Objectives and Plan of Work

Chapter 3
Chapter –III

Objectives and Plan of Work

3.1. OBJECTIVES OF THE STUDY

The objectives of the present study were to:

1. Design and develop a nasal nanodrug carrier for the controlled release and targeting of bioactive agents.

2. Study the \textit{in vitro} release profile of the incorporated drug from nanocarrier/nanoparticles.

3. Optimize the nasal nanoparticulate drug delivery system with various process variables.

4. Evaluate the developed nanotechnology based drug delivery system.

5. Stability studies on the optimized nanoparticulate system.


3.2. RATIONALE OF THE STUDY

Osteoporosis is the most common metabolic bone disease, affecting 75 million people in the United States, Europe, and Japan, including one-third of postmenopausal women and most of the elderly. In the United States, of the 25 million women with osteoporosis, 8 million have had a documented fracture. It is estimated that more than 1.3 million osteoporotic fractures occur each year in the United States, with an annual cost of approximately $13.8 billion to the U.S. health system. (Kleerekoper and Al-Khayer, 2004).

The most desirable and convenient method for drug administration is oral route but the failure through this route led to research on alternate routes of drug delivery. Raloxifene is SREM widely used for the treatment of osteoporosis but the major disadvantage related to the oral delivery of this molecule is low oral bioavailability because of its first pass metabolism. Thus nasal mucosa has been focused as an alternate route for systemic drug delivery in order to achieve faster and higher drug absorption. Intranasal delivery of drugs in the form of polymeric nanoparticles is the most promising approach for the delivery of drugs for systemic use as this provides following advantages:

- It opens tight junctions and enhances absorption of drug via nasal route
- Provides for high drug loading capacity
- Enhances bioavailability of drug by avoiding first pass effect
- Improves dissolution rate of drug
- Requires smaller dose as compared to other conventional formulation to obtain same pharmacological effect
- Provides economic benefit for both consumer and manufacturer
- Improves patient compliance
For intranasal drug delivery, chitosan nanoparticles have been selected. Chitosan have been intensively investigated for drug administration because of the favorable features in terms of biocompatibility, non-toxicity and bioadhesion (Mei et al., 2008). Presence of primary amine groups, render special properties that make chitosan very useful in pharmaceutical applications. The amino group in chitosan has a \( pK_a \) value of \( \sim 6.5 \), thus, chitosan is positively charged and soluble in acidic to neutral solution with a charge density dependent on \( \text{pH} \) and the % degree of deacetylation (DA) value. It is probably superior mucoadhesive due to an ability to develop molecular attraction by electrostatic interactions with the negative charges of sialic acid groups on the mucin. Such interactions encourage prolonged contact time between the drug substance and the absorptive surface, thereby permitting the absorption of drug molecules via the paracellular, transcellular pathway or through endoytosis and transcytosis. Thus, the mucoadhesive property of chitosan will retain the drug in the nasal mucosa for a prolonged period of time, preventing the muco ciliary clearance of the drug. In previous studies, chitosan and its derivatives has demonstrated enhanced penetration of drugs through the mucosa by opening the tight junctions between epithelial cells or by intracellular routes. Chitosan is biocompatible with living tissues since it does not cause allergic reactions and rejection. It breaks down slowly to harmless products (amino sugars), which are completely absorbed by the human body.

### 3.2.1. Choice and selection of drug candidate

Osteoporosis is the most common metabolic bone disease, which occurs due to imbalance between bone resorption and bone formation. With aging, less bone is formed by osteoblasts than is removed by osteoclasts. Estrogen deficiency increases osteoclast life span but decreases osteoblast life span, resulting in an increased rate of bone remodeling and a decreased rate of bone replacement by osteoblasts during menopause, which in turn increases the risk of osteoporosis in postmenopausal patients.

Raloxifene hydrochloride, a nonsteroidal benzothiophene, is the first selective estrogen receptor modulator to be approved by the Food and Drug Administration for the prevention and treatment of postmenopausal bone loss at a dose of 60 mg/day. It acts as estrogen agonist in bone and heart and as estrogen antagonist in breast and uterine tissue. Raloxifene inhibits vertebral bone loss by inhibiting the activity of cytokines, which stimulate bone resorption.

Raloxifene posses bioavailability limited to 2% by oral route because of extensive first-pass metabolism. As a result, it is very important to develop a non-gastrointestinal delivery system.
3.3. DRUG PROFILE OF RALOXIFENE HCl

Raloxifene hydrochloride was first approved by the Food and Drug Administration in 1997, as a selective estrogen receptor modulator for the treatment of osteoporosis. Due to the antiestrogenic and antiandrogenic effects, it is also applied to treat breast cancer, prostate cancer, benign prostate hypertrophy, and fibrocystic disease. It is commercially available under the trade name of Evista® (Eli Lilly and Company, Indianapolis, IN), which is one of the blockbuster drugs as a filmcoated tablet formulated with the excipients of povidone, polysorbate 80, and anhydrous lactose, etc. (Jansen, 2009). Osteoporosis is associated with lack of estrogens, therefore, understandably one of the treatment options in osteoporosis is a group of drugs known as selective estrogen receptor modulators (SERMs). They can act as an estrogen receptor agonist in some tissues, whereas as an antagonist in others. In relation to this antago-antagonistic action, SERMs have a positive effect on bones, the serum lipid profile and the cardio-vascular system. Moreover, they can protect against some estrogen-dependent neoplasm development. Raloxifene, which is currently in use, besides the reduction of vertebral fractures risk, has beneficial influence on endometrial and breast neoplasm development risk as well. On the other hand, raloxifene intensifies vasomotor symptoms and its bone-protecting effect is limited (Meczekalski et al., 2009).

Fig. 3.1. Structure of raloxifene

**Generic name:** Raloxifene Hydrochloride  
**Brand names:** Evista (Eli Lily), Keoxifene  

**3.3.1 Physicochemical properties** (www.drugbank.ca/drugs/)  
**Description:** Raloxifene HCl is off-white to pale-yellow non-volatile powder  
**State:** Solid  
**Drug Category:** Antihypocalcemic Agents, Bone Density Conservation Agents, Estrogen Antagonists, Osteoporosis Prophylactic, Selective Estrogen Receptor Modulators
Chemical Name: [6-hydroxy-2-(4-hydroxyphenyl)-1-benzothiophen-3-yl]-[4-(2-piperidin-1-ylethoxy) phenyl]methanone

Solubility: 627 µg/mL (in water) (Jansen, 2009)

CAS #: 84449-90-1

Molecular formula: C_{28}H_{27}NO_{4}S.HCl

Molecular weight: 510.05 g/mol

Partition coefficient: log P = 1323 ± 91 (1-octanol/water at pH 7) (Trontelj et al., 2005)

Melting point: 267.3-268.5 °C

3.3.2. Pharmacology (www.medicines.org.uk/emc/)

3.3.2.1. Mechanism of action

Raloxifene binds to estrogen receptors, resulting in differential expression of multiple estrogen-regulated genes in different tissues. Raloxifene produces estrogen-like effects on bone, reducing resorption of bone and increasing bone mineral density in postmenopausal women. Raloxifene also antagonizes the effects of estrogen on mammary tissue and blocks uterotrophic responses to estrogen. Raloxifene mechanism of action is explained on the basis of estrogens mechanism of action, particularly estradiol (E2), which enters the nucleus of the target organ cells in order to bind there to a series of unoccupied, inactive proteins, called ER. These proteins, has two different isoforms, ER\(\alpha\) (predominantly activating) and ER\(\beta\) (which inhibits the former), transform into active receptors with a different spatial configuration (E2-ER) when they bind to E2, enabling them to simultaneously dimerize and subsequently interact with a specific sequence of DNA known as the Estrogen Responding Element (ERE) (Fig. 3.2, column A, level 2). A specific group of genes responsible for synthesizing the cell proteins that are in charge of triggering the estrogen effect on target reproductive tissues such as the uterus and the breast depend on ERE (Fig. 3.2, column A, level 3) (Riggs and Hartmann, 2003).

There are two specific areas in the ER known as activation factors (AF); the first one, or AF-1, located at the site of interaction with the specific DNA sequence, and the second one, or AF-2, located at the site where the ligand binds. In order for the group of genes associated with the ERE to be activated and hence, for the proteins associated with them to be synthesized (gene expression), the E2 side-chain must interact with the AF-2 region (Fig. 3.2, column A, level 2) (Riggs and Hartmann, 2003).

It is also known that ER do not have a single molecular binding site, but that they present two different domains, one for estrogen-type ligands and another one for antiestrogen type
ligands and SERM. Thus, depending on the binding site of the ligand to the ER, different spatial structures would be generated which would determine, at least in part, whether the ligand had a pure estrogen agonist action, a partial estrogen agonist action, or a pure estrogen antagonist action (Fig. 3.2, columns A and B, level 1) (Riggs and Hartmann, 2003).

ER do not act in the same way in all target tissues and that, in all likelihood, their action depends on which ER (alpha or beta) subtype is predominant in the tissue, on the nature of the ligand that binds to them (estrogen, antiestrogen, or SERM), on the cell transcription machinery (ERE and AF), and on the presence or absence of “helper” or regulating proteins (HP) (Riggs and Hartmann, 2003).

The benzothiophene ring of the raloxifene molecule binds to the ER with an affinity similar to that of E2. This bond, in addition to preventing access of E2 to the ER, also brings about a change in the spatial configuration of the ER in charge of activating it and binding it to the ERE (Fig. 3.2, column B, level 2). However, unlike the intimacy with which this ring adapts to the ER, the basic side-chain of the molecule, which is large and inflexible, does not completely bind to it, making it “protrude” from the receptor area (Helix 12). This peculiarity provokes an alteration in the orientation of the molecule that blocks its possible interaction with AF-2, thereby preventing the associated genes from being activated and their gene transcription. In the opinion of some authors, this blockade would be the key to the estrogen antagonist effects of the drug on uterine and breast tissues (Grese et al., 1996; Bryant et al., 1996).

However, in contrast, in bone and in other non-reproductive tissues, raloxifene bound to the ER (RLX-ER) and with the help of a series of HP (activating, helping, and/or adapting proteins) would activate a specific sequence of DNA known as the Raloxifene Responding Element (RRE). A group of genes that bring about the synthesis of specific cell proteins that are responsible for the estrogen agonist effect of the drug on these non-reproductive tissues would depend on this element. Thus, it would be possible to explain how raloxifene imitates the effect of estrogens on non-reproductive tissues such as bone (estrogen agonist effect) (Fig. 3.2, column B, level 3) (Grese et al., 1996; Bryant et al., 1996).

Raloxifene exerts two other direct actions on bone tissue. The first, which depends on the activation of its binding to the ligand, appears to indicate that this drug is capable of decreasing osteoclastic resorptive activity by up to 50%, interleukin-6 (IL-6) production, and up to 30% of the production of tumor necrosis factor α (TNF-α) at 6 months in vitro as well as in vivo (Jilka et al., 1992; Yang et al., 1996; Gianni et al., 2004). Both of these latter substances constitute important mediators of bone resorption. The second, which depends in this case on the activation of the RRE, suggests that raloxifene is capable of increasing the production of transforming growth factor β3 (TGF-β3), thereby decreasing the number of
osteoclasts as well as their resorptive activity (Fig. 3.2, column C) (Kumar et al., 1987).

### Pharmacokinetics

**Absorption**

Raloxifene is absorbed rapidly after oral administration. Approximately 60% of an oral dose is absorbed. Presystemic glucuronidation is extensive. Absolute bioavailability of raloxifene is 2%. The time to reach average maximum plasma concentration and bioavailability are functions of systemic interconversion and enterohepatic cycling of raloxifene and its glucuronide metabolites.

**Distribution**

Raloxifene is distributed extensively in the body. The volume of distribution is not dose dependent. Raloxifene and the monoglucuronide conjugates are highly (95%) bound to plasma proteins. Raloxifene binds to both albumin and α1-acid glycoprotein, but not to sex-steroid binding globulin.

**Metabolism**

Raloxifene undergoes extensive first pass metabolism to the glucuronide conjugates: raloxifene-4'-glucuronide, raloxifene-6-glucuronide, and raloxifene-6, 4'-diglucuronide. No other metabolites have been detected. Raloxifene comprises less than 1% of the combined concentrations of raloxifene and the glucuronide metabolites. Raloxifene levels are maintained by enterohepatic recycling, giving a plasma half-life of 27.7 h.

Results from single oral doses of raloxifene predict multiple dose pharmacokinetics. Increasing doses of raloxifene result in slightly less than proportional increase in the area under the plasma time
concentration curve (AUC).

**Excretion**

The majorities of a dose of raloxifene and glucuronide metabolites are excreted within 5 days and are found primarily in the faeces, with less than 6% excreted in urine.

**3.3.2.3. Indications**

Raloxifene is indicated for the treatment and prevention of osteoporosis in postmenopausal women.

**3.3.2.4. Contraindications**

Raloxifene is contraindicated in patients with a known hypersensitivity to the active substance, must not be used in women with child bearing potential, active or past history of venous thromboembolic events (VTE), including deep vein thrombosis, pulmonary embolism and retinal vein thrombosis, Hepatic impairment including cholestasis, severe renal impairment and unexplained uterine bleeding.

**3.3.2.5. Precautions**

**Venous Thromboembolism:** Raloxifene is associated with an increased risk for venous thromboembolic events that is similar to the reported risk associated with current use of hormone replacement therapy. The risk-benefit balance should be considered in patients at risk of venous thromboembolic events of any etiology. Raloxifene should be discontinued in the event of an illness or a condition leading to a prolonged period of immobilization. Discontinuation should happen as soon as possible in case of the illness, or from 3 days before the immobilisation occurs. Therapy should not be restarted until the initiating condition has resolved and the patient is fully mobile.

**Cardiovascular Disease:** Raloxifene should not be used for the primary or secondary prevention of cardiovascular disease. Postmenopausal women with documented coronary heart disease or at increased risk for coronary events, no cardiovascular benefit was demonstrated after treatment with raloxifene for 5 years.

**Premenopausal Use:** There is no indication for premenopausal use of raloxifene. Safety of Raloxifene in premenopausal women has not been established and its use is not recommended.

**Renal Impairment:** Raloxifene should be used with caution in patients with moderate or severe renal impairment. Safety and efficacy have not been established in patients with moderate or severe renal impairment.

**Hepatic Impairment:** Raloxifene should be used with caution in patients with hepatic impairment. Raloxifene is metabolized primarily in the liver. Single doses of raloxifene given to patients with cirrhosis and mild hepatic impairment (Child-Pugh class A) produced plasma concentrations of raloxifene which were approximately 2.5 times the controls. The increase correlated with total bilirubin concentrations. Until safety and efficacy have been evaluated further in patients with hepatic insufficiency, the use of raloxifene is not recommended in this patient population. Serum total bilirubin, gamma-glutamyl transferase, alkaline phosphatase, ALT and AST should be closely monitored during treatment if elevated values are observed.

**Concomitant Estrogen Therapy:** The safety of concomitant use of raloxifene with systemic estrogens has not been established and its use is not recommended.
Chapter –III

Objectives and Plan of Work

History of Breast Cancer: The safety of raloxifene in patients with breast cancer has not been adequately studied. No data are available on the concomitant use of raloxifene and agents used in the treatment of early or advanced breast cancer. Therefore, raloxifene should be used for osteoporosis treatment and prevention only after the treatment of breast cancer, including adjuvant therapy, has been completed.

Unexplained Uterine Bleeding: Any unexplained uterine bleeding should be investigated as clinically indicated. Raloxifene-treated and placebo-treated groups had similar incidences of endometrial proliferation.

Breast Abnormalities: Any unexplained breast abnormality occurring during raloxifene therapy should be investigated. Raloxifene does not eliminate the risk of breast cancer.

Carcinogenesis, mutagenesis, impairment of fertility:
Carcinogenesis — In a 21-month carcinogenicity study in mice, there was an increased incidence of ovarian tumors in female animals given 9 to 242 mg/kg, which included benign and malignant tumors of granulosa/theca cell origin and benign tumors of epithelial cell origin. Systemic exposure (AUC) of raloxifene in this group was 0.3 to 34 times that in postmenopausal women administered a 60 mg dose. There was also an increased incidence of testicular interstitial cell tumors and prostatic adenomas and adenocarcinomas in male mice given 41 or 210 mg/kg (4.7 or 24 times the AUC in humans) and prostatic leiomyoblastoma in male mice given 210 mg/kg.
In a 2-year carcinogenicity study in rats, an increased incidence in ovarian tumors of granulosa/theca cell origin was observed in female rats given 279 mg/kg (approximately 400 times the AUC in humans). The female rodents in these studies were treated during their reproductive lives when their ovaries were functional and responsive to hormonal stimulation.
Mutagenesis-Raloxifene was not found genotoxic in any of the following test systems. The Ames test for bacterial mutagenesis with and without metabolic activation, the unscheduled DNA synthesis assay in rat hepatocytes, the mouse lymphoma assay for mammalian cell mutation, the chromosomal aberration assay in Chinese hamster ovary cells, the in vivo sister chromatid exchange assay in Chinese hamsters, and the in vivo micronucleus test in mice.

Pediatric Use: Safety and effectiveness in pediatric patients have not been established.

Geriatric Use: There is no need for dose adjustment for geriatric patients.

3.3.2.6. Drug- drug interactions

Cholestyramine
Raloxifene should not be co-administered with cholestyramine (or other anion exchange resins), which significantly reduces the absorption and enterohepatic cycling of raloxifene.

Warfarin
Co-administration of raloxifene and warfarin does not alter the pharmacokinetics of either compound. However, modest decreases in the prothrombin time have been observed, and if raloxifene is given concurrently with warfarin or other coumarin derivatives, the prothrombin time should be monitored. Effects on prothrombin time may develop over several weeks if raloxifene treatment is started in patients who are already on coumarin anticoagulant therapy.
Other Highly Protein-Bound Drugs
Raloxifene should be used with caution with certain other highly protein-bound drugs such as diazepam, diazoxide, and lidocaine.

Systemic Estrogens
The safety of concomitant use of raloxifene with systemic estrogens has not been established and its use is not recommended.

Other Concomitant Medications
Raloxifene can be concomitantly administered with ampicillin, amoxicillin, antacids, corticosteroids, and digoxin. Peak concentrations of raloxifene are reduced with co-administration with ampicillin. However, since the overall extent of absorption and the elimination rate of raloxifene are not affected, raloxifene can be concurrently administered with ampicillin. Concurrent administration of either calcium carbonate or aluminium and magnesium-hydroxide containing antacids do not affect the systemic exposure of raloxifene. Raloxifene has no effect on the pharmacokinetics of methylprednisolone given as a single dose. Raloxifene does not affect the steady-state AUC of digoxin. The $C_{\text{max}}$ of digoxin increased by less than 5%.

3.3.2.7. Adverse effects
The adverse reactions associated with the use of raloxifene in osteoporosis are given below:

- Vascular disorders
  - Very common: Vasodilation (hot flushes)
  - Uncommon: Venous thromboembolic events, including deep vein thrombosis, pulmonary embolism, retinal vein thrombosis Superficial vein thrombophlebitis

- Musculoskeletal and connective tissue disorders
  - Common: Leg cramps

- General disorders and administration site conditions
  - Very common: Flu syndrome
  - Common: Peripheral oedema

The adverse reactions reported in post-marketing experience and are below.

- Blood and lymphatic system disorders
  - Very rare: thrombocytopenia

- Gastrointestinal disorders
  - Very rare: Gastrointestinal symptoms such as nausea, vomiting, abdominal pain, dyspepsia

- General disorders and administration site conditions
  - Rare: peripheral oedema

- Investigations
  - Very rare: Increased blood pressure

- Nervous system disorders
  - Very rare: Headache, including migraine

- Skin and subcutaneous tissue disorders
  - Very rare: Rash

- Reproductive system and breast disorders
  - Very rare: Mild breast symptoms such as pain, enlargement and tenderness
Vascular disorders
  - Rare: venous thromboembolic reaction
  - Very rare: arterial thromboembolic reaction

3.3.2.8. Dosage and administration
The recommended dose is one tablet daily by oral administration, which may be taken at any time of the day without regard to meals. No dose adjustment is necessary for the elderly. Due to the nature of this disease process, it is intended for long term use.

Generally calcium and vitamin D supplements are advised in women with a low dietary intake.

Renal & hepatic impairment:
Raloxifene should not be used in patients with severe renal impairment and hepatic impairment. In patients with moderate and mild renal impairment, it should be used with caution.

3.3.2.9. Overdosage
Overdosage with raloxifene hydrochloride in adults leads to leg cramps and dizziness. In children, symptoms of accidental overdose included ataxia, dizziness, vomiting, rash, diarrhea, tremor, and flushing, and elevation in alkaline phosphatase. No fatalities associated with overdose have been reported. There is no specific antidote for raloxifene.

3.3.2.10. Presentation and storage condition
Raloxifene film-coated, elliptically shaped, white tablets imprinted with the code '4165', containing 60 mg raloxifene hydrochloride, packaged either in PVC/PE/PCTFE blisters or in high density polyethylene bottles. Blister boxes contain 14, 28, or 84 tablets. Bottles contain 100 tablets.

Storage Conditions - Store in the original package. Do not freeze.

3.4. PLAN OF WORK
1. Physicochemical characterization and identification of raloxifene HCl
   a) Organoleptic properties
   b) Solubility
   c) Partition coefficient
   d) Loss on drying
   e) IR spectroscopy
   f) Differential scanning calorimetry (DSC)
2. Drug excipients compatibility studies.
3. Analytical method development for raloxifene HCl.
   a) Analytical method development and validation for determining drug entrapment efficiency, drug loading, in vitro release using HPLC.
   b) Analytical method development and validation for stability studies using UPLC.
   c) Bioanalytical method development and validation for quantification of raloxifene from plasma and tissue homogenates using LC-MS/MS.
4. Selection and evaluation of polymers for the formation of nanoparticles.
5. Preparation of nanoparticles by using various techniques as:
Chapter –III

Objectives and Plan of Work

1. Emulsion evaporation
2. Salting out
3. Ionic gelation
4. Emulsification diffusion etc.

6. Selection and evaluation for the effect of coating on nanoparticles.

7. Evaluation of nanoparticles on the basis of process parameters.


9. Formulation, optimization and evaluation of chitosan nanoparticulate formulation.
   a) Formulation of placebo chitosan nanoparticles.
   b) Formulation of drug loaded chitosan nanoparticles
   c) Optimization of drug loaded chitosan nanoparticles using drug design expert

10. Characterization of the optimized raloxifene HCl loaded chitosan nanoparticulate formulation
   • Surface morphology and shape
     - Transmission electron microscopy (TEM)
     - Scanning electron microscopy (SEM)
   • Particle size, particle size distribution and Zeta potential
     - Photon correlation spectroscopy (PCS)
   • Crystallinity
     - Differential scanning calorimetry (DSC)
     - X-ray diffraction (XRD)
   • Interaction studies
     - Fourier Transform Infrared Spectroscopy (FTIR)

11. Animal Studies after getting approval from CPCSEA (Committee for the purpose of controlled and supervision of experimentation animals) which includes
   • Pharmacokinetic studies
   • Biodistribution studies


   These studies determine the effect of the presence of polymers, excipients and accelerated storage conditions of temperature and humidity under specified guidelines determine the physical stability of the formulations.