Concentration of Span 80	X_1	%w/w	5	10	3.3	12	
Homogenization speed for primary emulsion W/O	X_2	rpm	2000	4000	1318	4700	Globule size (μ) R_1 (%) Creaming R_2 (%) Encapsulation efficiency R_3 % drug permeated R_4
Time of homogenization for primary emulsion W/O	X_3	mins	10	20	7	23.5	

TABLE 3.10 Matrix showing formulation with low and high values of factors

Formulation	Factors		
	X_1	X_2	X_3
F1	7.50	3000	23
F2	3.30	3000	15
F3	11.70	3000	15
F4	10.00	2000	20
F5	7.50	3000	7
F6	7.50	3000	15
F7	10.00	2000	10
F8	5.00	2000	20
F9	7.50	3000	15
F10	10.00	4000	10
F11	7.50	3000	10
F12	7.50	2000	15
F13	5.0	1300	10
F14	10.00	4000	20
F15	5.00	4000	20
F16	7.50	4700	15
F17	7.50	3000	15
F18	5.00	4000	10

TABLE 3.11 Formulations of RIS w/o/w multiple emulsion

Component/ Formulation code	F1	F2	F3	F4	F5	F6	F7	F8	F9
RIS	25	25	25	25	25	25	25	25	25
IPM	5	5	5	5	5	5	5	5	5
Span 80	7.5	7.5	7.5	7.5	5	10	5	7.5	5
W_1	5	5	5	5	5	5	5	5	5
Tween 80	1	1	1	1	1	1	1	1	1
W_2 (q.s)	20	20	20	20	20	20	20	20	20

TABLE 3.12 Formulations of RIS w/o/w multiple emulsion

Component/ Formulation code	F10	F11	F12	F13	F14	F15	F16	F17	F18
RIS	25	25	25	25	25	25	25	25	25
IPM	5	5	5	5	5	5	5	5	5
% Span 80	10	7.5	5	10	7.5	10	10	5	7.5
W_1	5	5	5	5	5	5	5	5	5
%Tween 80	1	1	1	1	1	1	1	1	1
W_2 (q.s)	20	20	20	20	20	20	20	20	20

3.3.3 Optimization and validation of w/o/w RIS multiple emulsion

Optimization of formulation was carried out by numerical and graphical method. In addition to evaluation tests as described for preliminary formulation in-vitro release study, in-vivo absorption study and stability study was also conducted for optimized batch.

3.3.3.1 Numerical method

Optimization of the RIS multiple emulsions were made with numerical method. Criteria for the optimizations are tabulated in Table 3.13 and 3.14.

TABLE 3.13 Criteria for RIS w/o/w multiple emulsion optimization

Significant factor	Goal	Lower	Upper
A	In range	7.5	10
B	In range	3000	4000
C	In range	10	20

TABLE 3.14 Set responses for optimization

Response	Goal	Lower	Upper
R1	In range	2.1	3
R2	Maximize	10	12
R3	Minimize	65	12
R4	Maximize	18	20.5

3.3.3.2 Graphical method

Optimization of the RIS multiple emulsion was also made with graphical method. Criteria for the optimizations are tabulated in Table 3.15.

TABLE 3.15 Set responses for optimization

Response	Goal	Lower	Upper
R1	In range	2.1	2.2
R2	Maximize	11	12
R3	Minimize	65	70
R4	Maximize	18	20.5

3.3.3.3 Validation of the optimized batch

Optimized batch was validated by reproducing it as the formula derived from the optimization and evaluated for selected responses.

3.3.4 Stabilization of optimized RIS W/O/W multiple emulsion

Optimized RIS multiple emulsion formulation was stabilized by multiple approaches. Phase volume ratio, surfactant blend and addition of thickening agent in external aqueous phase. Table 3.16 presents the formulation compositions of stabilized batches.

TABLE 3.16 Stabilized formulation composition

Formulation	Component/ Formulation code	F19A	F19B	F19C	F19D
Primary emulsion W1/O	RIS	25	25	25	25
	IPM	5	5	5	5
	Span 80	10	10	10	10
	W_1	5	5	5	5
Secondary emulsion W1/O/W2	Primary emulsion W1/O	10	10	10	10
	Span 80/tween 80 (32.5/67.5)	1	1	1	1
	Xanthun gum (%)	0.25	0.5	0.25	0.5
	Methyl parabene (%)	0.18	0.18	0.18	0.18
	Propyl parabene (%)	0.02	0.02	0.02	0.02
	W_2 (q.s)	20	40	40	20

3.3.5 Evaluation of multiple emulsions

3.3.5.1 Organoleptic characteristics

Freshly prepared primary and multiple emulsions were investigated organoleptically (colour, appearance, odour). Organoleptic characteristics of both primary and multiple emulsions kept at different storage conditions were noted at various intervals, i.e. 0 h, 1 h, 1 day, 3 days, 7 days, 14 days, 21 days and 28 days for 28 days [29].

3.3.5.2 Type of emulsion

DILUTION TEST: Test was carried out to find the type of emulsion and performed by addition of water and oil to the preparation separately and state of the preparation was checked [100].

CONDUCTIVITY TEST: The test confirms the type of emulsion and performed by addition of small fraction of an electrolyte in preparation under test and change in conductance was measured [48].

pH DETERMINATION: Previously calibrated pH meter was used to measure pH. Prepared formulation was filled in glass beaker and pH electrode was dipped in it and allowed to stabilize. Value displayed was noted.

3.3.5.3 Structure analysis and globule size measurement

A trinolcular microscope (Zeiss Instruments) with camera device was used for the microscopic observation. Small sample of emulsion with appropriate dilution was mounted on the slide and observed under microscope to determine average globule size and distribution. The oil droplets and the inner water droplets were counted and the diameter of the droplets was determined in all cases. The number of simple and multiple droplets were counted per slide, and the multiple character was calculated in %.The homogeneity was characterized by means of the droplet diameters [29] [97].

3.3.5.4 Viscosity determination

Viscosity was determined using rotating spindle viscometer (Brookfield viscometer DV II+Pro). Viscosity was determined at RT using spindle no.63 at 60 rpm. The viscosity was reported as mean and standard deviation of the mean of three determinations [98] [99].

3.3.5.5 Creaming

W/O/W emulsions were evaluated by visual observation for the creaming process. The emulsion samples were poured into 100 ml glass cylinders instantly after preparation. Multiple emulsions were observed for phase separation i.e., three phases; oil phase, unseparated emulsion and water phase from the top. The volume ratios of each separated phase were measured [101].

3.3.5.6 Entrapment efficiency

Small sample from the required quantity of test sample was withdrawn and analysed by spectroscopic method to find out the amount of drug present and subtracted from the total amount incorporated to know entrapment as shown in Equation (3.1) [102].

$$\textbf{Entrapment efficiency} = \frac{\textbf{(total amount of drug} - \textbf{free (unentrapped) drug)}}{\textbf{total amount of drug}} \times \textbf{100} \qquad (3.4)$$

3.3.5.7 Osmotic behaviour

Osmotic behaviour of the formulations were determined by dispersing the formulation in distilled water having 1% sodium chloride in internal aqueous phase and percentage change in globule size was noted by microscopy [46].

3.3.5.8 In vitro drug release study

The drug release study was performed by Dialysis method. 5 ml of the emulsion was placed in a dialysis tube covered by a cellophane membrane at both ends that was then placed in 200 mL of dissolution media phosphate buffer pH 7.4 at 37±1˚c in a rotating basket United States Pharmacopoeia Type I dissolution apparatus. A sink condition was maintained throughout the duration of the study. Samples were withdrawn at different time intervals, and the volume of the dissolution media was kept constant by replacing it with an equal volume of fresh media [35]. Samples were analyzed by the UV spectroscopy method at 262nm λmax and cumulative drug release was calculated [94].

3.3.5.9 *Ex-Vivo* permeation study

Ex-vivo permeation study was carried out using Franz diffusion cell. 20 ml phosphate buffer pH 7.4 was filled in receiver compartment containing magnetic stirring bar. Donor

and receiver compartment were separated by treated goat sublingual mucosa which was stored at 2-8°C in refrigerator in ringer's solution to preserve its biological characteristic [103]. Experiments were approved by the IAEC in accordance to proposal no. PhD/13-14/22. Entire assembly was set on magnetic stirrer and slowly temperature was raised and maintained at 37°C. Multiple emulsions containing Risedronate sodium were applied in donor compartment and 2 ml samples from receiver compartment were collected through sample withdrawal tube and equal volume of phosphate buffer was replaced at the interval of 10 minutes for one hour. Procedure was repeated for each spray formulation. Absorbance of Withdrawn samples was measured at 262 nm by UV spectroscopy [94].

3.3.5.10 Zeta potential measurement

Zeta potential was measured to know stability of the dispersion as it indicates the kind of force acting between the nearby globules on [Malvern zetasizer nano] [104].

3.3.5.11 *In Vivo* absorption (permeation) study

EXPERIMENTAL ANIMALS: The study protocol was approved by the IAEC proposal no. PhD/13-14/22. The study was carried out on healthy male Wistar rats weighing 200-250 g. Rats were housed in polypropylene cages, maintained under standard condition (12 h light/dark cycle, 25°C, 35-55 % humidity) and allowed free access to diet. The animals were fasted at least 12 h prior to dose administrations and for 4 h after dosing with free access to water.

EXPERIMENTAL DESIGN: Animals were divided into four groups each consisting of 3 animals. All animals were given different formulation group wise as described underneath.

1. Group I: Control group (Plain RIS solution, 5mg/kg, p.o.)
2. Group II: Optimized multiple emulsion Formulation equivalent to RIS to 5mg/kg, p.o.
3. Group III: Optimized sublingual spray formulation equivalent to RIS to 5mg/kg, p.o.
4. Group IV: Marketed tablet formulation made in suspension form to RIS to 5mg/kg, p.o.

Serial blood samples (0.5ml) were withdrawn through capillary inserted in to retro orbital plexus under mild ether anaesthesia at a time interval of predose 1, 2, 4, 8, 12 and 24 h post dose.

SAMPLE EXTRACTION PROCEDURE: Blood samples were collected in micro centrifuge tubes containing anticoagulant (1.2% w/v EDTA disodium). The samples were centrifuged at 3000 RPM at room temperature for 10 min, and then 150 µl of plasma were

collected and stored at -18^0c until analysis. Three rats were employed for each single administration.

PREPARATION OF STANDARDS: A Risedroante sodium stock solution was prepared at a concentration of 1mg/ml in deionized water. Working standards, at concentration of 0.1, 0.5, 1.0, 2.5 and 5.0 µg/ml of risedronate, were obtained from stock solutions by serial dilutions with water. Drug-free plasma samples were spiked with Stock solutions before protein denaturation.

BIO ANALYTICAL METHOD: Chromatographic technique was used as described in earlier work. HPLC system consisted of a Series I binary gradient system, a model 525 Dual-wavelength UV detector, Hypersil C18 reverse phase column (i.d. 5mm, 4.6mm×250 mm, ODS-2) and a Hypersil guard column (5 µ, 4.6mm×10 mm) at room temperature. The mobile phase for separation of risedronate sodium in samples consisted of buffer (5mMTBABion-pair reagent, 11mMsodium phosphate and 1.5mM EDTA-2Na) – methanol (88:12, v/v), adjusted to pH 6.75with 0.2 M NaOH and was pumped at a flow rate of 1.0 ml $\min^{-1}$. The injection volume was 10 µl and the detection wavelength was 262 nm. Peak areas were used for quantitative analysis [26] [105].

3.3.5.12 Pharmacokinetic data analysis

PK solver 2.0 add-in program for Microsoft Excel was used for the estimation of Pharmacokinetic parameters. Various parameters like maximum plasma concentration (C_{max}), time for achieving maximum plasma concentration (T_{max}), Area under curve [AUC] $_{0-8}$ and relative bioavailability (F) were determined. Each experiment was carried out in triplicate and treated statistically.

3.3.5.13 Stability test

Optimized RIS formulation was stored for 1 month with exposure to daylight and at 4 ^{o}c in refrigeration [106].

3.4 RIS Sublingual Spray formulations

3.4.1 Preliminary study

3.4.1.1 Container specifications and light transmission study

Physical dimensions of pump spray container were measured by vernier caliper. Light transmission study was conducted to check light protection property. Selected circular sections to represent the average wall thickness were cut from three areas of the container and trimmed them as necessary to give segments of a size convenient for mounting in the spectrophotometer. A Scan was carried out between 290 to 450 nm and transmittance was recorded [107].

3.4.1.2 Model batch for RIS spray approximation

Approximately 5 ml distilled water was poured in a glass beaker. Other components were included at lower level and added one by one and stirred to get homogeneous solution. Poloxamer 180 was added in this and stirred to dissolve. Finally volume was made with distilled water to 15 ml. Prepared solution was further filled in pump spray container. Table 3.17 presents model formulation composition. Preparations were evaluated for further formulation [108].

TABLE 3.17 Model spray formulation of RIS

Sr no.	Component	Quantity
1	RIS	50 mg
2	Propylene glycol	10%
3	Poloxamer	0.5 %
4	Alcohol	10%
5	Distilled water	q.s to 15 ml

3.4.1.3 Excipient compatibility study

Excipient compatibility study was performed to identify any possible interaction between components. Model formulation was stored in humidity chamber away from light for 1

month to identify any possible interaction between product components. After completion of storage period sample was analyzed for λmax of RIS by UV spectroscopy [108].

3.4.1.4 Container and product compatibility study

Model formulation was filled in pump spray container and stored in humidity chamber away from light for 1 month to identify any possible interaction between product concentrate and container system [108].

3.4.1.6 Content of RIS emitted per shot

Content per spray was determined by shots of two sprays in a beaker containing 0.01M HCl. This solution was shaken for 5 minutes and the drug content was determined at 262 nm by the UV-spectroscopy as described in subsection 3.2.1.2.

3.4.1.7 Preliminary batches of RIS sublingual spray formulation

Preliminary batches of RIS sublingual spray formulation made for identifying the significant factors. Fractional factorial design (1/4) was applied to generate formulation batches using Minitab software 10. Table 3.18, 3.19 and 3.20 represents the selected factors, levels and responses.

TABLE 3.18 Preliminary formulation composition for RIS spray

Sr no.	Component	Quantity
1	RIS	1 to 2%
2	Propylene glycol	5 to10 %
3	Poloxamer	1 to 5%
4	Alcohol	10 to 20%
5	Oleic acid	1 to 2%
6	Distilled water	q.s to 15 ml

TABLE 3.19 Experimental design

Causative factor	CODE	Unit	Low level (-)	High level (+)	Responses
RIS	X_1	%	1	5	
Propylene glycol	X_2	%	10	20	
Poloxamer	X_3	%	1	5	Residence time min Y1
Alcohol	X_4	%	10	20	% drug permeatioedY2
Oleic acid	X_5	Ml	0	1	

TABLE 3.20 Design table (randomized)

Formulation	BLK	X_1	X_2	X_3	X_4	X_5
1	1	+	+	-	+	-
2	1	-	-	+	+	-
3	1	-	-	-	+	+
4	1	+	+	+	_	+
5	1	+	-	-	-	-
6	1	-	+	+	-	-
7	1	-	+	-	-	+
8	1	+	-	+	-	+

3.4.2 Formulation of sublingual spray formulation

Face centered composite experimental design was used to prepare the spray formulations as shown in Table 3.21. Concentration of Risedronate sodium (X_1) and Propylene glycol (X_2) and Poloxamer 188 (X_3) were selected as the causal factors and their effects were seen on residence time (Y_1), % drug released (Y_2) and % drug permeated (Y_3) shown in Table 3.21, 3.22 and 3.23 In present study, Total 16 formulations were prepared as presented in matrix table. Amount of alcohol and oleic acid were not varied. Formulation were made as described earlier and filled in pump spray containers for evaluation [108].

TABLE 3.21   Formulation of RIS sublingual spray

Sr no.	Component	Quantity
1	RIS	1 to 5%
2	Propylene glycol	10 to 20 %
3	Poloxamer	1 to 5%
4	Alcohol	20%
5	Oleic acid	1%
6	Distilled water	q.s to 15 ml

TABLE 3.22 Operational parameters

Causative factor	CODE	Unit	Low level (-)	High level (+)	Responses
RIS	X_1	%	1	5	Residence time min Y1
Propylene glycol	X_2	%	10	20	% drug released Y_2
Poloxamer 188	X_3	%	5	10	% drug permeated Y_3

TABLE 3.23 Matrix showing formulation with low and high values of factors

Formulation	Factors		
F	X_1	X_2	X_3
1	1	10	5
2	1	20	10
3	1	10	10
4	5	20	5
5	5	10	5
6	3	15	5.0
7	0.4	15	7.5
8	3	15	7.5
9	3	15	7.5
10	3	21.5	7.5

TABLE 3.23 Matrix showing formulation with low and high values of factors (continued)

11	1	10	5
12	3	15	10.
13	3	8.5	7.5
14	5	10	10
15	5	20	10
16	5.6	15	7.5

3.4.3 Optimization and validation of RIS formulation

Optimization of formulation was carried out by numerical and graphical method. In addition to evaluation tests as described for in-vivo absorption study and stability study was also conducted for optimized batch.

3.4.3.1 Numerical method

Optimization of the RIS spray formulation was made with numerical method. Criteria for the optimizations are tabulated in Table 3.24and 3.25.

TABLE 3.24 Criteria for RIS spray optimization

Significant factor	Goal	Lower	Upper
X_1	In range	1	5
X_2	In range	10	15
X_3	In range	5	10

TABLE 3.25 Set responses for RIS spray formulation

Response	Goal	Lower	Upper
Y_1	In range	25	30
Y_2	Maximize	90	95
Y_3	Maximize	45	50

3.4.3.2 Graphical method

Optimization of the RIS multiple emulsion was also made with graphical method. Criteria for the optimizations are tabulated in Table 3.26.

TABLE 3.26 Criteria for graphical optimization

Response	Goal	Lower	Upper
Y_1	In range	25	30
Y_2	Maximize	90	95
Y_3	Minimize	45	50

3.4.3.3 Validation of the optimized batch

Optimized batch was validated by reproducing it as the formula derived from the optimization and evaluated for selected responses.

3.4.4 Evaluation of formulations

3.4.4.1 Spray pattern

For the spray pattern test two third volume of each spray formulation was removed from the corresponding spray container and stored in labelled glass beaker for time being. One third volume of each Risedronate spray formulation was mixed with patent blue V dye and sprayed over Whatmann filter paper. Spray pattern was determined by Ovality ratio which was calculated using Equation (3.5).

$$\textbf{Ovality Ratio} = \frac{\textbf{Dmax}}{\textbf{Dmin}} \qquad (3.5)$$

Where, D_{max} and D_{min} are the maximum and minimum diameters of the spray pattern respectively

3.4.4.2 Prime test

Test for priming was done to support the number of actuations (priming actuations) that should be fired to waste solution prior using the product. Priming actuations was counted for container to release drug that would come out per actuation as per experimental design

3.4.4.3 Average weight per meter dose

This test was performed to find out the amount of solution delivered per spray. Initial weight of the container was recorded (W_i). Ten successive deliveries were sprayed from the container. Container was weighed again (W_f). Equation (3.6) was used o calculate weight emitted.

$$\textbf{Avg. wt per meter dose} = \frac{(W_i - W_f)}{n} \qquad\qquad (3.6)$$

Where, W_f = final weight of the container,

$\quad\quad\;\; W_i$ = initial weight of the container and

$\quad\quad\;\; n$ = no. of deliveries.

3.4.4.5 Drug content per spray

Content per spray was determined by shots of two sprays in a beaker containing 0.01m HCl. This solution was shaken for 5 minutes and the drug content was determined at 262 nm by the UV-spectroscopy as described in subsection 3.2.7.

3.4.4.6 Net content

Empty containers were weighed before filling and then reweighed after packed containers and the difference obtained was the net content.

3.4.4.7 Density

Empty pycnometer was weighed and then filled with 25ml of the product and reweighed. Difference in the weight of filled pycnometer to empty pycnometer was divided by the volume filled to get the density of the product.

3.4.4.8 Spray profiling (delivered dose uniformity)

Reproducibility of dosage was determined using this test as per USP. The average amount of active ingredient delivered through the actuator per spray was assayed. Uniformity of content was validated by performing the test at three different points i.e. starting, intermediate and ending point approximately.

3.4.4.9 Spray angle

The method of impingement of spray on a piece of paper was used for the study. Patent blue V (10 mg) was dissolved in formulation to facilitate visualization. The sprays were actuated in horizontal direction onto a white paper mounted at a distance of 1 cm from the nozzle. The radius of the circle, formed on the paper, was recorded for minimum and maximum diameters. Spray angle (θ) was calculated by Equation (3.7) [108]

$$\theta = \tan^{-}\left(\frac{h}{r}\right) \qquad (3.7)$$

θ= angle in degree, h= height of triangle and r radius of circle

3.4.4.10 Sublingual mucosal residence time of emitted spray (visualization with dye)

Sublingual mucosa was procured from local licensed slaughter house. It was washed with phosphate buffer and was mounted on 4×4 cm glass slide. A small portion of spray formulation was mixed with oil soluble dye sprayed on the mucosa surface and kept in plastic beaker in slanting position. Simulated salivary fluid was allowed to pass over the sprayed portion and time of disappearance of the colour spots was observed [110].

3.4.4.11 *ex-vivo* drug permeation study (diffusion cell)

Ex-vivo permeation study was carried out using Franz diffusion cell. 20 ml phosphate buffer pH 7.4 was filled in receiver compartment containing magnetic stirring bar. Donor and receiver compartment were separated by treated goat sublingual mucosa which was stored at 2-8 ^{o}C in refrigerator in ringer's solution to preserve its biological characteristic. Experiments were approved by the IAEC in accordance to proposal no. PhD/13-14/22. Entire assembly was set on magnetic stirrer and slowly temperature was raised and maintained at 37°C. Spray formulations containing Risedronate sodium were applied in donor compartment and 2 ml samples from receiver compartment were collected through sample withdrawal tube and equal volume of phosphate buffer was replaced at the interval of 10 minutes for one hour. Procedure was repeated for each spray formulation. Absorbance of Withdrawn samples was measured at 262 nm by UV spectroscopy [19,111].

3.4.4.12 Flux and apparent permeability determination

Flux and apparent permeability were calculated using following equations (3.8) and (3.9).

$$\textbf{Flux } (J_{ss}) = \Delta Q_t / \Delta t \textbf{ x S} \qquad (3.8)$$

Where, $\Delta Q_t/S$ is the cumulative drug permeation per unit of mucosal surface area (µg/ cm^2), t is time expressed in h

$$\textbf{Permeability } (P) = J_{ss} / C_d \qquad (3.9)$$

Where, Jss is steady state Flux and C_d is the concentration of drug in donor compartment [23].

3.4.4.13 Stability study of the optimized formulation

short term stability study was carried out according to ICH guidelines Q1C in humidity chamber (40°C ± 2 °C/75 ± 5% RH) for 3 month for optimized batch. At the end of studies, responses were measured which had been used to optimize the formulation [23].

3.4.4.14 In *Vivo* absorption study

In vivo absorption study was conducted over controlled rats to measure the extent of drug permeated from optimized formulation compared to plain drug solution and conventional formulation. The study was carried out as described in subsection 3.3.5.11.

CHAPTER 4

Result and Discussion

4.1 Preformulation studies

4.1.1 Identification of RIS

4.1.1.1 Organoleptic properties

Received drug sample was evaluated visually and tested for colour, odour and taste. Table 4.1 represents the observations. This qualitative evaluation of the drug is an important characteristic in oral drug delivery systems i.e., palatability. Patient compliance is dependent on favourable taste determined by the target audience. Formulations under consideration in this study are intended to administer orally where taste of drug may influence the formulation composition. If, the drug is bitter than it may require taste masking to make it palatable.

Table 4.1 Organoleptic properties of drug sample observed

Property	RIS standard	RIS sample
Colour(appearance)	white crystalline solid powder	white crystalline powder solid
Odour	Odourless	Odourless
Taste	Tasteless	Tasteless
Clarity of solution	clear and colorless	clear and colorless

Observed characteristics are relevant to the reported data of RIS [114]. Closeness of the reported organoleptic properties with the experimental one has met the part of identification tests and needs further investigation.

4.1.1.2 Melting point

Melting point information can be used for compound identification or in estimation of purity. It is general rule that pure substances will exhibit sharp melting points, while impure materials (or mixtures) will melt over a broad range of temperature. Melting point of Risedronate sodium was found to be 255-257 °C, confirming the purity of the drug.This suggested that the received sample could be monosodium risedronate [115].

4.1.1.3 FT IR analysis

Identity of the drug sample RIS was confirmed from the FTIR spectra obtained by KBr pellet method. Fig. 4.1 shows FTIR spectra of drug sample, where in bending vibrations in Fig. 4.1 finger print region i.e., 629-669 cm^{-1}, 933-910 cm^{-1}, 1100-900 cm^{-1}, 1130 cm^{-1}, 1207 cm^{-1} and 1514-1429 cm^{-1} attributed to C-P stretching, C-H stretching, P-OH stretching, C=N stretching, P=O stretching and C-C ring stretching accordingly. Observed bending vibrations in sample fits to reference standard as shown in Fig. 4.2 which confirmed the identity of sample as RIS [116].
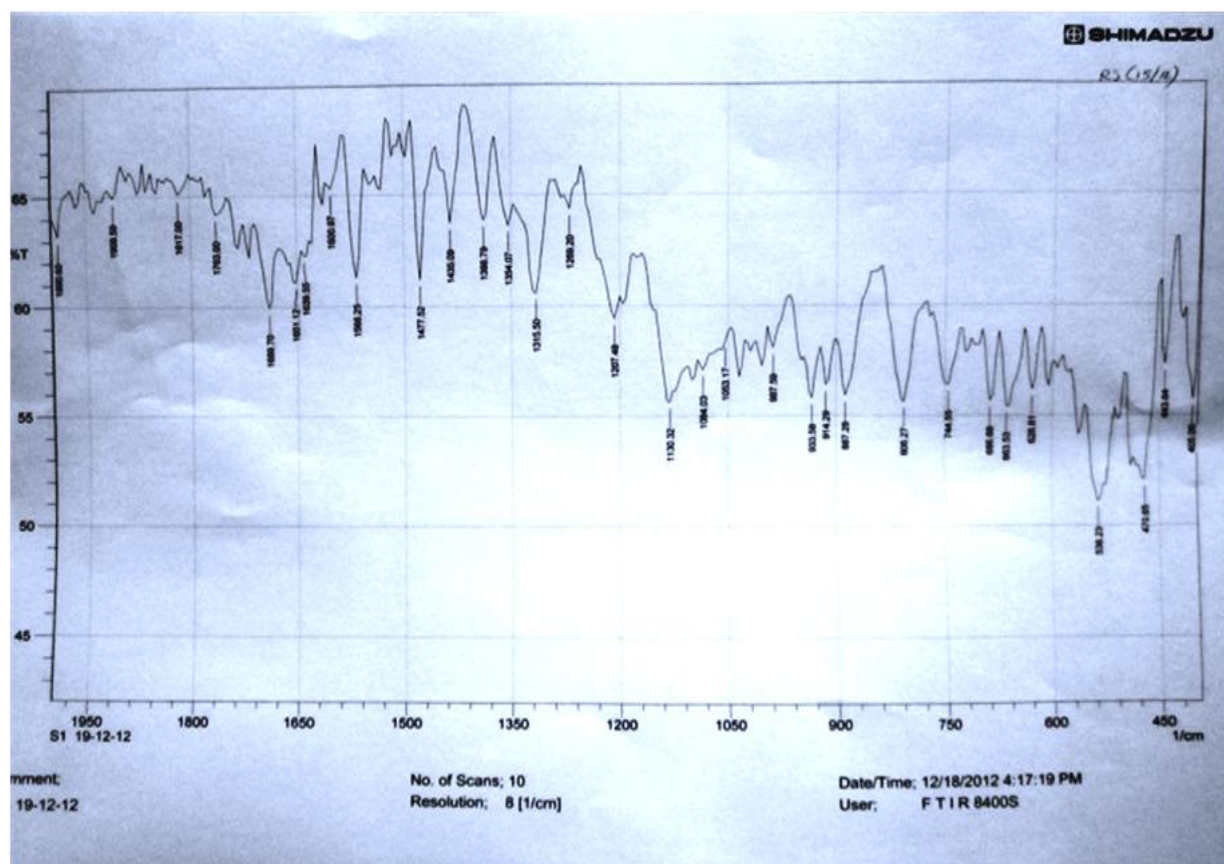
FIGURE 4.1 FTIR spectra of RIS (sample)
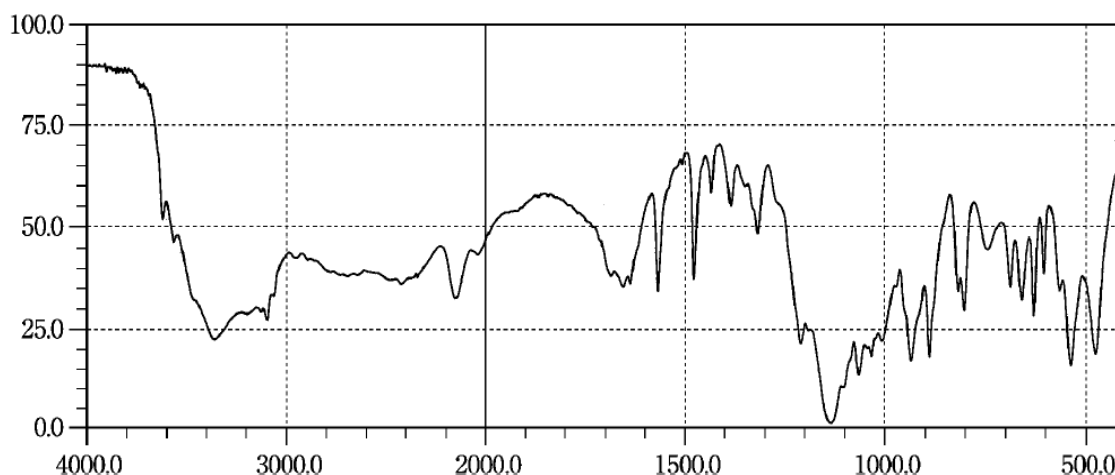
FIGURE 4.2 FTIR spectra of RIS (reference)

4.1.1.4 UV Visible Spectrophotometry determination of absorption maximum by ΔA method

RIS exhibited maximum absorption at 262 nm in 0.01 $moll^{-1}$ hydrochloric acid with absorbance 0.98 and in 0.01 $moll^{-1}$ sodium hydroxide with absorbance 0.58 as shown in Fig 4.3. Higher absorbance value in acidic condition could be due to protonation of pyridyl group (chromophore) present in RIS in an acidic medium (0.01 $moll^{-1}$ hydrochloric acid), than in the alkaline medium (0.01 $moll^{-1}$ sodium hydroxide). Observed absorption maximum (λ max) was identical to reported absorption maxima 262 nm for RIS hence from this it was also confirmed that received sample was RIS [117].
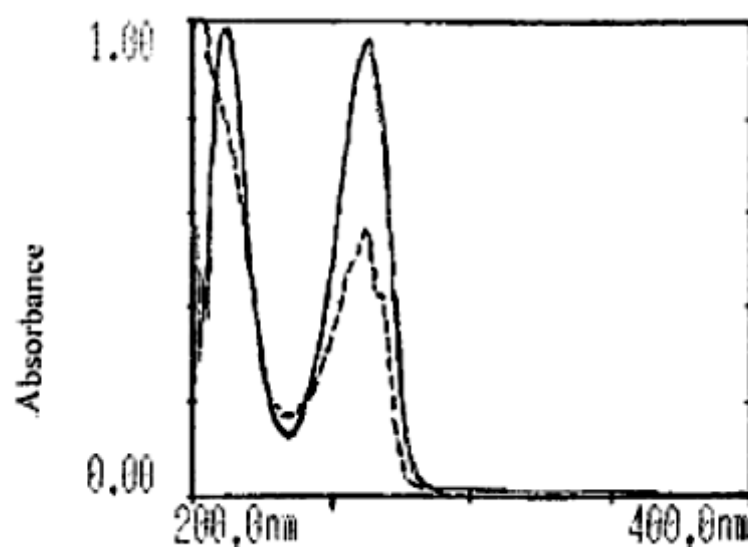
FIGURE 4.3 Absorption maxima of RIS

4.1.2 Analytical method for quantitative determination of RIS

Spectrophotometry method for estimation of RIS based difference in absorbance (ΔA) method for the determination of RIS was used and validated.

4.1.2.1 Linearity and range

The method was tested for linearity, accuracy, precision, LOD and LOQ. Stock solution was serially diluted to get 15 µg/ml to 150 µg/ml concentrations of RIS in0.01 moll^{-1} hydrochloric acid and 0.01 moll^{-1} sodium hydroxide. Mean values of absorbance obtained from triplicate observations in acidic and alkaline solution and subsequent difference were plotted against respective concentrations to get linearity plot and linear regression equation. The regression plot showed a linear dependence of the absorbance over the Beer's law range given in Table 4.2

TABLE 4.2 Linearity range of RIS

RIS concentration (µg/ ml)	ΔAbsorbance n=3
	Average± S.D.
15	0.0567± 0.0045
45	0.161± 0.01473
60	0.225± 0.00849
90	0.3183± 0.0116
120	0.409± 0.01253
150	0.485± 0.01258
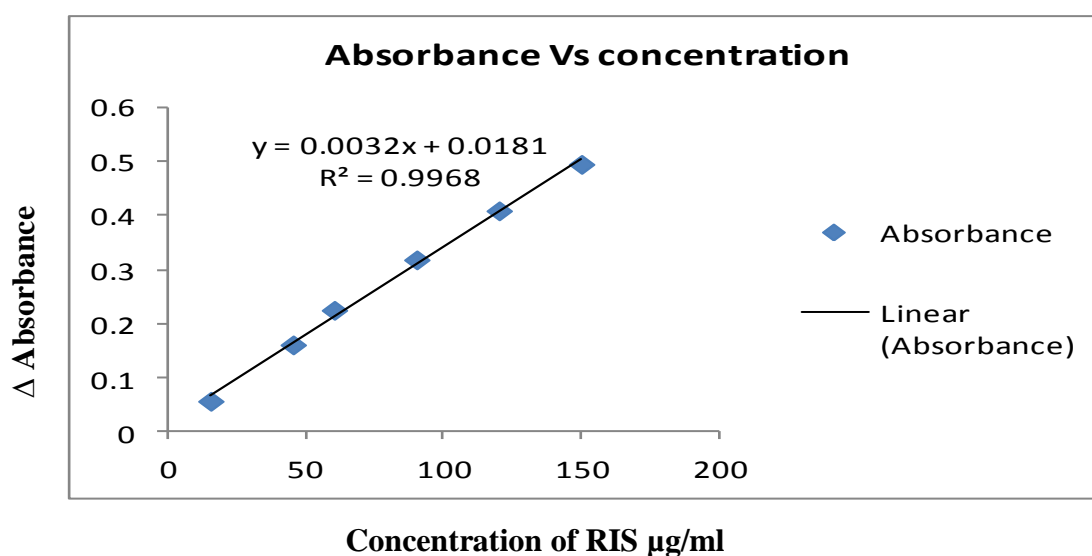
FIGURE 4.4 Linearity plot of RIS by UV

The ΔA method depended on measuring the difference in absorbance of risedronate sodium solution in 0.01 moll^{-1} hydrochloric acid as a test and the drug in 0.01 moll^{-1} sodium hydroxide as a blank as shown in Fig. 4.4. The difference in the absorbance ΔA was proportional to the concentration of drug. Linearity plot was generated using Microsoft excel 2007 and regression coefficient value was found to be 0.9968.

4.1.2.2 Precision (Repeatability)

Relative standard deviations (RSD) for intra-day and inter-day precision study are presented in Table 4.3. The smaller values of intra-day and inter-day variation in the analysis show that the method was precise.

TABLE 4.3 Intraday and Interday precision

Concentration µg /ml of RIS	Intraday precision data (n=6) % RSD	Interday precision data (n=6) % RSD
15	1.62±0.0154	1.59±0.0164
90	1.58±0.0274	1.58±0.0264
150	1.23±0.0150	1.60±0.0154

4.1.2.3 Accuracy of Method

The accuracy of the method was checked by carrying out recovery studies at three different additions of original amount of RIS i.e., 50, 100, and 150 %. It can be seen from Table 4.4 that % recovery of the method was in the range 98 % - 102 % and % RSD was below 2 which suggest that the method is accurate.

TABLE 4.4 Results of accuracy

Amount Taken (µg/ml)	Amount added (% of original amount)	Conc. Obtained (µg/ml)	%Recovery* n=3	SD	%RSD
10	50	15.2	101.3	1.58	1.56
20	100	40.5	101.2	1.33	1.32
30	150	74.8	99.73	1.19	1.20

*Avg. of three determinations, SD is standard deviation, R.S.D. is relative standard deviation

4.1.2.4 LOD and LOQ

The values of LOD and LOQ of the method are shown in the Table 4.5.

TABLE 4.5 LOD and LOQ for RIS

Parameter	RIS
Wavelength	262 nm
LOD (µg/ml)	5.31
LOQ(µg/ml)	15.57

4.1.3 Bio analytical method for estimation of RIS RP-HPLC analysis

4.1.3.1 Linearity and plot

The linearity of the plasma assay was performed with a six-point calibration curve. The slope and the intercept of the calibration graph were calculated by linear regression of the risedronate concentration. The experimental peak area is interpolated on the calibration curve and the concentrations were back calculated. The intercept, slope and coefficient of correlation (*r*) were evaluated and linearity of the method between the peak area and the concentration of risedronate in plasma was studied over the range 0.1–5 µg ml^{-1} as presented in Fig. 4.5. The method was found to be linear over the examined concentration range 0.1–5 µg ml^{-1}Fig. 4.6. The average calibration equation could be described by Equation (4.1) with an average correlation coefficient of 0.9995.

$$\mathbf{y = 314.48x + 2.2469} \tag{4.1}$$
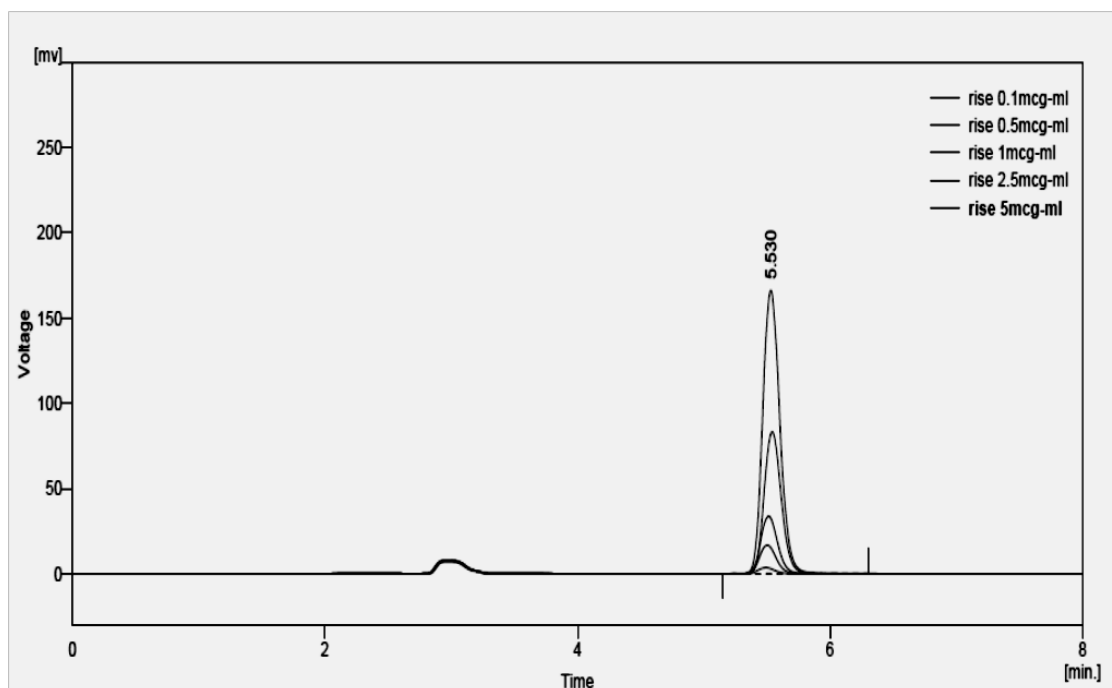
FIGURE 4.5 RP-HPLC peaks of RIS

TABLE 4.6 Linearity of RIS

Sr No.	Conc.(μg/ml)	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.1	5.493	34.130	3.666	100.00
2	0.5	5.503	157.898	16.858	100.00
3	1	5.513	317.449	33.794	100.00
4	2.5	5.540	789.303	83.205	100.00
5	5	5.530	1574.230	166.180	100.00

Concentration of RIS in μg/ml
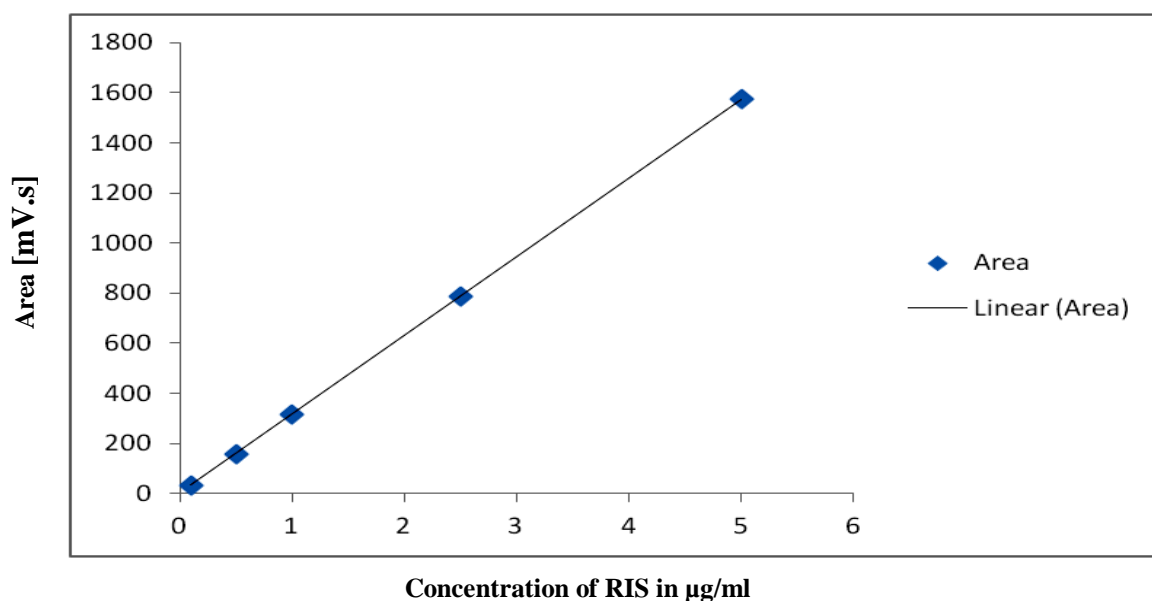
FIGURE 4.6 Linearity plot for RIS

4.1.3.2 Precision

A measurement of intra- and inter-day precision of the method was carried out by analyzing six replicates of three quality controls (0.1, 2.5 and 5 µg ml^{-1}) on three consecutive days. Precision is expressed as relative standard deviations (RSD) are summarized in Tables 4.7. The intra and inter-precision RSD values were less than 2% at all levels. Accordingly the precision of the method was found to be satisfactory.

TABLE 4.7 Intraday and Interday precision data

Concentration µg/ml of RIS	Intraday precision data (n=6) % RSD	Interday precision data (n=6) % RSD
0.1	1.40	0.59±0.0164
2.5	0.58	0.38±0.0264
5.0	0.23	0.20±0.0154

4.1.3.3 Accuracy of method

The accuracy of the method was determined by adding RIS at three different concentrations and expressed as the percent deviation between amount found and amount added at the three concentrations examined. Table 4.8 summarizes the results.

TABLE 4.8 Results of accuracy

Amount Taken (µg/ml)	Amount added (% of original amount)	Actual drug Content (µg/ml)	Conc. Obtained (µg/ml)	%Recovery* n=3	SD	%RSD
0.1	50	0.15	0.151	100.6	0.86	0.86
1	100	2.0	2.02	101.0	0.63	0.62
2	150	5.0	5.07	101.4	0.61	0.60

*Avg. Of three determinations, SD is standard deviation, R.S.D. is relative standard deviation

4.1.3.4 Extraction recoveries

The extraction recoveries of RIS from rat plasma were determined by comparing the absolute peak areas of samples spiked with RIS with appropriate concentrations of RIS. The extraction recoveries of RIS in plasma are shown in Table 4.9.

TABLE 4.9 Extraction recoveries of RIS

Estimated concentration of RIS after extraction µg/ml	Mean recovery concentration after extraction µg/ml	% Recovery	% RSD
0.1	0.0921	92.1%	0.66
2.5	2.39	95.8%	0.32
5.0	4.94	98.9%	0.24

4.1.4 Physicochemical test

4.1.4.1 Solubility

Solubility of RIS in distilled water was found 10.6 mg/ml while reported is 10.4 mg/ml Table 4.10 [118]. Study includes formulation of w/o/w multiple emulsion and sublingual spray formulation where aqueous phase would be major component. In the case of w/o/w multiple emulsion RIS is to be added in inner aqueous phase and determined solubility value made feasible to incorporate RIS in w/o/w multiple emulsion. On the other hand addition of RIS in aqueous vehicle would yield clear spray formulation.

TABLE 4.10 Solubility data of RIS

Component	Reported solubility of RIS mg/ml	*Determined solubility of RIS mg/ml
Distilled water	10.4	10.6

*n=3 determination

4.1.4.2 Initial drug stability studies

Aqueous solubility of the RIS made it feasible to include in both formulation i.e., w/o/w multiple emulsion and sublingual spray solution. In order to ensure compatibility with aqueous phase, stability was performed prior to formulation. Concentrations of the RIS at initial stage of study and after the completion of the study were determined are presented in Table 4.11.

TABLE 4.11 Concentrations of RIS

Conditions	Initial concentration of RIS in aqueous phase at time "0"	*Concentration of RIS in aqueous after 1 month (n=3)
1 % w/v RIS dissolved in distilled water and stored at 40±2°C/75±5% RH in amber colour bottle in day light	200 µg/ml	198 µg/ml

* Average of three samples

Initial stability study indicated that RIS did not interact with water and no degradation observed. Absence of sign of chemical incompatibility with aqueous phase supported the study design.

4.1.4.3 Octanol/water coefficient ($K_{o/w}$)

Ko/w allows predicting drug partition in the biological membrane since higher value indicates lipophilic nature while low value indicates hydrophilic nature. Table 4.12 shows results of the test for RIS Octanol /water coefficient.

TABLE 4.12 Octanol water coefficient of RIS

Ko/w	Reported value of Ko/w	*logP	Experimental value of Ko/w	*logP
Ratio of Concentration of RIS in Octanol/water	2.52×10^{-4}	-3.6	2.46×10^{-4}	-3.61

* Ko/w is Octanol water coefficient, Log p frequently used to represent log value of Ko/w

RIS is bcs class III drug having high solubility and poor permeability. From the result it is clear that RIS had almost partitioned in aqueous layer than octanol layer indicating its

hydrophilic nature. In addition negative value of logP -3.61 indicates that drugs needs permeability enhancement in order to get absorbed through biological membrane.

4.1.4.4 pH of the RIS solution

pH of 1%w/v solution of RIS in water was found 5.8 indicating acidic nature.

4.2 Formulation of RIS W/O/W multiple emulsion

4.2.1 Preliminary batches of RIS w/o/w multiple emulsions

Preliminary batches of the RIS w/o/w multiple emulsions were prepared with different oils namely arachis oil, olive oil, sunflower oil and isopropyl myristate. In order to find out effect of formulation components and operational parameters of manufacture, preliminary study was conducted and different formulations were made using the scheme generated by plackett-burmen design from Minitab 16 software. Total 12 formulations containing each oil were prepared where concentration of span 80, homogenization speed for primary emulsion W/O, time of homogenization for primary emulsion W/O, concentration of tween 80 and homogenization speed for secondary emulsion were selected as independent factors and responses to them where average globule size, % creaming, % encapsulation efficiency. The independent factors and responses were coded as A, B, C, D, E and R1, R2, R3 respectively. All 12 batches were also evaluated for organoleptic properties, type of emulsion, structure, viscosity, pH, pour ability.

4.2.2 Evaluations of preliminary batches of RIS multiple emulsions

4.2.2.1 Organoleptic properties of RIS w/o/w multiple emulsion

Patient acceptability of oral formulations is largely depended over organoleptic properties of the product. Hence freshly prepared multiple emulsions were tested for appearance, colour and odour. Primary emulsions i.e., w/o emulsions made up of different oils were sticky, glossy and off-white to white colour having characteristics odour of oils. Results of the secondary emulsions i.e., w/o/w multiple emulsions stored between o to 14 days are presented in Table 4.13while at the end of the 28 days are presented in Table 4.14.

TABLE 4.13 Organoleptic properties of w/o/w emulsions for o to 14 days storage

Oil phase in w/o/w multiple emulsion (Formulation batch)	Appearance	Odour	Colour
Arachis oil (F1-F12)	Milky	Medium peanut aroma	Off white to white
Olive oil (F13-F24)	Cloudy	Faint phenolic	Faint yellow
Sunflower oil (F25-F36)	Milky	Pleasant	white
IPM (F37-F48)	Milky	odourless	white

Table 4.14 Organoleptic properties of w/o/w emulsions on 28th day storage

Oil phase in w/o/w multiple emulsion (Formulation batch)	Appearance	Odour	Colour
Arachis oil (F1-F12)	cloudy	mild peanut aroma	Off white to white
Olive oil (F13-F24)	cloudy	rancid	Faint yellow
Sunflower oil (F25-F36)	milky	mild Pleasant	White
IPM (F37-F48)	milky	odourless	White

Generally all oil can form the multiple emulsions however they can markedly affect the behavior of multiple emulsion system. The components of oil can have effect over stability of oil. Studies have shown a better yield and better stability with mineral oils and fatty acid esters compared to vegetable oils (Florence et al., 1982) [34]. From the above results it is seen that emulsion containing arachis, sunflower and IPM remained unaltered in their organoleptic properties during storage while emulsion containing olive oil had rancid oil odour after 28 days storage which could be due to oxidation of phenolic compounds of oil [119].

4.2.2.2 Type of emulsion

Study was aimed to formulate RIS w/o/w multiple emulsions since RIS was incorporated in inner water phase of w/o primary emulsion using span 80 as internal emulsifier covered by outer water phase having tween 80 to yield w/o/w emulsion. Selection of surfactant was based on HLB scale i.e., span 80 has HLB value 4 which usually forms w/o emulsion while tween 80 has HLB value 14 forms o/w emulsion. Type of the emulsion was determined by dilution and conductivity test for secondary emulsion. In dilution test 2 ml of all the formulations of each oil were mixed with equal portion of water, which formed the diluted product. There was no distinct layer observed which could be due to miscibility of outer aqueous phase of the emulsion with added water confirming the formation of w/o/w emulsion.

Conductivity tests merely indicates the presence of outer phase in emulsion i.e., oil or aqueous and to know the type of emulsion conductivity test was performed after primary and secondary emulsification. All primary emulsion showed negligible conductivity in range of 2-5 µs/cm and secondary emulsions showed conductivity in 20-25 µs/cm. Results proved that in primary emulsion oil was outer phase however very small values of conductivity could be due to leakage of aqueous phase to outside while large conductivity values in secondary emulsion showed that water was outer phase. This supported the fact that oil is bad conductor of electricity and water is good conductor of electricity.

4.2.2.3 pH determination

pH of the formulations was near to neutral region. Results are presented in Table 4.15 Results shown in table revealed that RIS could have leaked from inner water phase in outer

TABLE 4.15 pH of w/o/w multiple emulsion

Oil phase in W/O/W multiple emulsion (Formulation batch)	pH
Arachis oil(F1-F12)	6.1-6.3
Olive oil(F13-F24)	6.0-6.4
Sunflower oil(F25-F36)	6.0-6.2
IPM(F37-F48)	6.0-6.2

continuous water phase in the formulations.

4.2.2.4 Structure analysis and globule size determination

A computerized image analyzing device connected to a trinocular microscope was used for the microscopic observations (Zeiss Instruments). Images of the field showed existence of multiple structures as shown in Fig 4.7 (a), Fig 4.7 (b), Fig. 4.8 (c), and Fig. 4.8 (d).

All formulation made of different oils formed the multiple droplet structure specifically w/o/w. Multiple droplet structure for each formulation was determined by counting 100 globules in selected field and are present as % table 4.16

TABLE 4.16 Multiple structure of emulsion

Oil phase in W/O/W multiple emulsion (Formulation batch)	*Single droplets (%)	*Multiple droplets (%)
Arachis oil (F1-F12)	18.60±8.53	80.40±8.44
Olive oil (F13-F24)	22.50±5.38	77.00±5.44
Sunflower oil (F25-F36)	18.25±4.70	82.25±2.06
IPM (F37-F48)	09.00±0.86	89.50±5.29

* Range of 12 formulation batches

Multiple droplet structure formation was observed in decreasing pattern with different oils as IPM>sunflower oil>Arachis oil>olive oil.
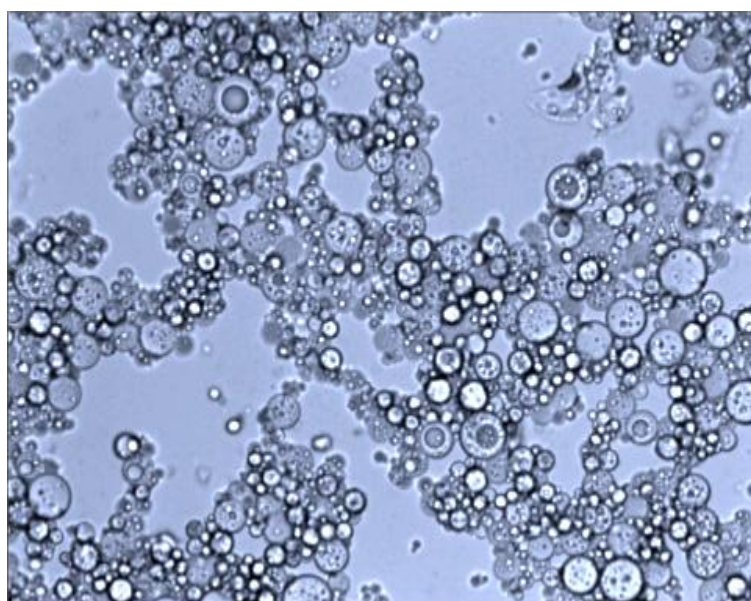
FIGURE 4.7 (a) Multiple structure of Arachis oil emulsion
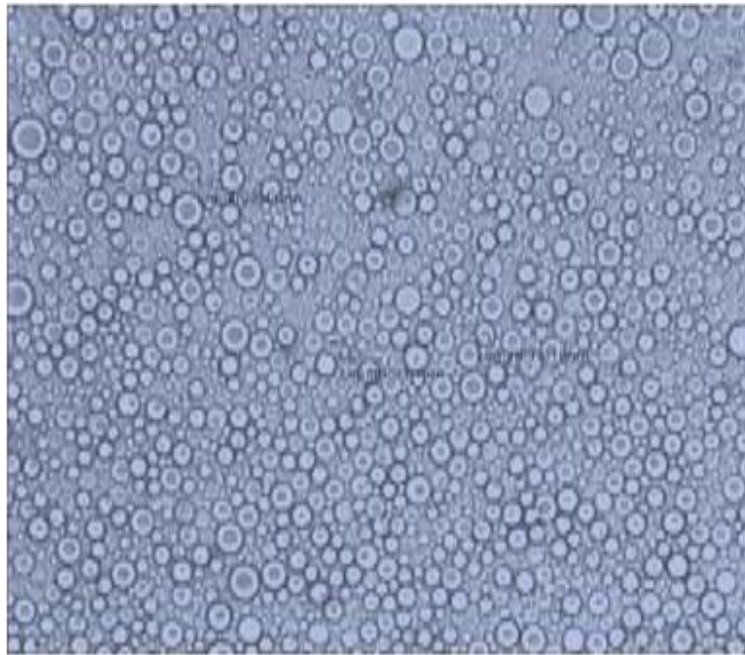
FIGURE 4.7 (b) Multiple structure of Olive oil emulsion
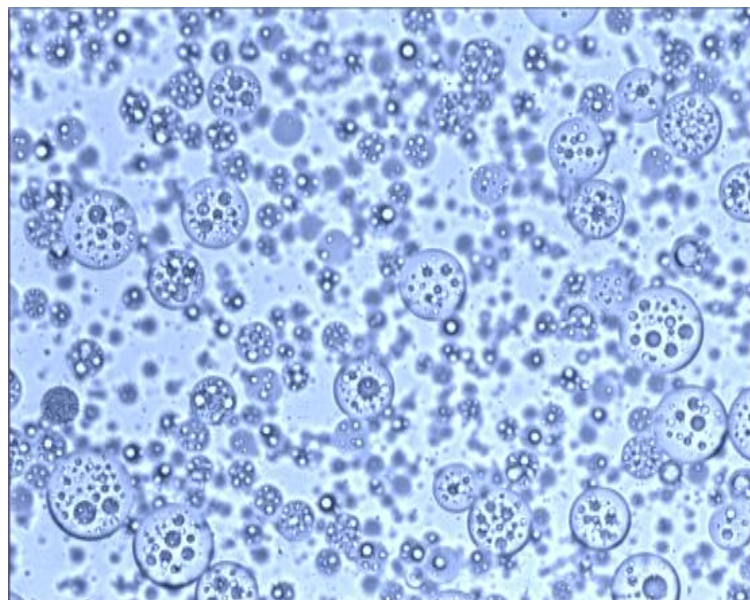
FIGURE 4.8 (c) Multiple structure of IPM oil emulsion
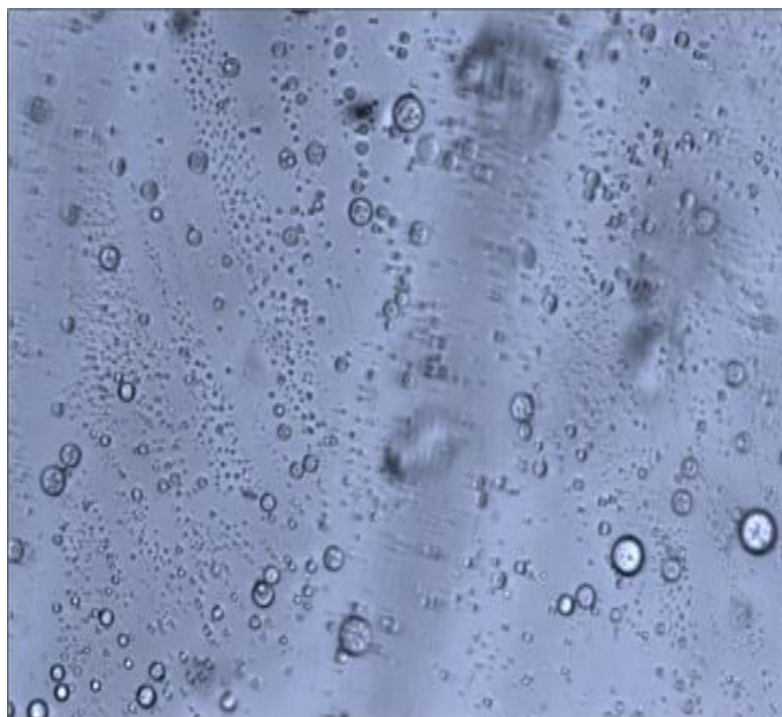
FIGURE 4.8 (d) Multiple structure of Sunflower oil emulsion

4.2.2.5 Viscosity determination

Viscosity of the multiple emulsion indicates nature of outer phase as well as its volume fraction.

TABLE 4.17 Viscosity of preliminary batches

Oil phase in W/O/W multiple emulsion (Formulation batch)	*Viscosity (mPa)
Arachis oil(F1-F12)	11.60±3.53
Olive oil(F13-F24)	12.50±4.38
Sunflower oil(F25-F36)	08.25±3.70
IPM(F37-F48)	08.00±1.86

* Range of 12 formulations

Table 4.17 represents viscosity values and it is inferred from the data that due to higher density of the of Arachis oil, olive oil and sunflower oil compare to IPM, formulations made up of these oils have slightly higher viscosity than IPM composed emulsions. In

w/o/w multiple emulsion water is outer phase which is less viscous compare oil and also desirable for pour ability and patient acceptability.

4.2.2.6 Osmotic behavior

Formulations were added in external water phase with 1 and 10% amount of sodium chloride and studied for globule size variation. It was seen from the results that as the amount of electrolyte increased in outer phase the dispersed oil globules shrunk and became smaller than initial indicating drainage of internal water phase to external Table 4.18.

TABLE 4.18 Osmotic effects on emulsion

Oil phase in W/O/W multiple emulsion (Formulation batch)	*Globule size (Initial)	*Globule size (after addition of sodium chloride)
Arachis oil(F1-F12)	5.90 ±2.32	2.90 ±2.32
Olive oil(F13-F24)	6.00±3.31	3.40±3.31
Sunflower oil(F25-F36)	5.09 ±2.53	2.09 ±2.53
IPM(F37-F48)	3.85±1.14	1.85±1.14

* Range of globule size

4.2.2.7 Effect over globule size (R1)

Overall effect of the independent factors A, B, C, D and E over globule size can be seen from the main effect graph generated from Minitab 16 software Table 4.19. Average globule size of the multiple (R1) droplets has decreased markedly from 5.3 to 2.8 μ with increasing emulsification speed for primary emulsion (B). Decrease in the globule size could be due to high shear applied and subsequent reduction in droplet size. Concentration of the span 80(A) and Time for primary emulsification(C) also has moderate decreasing effect on R1. This effect could be due to higher strength provided by the internal emulsifier with more time for emulsification. While concentration of tween 80 (D) and homogenization speed (E) has moderate increasing effect on R1 as shown in Fig.4.9. Higher amount of tween 80 could have extracted some amount of the internal emulsifier leading to swelling of the globule.

TABLE 4.19 Effect of A, B, C, and D on R1, R2 and R3

Oil phase in W/O/W multiple emulsion (Formulation batch)	Globule size* (R1)	% Creaming* (R2)	% Encapsulation* efficiency (R3)
Arachis oil (F1-F12)	5.90 ±2.32 to 5.98±1.02	24.00±1.12 to 25.01±2.52 to	60.11±2.52 to 56.01±1.42
Olive oil (F13-F24)	6.00±3.31 to 6.20±2.40	26.83±5.52 to 28.62±3.11	49.01±3.42 to 59.21±2.52
Sunflower oil (F25-F36)	5.09 ±2.53 to 5.39 ±1.13	23.65±3.52 to 25.85±1.52	56.29±2.72 to 60.22±4.02
IPM (F37-F48)	2.85±2.04 to 3.85±1.14	28.23±2.02 to 30.60±2.02	61.25±2.52 t0 66.20±2.22

* Range observed in 12 formulations

R1 increment was also observed with higher E i.e., high speed could disrupt the small structure to form larger one.
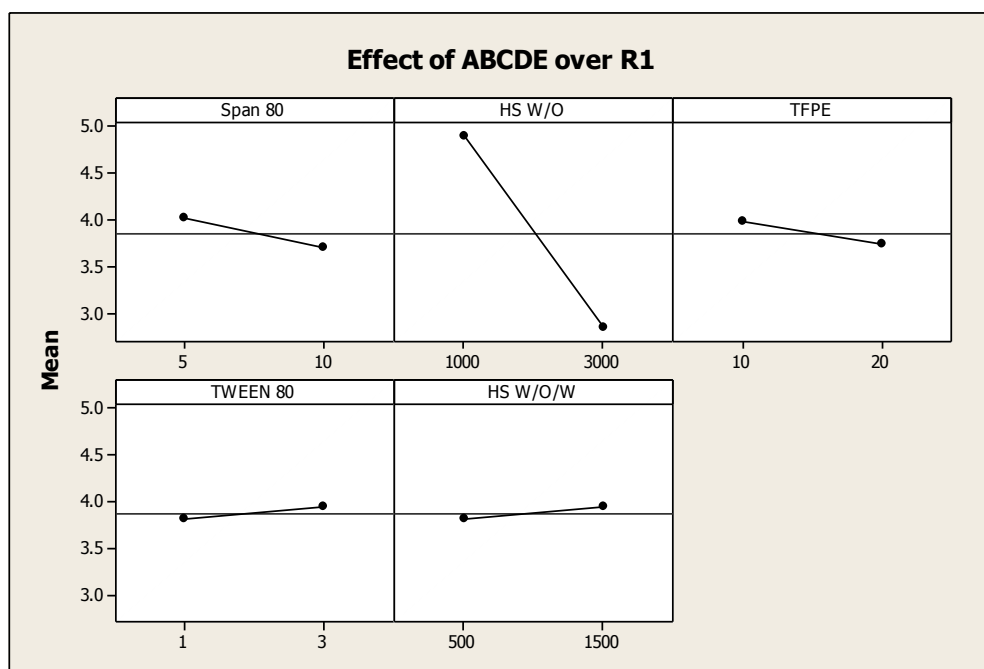
FIGURE 4.9 Effects of independent factors A, B, C, D and E on R1

4.2.2.8 Effect over % creaming (R2)

Multiple emulsions are multicompartment system and often suffers instability problem. Major instability marker is creaming but it is reversible i.e., coalescence of the oil globules leads to concentrated layer and get deposited on top region of the bulk and whenever bulk is shaken these gathered globules rearrange themselves to give homogeneous mass. With this study it was evident from the graph and result shown in Table 4.18 and the % creaming observed for different formulation of oils are in order i.e., IPM> olive oil>Arachis oil>sunflower oil Table 4.19. From this it could be said that fixed oils obtained from vegetable sources have higher density value (density 0.92-.95gm/ml) than IPM (density 0.85gm/ml) which are dispersed phase in multiple emulsion and water (density 1.00 gm/ml) is outer continuous phase [120]. According to stroke's law more is density difference between dispersed phase and continuous phase higher is sedimentation.
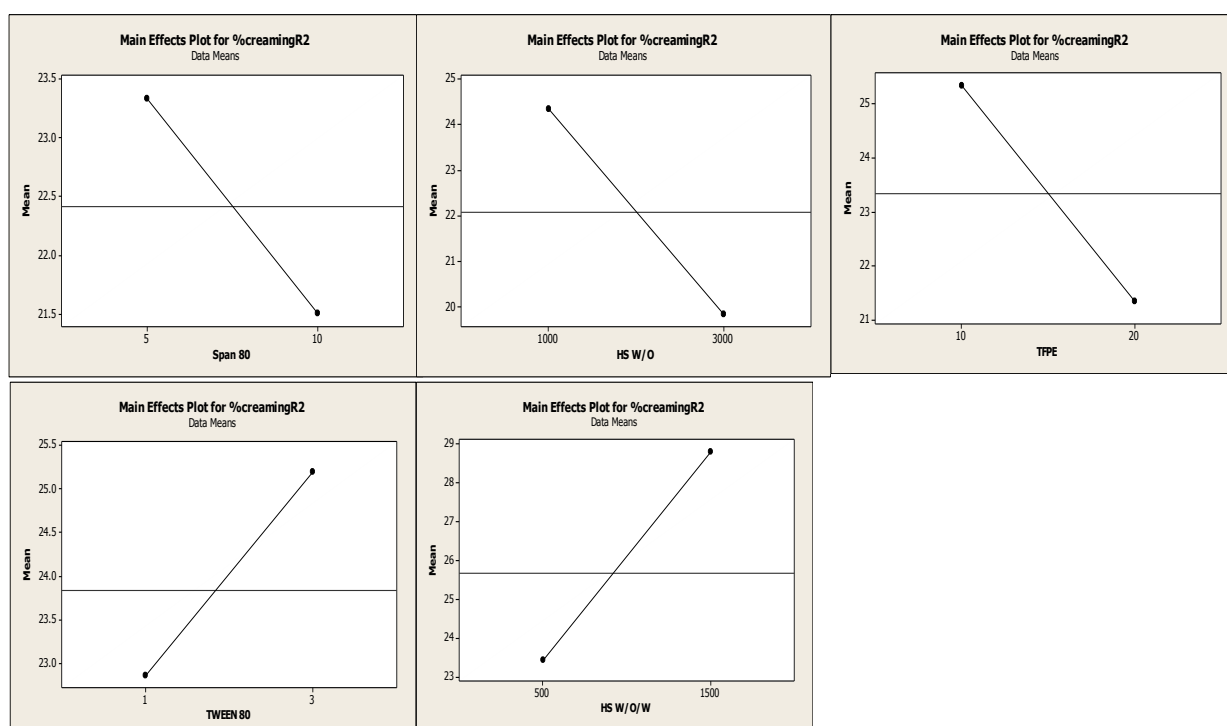
FIGURE 4.10 effects of independent factors A, B, C, D and E on R2

From the Fig 4.10 it is also evident that A,B,C has decreasing effect on creaming which could be attribute to presence of smaller oil globules due to higher shear applied, more time for emulsification and greater stability of oil globules imparted by internal emulsifier. Larger globules tend to coalescence rapidly than the small as explained by stokes's equation for sedimentation [100]. While with E and D has increasing effect on % creaming

because of possible removal of the internal emulsifier from the interfacial region leading swelling of oil globules and further disrupting it by higher speed of emulsification Fig 4.10.

4.2.2.9 Effect over % entrapment efficiency (R3)

Incorporation of RIS in inner water phase imparts lipophilic nature and increase permeability and for that it has to be entrapped in inner water phase. More entrapment of RIS in inner water phase of w/o/w multiple emulsion may ultimately increase absorption and permeation.

In present study effect of A, B, C, D and E on % entrapment efficiency of the drug were seen and data are presented in Table 4.19 and Fig 4.11. From the table it is evident that nature of oil didn't have any remarkable effect. With respect to A i.e., concentration of the span 80 has incremental effect on % entrapment which could be due to stable formation of interfacial film between oil and water phase. B, C, D, and E have lowering effect on % entrapment.
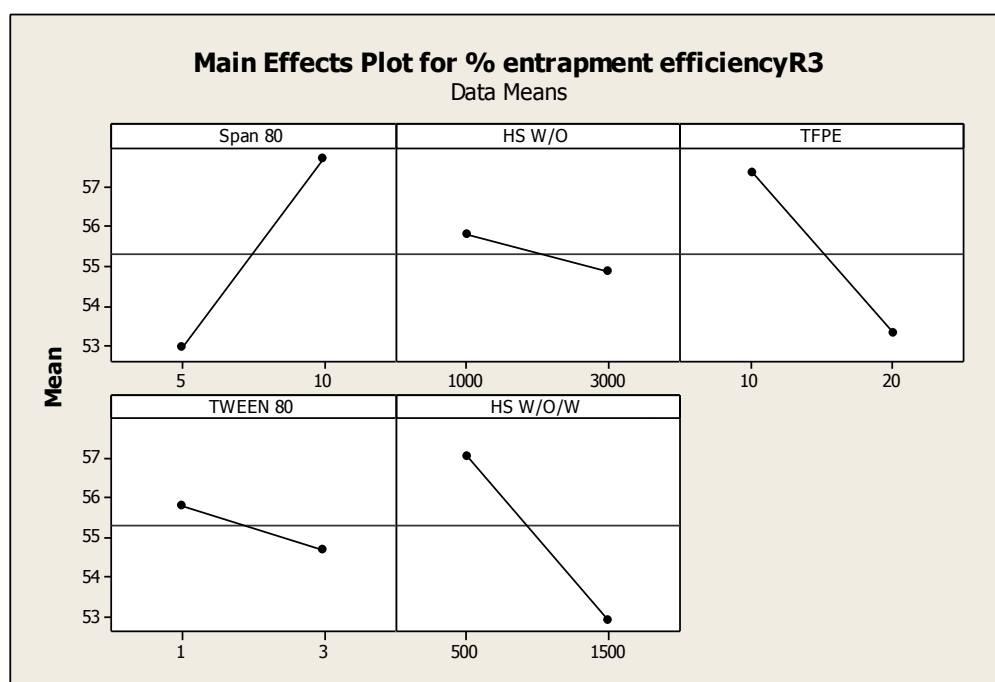
FIGURE 4.11 effect of A, B, C, D and E on % entrapment efficiency

Higher magnitude of B, C and E could have acted as driving force for escaping RIS from inner water phase while higher D as said earlier could have extracted internal emulsifier leading swelling of globules and subsequent leakage of drug in continuous phase.

4.2.2.10 Summary of preliminary formulation batches

Preliminary formulations were prepared and evaluated for screening the significant factors and eliminating non significant factors. From all the observations it was decided to go with IPM as oil phase and leaving behind other oils. Apart from observations, another cause is IPM has no colour and odour requires no additional additives. Concentration of Span 80 (A), Homogenization speed for primary emulsion W/O (B), Time of homogenization for primary emulsion W/O (C) were found significant and included in further formulation study while concentration of Tween 80 (D) and Homogenization speed for secondary emulsion (E) were fixed to minimum level for further formulation and optimization study. Observed responses i.e., average globule size (R1), % creaming (R2), % encapsulation efficiency (R3) were carried forward and one more response % drug permeated was added ahead of study.

4.2.3 Formulation of RIS w/o/w multiple emulsion

Total 18 formulation batches were prepared and evaluated for different tests as described in earlier chapter 3. Design expert 7.0 software was used to generate formulation batches as presented in Table 3.11 and 3.12. Two step emulsification method was adopted as described in chapter 3. Results of the formulation study are presented in Table 4.20 and 4.21.

4.2.4 Evaluation of formulation batches

4.2.4.1 Organoleptic properties

Final preparations were all milky in appearance with white colour, odourless and pourable.

4.2.4.2 Type of emulsion

Dilution test and conductivity test carried out for determining type of emulsion revealed that formulation were w/o/w.

TABLE 4.20 Results of formulation study

Formulation (F)	Type of emulsion		*Droplet structure		*Viscosity mPa
	Dilution test with water	Conductivity μs/cm	%single	%multiple	
1	Diluted	22	8.80±2.23	92.50±1.24	10.11±2.43
2	Diluted	21	9.84±3.24	90.22±2.10	12.22±1.22
3	Diluted	23	10.11±1.09	91.24±1.20	10.86±1.43
4	Diluted	22	13.70±2.27	85.78±1.86	12.22±1.65
5	Diluted	22	18.80±1.89	80.02±3.09	09.22±2.77
6	Diluted	21	10.00±3.56	89.10±2.27	10.45±2.44
7	Diluted	20	14.80±1.54	84.21±1.35	11.65±1.53
8	Diluted	22	16.54±1.33	83.22±3.89	9.08±2.98
9	Diluted	23	10.21±2.09	89.19±3.21	11.76±1.78
10	Diluted	23	7.60±1.55	93.18±2.45	13.21±2.40
11	Diluted	22	9.81±5.44	90.02±2.09	11.09±2.41
12	Diluted	21	19.65±3.64	78.11±2.59	08.98±1.63
13	Diluted	22	15.78±1.98	83.14±2.87	10.01±1.55
14	Diluted	23	8.80±2.27	91.22±1.16	14.21±2.66
15	Diluted	23	9.80±1.24	90.00±1.28	13.69±2.78
16	Diluted	23	6.60±2.24	93.50±1.45	14.22±2.32
17	Diluted	22	10.80±2.32	90.00±2.04	10.11±1.55
18	Diluted	22	9.80±2.01	91.14±3.49	13.31±1.89

*n=3

TABLE 4.21 Factors and their effects on responses

Formulation batch(F)	*A (%)	*B (rpm)	*C (min)	R1 (μ)	R2 (%)	R3 (%)	R4 (%)
1	07.50	3000	23.41	3.8	22	57.12	32.11
2	03.30	3000	15.00	5.7	33	45.23	27.09
3	11.70	3000	15.00	2.0	18	71.06	41.44
4	10.00	2000	20.00	5.8	34	69.50	28.22
5	07.50	3000	6.59	5.7	32	52.11	27.90
6	07.50	3000	15.00	6.0	35	59.00	25.09
7	10.00	2000	10.00	5.7	24	69.55	26.89
8	05.00	2000	20.00	5.5	23	56.70	26.90
9	07.50	3000	15.00	5.5	32	59.23	25.55
10	10.00	4000	10.00	4.2	27	69.98	23.21
11	07.50	3000	15.00	5.0	26	60.11	28.90
12	07.50	1300	15.00	4.2	26	58.65	22.34
13	05.00	2000	10.00	1.8	19	53.68	40.09
14	10.00	4000	20.00	1.9	19	70.22	38.90
15	05.00	4000	20.00	1.8	20	55.12	41.55
16	07.50	4700	15.00	4.3	26	58.09	30.90
17	07.50	3000	15.00	4.2	25	59.90	31.22
18	05.00	4000	10.00	4.3	28	56.54	32.44
Plain drug solution							05.10

A, B and C are X_1, X_2 and X3

4.2.4.3 Droplet structure

Multiple structure of the w/o/w emulsion was determined as described earlier. It was inferred from the results that with higher emulsification speed in formation of primary emulsion % multiple droplets were higher compared to lower emulsification speed. This could be due to more shear applied during the emulsification which caused more dispersion of water phase in smaller droplets. Result data also proved that increase in dispersion time increased the multiple structures.

4.2.4.4 Viscosity

Viscosity of the formulations showed noticeable variation between formulations prepared with different emulsification speed at primary emulsification. Higher value of the viscosity at higher speed of primary emulsification indicated more shear stress which could be due to presence of more multiple structures. Rajinder Pal (2008)in his review concluded that increase in multiple structure droplet in dispersed phase causes increase in viscosity of emulsion because increase in the distortion of flow pattern around the droplets, resulting in an increase in viscosity of multiple emulsion[121].

4.2.4.5 Effect on globule size R1

Effect of independent factors A, B and C on globule size was analyzed using design expert software 7.0. ANOVA for Table 4.22 for R1 showed that the quadratic model was significant. F value 13.65 implies that model is significant while $p < 0.05$ value for term B and C indicates significant factors. As shown in Table 4.22 speed of homogenization B and time C for homogenization during formation of primary emulsion significantly affected the globule size R1. While speed of emulsification (500 rpm) and time (5 min) for second emulsification were fixed previously and kept unchanged. It was also revealed from the ANOVA table that (A) concentration of primary (internal) emulsifier did not affect the globule size

TABLE 4.22 ANOVA table for dispersed phase globule size R1

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	36.4	10	3.64	13.65	0.0012 significant
A-A	7.322×10^{-4}	1	7.322×10^{-4}	2.685×10^{-3}	0.9601
B-B	35.32	1	35.32	129.52	0.0001
C-C	0.54	1	0.54	1.98	0.0020
AB	1.250×10^{-3}	1	1.250×10^{-3}	4.583×10^{-3}	0.9479
AC	0.031	1	0.031	0.11	0.7449
BC	1.250×10^{-3}	1	1.250×10^{-3}	4.583×10^{-3}	0.9479
A2	0.091	1	0.091	0.33	0.5819
B2	0.46	1	0.46	1.69	0.2349
C2	2.496×10^{-3}	1	2.496×10^{-3}	9.151×10^{-3}	0.9265
ABC	1.250×10^{-3}	1	1.250×10^{-4}	4.583×10^{-3}	0.9479
Error	1.91	7	0.27		
Total	38.31	17			

From the observation table it was seen that as the speed of the homogenization (B) increased the average globules size R1 of the dispersed phase decreased from 6.0 to 1.8 μ. High shear rate with longer time (C) could have provided disrupting force for dispersion and formed smaller globules than at low shear rate. At low shear rate larger average globules were formed which ultimately can lead to instability and tendency toward coalescence.

Shear time is another important parameter for preparing stable multiple emulsions. As it was clear from the results that longer time for emulsification (C) produced smaller globules compare to short time. However high shear employed for longer periods can lead to incorporation of air within the emulsion. It was reported that excessive frothing caused

by air entrapment causes displacement of the surfactant from the interface. Such an emulsion system would be unstable ((Florence et al., 1982) [34]. Therefore, an optimal shear rate and time is vital for the production of a stable multiple emulsion.

Final equation in terms of coded factors:

GLOBULE SIZE R1 = +4.24 +7.322E-003A -1.61B -0.20C-0.012 A B +0.063 AC-0.012 BC-0.085A^2-0.19 B^2-0.014 C^2-0.013 A BC   (4.2)

Negative coefficient of the B and C indicates negative effect on the R1 i.e., with increase in B and C, R1 decreases while very small coefficient of A indicates negligible effect on R1. Negative coefficient of quadratic terms also showed rectilinear relationship between factors and response. Overall A, B and C had negative effect on R1.
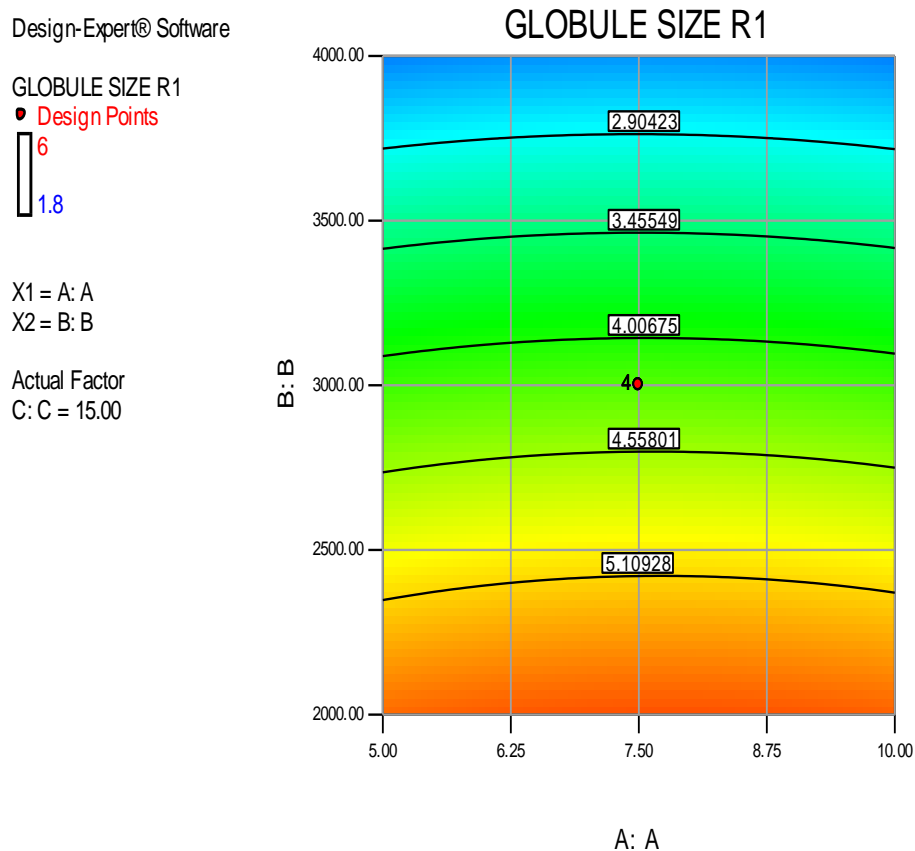
FIGURE 4.12 (a) contour plot R1
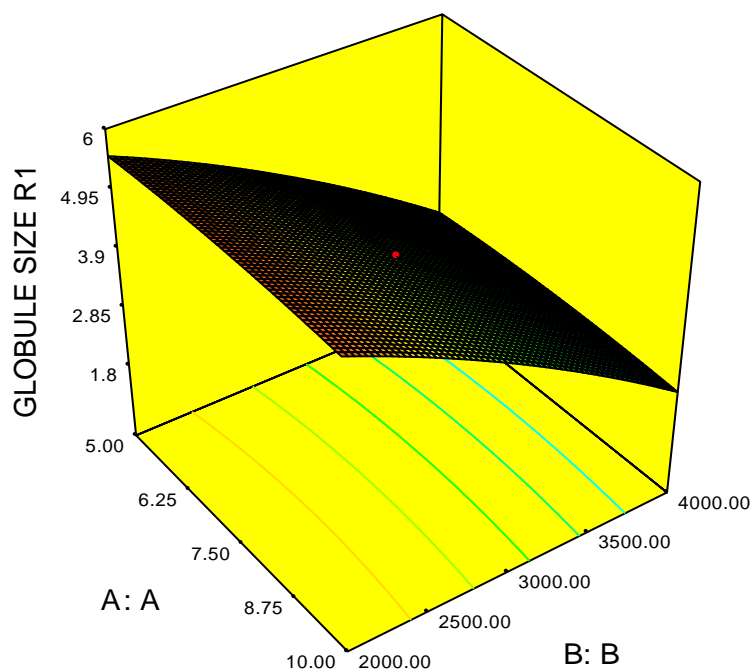
FIGURE 4.12 (b) 3D plot of R1

Broader contour lines in Fig 4.12 (a) explain the robustness of the formulation as small change in the A, B and C would not affect the stability of the formulation drastically. 3D surface graph Fig. 4.12 (b) indicates that increase in B and C cause proportional decrease in globule size.

4.2.4. 6 Effect on % creaming R2

Creaming is reversible process where concentrated portion of oil layer gets deposited over top of the bulk. This layer gets dispersed whenever system is shaken. The Stokes's law says that smaller globules would yield less creaming than larger globules. It was evident from the ANOVA Table 4.23 that B and C significantly affected R2 since both factors as described in previous discussion decreases the globule size. It is also reported that small droplet size causes reduction in mass and gravity force, and Brownian motion may be sufficient for overcoming gravity prevents creaming, thus offering increased physical stability (Tadros et al., 2004)[122].

TABLE 4.23 ANOVA table for % creaming R2

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	474.01	10	47.40	13.31	0.0012 significant
A-A	6.86	1	6.86	1.93	0.2076
B-B	433.11	1	433.11	121.60	< 0.0001
C-C	8.43	1	8.43	2.37	0.1679
AB	4.50	1	4.50	1.26	0.2981
AC	8.00	1	8.00	2.25	0.1776
BC	0.50	1	0.50	0.14	0.7190
A2	3.08	1	3.08	0.87	0.3833
B2	5.68	1	5.68	1.60	0.2470
C2	1.93	1	1.93	0.54	0.4859
ABC	0.50	1	0.50	0.14	0.7190
Error	24.93	7	3.56	0.69	
Total	498.94	17			

ANOVA table showed that term B i.e., speed of emulsification affects the creaming phenomenon since higher B yields smaller globules and subsequently higher rate of creaming was observed. Other terms were found insignificant.

Final equation in terms of coded factors:

$$R2\ CREAMING = +25.47 - 0.71A - 5.63\ B - 0.79\ C - 0.75\ AB + 1.00\ AC - 0.25\ BC \qquad (4.3)$$
$$+0.49A^2 + 0.67\ B^2 - 0.39\ C^2 + 0.25\ A\ BC$$

linear terms in the equation suggested that they have negative effect on rate of creaming up to a certain extent beyond that they have positive effect on the creaming. This can be inferred from the observation that as the shear rate and time increased the size of droplets

decreased but due to prolong emulsification caused coalescence of globules lead to formation of larger globules and creaming. At lower speed larger globules are formed wich gave rise to creaming.
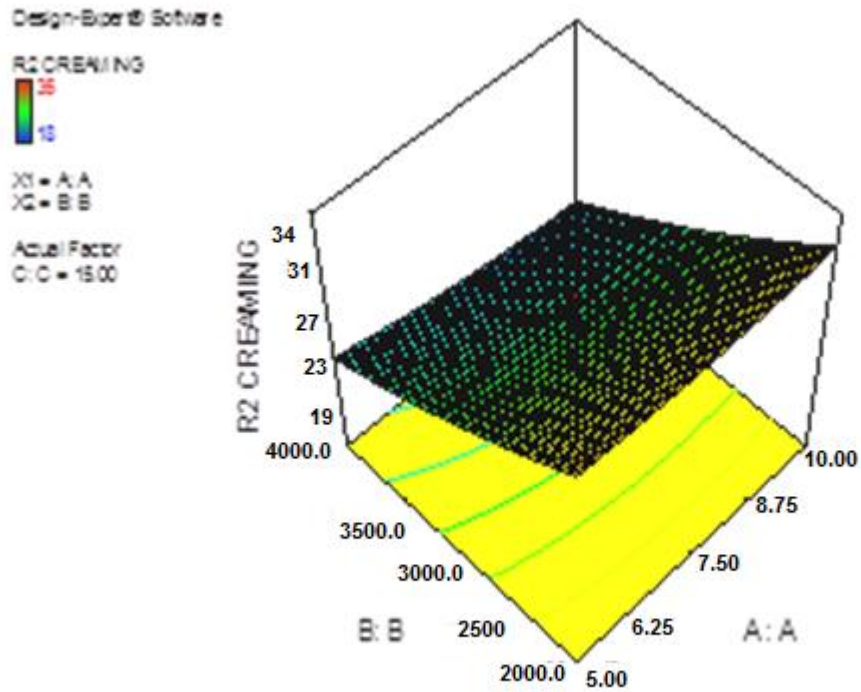
FIGURE 4.13 (a) contour plot
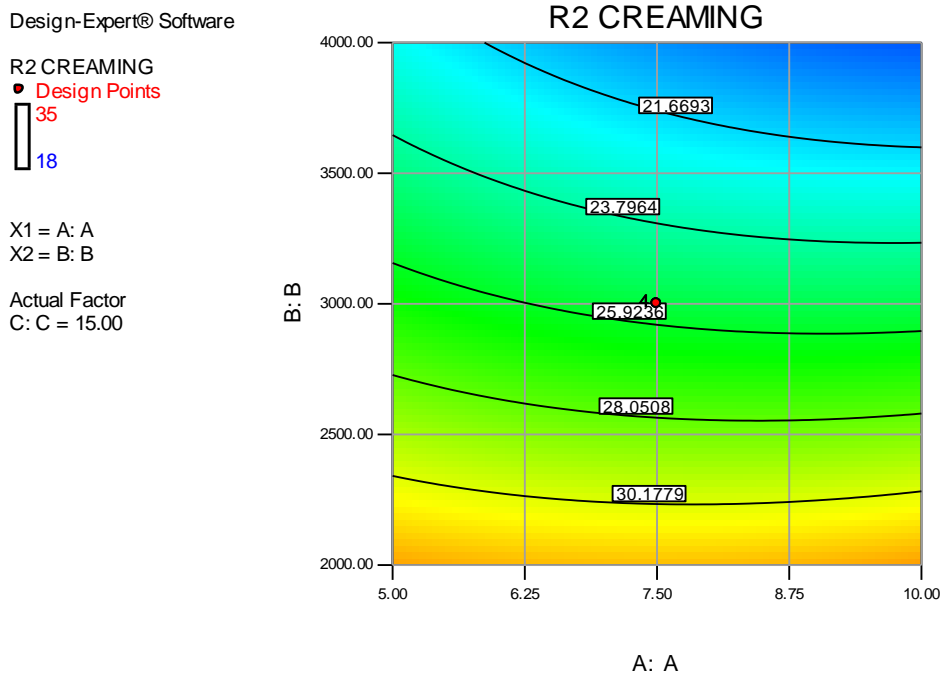
FIGURE 4.13 (b) 3D plot of R2

Fig. 4.13(a) and (b) presents the 3D surface plot and contour plot for R2. Plot showed that as the with increasing B and C globule size decreased which in turn caused lower % creaming while at lower speed and time, larger globules were formed lead to faster coalescence and creaming. The wide contour lines indicated formulation stability towards operational parameters' variation.

4.2.4.7 Effect on % entrapment efficiency (EE) R3

The EE is defined as the fraction (% drug) of the drug loaded in the primary emulsion which remains entrapped in the final W/O/W emulsion.

TABLE 4. 24 ANOVA table for EE R3

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	501.40	10	50.14	9.59	0.0033 significant
A-A	01.17	1	1.17	0.22	0.6503
B-B	341.30	1	341.30	65.30	< 0.0001
C-C	83.97	1	83.97	16.07	0.0051
AB	4.50	1	4.50	0.86	0.3843
AC	0.00	1	0.000	0.00	1.0000
BC	24.50	1	24.50	4.69	0.0671
A2	21.35	1	21.35	4.09	0.0830
B2	21.35	1	21.35	4.09	0.0830
C2	15.94	1	15.94	3.05	0.1243
ABC	4.50	1	4.50	0.86	0.3843
Error	36.58	7	5.23	2.39	0.2505
Total	538	17			

From the ANOVA Table 4.24 it was clear that independent factor A had significant effect on the RIS entrapment. Factor A i.e., span 80 is lipophilic emulsifier and forms w/o emulsion in which water is dispersed phase containing RIS.

Final equation in terms of coded factors:

ENTRAPMENT EFF R3 = +58.14+7.69 A-0.025 B+0.10 C+0.79 AB+0.21 AC-0.41 BC+0.42 A^2 +1.12 B^2+1.30 C^2+0.34 ABC   (4.4)

Linear term A and C had positive effect on the R3 while B had negative effect but all qudratic terms had positive effect on the R3. Small magnitude of coefficient of terms indicate small effect while higher coeffcient values indicate higher effect on the R3.
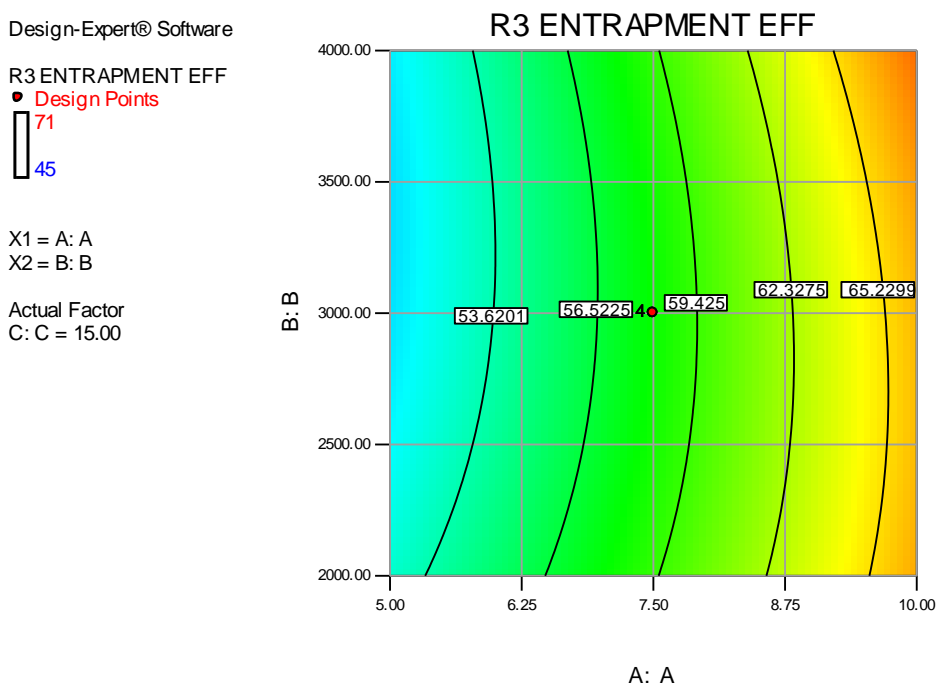
FIGURE 4.14 (a) contour plot for R3

Design-Expert® Software

R3 ENTRAPMENT EFF

71

45

X1 = A: A
X2 = B: B

Actual Factor
C: C = 15.00
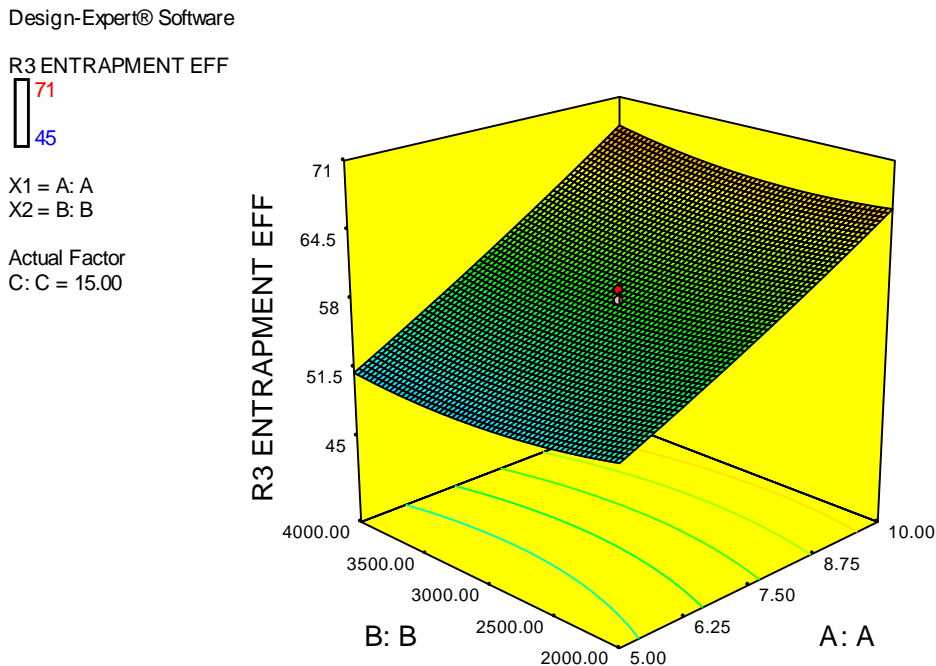
FIGURE 4.14 (b) 3D surface plot for R3

represents contour and 3D surface plots showed that R3 increased with increase in A showed in Fig. 4.14 (a) and Fig. 4.14 (b). In RIS w/o/w multiple emulsion outer phase is water which provides space for the escape of RIS from internal water droplets to outside. However, increased R3 could be due to formation of stable interfacial film by span 80 surrounding oil globules which avoided RIS migration from enclosed water droplets.

4.2.4.8 Effect on % drug permeated R4

Ultimate goal of the study was to increase permeation of the poorly permeable RIS. Percent drug permeation is the measure of drug passage across membrane from site of absorption to system. ANOVA Table 4.25 shows that R4 was significantly affected by B.

TABLE 4. 25 ANOVA table for %drug permeated R4

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	88.89	10	8.89	4.43	0.0303 significant
A-A	0.29	1	0.29	0.15	0.7139
B-B	83.52	1	83.52	41.59	0.0004
C-C	1.57	1	1.57	0.78	0.4054
AB	0.50	1	0.50	0.25	0.6331
AC	0.000	1	0.000	0.000	1.0000
BC	2.00	1	2.00	1.00	0.3516
A2	0.36	1	0.36	0.18	0.6843
B2	7.735×10^{-4}	1	7.735×10^{-4}	3.851×10^{-4}	0.9849
C2	0.43	1	0.43	0.21	0.6572
ABC	0.000	1	0.000	0.000	1.0000
Error	14.06	7	2.01		
Total	102.94	17			

ANOVA table showed that factor B significantly affected % drug permeation. Increase in B decreased globule size. During the ex vivo permeation study the formulation containing RIS entrapped in internal water droplets of oil globules having small globule size would have diffused more than the larger globules. The higher diffusion could also be due inherent permeation enhancement property of the IPM.

Final equation in terms of coded factors:

% DRUG PERMEATED R4= +37.96-0.15A+2.47 B-0.34C-0.25AB   (4.5)
+0.0001 A C-0.50BC+0.17A^2-7.820E-003B^2- 0.18C^2+0.0001ABC

Positive coefficient of term B indicated that it had positive effect on R4. Linear and

Design-Expert® Software

R4 DRUG PERMEATED
• Design Points
43
34

X1 = A: A
X2 = B: B

Actual Factor
C: C = 15.00
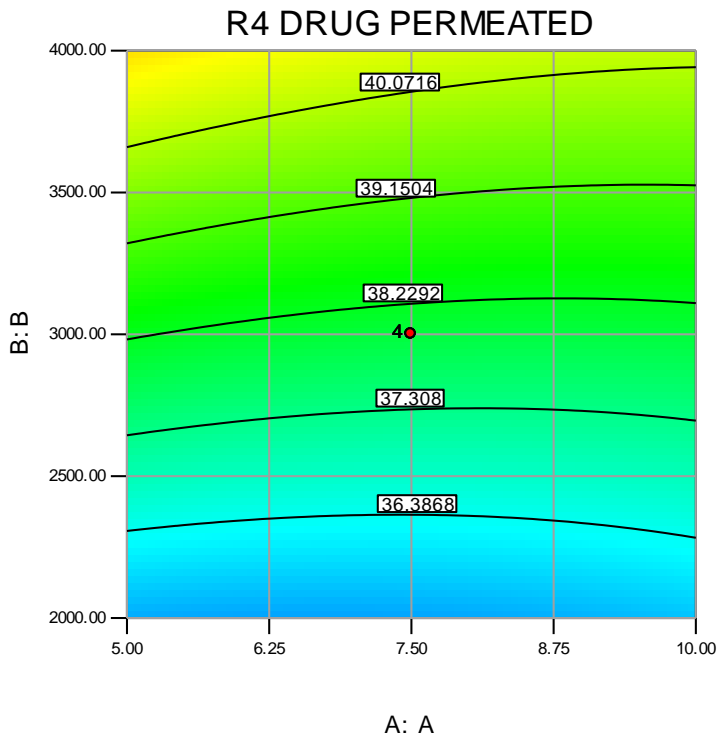
FIGURE 4.15 (a) contour plot for R4

Design-Expert® Software

R4 DRUG PERMEATED
43
34

X1 = A: A
X2 = B: B

Actual Factor
C: C = 15.00

(b)
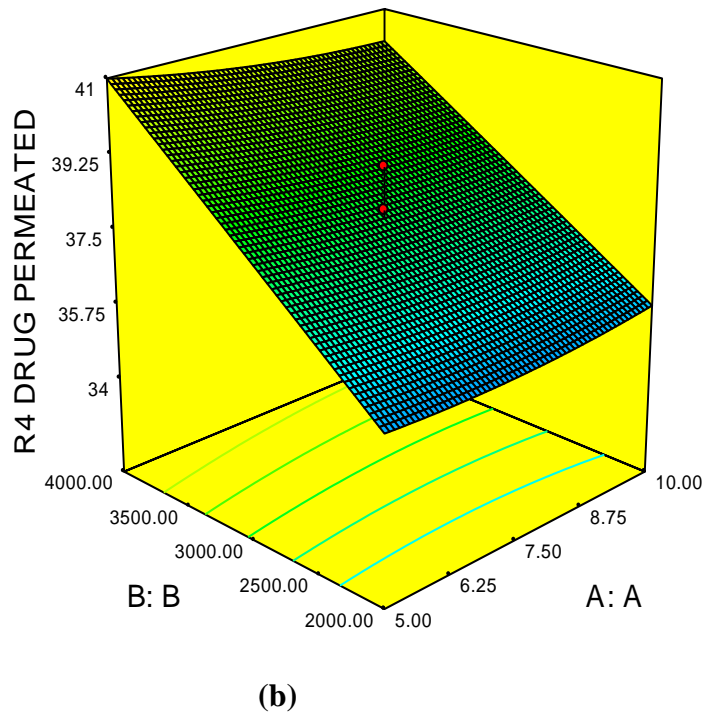
FIGURE 4.15 (b) 3D surface plot for R4

quadratic terms A and C had negative effect on R4. Broadening of the contour lines with increase in magnitude of A and B indicated increases R4 in that region as shown in Fig 4.15(a). 3D surface plot also supports that there was little bit rectilinear relationship between the terms as shown 4.15(b).

4.2.5 Optimization of w/o/w RIS multiple emulsion

Based on the results of face centered central composite experimental design study, optimum formulation of the emulsion, which had small droplet size, low creaming, high drug entrapment and permeation as a pharmaceutical formulation, was developed. To begin with as described in chapter 3 numerical and graphical method was adopted considering optimization criteria as shown in Fig 4.16 and Fig. 4.17. Table 4.26 represents optimize formula and predicted response for RIS w/o/w multiple emulsion and under given formula was used to prepare optimize batch and evaluated for selected responses.

TABLE 4.26 Optimized formulation of RIS w/o/w multiple emulsion

Formulation	A	B	C	R1	R2	R3	R4	Desirability
F19	10	3800	12	2.6	20	68.42	40.34	0.9
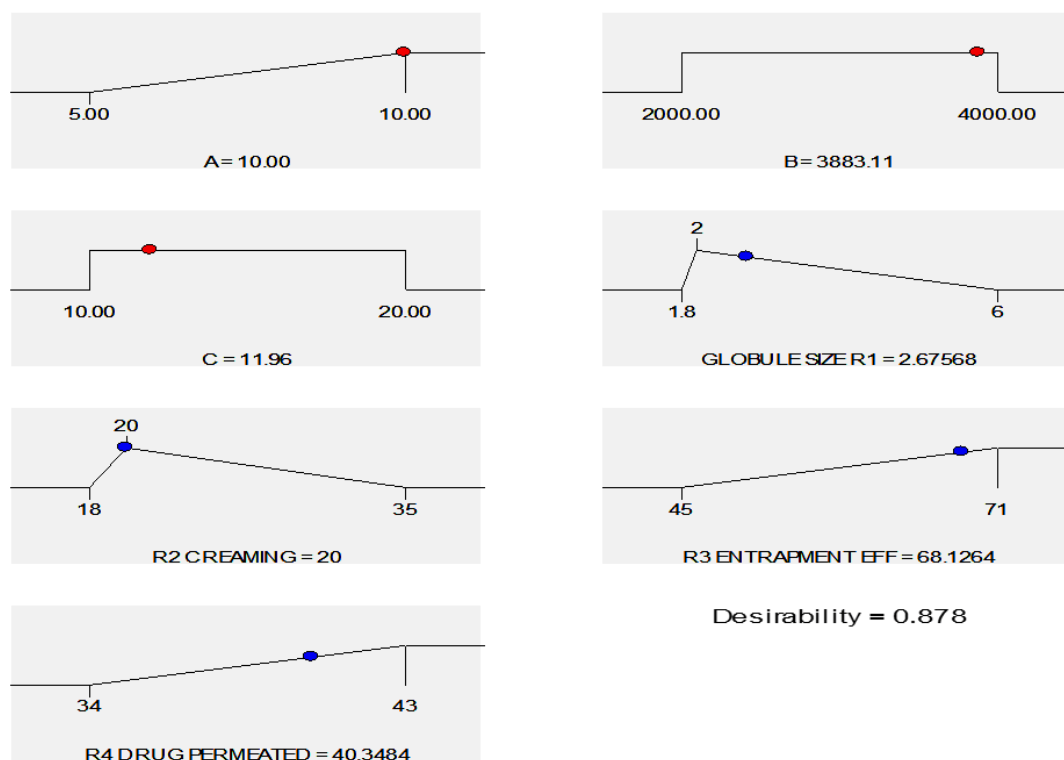
FIGURE 4.16 Ramps for the optimized formulation F19

Desirability
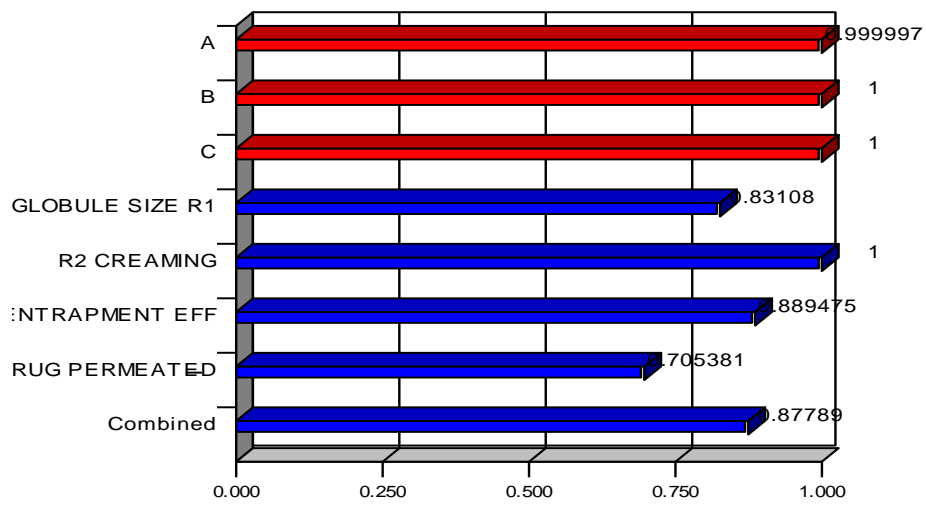
FIGURE 4.17 Desirability for the optimized formulation F19
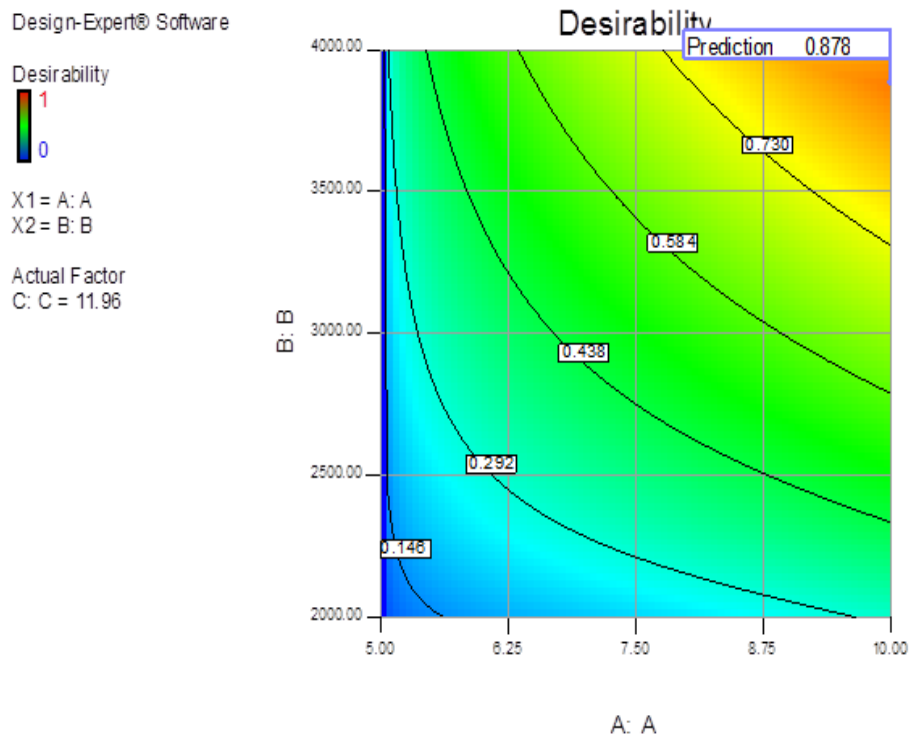
FIGURE 4.18 (a) Contour for optimized batch F19

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Desirability

1
0

X1 = A: A
X2 = B: B

Actual Factor
C: C = 11.96
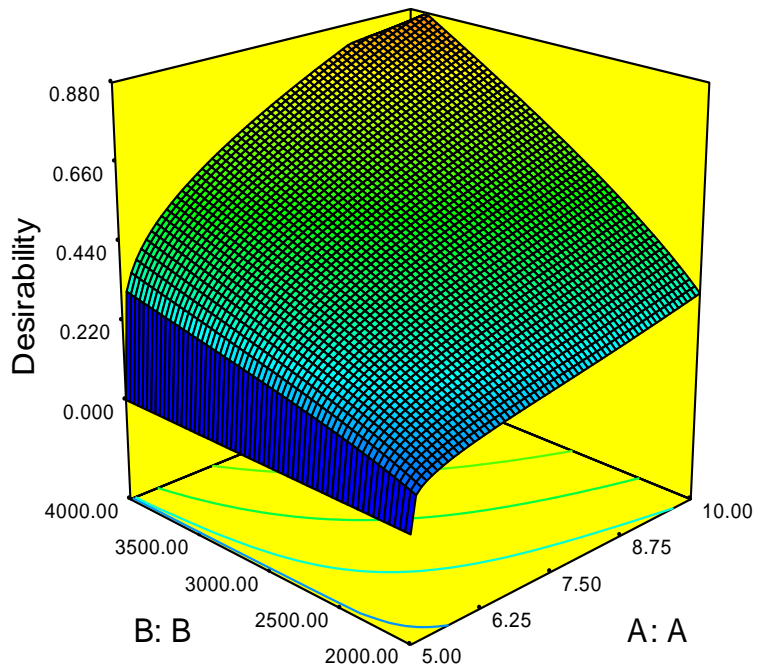
(b)

FIGURE 4.18 (b) 3D surface plot for optimized batch F19

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Overlay Plot

GLOBULE SIZE R1
R2 CREAMING
R3 ENTRAPMENT EFF
R4 DRUG PERMEATED

X1 = A: A
X2 = B: B

Actual Factor
C: C = 12.43

Overlay Plot

R4 DRUG PERMEATED: 40

GLOBULE SI	2.66363
R2 CREAMI	20.0447
R3 ENTRAP	68.0072
R4 DRUG	40.2924
X1	10.00
X2	3882.38

R3 ENTRAPMENT EFF: 60

A: A
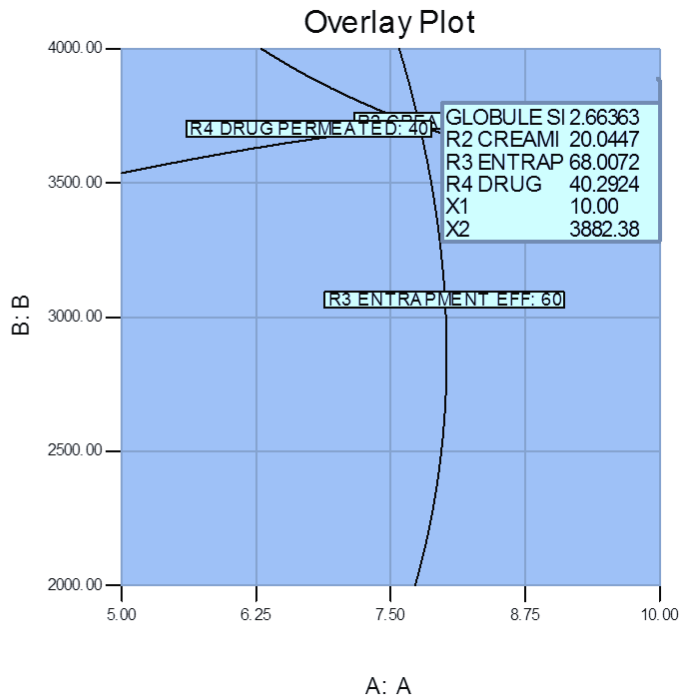
FIGURE 4.19 Overlay plot for optimized batch

Optimized batch 19 was evaluated for selected responses which were used to validate the optimization model. From the observed values it was clear that predicted values were in agreement with experimental values as stated in Table 4.27. This reveals that the model satisfies and yields desired formulation with reproducibility for selected condition within the design range. Fig. 4.18 (a) and (b) represents the contour plot and surface plot for the optimized batch. Fig. 4.19 represents overlay plot for the graphical optimization of the optimum batch.

TABLE 4.27 Predicted and actual responses

Formulation	Predicted responses				*experimental responses			
	R1 μ	R2 %	R3 %	R4 %	R1 μ	R2 %	R3 %	R4 %
F19	2.6	20	68.42	40.34	2.4±0.55	18.60± 2.20	70±3.12	35.22± 2.45

*Mean ± S.D. of three determinations.

4.2.5. 1 Zeta potential

Zeta potential value – 38.5 mv of the optimized batch F19 showed stability of the formulation as shown in Fig 4.20. The general dividing line between stable and unstable emulsion is generally taken at either +30 or -30 mV. Particles with zeta potentials more positive than +30 mV or more negative than -30 mV are normally considered stable. Both particle size and zeta potential are directly related to the repulsion potential..Measurements of zeta potential have shown a relationship to dispersion stability such that higher the magnitudw of zeta potential higher the stability of dispersion. The value of the zeta potential supports that the formulation has moderate stability and composed of the non-ionic surfactansts. Higher the values of the zeta potential higher the double layer formation around the particles causing more repulsion between the particles causing less settling.

FIGURE 4.20 Zeta potential report of F19

4.2.5.2 Stability study

Optimized formulation F19 was subjected for stability study as described in chapter 3. Percent creaming was considered as instability marker. Immediately after manufacture the formulation showed 10% creaming with average globule size of 2.3µ. However, when it was kept for stability period of 1 month, 18% creaming was observed with increase in globule size to 6.0µ. Increase in % creaming could be due to coalescence of the oil droplets forming aggregate and finally lead creaming. Another cause of instability might be removal of internal emulsifier by the external emulsifier leading swelling of the globules. Creaming is reversible phenomenon but can result in cracking in long-term storage which cannot be reversed.

4.2.6 Stabilization of optimized RIS w/o/w multiple emulsion

Optimized formulation F19 was found less stable in stability study and needed stability enhancement. Stability of the F19 was addressed by multiple approaches i.e., by changing phase volume ratio, addition of viscosity enhancing polymer in outer water phase and using surfactant blend in secondary emulsification.

4.2.6.1 Effects on stability of multiple approaches

Optimized formulation F19 was stabilized by adopting multiple approaches. Total four stabilized formulation F19A, F19B, F19C and F19D were formulated by varying concentration of viscosity enhancer and phase volume ratio at two levels and keeping surfactant blend fixed. The aqueous to oily phase ratio is an important parameter that

TABLE 4.28 Effect of stabilizing approaches

Formulation batch	*average globule size (R1)	*% creaming (R2)
F19 A	2.90 ±2.32	15.01±2.52
F19 B	2.00±3.31	11.83±5.52
F19 C	3.09 ±2.53	13.65±3.52
F19 D	3.85±1.14	13.23±2.02

* At the end of 1 month

influences the stability of multiple emulsions. Stable multiple droplets are produced at low volume fractions only (Matsumoto et al., 1976). It was decided to vary phase volume ratio from initial 1:2 to 1:4 by increasing outer water phase volume to 40 ml. Effect of these was seen as decreased in creaming F19 B which could be because of under crowding of dispersed phase reducing chances of collision of nearby globules during emulsification and storage.

HLB of the oil and selected surfactant can markedly influence the stability of the emulsion. As span 80 (HLB value 4.3) was used as internal emulsifier to produce primary emulsion w/o. This in turn actually formed stable w/o emulsion. In secondary emulsification tween 80 (HLB 15) was used to give w/o/w emulsion. However, HLB of the IPM (oil phase) is 11-12. Now in order to form stable oil in water emulsion, an emulsifier should have HLB value close to HLB value of IPM. A surfactant blend of span 80 and tween 80 in proportion 32.5/67.5 was used to match the HLB value of IPM. Effect of this was seen in increase stability of the F19B compare to other formulations.

Viscosity enhancing (thickening) agent was added in external water phase to increase viscosity. It was evident from the result that F19B had good characteristics with 11.83 % creaming and small globule size 5.52 μ. Reduction in the % creaming can be understood by Stoke's equation. Viscosity of the dispersion medium influences the creaming process inversely. Increase in viscosity of dispersion medium causes reduction in globule velocity for creaming and further aggregation which can ultimately cause cracking.

4.2.7 In-vivo absorption study

In vivo absorption studies were conducted in rats along with sublingual spray formulation and explain in 4.1.6.3.

4.3 RIS SUBLINGUAL SPRAY FORMULATION

4.3.1 Preliminary study

4.3.1.1 Container specifications and light transmission study

Physical dimensions of pump spray container were measured by vernier caliper. Dimensions were noted and tabulated in Table 4.29. Light transmission study was conducted to check light protection property within 290 to 450 nm.

TABLE 4.29 Physical dimensions of pump spray containers

Particulars	*Specification
Capacity	20 ml
Actuator	Made of Polyethylene
Valve body	Made of Polyethylene
Height of Container	38.00±1.0 mm
Outer diameter	13.54±0.8 mm
Diameter of actuator orifice	13.52 ±2.0μm
Dip tube length	41.00±1.1 mm
Valve discharge volume	0.15±0.5 ml

* n=3 Mean ±SD, SD is standard deviation

The transmittance observed 0.4% which was quite below 12%, limit for transmittance specified for Container in USP 37[123].

4.3.1.2 Excipient compatibility study

Excipient and drug compatibility is prime importance in product development since incompatibility between the drug and excipients causes alteration of pharmaceutical properties of drug and product becomes not consumable. Study can guide for selection of alternative excipients which would be suitable for inclusion. In present study a model formulation was formed and a sample was withdrawn from glass container after 1 month storage period and FT-IR study of it was carried out and compared with FT-IR of pure RIS.

The excipients selected were found nonreactive and had not interacted with RIS which was evident from the presence of the IR peaks of RIS even after storage period. Figure 4.32 (a) shows FTIR spectra of drug sample, where in bending vibrations in figure print region i.e., 629-669 cm^{-1}, 933-910 cm^{-1}, 1100-900 cm^{-1}, 1130 cm^{-1}, 1207 cm^{-1} and 1514-1429 cm^{-1} attributed to C-P stretching, C-H stretching, P-OH stretching, C=N stretching, P=O stretching and C-C ring stretching accordingly. Figure 4.32 (b) shows FTIR spectra of model formulation with appearance of all vibration bends of RIS in finger print region. The IR spectra of the drug with excipients showed neither shift nor disappearance of characteristic peaks suggesting that there was no interaction between drug and excipients and they were very close in conformity with the standard reference spectra.
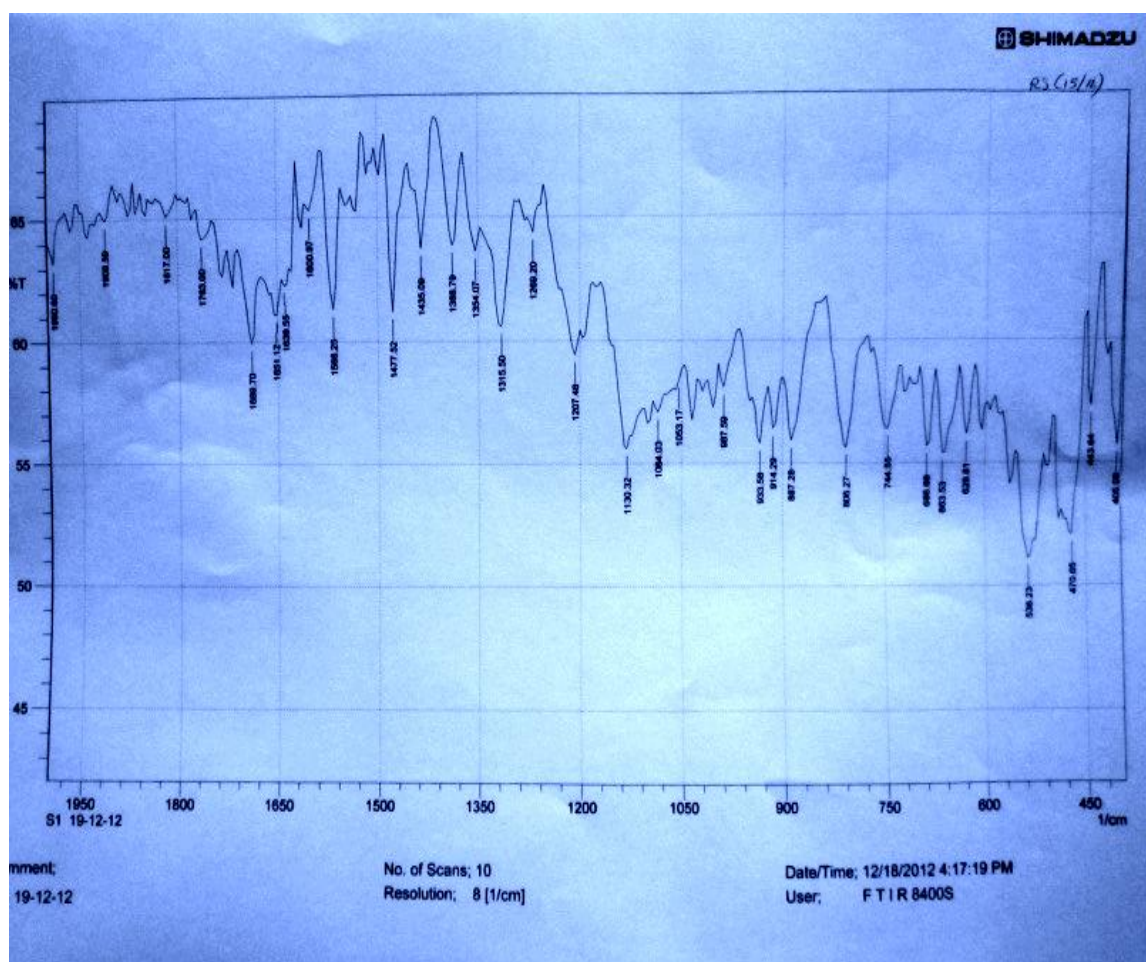
FIGURE 4.21 (a) FT-IR of pure RIS
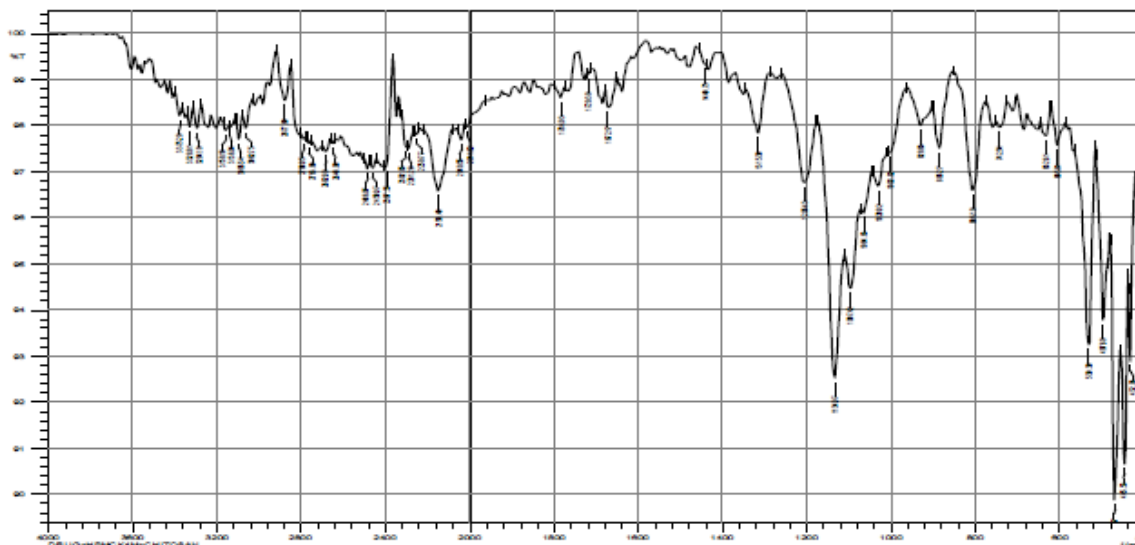
FIGURE 4.21 (b) FT-IR of Model formulation of RIS spray

4.3.1.3 Container and product compatibility study

Container plays crucial role in stability of the formulation. Selected container should not react with enclosed formulation components in any way. From the observation Table 4.30 it was revealed that selected packaging components were compatible with dosage form and didn't interact to cause unacceptable changes in the quality of either the dosage form or the packaging component.

\

Table 4.30 Compatibility study of product and container closure system

Test parameter	Before storage	After one month of storage
Precipitation	Clear solution	Clear solution
changes in drug product pH	6.8	6.8
discoloration of either the dosage form or the packaging component	Clear solution with white surface from inside, dip tube clear transparent	Clear solution with white surface from inside container, dip tube clear transparent
brittleness of the packaging component	Flexible	Non brittle
Concentration of RIS	1 mg/ml	0.99 mg/ml

It was also come to know that there were no interactions between RIS and excipients which would cause loss of potency due to absorption or adsorption drug substance. Absence of precipitates, discolouration and pH shift of the formulation after one month storage period suggested that there were no degradation of drug substance induced by a chemical entity leached from a packaging component.

4.3.1.4 Content of RIS emitted per shot

After satisfactory results from the excipients and container compatibility study a test was conducted to determine the amount of RIS emitted per actuation of the spray container in order to formulate for further study. Test sample of model formulation was collected after two priming in glass beaker and analyzed for quantity of RIS and tabulated in Table 4.31.

TABLE 4.31 Content emitted per actuation

Actuation	*Volume emitted(ml)	*Content of RIS emitted (mg)
3	0.153±0.011	1.46 ±0.058

*n=3, mean±SD, SD is standard deviation

4.3.2 Preliminary formulation batches of RIS sublingual spray formulation

Preliminary study was carried out to find significant factors affecting dosage form residence time (Y1) and % drug diffused (Y2). Results obtained are tabulated in table 4.32. Total 8 batches were prepared according to design generated from fractional factorial design. Other evaluation test for device performance were also carried out and presented in table

4.3.3 Evaluation tests of preliminary batches of RIS spray

Preliminary batches were evaluated for the device and biological performance tests. The result of these batches could become the base for further development of the formulation.

4.3.3.1 Effect on residence time Y1

Sublingual route offers rapid permeation and quick therapeutic action of drug. Drugs having lipophilic nature can easily pass through sublingual mucosal barrier while drugs

having poor permeability and hydrophilic nature finds difficult to cross the barrier in short time. In order to improve their absorption these drug should stay longer time over sublingual mucosal surface thereby increasing absorption rate. Due to slow and continual salivary secretion major portion of applied drug would drain to stomach. This can be circumvented by prolonging the residence of the applied dosage to sublingual region by addition of mucoadhesive polymer and increasing viscosity. It was evident from the results that formulation containing higher amount of poloxamer stayed on sublingual mucosal tissue for longer time than the formulation with low amount.

TABLE 4.32 Results for Y1 and Y2

Formulation Batch	Residence time (min) Y1	Drug permeated (%) Y2
1	25	60
2	40	49
3	24	53
4	36	65
5	22	57
6	46	50
7	23	51
8	42	61
Plain drug solution	03	06

F6 having 5% poloxamer X3 showed 46 minute stay while F5 with 1% showed 22 min stay. Poloxamer 188 is a mucoadhesive polymer and its solution forms gel at body temperature. When the formulation sprayed from the device it was split in tiny droplets and landed on mucosa which could have soon turned in thin film of the gel structure providing mucoadhesion and reservoir for drug. In comparison, this plain drug solution could not stay more than 3 mins showed absence of mucoadhesion.
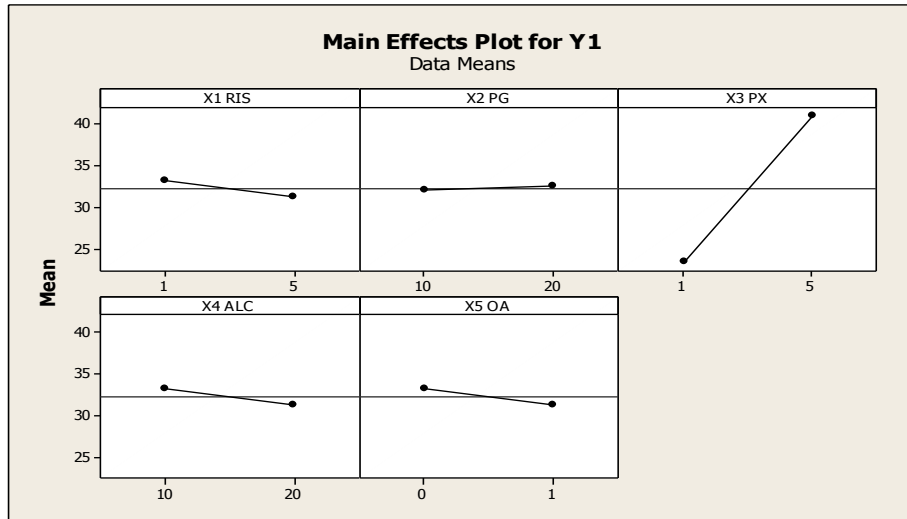
FIGURE 4.22 Main effects plot of Y1

Further F6 had 20% propylene glycol X2 and showed incremental effect on residence time. X1, X4 and X5 had slightly negative effect on the Y1 which could be seen as cumulative effect of all variables as shown in Fig. 4.22.

4.3.3.2 Effect on % drug permeation Y2

High permeability of the sublingual mucosa makes it appropriate for application of poorly absorbed drug from GIT.
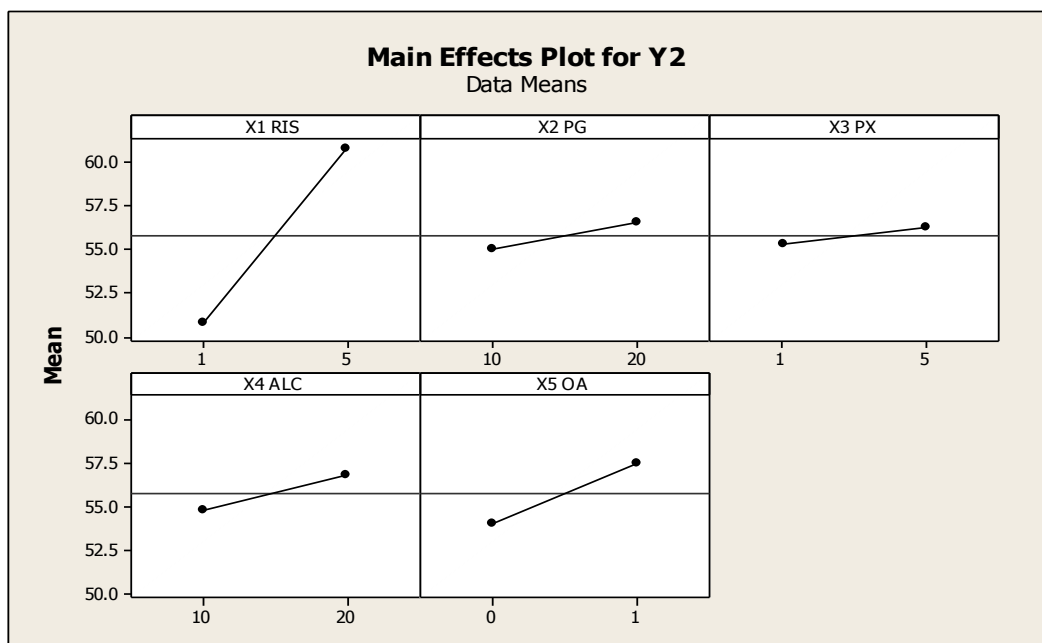
FIGURE 4.23 Main effects plot of Y2

Lipophilic drug can favorably partition through membrane while hydrophilic drugs often difficult to pass because of their polar nature and charged status. It was evident from the Table 4.32 and Fig. 4.23 that plain drug solution had very low % drug permeation compare to formulations F1 to F8. F1 to F8 showed % drug permeation in range of 49 to 65%. F4 showed 65 % drug permeation which could be due to higher level of co-solvent i.e., propylene glycol X1, higher concentration of X2 and oleic acid X5. Amongst them, X3 and X4 also contributed in permeation enhancement but lower than X1, X2 and X3.

4.3.3.3 Performance test for spray device

Device performance test were conducted for all spray formulations F1 to F8 and results are presented in Table 4.33 (a) and (b). All formulation when sprayed from the devices showed circular spray pattern i.e., the ovality ration was near to 1. It was inferred from the result that the formulation components had not influenced spray pattern of the emitted formulations.

Priming means the number of actuations and readiness of the device required to deliver first shot having required drug content. Property of the formulation may have impact over priming and it was seen that formulations F2, F3 and F5 having low X2 and X3 delivered first shot after two prime while F1, F4, F6, F7 and F8 having higher X2 and X3 required three prime indicating that variation in formulation composition caused variation in viscosity and flow ability of the formulation which could have affected the priming number.

Net Average weight of the product emitted per shot varied moderately because of the formulation composition and was between 1.12 to 1.32 gm/ml. Drug content per spray shot was found within 88 to 102%. Density of the formulation varied with respect to variation in propylene glycol X2 and alcohol X4. Formulations F6 and F8 had higher density because of higher X2 and lower X4. Other formulation components had little effect on density.

Net content of each filled container was found within acceptable limit. Formulations F4 and F6 showed near to lower limit of acceptance and this could be due to viscous nature of the formulations caused adherence of formulation to container wall surface and uncompleted draining.

Table 4.33 (a) Device performance results

Formulation Code	Spray Pattern (ovality ratio)	Primes (No of press)	Pump delivery (gm)	Drug content* per spray mg
F1	1.15	3	0.15	1.12
F2	1.05	2	0.16	1.08
F3	1.09	2	0.14	1.22
F4	1.19	3	0.16	1.09
F5	1.19	2	0.15	1.05
F6	1.19	3	0.14	1.32
F7	1.19	3	0.16	1.10
F8	1.19	3	0.14	1.14

* n=3 samples

Table 4.33 (b) Device performance results (continued)

Formulation Code	Spray profiling for device performance (Drug content per spray mg)			Spray angle θ
	Beginning	Mid	Tail off	
F1	1.1	1.05	1.1	50.0
F2	0.95	1.0	1.0	60.0
F3	1.1	1.2	1.0	69.0
F4	1.0	0.95	1.0	51.1
F5	0.95	1.0	1.0	61.0
F6	1.10	1.10	1.10	53.0
F7	1.0	1.10	1.10	54.0
F8	1.0	1.1	0.95	62.0

* n=3 samples

All the formulations F1 to F8 showed acceptable spray profiling which revealed that formulations were appropriately made and in homogeneous solution state. This was also supported by the fact that appropriate functioning of the valve assembly. Uniform spray shots emitted in beginning, midway and tail off state ensured repeatable and accurate emission of dose during product usage.

Spray angle of the formulations F1, F4, F6 and F7 was in the range of 50-54º compared to other formulations. This deviation in the spray angle was due to viscous nature of these formulations. However, the spray angles obtained from the all formulation devices were appropriate for application to sublingual mucosal surface.

4.3.3.4 Summary of preliminary work

The preliminary study involving drug residence time and permeation study indicated that concentration of RIS X1, propylene glycol X2 and poloxamer X3 were significant causative factors because they had markedly affected the residence time Y1 and % drug permeation Y2. For the maximum effectiveness of the formulation both these responses are quite important which ultimately influence therapeutic response. Alcohol X4 and oleic acid X5 had little positive effect on the % drug permeation Y2. It was evident from the study that concentration beyond 2% of X5 had not increased permeability of the polar compound. For the further studies to develop RIS spray formulation these formulation components, Alcohol X4 and oleic acid X5 were fixed to a level and X1, X2 and X3 were varied for optimization. Selected spray devices were found suitable, durable and performed very well. All other tests such as spray angle, spray pattern, prime test, pump delivery, drug content per spray and spray profiling were also satisfactory.

4.3.4 RIS sublingual spray formulations

After conducting preliminary work, formulation components of RIS spray formulation were varied at different level and designed through Design expert 7.0 software. Face centered design was selected for the generating formulation batches.

4.3.5 Evaluation tests for RIS sublingual spray formulations

Total 16 formulations were prepared and evaluated for residence time Y1, % drug release Y2 and % drug permeated Y3. Other evaluation tests for device performance were also performed.

4.3.5.1 Effect of selected variables on residence time Y1

As discussed in preliminary study residence time of the dosage form at the site of absorption affects the avability of drug for absorption. In preliminary study the Poloxamer 188 was having 1 to 5% level.

TABLE 4.34 Test results of RIS spray formulation batches

Formulation F	RIS concen. X1 mg	Propylene glycol X2 %	*P 188 X3 %	Residence time Y1min	Drug release Y2 %	Drug permeated Y3 %
1	3.00	10.00	7.50	53	90	54
2	3.00	15.00	5.00	49	90	63
3	1.00	10.00	10.00	58	87	50
4	1.00	15.00	7.50	53	87.9	51.5
5	1.00	20.00	10.00	58	92.3	51
6	5.00	20.00	10.00	59	92	67
7	3.00	15.00	7.50	52	88	62.5
8	5.00	15.00	7.50	53	89	56
9	3.00	15.00	7.50	53	88.8	63
10	3.00	15.00	10.00	55	87.5	62
11	5.00	20.00	5.00	46	93	66
12	1.00	20.00	5.00	45	90	51
13	3.00	20.00	7.50	52	89.5	63
14	5.00	10.00	10.00	57	87	57
15	5.00	10.00	5.00	45	91	57
16	1.00	10.00	5.00	44	90	52

*P 188 is poloxamer 188

This level of the poloxamer 188 could not give the sufficient residence time for the absorption of the poorly absorbed drug RIS. In order to increase the residence time of the sprayed formulations over sublingual mucosal surface poloxamer level was increased to 5

to 10%. Result Table 4.34 indicated that increase in poloxamer 188 concentration increased residence time of the sprayed droplets impressively. F 16 showed 44 min of stay while maximum residence time was observed for F6 formulations. All other formulations showed varied residence time according to their poloxamer contents. Poloxamer 188 is a polymer and has gelation property and because of this sprayed droplet of the formulation adheres over sublingual mucosa and provides longer residence for drug absorption. It also prevents draining of the drug from the droplets by providing polymeric gel structure.

Effect of the variables on the Y1 presented in ANOVA Table 4.35. The Model F-value of 7.79 implies the model was significant. Poloxamer 188 X3 was found significant amongst and affected residence time of sprayed droplets. Term X2 had affected the residence time Y1 due to its viscous nature and found nearly significant. The "error F-value" of 10.79 implies the error is not significant relative to the pure error.

TABLE 4.35 ANOVA table for Y1

Source	Sum of Squares	df[a]	Mean Square	F Value	p-value Prob > F
Model	343.92	10	34.39	7.79	0.0176*
A-X1	0.40	1	0.40	0.091	0.7755
B-X2	0.90	1	0.90	0.20	0.05105
C-X3	336.40	1	336.40	76.19	0.0003*
AB	0.50	1	0.50	0.11	0.7501
AC	0.50	1	0.50	0.11	0.7501
BC	0.000	1	0.000	0.000	1.0000
A^2	0.020	1	0.020	4.438×10^{-3}	0.9495
B^2	0.45	1	0.45	0.10	0.7621
C^2	2.20	1	2.20	0.50	0.5116
ABC	0.50	1	0.50	0.11	0.7501
Error	22.08	5	4.42	10.79	0.2240
Total	366.00	15			

a is degree of freedom, * significant term p < 0.05

Final Equation in Terms of Actual Factors (4.7)

Y1 residence time = $+19.04655+1.09569X1+0.70655X2+5.11310X3-0.050000X1X2-0.20000X1X3-0.030000X2X3+0.021552X1^2-0.016552X2^2-0.14621X3^2+1.00000E-002$ X1 X2 X3

From equation positive and bigger coefficient of X3 suggested that it had significant effect on response Y1. Other term X1 and X2 had little positive effect on Y1.
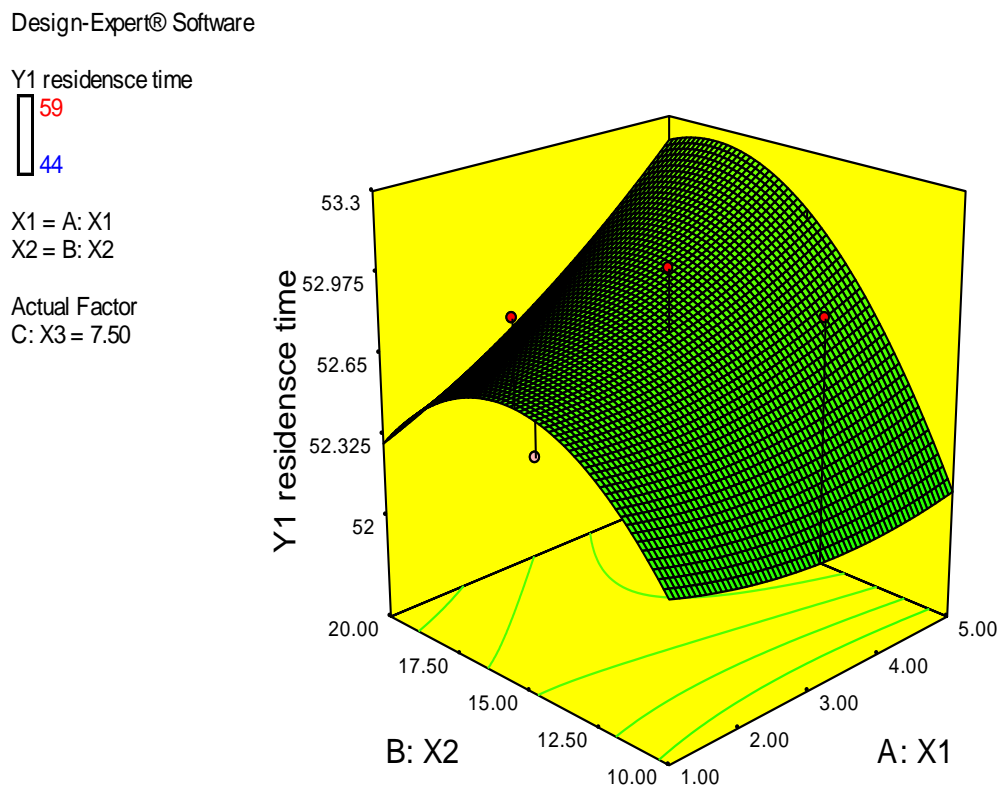
FIGURE 4.24 (a) Surface plot of Y1

Small negative coefficient of interactive terms X1X2, X1X3 and X2X3 indicated decreasing effect on Y1. Negative coefficient of quadratic terms $X2^2, X3^2$ showed that would be rectilinear response at some point with gradual increase and then decrease. From the contour plot Fig.4.24 (b) it was evident that contour lines were not parallel running from one end to other but some were curved and broad to narrow type. These types of lines indicate the importance of the selection of the factor and possible effect on response. Surface response plot Fig.4.24 (a) indicated rectilinear relationship between model terms.

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Y1 residensce time
• Design Points
59

44

X1 = A: X1
X2 = B: X2

Actual Factor
C: X3 = 7.50
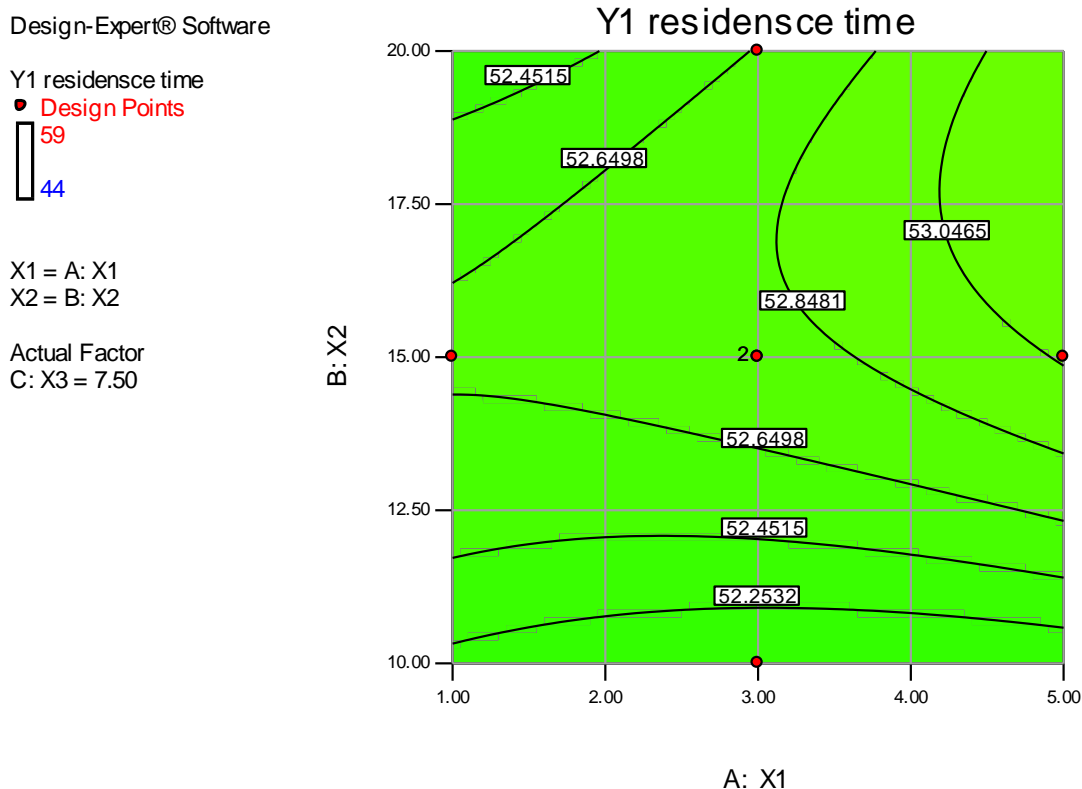
FIGURE 4.24 (b) contour plot of Y1

4.3.5.2 Effect of selected variables on % drug release Y2

Bioavability of the drug might be influence by its release and absorption at the site of application. Sublingual site provides greater chances of absorption for poorly permeable drugs both because of high permeability of membrane and avoidance of food interaction.

Absorption of the drug depends on the avability of drug in molecular form at site which in turn is related to drug release. Poloxamer 188 is a mucoadhesive gel forming polymer and used to retain drug at sublingual mucosa. Higher amount of poloxamer X3 caused formation of the gel matrix which could pause more resistance to drug release than with lower X3 leading to slower drug release. ANOVA table 4.36 showed that X3 was found significant term had dominant effect on drug release.

TABLE 4.36 ANOVA table for Y2

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	187.49	10	18.75	4.57	0.050
A-X1	29.24	1	29.24	7.13	0.0443*
B-X2	2.03	1	2.03	0.49	0.5136
C-X3	133.23	1	133.23	32.49	0.0023*
AB	0.50	1	0.50	0.12	0.7412
AC	8.00	1	8.00	1.95	0.2213
BC	0.000	1	0.000	0.000	1.0000
A^2	3.49	1	3.49	0.85	0.3988
B^2	0.32	1	0.32	0.079	0.7902
C^2	1.90	1	1.90	0.46	0.5258
ABC	0.50	1	0.50	0.12	0.7412
Error	20.50	5	4.10	15.77	0.1864
Total	207.99	15			

* Significant term p < 0.05

Term X1 was also found significant which could be explained as concentration gradient was higher more drug release found compare to low concentration gradient of drug. Other terms did not have any profound effect on the drug release.

Final equation in terms of actual factors   (4.8)

Y2 % drug release =+91.84750+0.42000 * X1+0.21000 * X2-0.47000 * X3+0.100000 * X1 * X2+0.35000 * X1 * X3+0.030000 * X2 * X3-0.28750 * X1^2-0.014000 * X2^2-0.13600 * X3^2-1.00000E-002 * X1 * X2 * X3

Equation suggested that Y2 was markely affected by X3 i.e., highe X3 lower was Y2 while X1 and X2 were found in opposite way. Negative coefficient of quadratic term indicated rectilinear behaviour.

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Y2 drug release
● Design Points
93

79

X1 = A: X1
X2 = B: X2

Actual Factor
C: X3 = 7.50

Y2 drug release

87.238 87.9631
88.6874
89.4118
86.5144

B: X2 (20.00, 17.50, 15.00, 12.50, 10.00)
A: X1 (1.00, 2.00, 3.00, 4.00, 5.00)

FIGURE 4.25 (a) contour plot of Y1

Design-Expert® Software

Y2 drug release
93

79

X1 = A: X1
X2 = B: X2

Actual Factor
C: X3 = 7.50

Y2 drug release
90.2
89.075
87.95
86.825
85.7

B: X2 (20.00, 17.50, 15.00, 12.50, 10.00)
A: X1 (5.00, 4.00, 3.00, 2.00, 1.00)

FIGURE 4.25 (b) 3D surface plot for Y2

123

Contour plot Fig 4.25 (a) showed broader lines that indicated small deviation in terms would not affect drug release greater. 3D surface plot Fig.4.25 (b) confirmed the rectilinear behaviour and probable interaction. Plot also showed that increase in poloxamer X3 caused reduction if drug release. X1 and X2 increased the drug release.

4.3.5.3 Effect of selected variables on % drug permeation Y3

Drug permeation is a rate limiting step for poorly absorbed drug. % drug permeation Y3 was found significantly affected by RIS concentration X1 and propylene glycol X2. The values from ANOVA Table 4.37 showed that the X1 and X2 significantly affected Y2. It is well-known fact that the concentration gradient is one of the driving forces for diffusion and the same was observed in Table 4.34. Formulations having higher concentration of RIS showed higher % drug permeation compare to those having lower.

TABLE 4.37 ANOVA table for Y3

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	481.05	10	48.10	6.46	0.0263*
A-X1	225.62	1	225.62	30.32	0.0027*
B-X2	78.40	1	78.40	10.54	0.0228*
C-X3	0.40	1	0.40	0.054	0.8258
AB	45.13	1	45.13	6.06	0.0570
AC	1.13	1	1.13	0.15	0.7134
BC	1.13	1	1.13	0.15	0.7134
A^2	96.55	1	96.55	12.98	0.0155
B^2	4.47	1	4.47	0.60	0.4735
C^2	19.19	1	19.19	2.58	0.1692
ABC	0.13	1	0.13	0.017	0.9019
Error	37.20	5	7.44	74.16	0.0868
Total	518.25	15			

* Significant term p<0.05

Propylene glycol X2 being solvent also has permeation enhancement property particularly for the hydrophilic molecules. Propylene glycol could have acted by opening the water channels of the mucosal cell barrier there by increasing the passage of the drug. Other formulation components were not affected the % drug permeation remarkably.

Final Equation in Terms of Actual Factors: (4.9)

Y3 % drug permeated =

$+62.24957+6.76509 X1+1.07207 X2-7.45586 X3+0.27500 X1 X2+0.15000 X1 X3+0.045000 X2 X3-1.51293 X1^2-0.052069 X2^2+0.43172X3^2-5.00000E-003 X1 X2 X3$

Equation suggested that X1 and X2 affected Y3 strongly than X3. Negative coefficent of the quadratic term X1 and X2 showed that there was interaction behaviour in higher order.
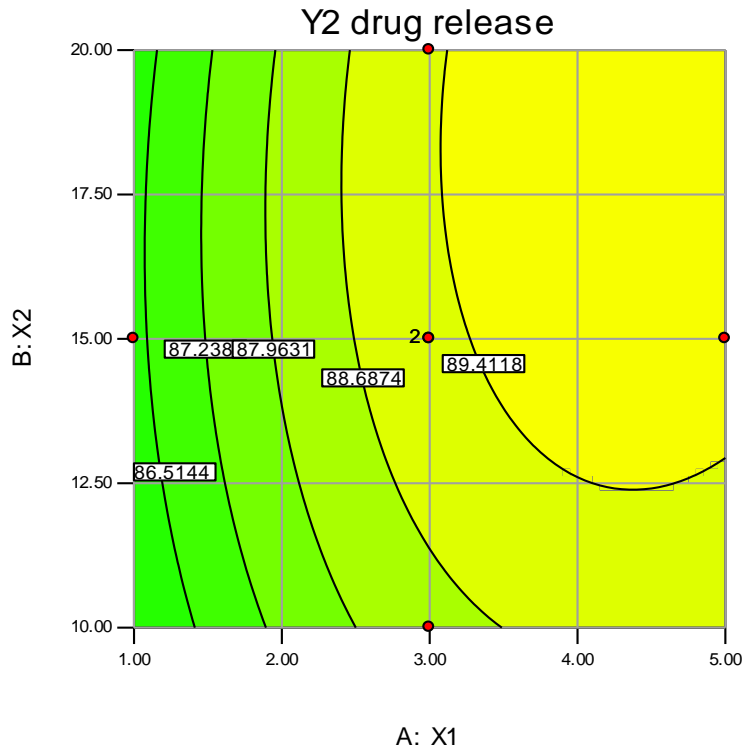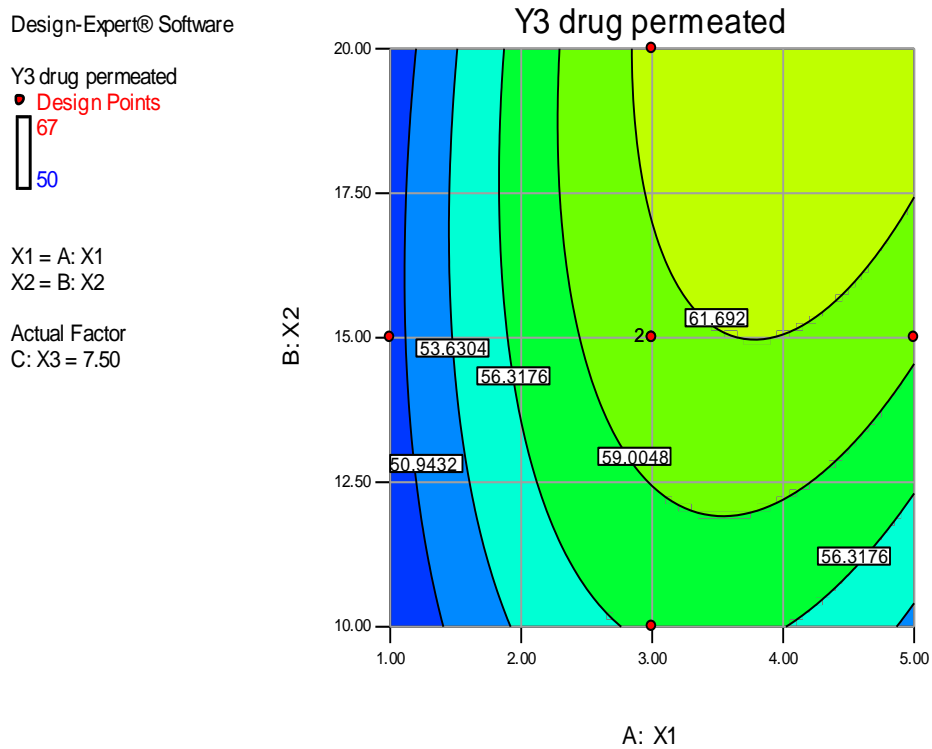

FIGURE 4.26 (a) contour plot of Y3

Design-Expert® Software

Y3 drug permeated

67

50

X1 = A: X1
X2 = B: X2

Actual Factor
C: X3 = 7.50

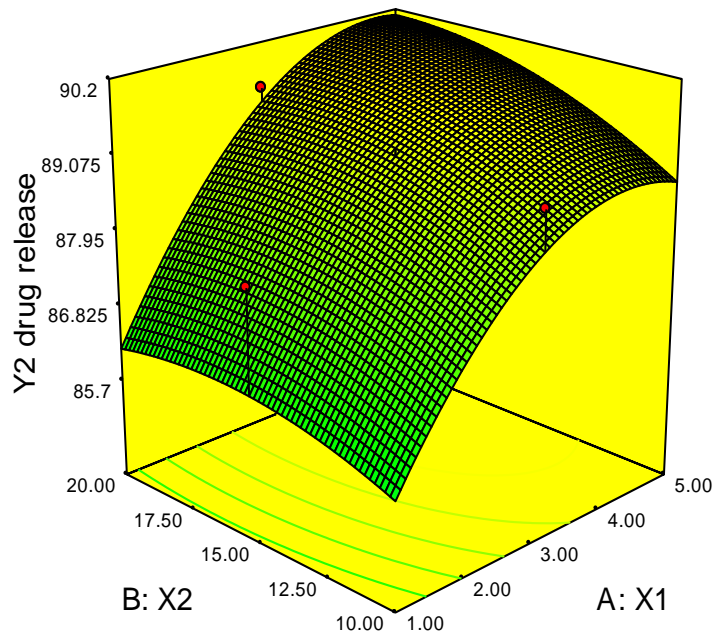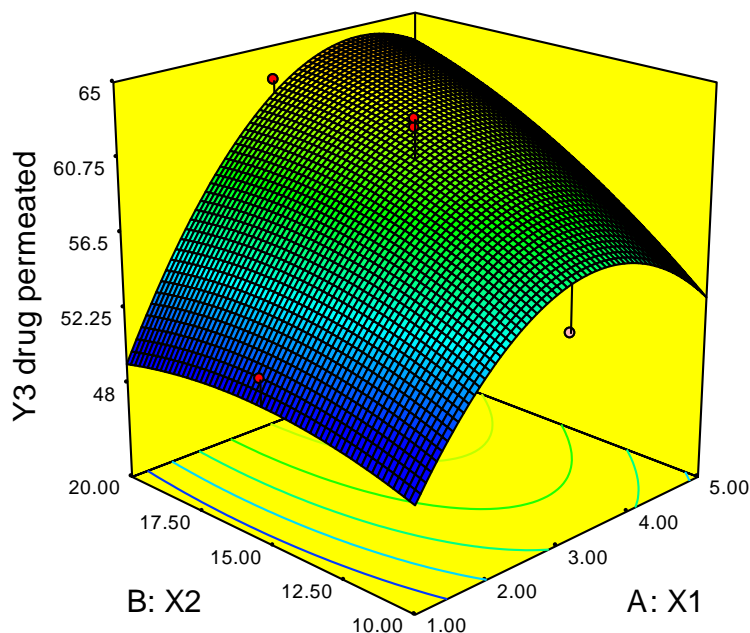**FIGURE 4.26 (b) 3D surface plot of Y3**

Contour plot Fig 4.26 (a) and 3D surface Fig 4.26 (b) showed that there was interaction between X1 and X2 terms giving curved shaped plot. Y3 % drug permeation increased with increase in X1 and X2 upto midway later on turned downwards. Y3 % drug permeation depends on lipophilicity of the molecule. RIS being hydrophilic drug has poor permeability and need permeability enhancement. X1 acted as driving force for permeation causing more penetration but could not be considered sole p-factor. Propylene glycol (X2) used as co solvent has given small viscous nature to product and has permeation enhancement property. It works by changing the thermodynamic activity of the drug in solution, increasing its concentration and facilitating partition of the drug into the membrane, and promoting passive diffusion. As ethanol and propylene glycol penetrate into mucosa, drugs dissolved in these co solvents are expected to be carried with them [112]. In most studies, the vehicle is used in combination with a permeation enhancer to further increase absorption. A combination of oleic acid (1%) appreciably enhanced the ex vivo permeation of a model peptide across porcine buccal mucosa [113].

4.3.5.4 Spray device performance Tests

Device performance test were conducted for all spray formulations F1 to F16 and results are presented in Table 4.38.

TABLE 4.38 Device performances

Formulation Code	Spray Pattern Ovality Ratio	Primes No of press	Pump delivery Weight in gm	Drug content per spray (n=3) mg	Spray profiling for device performance			Spray angle θ
					Beginning	Mid	Tail off	
F1	1.10	2	0.15	1.11	1.1	1.05	1.1	50.0
F2	1.05	2	0.15	1.09	0.95	1.0	1.0	60.0
F3	1.09	2	0.14	1.20	1.1	1.2	1.0	69.0
F4	1.03	2	0.15	1.09	1.0	0.95	1.0	51.1
F5	1.05	2	0.16	1.06	0.95	1.0	1.0	61.0
F6	1.09	2	0.16	1.30	1.10	1.10	1.10	53.0
F7	1.12	3	0.15	1.09	1.0	1.10	1.10	54.0
F8	1.02	3	0.15	1.14	1.0	1.1	0.95	62.0
F9	1.09	2	0.15	1.08	1.1	1.05	1.1	55.0
F10	1.12	2	0.14	1.09	0.95	1.0	1.09	54.0
F11	1.02	3	0.16	1.20	1.1	1.2	1.14	53.0
F12	1.09	3	0.15	1.09	1.1	1.05	1.08	52.2
F13	1.12	3	0.15	1.06	1.1	1.09	1.09	50.5
F14	1.02	2	0.14	1.08	0.95	1.14	1.20	57.0
F15	1.12	2	0.14	1.09	1.08	1.20	1.09	58.0
F16	1.02	2	0.14	1.20	1.09	1.09	1.14	60.0

SPRAY PATTERN: In the spray pattern test, each spray formulation was sprayed from the device and spray pattern was recorded over treated paper. Minimum and maximum diameter of the spray portion of each spray formulation was measured and ovality ratio was calculated. Observed spray pattern was circular i.e., the ovality ration was near to 1 as shown in Fig. 4.27. It was inferred from the result that the formulation components had not influenced spray pattern of the emitted formulations like in the preliminary studies.

PRIME TESTS: Results obtained in priming test were similar to that of preliminary tests i.e., F1-F6, F9-F10 F14- F16 formulations having low amount of propylene glycol X2 and poloxamer 188 X3 were emitted at the end of the two primes and those having higher amount took three primes. This difference in prime number could be due to difference in flow ability of the product through dip tube.

NET AVERAGE WEIGHT OF THE PRODUCT EMITTED PER SHOT: After the required number of priming was between 0.14 to 0.16 gm/ml and varied fairly. This indicated that the formulation composition would not affect the shot emitted remarkably.

DRUG CONTENT PER SPRAY SHOT: In the test for content emitted in one shot it was found that RIS content per shot was in range of 88 to 102%. This difference in the content could be due to difference in amount of the product emitted per shot and handling error. However, obtained result proved that each spray device performed in acceptable manner.

DENSITY OF FORMULATIONS: Formulates RIS spray formulations were filled in pump spray device and density of the product would not affect the product emitting in large magnitude. The density of the formulations varied with respect to variation in propylene glycol X2 and alcohol X4. Other formulation components had little effect on density.

SPRAY PROFILING: All the formulations F1 to F16 showed acceptable spray profiling as mentioned in Table 4.38 which revealed that formulations were appropriately made and in homogeneous solution state. This was also supported by the fact that appropriate functioning of the valve assembly. Uniform spray shots emitted in beginning, midway and tail off state ensured repeatable and accurate emission of dose during product usage.

SPRAY ANGLE: The formulations F1 to F16 showed spray angle in the range of 50-69º s. This deviation in the spray angle was due to difference in the consistency of these formulations as well as device components. However, the spray angles obtained from the all formulation devices were appropriate for application to sublingual mucosal surface.
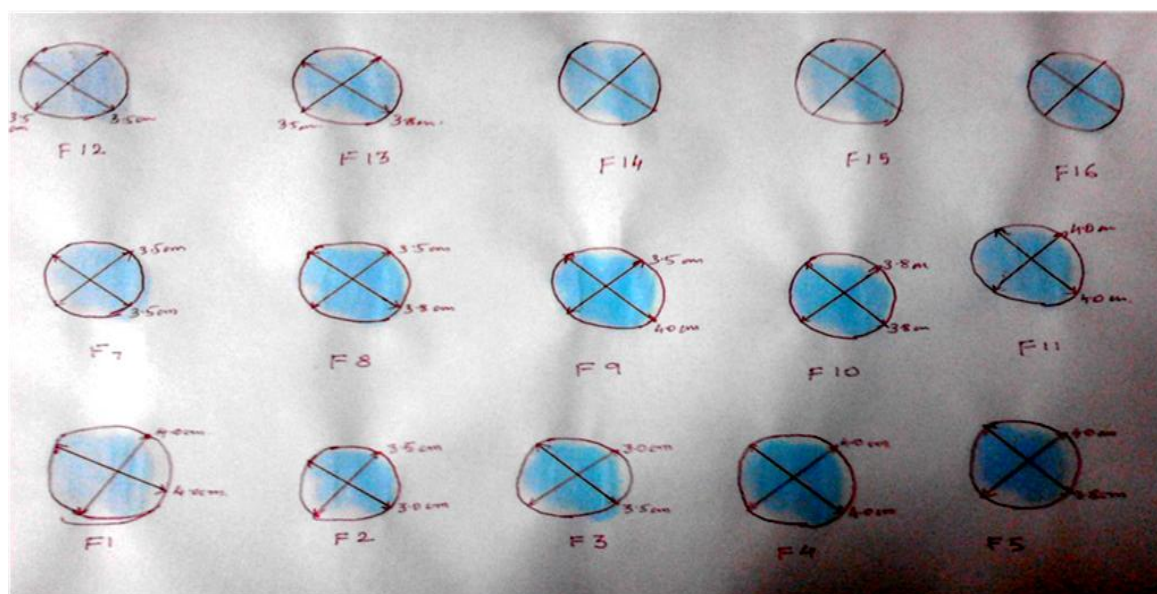
FIGURE 4.27 Spray pattern for F1 to F16

4.3.5.5 Ex-vivo permeability test

Ex-vivo permeability test was conducted over goat sublingual mucosa which was procured freshly before the evolutions and kept in deep freezer for avoiding decomposition of the tissue. Ex-vivo permeability test was aimed to know the effect of the formulation components on permeation of RIS. Effect of the variables is discussed in subsection 4.3.5.3. Apparent permeability of any molecule is predicted form permeability coefficient the penetrating molecule. It could be seen from the result Table 4.39 that the formulation F9 showed maximum permeability coefficient (P) 5.5×10^{-5} (cm/s) while F3 showed minimum P 4.10×10^{-5}. Permeability coefficient of the plain drug solution was found 1.09×10^{-6} which confirms poor permeability of RIS. Concentration of RIS and propylene glycol in the F9 was at higher level and both acted synergistically to increase the permeation of RIS. Concentration gradient of RIS across the sublingual mucosa caused favoured partition through the barrier and more permeation while propylene glycol acted by opening water channels and activity of the drug. RIS being hydrophilic molecule preferably pass through these water channels and partitioned across mucosa.

TABLE 4.39 Permeability coefficients of spray formulations

Formulation batch	Permeability coefficient P (cm/s)	Formulation batch	Permeability coefficient P (cm/s)
F1	4.45×10^{-5}	F9	5.5×10^{-5}
F2	5.1×10^{-5}	F10	5.1×10^{-5}
F3	4.10×10^{-5}	F11	5.41×10^{-5}
F4	4.22×10^{-5}	F12	4.18×10^{-5}
F5	4.18×10^{-5}	F13	5.1×10^{-5}
F6	5.5×10^{-5}	F14	4.67×10^{-5}
F7	5.13×10^{-5}	F15	4.67×10^{-5}
F8	5.08×10^{-5}	F16	4.26×10^{-5}
		Plain drug	1.09×10^{-6}

Above table showed that the permeability coefficient of the spray formulation was increased by 10 fold compared to plain drug.

4.3.6 Optimization and validation of RIS sublingual spray:

Numerical and graphical method was used to generate optimum formula. Table 4.40 show optimum formula and Fig. 4.28 (a) and (b) shows ramps and bar graph for optimumized batch.

TABLE 4.40 Optimized formula for RIS spray

Formulation	X1	X2	X3	Y1	Y2	Y3	Desirability
FO	4.5	20	9.87	57.77	91.54	67.01	0.891

Optimized formulation FO was composed according to Table 4.40 i.e., the values obtained for each component and evaluated for the Y1, Y2 and Y3. Experimental responses were compared with predicted responses.
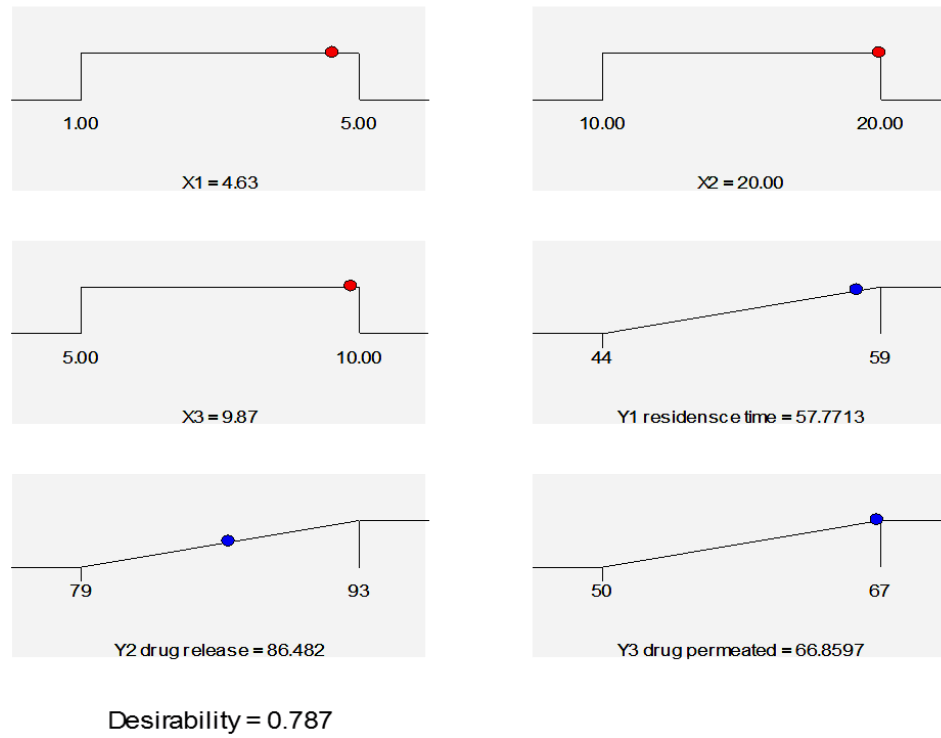
FIGURE 4.28 (a) Ramps graph for optimized batch FO

Desirability
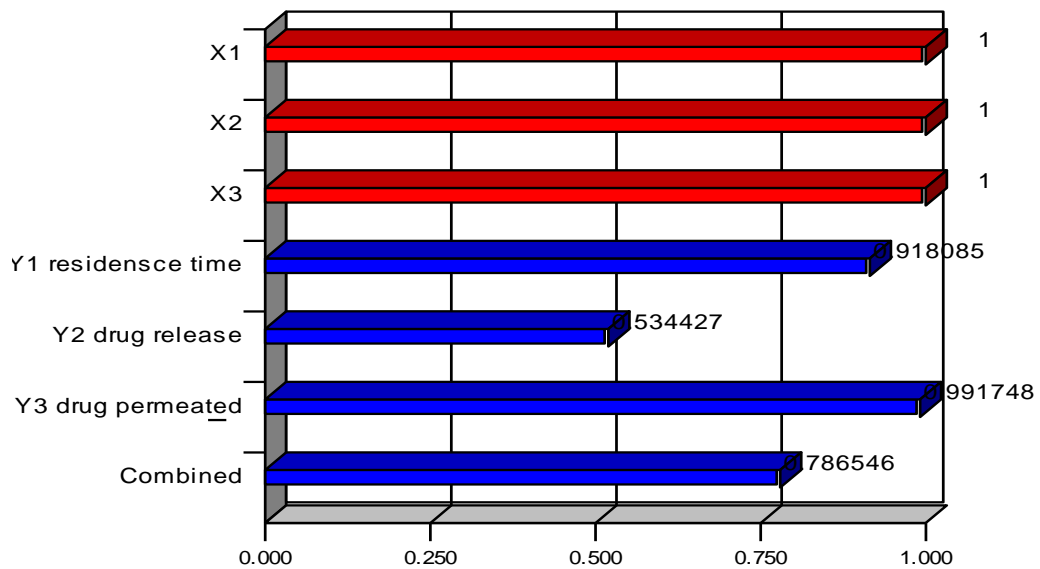
FIGURE 4.28 (b) bar graph for optimized batch FO
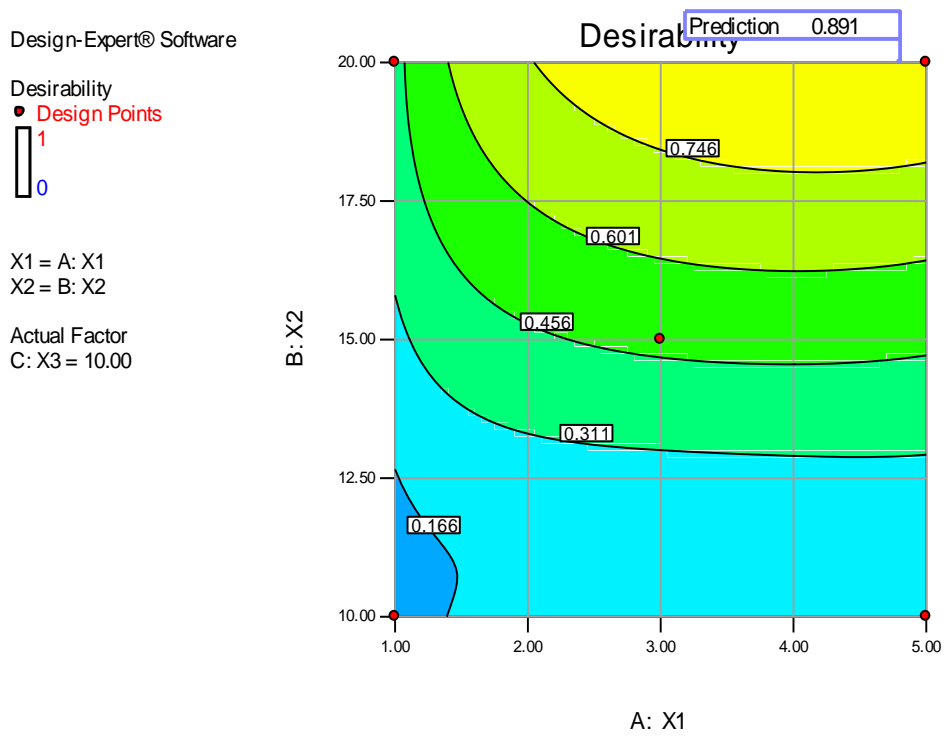
FIGURE 4.29 9 (a) Contour plot for FO
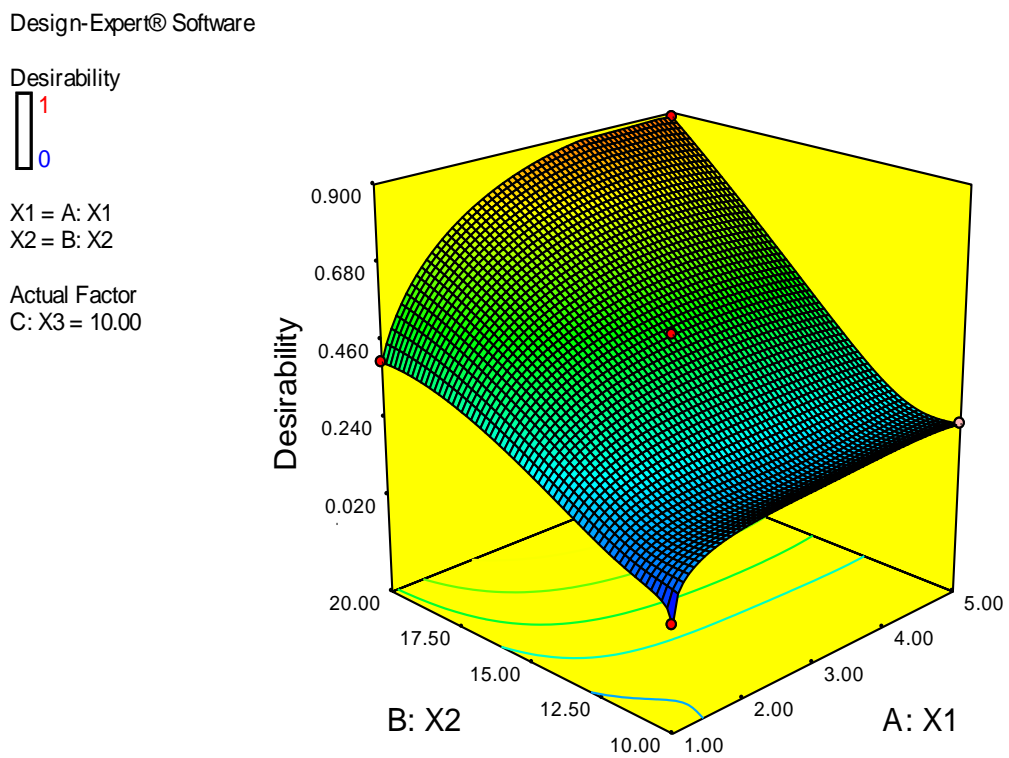
FIGURE 4.29 (b) 3D surface plot for FO

Design-Expert® Software

Overlay Plot

Y1 residensce time
Y2 drug release
Y3 drug permeated
● Design Points

X1 = A: X1
X2 = B: X2

Actual Factor
C: X3 = 10.00

Overlay Plot

Y1 residensc	58.0238
Y2 drug rele	91.5408
Y3 drug perm	67.0002
X1	4.80
X2	20.00

Y2 drug relea

Y3 drug permeated: 60
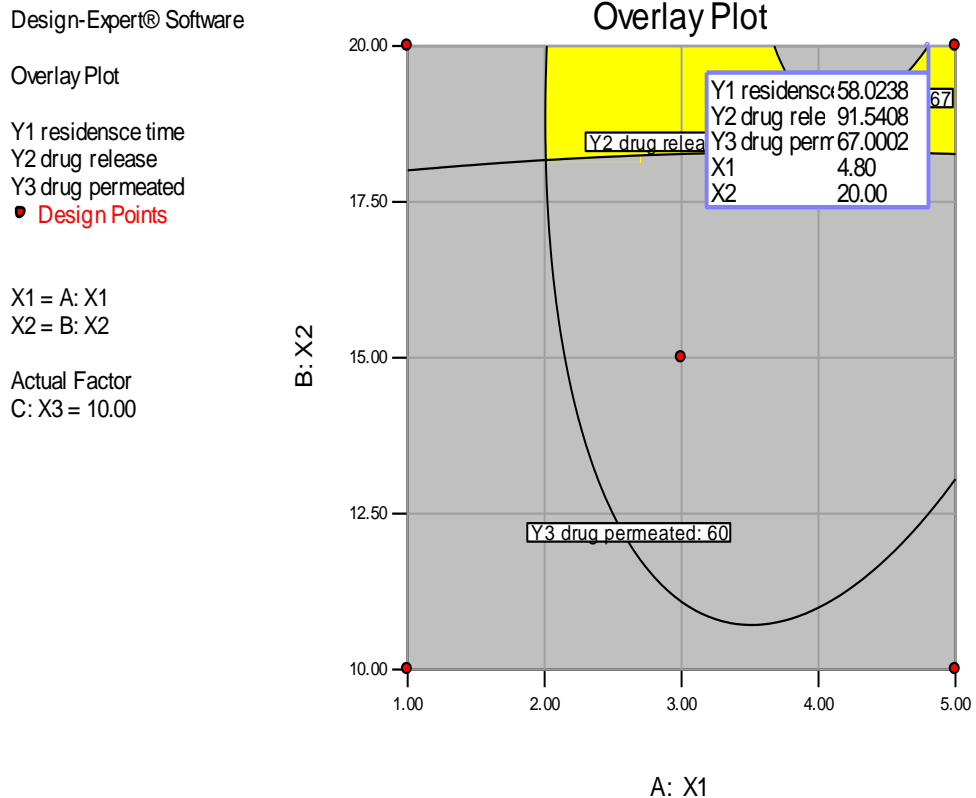
FIGURE 4.30 overlay plot

Overlay plot showed yellow region indicating desirable formulation compositions for optimum formulation. Any composition within this region would yield desirable formulation. Fig. 4.29 (a) and (b) represents contour and surface plots while Fig.4.30 shows overlay plot for optimized batch. From the plots it was evident that optimized formulation was stable and effective.

VALIDITY OF MODEL: Optimized formulation FO was prepared and tested for the responses Y1, Y2 and Y3. Observed values for these responses were near about same to those obtained from the model equations generated from experimental design i.e., experimental responses were close to predicted responses confirming the validity of the model as showed in Table 4.41. Permeability coefficient P of the FO was found 5.45×10^{-5} cm/sec and quite near to F9. Closeness of the P value additionally provides information that the optimized formulation FO from the design showed equal permeation characteristic in animal tissue.

TABLE 4.41 Predicted versus actual

Formulation	Predicted			Actual			Desirability
	Y1	**Y2**	**Y3**	**Y1**	**Y2**	**Y3**	
FO	57.25	86.48	66.85	55.21	87.32	66.22	0.787

4.3.7 Stability of the optimized batch FO

Finally optimized RIS spray formulation was investigated for stability. Stability study revealed that the values of responses Y1, Y2 and Y3 after storage period very close to those observed before. Proximity between before and after the stability test results AS showed in Table 4.42 proved that the FO was stable during the storage period and there was no reaction within FO components and container closure system.

TABLE 4.42 Stability data for FO

Formulation	Before			After 1 month			inference
	Y1	**Y2**	**Y3**	**Y1**	**Y2**	**Y3**	
FO	55.21	87.32	66.22	56.09	87.00	60.00	stable

There was no change in appearance of the formulation and container closure system indicating absence of incompatibility as per Table 4.43. FO was also subjected for container and product interaction. Photographs for before study as presented in Fig. 4.31 (a), 4.32 (a), Fig.4.33 (a) and after stability test study Fig. 4.31 (b), 4.32 (b), Fig.4.33 (b) suggested that there were no signs of interaction between the product and container system as was found in preliminary studies. There was no remarkable shift in the pH of the spary formulation FO after storage period and showed that selected container was suitable for the formulation. Container was cut vertically to observe internal surface and found smooth, plain and without any sign of interaction. Dip tube and valve was also found plain and transparent . Overall the stability study proved that the spray solution FO and spray container system was compatible with each other.

TABLE 4.43 Stability data for FO

Product or component	Observation At 0 Day	Observation after 15 days
Ph	7.13	7.16
Drug formulation solution	Clear	Clear
Container	Smooth internal surface with no signs of corrosion	Plastic containers had internal surface smooth with no signs of corrosion
Valve assembly	Transparent dip tube. No valve softening and cracking.	Dip tube was not discoloured and no elongation was observed. There was no softening and cracking of valve.
Content per Spray	1.48 mg/ml	1.50 mg/ml

(a)   **(b)**

FIGURE 4.31 (a) Initial and (b) after storage period clarity of Spray formulation FO

<div align="center">(a)   (b)</div>
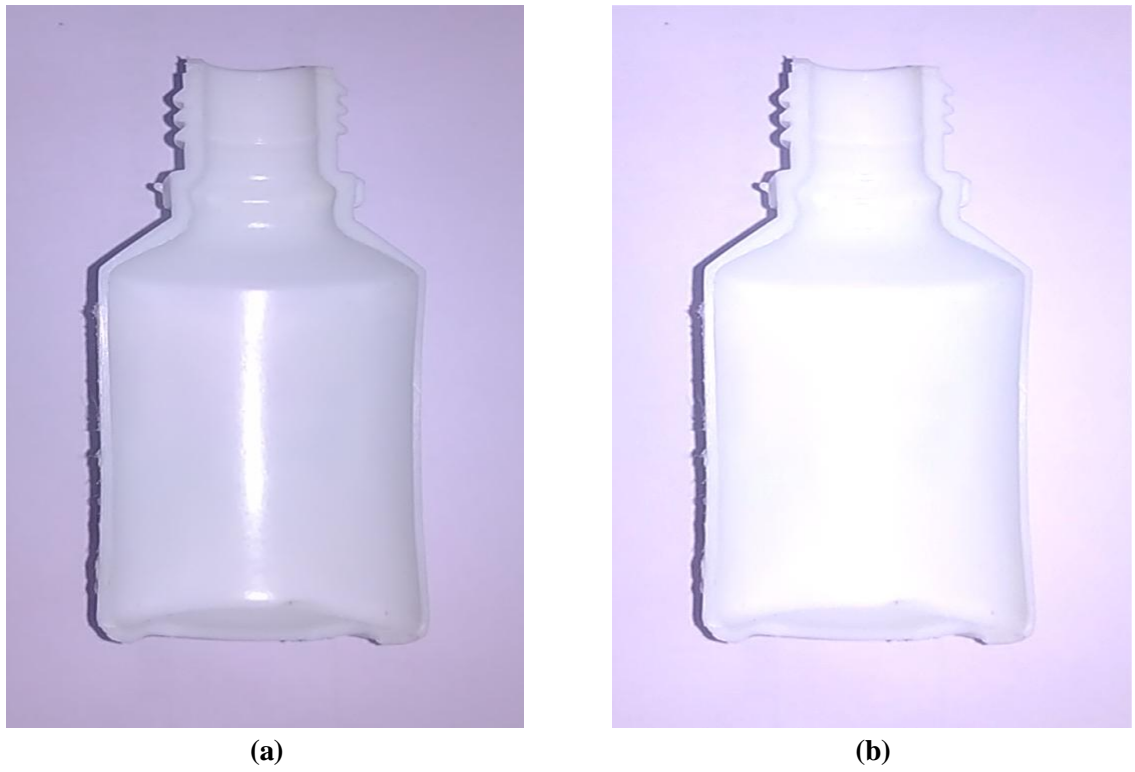
FIGURE 4.32 (a) Initial and (b) after storage Spray FO inside wall surface of container
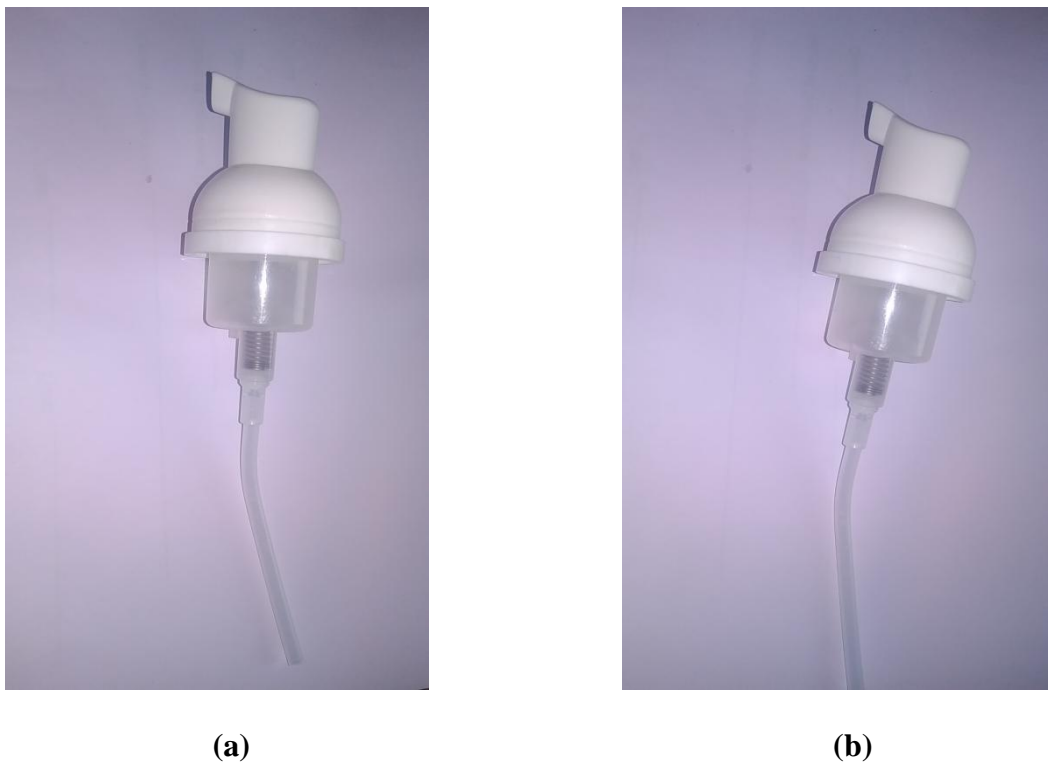

<div align="center">(a)   (b)</div>

FIGURE 4.33 (a) Initial and (b) after storage Spray FO container valve

4.3.8 In vivo absorption Study

RIS being a BCS-III drug has high solubility and low permeability, which often results in poor absorption and low oral bioavailability of drug. RIS was formulated in w/o/w multiple emulsion to increase the lipophilicity around the drug molecule which may enhance the permeability and increase absorption through intestinal track and lymphatic uptake. On the other hand RIS was also formulated in sublingual spray since high permeability of sublingual mucosa allows permeation of poorly permeable drug. Study results obtained after *in vivo* absorption study in rats were presented in Table 4.44 and graphically in Fig.4.34.

TABLE 4.44 Pharmacokinetic parameters of RIS administered in rats

Sample	C_{max} ng/ml	T_{max} hr	AUC_{0-8} ng*hr/ml	F
Plain drug solution	420 ±20	8	1630± 80	--
Convention formulation	460±23	6	1855±0.10	1.13
Drug loaded W/O/W Multiple emulsion (F19B)	1300.10 ± 50 *	4	4425±0.20 *	2.71
Sublingual spray formulation (FO)	920 ±0.045*	5	3710 ±0.18 *	2.27

Value are expressed as mean±SD; n=3, F–Relative bioavailability, *p<0.05 compared with plain RIS solution

The effect of the formulation on bioavailability of RIS was determined by the total amount of drug present in the plasma after oral administration of different formulations of the drug to rats. As summarized in Table 4.44 and Fig. 4.34 compared with plain drug solution and the conventional formulation (untreated drug), the multiple emulsion formulation and sublingual formulation significantly (P<0.05) improved drug absorption in rats. In particular, drug loaded multiple emulsion showed 3 fold increases in the total amount of drug present in the plasma in rats compared with the plain solution of drug and conventional formulation, whereas sublingually administered drug showed increase it by 1.9-fold. *In vivo* absorption study was aimed to know the enhancement in permeability of optimized and stabilized RIS formulations FO and F19B after oral administration in rats and the extent of absorption was determined. Rats were divided in different four groups and blood samples were collected at regular interval for 8 hours. Various pharmacokinetic parameters like C_{max}, T_{max} and AUC were found 1300.10 ± 50 ng/ml, 4 hr and 4425±0.20

ng.hr/ml for optimized multiple emulsion formulation F19B and 920 ±0.045ng/ml, 5 hr and 3710 ±0.18 ng.hr/ml for sublingual formulations FO.
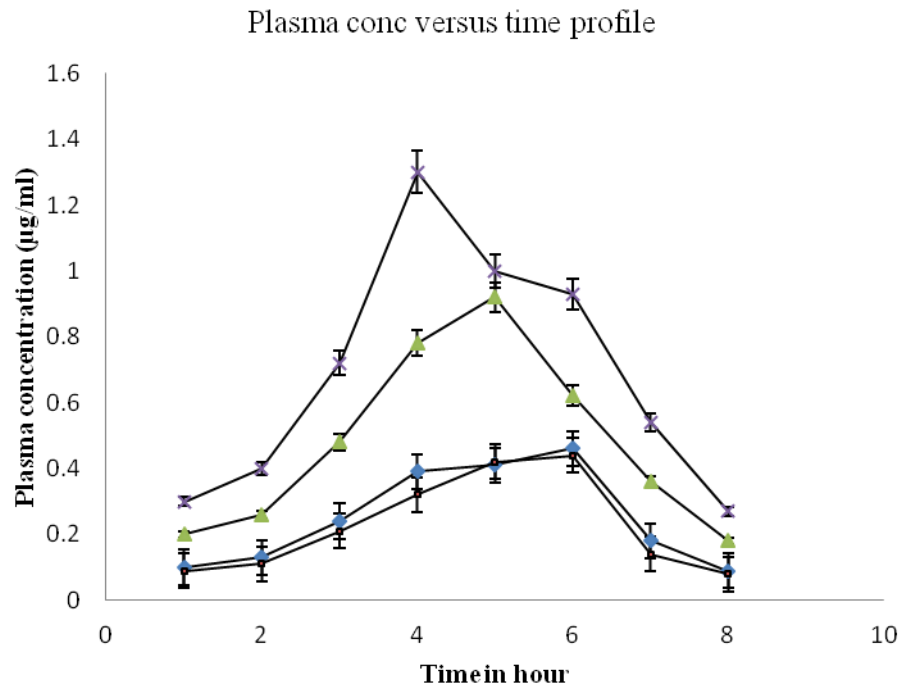
FIGURE 4.34 Plasma concentrations versus time
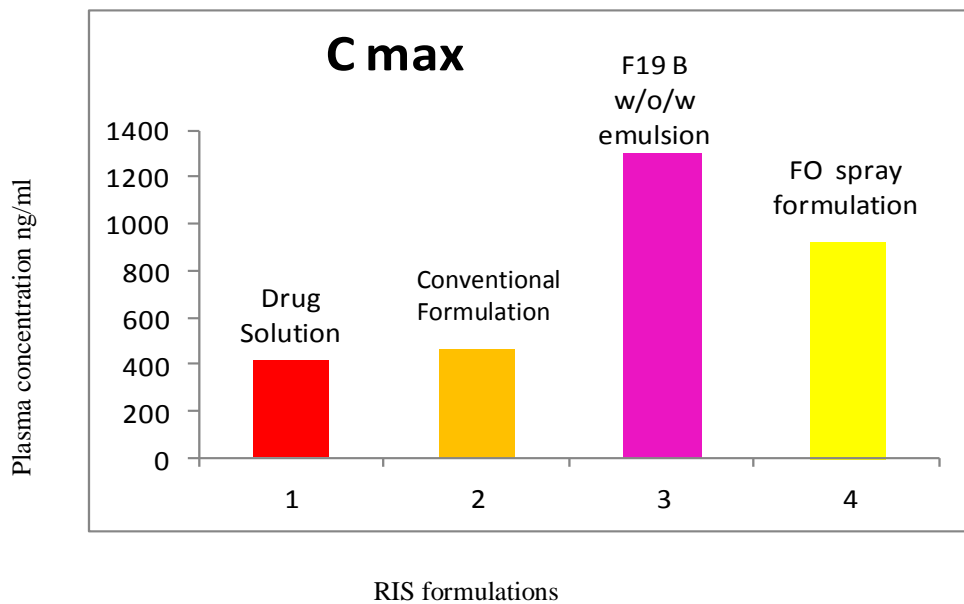
FIGURE 4.35 Plasma concentrations (C max) for different RIS formulations
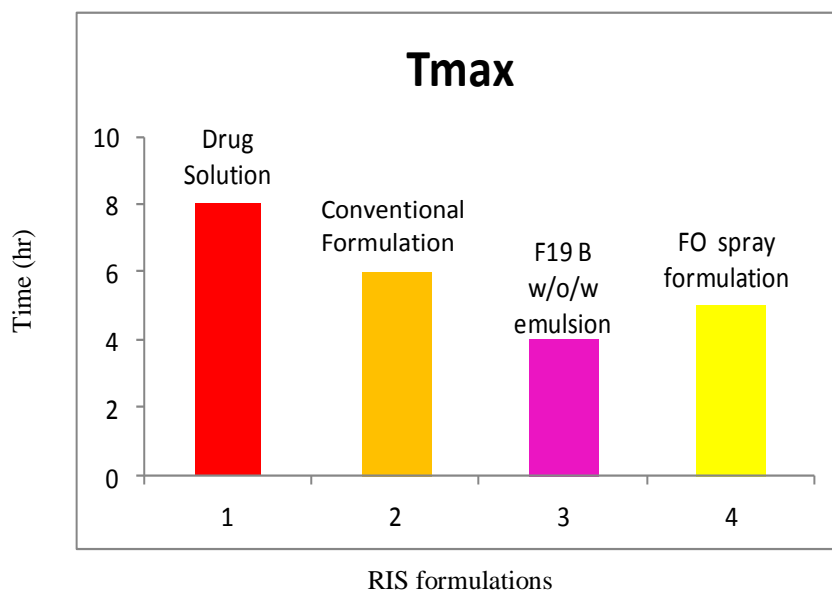
FIGURE 4.36 T max for different RIS formulations
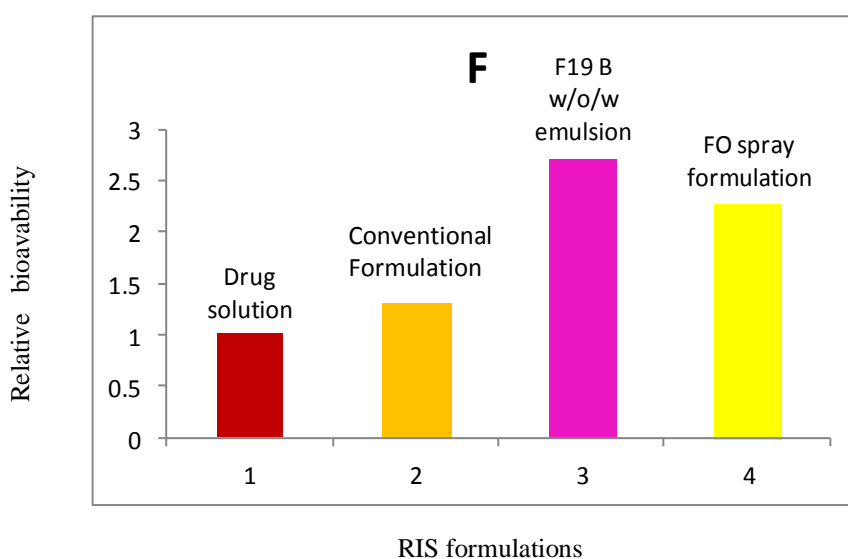
FIGURE 4.37 Relative bioavability (F) for different RIS formulations

The enhancement in permeability of RIS was evident from pharmacokinetic data when compared with drug solution and conventional preparation which were 420 ±20ng/ml, 8 hr, 1630± 80 ng.hr/ml and 460±23 ng/ml, 6 hr, 1855±0.10 ng.hr/ml accordingly for C_{max}, T_{max} and AUC_{0-8} are present in Fig 4.35 and Fig. 4.36. Relative bioavability of RIS multiple emulsion formulation was 3 fold and sublingual formulation was 2 fold than the plain drug solution and conventional preparation as presented in Fig 4.37.

This result may be explained by several assumptions. Enclosing the hydrophilic charged drug RIS in the inner oil phase could have increased the lipophilicity there by increasing the permeation across gastric lumen in addition slow release of drug from the oil globules might have allowed prolong absorption of the drug. Increase in uptake of sublingually administered drug could be due to high permeability of that region and avoidance of the food interaction. Longer residence of the spray droplets as result of the addition of poloxamer 188 could have increased the permeation of the drug. The study suggested sublingual spray and multiple emulsion formulation may be an alternative way of administration of RIS, providing enhanced bioavailability and also opened future scope for incorporation of other bisphosphonates.

CHAPTER 5

Conclusion And Future Scope

Risedronate sodium (RIS) is a bisphopshonate and has osteoclast inhibitory action. It is a current arsenal against osteoporosis and other bone diseases, and it is the mainstay of drug therapy for patients with osteoclast mediated osteoporosis. However, it is difficult to deliver RIS via the gastrointestinal tract because of poor bioavability due to poor absorption, poor membrane permeability and low lipophilicity. In addition RIS has oesophagus and gastric adverse effects. Patient needs to take the medicine with a glass of water preferably with empty stomach remaining to stay in vertical position. All these restrictions causes poor adherence to therapy. All current RIS preparations are limited to the tablet and capsule dosage forms, so an alternative oral delivery system of RIS has been desired for a long time having improved absorption with minimum or no adverse effects associated with oral dosage forms. In present study two different approaches were adopted for improvement of poor absorption of RIS namely w/o/w multiple emulsion and sublingual spray.

W/o/w multiple emulsion is thought to be an efficient drug carrier because it can accommodate hydrophilic drugs like RIS within inner water phase avoiding direct contact of drug to gastric membrane reducing drug associated gastric adverse effects. It also protects the drug from food content present in stomach obviating food-drug interaction. In addition these emulsions are safer to administer and easier to prepare than others since the w/o/w emulsions do not need organic solvents in their manufacture. However, because of some problems such as difficulty in particle size reduction and low entrapment efficiency for low-molecular-weight drugs, the w/o/w emulsions are limited for application compared to other formulations.

An attempt was made to incorporate RIS in inner water phase of the w/o/w multiple emulsion to deliver it by oral route. Initially Preformulation studies were performed to verify the physicochemical properties of the RIS. All the properties of the RIS were found close to reported values and further it was investigated for compatibility with formulation excipients. RIS was found stable with no interaction with excipients. Preparation of multiple emulsions was carried out using two step emulsification method. In preliminary study, w/o/w multiple emulsions of RIS were made with different fixed oils like arachis oil, olive oil, sunflower oil and fatty acid ester like isopropyl myristate (IPM). Formulation batches composed of fixed oils were shown to be w/o/w type and had reasonable characteristics like appearance, multiple structure, globule size, viscosity etc., immediately after preparation but could not retain the same till the end of preliminary evaluation. RIS w/o/w emulsion prepared with IPM was found to be superior to fixed oil preparations and was shortlisted for further optimization. Optimized formulation F19 showed 70% drug entrapment and 35% permeation during ex vivo permeation study with average globule size of 2.3 μ. However F19 showed 18 % creaming as a result of aggregation of oil dispersed phase, indicating need for stabilization. Stabilization of w/o/w multiple emulsion has been a challenging job because of their inherent thermodynamic [115]. Stability of the F19 was significantly enhanced by various approaches viz. phase volume ratio, viscosity built up of external water phase and use of external emulsifier surfactant blend were the approaches applied for stabilization. An improvised RIS w/o/w multiple emulsion with average globule size 2.0 μ, 11.83% creaming and elegant with pour ability was designed as 19B. Addition of viscosity enhancing agent in outer water phase could have decreased creaming rate and increased phase volume ratio reduced coalescence between dispersed phase. It can be concluded that most stable emulsion systems usually requires blends of two or more emulsifiers, one portion having lipophilic tendencies, the other hydrophilic.

A simple still effective administration of RIS was also attempted by formulating sublingual spray. It is well known that sublingual mucosa offers rapid and greater therapeutic effect for drugs having poor absorption in GIT. RIS sublingual spray showed acceptable device and biological performance. Inclusion of oleic acid enhanced permeation of the poorly permeable drug RIS across sublingual mucosa probably by increasing lipophilicity of the molecule and propylene glycol by opening the water channels of mucosal membrane. Increasing the time of residence of the sprayed droplets on mucosa due to the bioadhesive

effect of poloxamer 188 could have provided sufficient time for absorption. Application of RIS spray to sublingual mucosa would reduce chances of GIT associated adverse effects and food drug interaction. It would also increase patient compliance because of ease of administration. In the present research study, MINITAB 16 software and Experimental design 7 were used to design, analyze and optimize the formulations. Involvement of numerous factors which may affect the formulation and screening such significant factors from less number of trials is a great task for researcher and requires enough time because of economic and effective use of resources. This difficult task was made easy by designing formulation batches using experimental design namely factorial design in the initial stage of formulation development for RIS w/o/w multiple emulsion and sublingual spray. In the case of RIS multiple emulsion, Placket-Barman design was used to screen the significant factors that can affect the responses such as globule size, creaming entrapment efficiency and ex-vivo permeation rate. While for preliminary study of RIS sublingual spray fractional factorial design (1/4) was used. Using these designs moderate number of formulations were developed to screen factors that might have an influence on formulation characteristics and performance. Finally identified significant causative factors were further investigated for optimizing formulations by response surface method.

Finally in vivo absorption study of the RIS optimized and stabilized formulations of multiple emulsion and sublingual spray formulation was investigated for increment in the absorption of drug through oral route in comparison to plain drug solution and conventional marketed formulation. Results of the study in rats showed different amount of RIS absorption. Absorption of RIS was in order of w/o/w multiple emulsion > sublingual spray > conventional dosage form > plain drug solution. Many fold absorption of RIS from both formulation was attributed to increased permeation. Absorption from w/o/w multiple emulsion was thought because of increased residence time in stomach and lymphatic uptake. While moderate absorption in sublingual spray formulation could be due to hydrophilic nature of the drug and less residence time in mucosal surface.

FUTURE SCOPE: The present study provides sufficient experimental evidence that w/o/w multiple emulsion and sublingual spray approaches can be favourably applied for the poorly absorbed drugs through oral route having high solubility and poor permeability i.e., BCS class III drugs. W/O/W multiple emulsion would offer a deliver system for drugs having stomach degradation and gastric adverse effects. In addition w/o/w multiple emulsions can also be used as controlled drug delivery system for these drugs. Sublingual

spray formulation can be used for delivery of drugs having poor gastric absorption and unstable in stomach environment.

There is however a need to further corroborate the findings of this study on living systems and therefore in future investigations on disease models and clinical trials may be planned. The new formulations shall be a great help to the suffering humanity as the drug will be more efficacious and bear less side effects.

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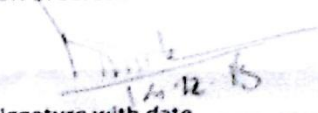
Appendix-A

CERTIFICATE

This is to certify that the experimental protocol titled "Formulations and Evaluation of oral delivery systems for poorly absorbed drugs used in osteoporosis" and bearing the proposal number **Phd/13-14/22** has been approved by the IAEC vide its meeting held on **14th December 2013**.

Name of Chairman, IAEC:
S.S Pancholi

Signature with date

Name of CPCSEA nominee, IAEC: Dr.
Dr. B. Suresh

Signature with date

Name of the Principal Investigator :
Mr. Milind M.Thosar