

FORMULATION AND EVALUATION OF PRNOSOMES FOR ANTICANCER DRUGS

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FORMULATION AND EVALUATION OF PRNIOSOMES FOR ANTICANCER DRUGS

1. INTRODUCTION

Cancer is a leading cause of death worldwide; There were an estimated 14.1 million cancer cases around the world in 2012, of these 7.4 million cases were in men and 6.7 million in women .More than half of these – 8 million – occurred in economically developing countries.¹ Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030². The four most common cancers occurring worldwide are lung, female breast, bowel and prostate cancer. These four account for around 4 in 10 of all cancers diagnosed worldwide.³

Breast cancer is the most frequently diagnosed cancer and the primary cause of cancer-related death in women worldwide.The incidenceand mortality of breast cancer have been rising in low to middle-income countries.⁴

Today the challenge for the pharmaceutical formulators is to work and investigate to deliver the drug using promising drug carriers including biodegradable polymers. The systems that are capable of releasing the therapeutic agents by well defined kinetics are available at present. But in many cases these do not yet represent the ultimate therapy to needs of recipient. Hence attention should also be focused to fabricate controlled, modulated drug delivery system that are capable of receiving the physiological feedback information and adjusting the drug output and system that are capable of precisely targeting the specific tissue or cells. Current attempts to overcome these limitations include the development of novel drug delivery systems that can improve the efficacy of existing anti cancer drugs.

It is almost impossible to deliver anticancer drugs specifically to the tumour cells without damaging healthy organs or tissues⁵.Drug delivery systems using colloidal particulate carriers such as liposomes or niosomes⁶have distinct advantages over conventional dosage forms. These systems can act as drug reservoirs, and provide controlled release of the active substance. In addition, modification of their composition or surface can adjust the drug release rate and/or the affinity for the target site. Liposomes or niosomes in dispersion can carry hydrophilic drugs by

encapsulation or hydrophobic drugs by partitioning of these drugs into hydrophobic domains⁷. Colloidal drug delivery carriers are easily phagocytised by macrophages. Therefore, they can facilitate the uptake of drugs by these cells and may enable a considerably improvement of cancer therapy.

Niosomes are non-ionic surfactant based vesicles that had been developed as alternative controlled drug delivery systems to liposomes in order to overcome the problems associated with sterilization, large scale production and stability⁸. The advantages of niosomal drug delivery systems include the structure and properties that are similar to those of liposomes, the chemical stability of niosomes and the relatively low cost of the materials that form them make niosomes more attractive than liposomes for industrial manufacturing. Many drugs, those currently available in the market and those under development, have poor aqueous solubility that results in variable bioavailability. This problem can be overcome by entrapping the drug into niosomes⁹.

Though the niosomes exhibit good chemical stability during storage, there may be problems of physical instability in niosome dispersions. Like liposomes, aqueous suspensions of niosomes may exhibit aggregation, fusion, leaking of entrapped drugs, or hydrolysis of encapsulated drugs, thus limiting the shelf life of the dispersion¹⁰. The niosomes are very difficult to sterilize, transport, distribution, storage uniformity of dose and scale up. There is a possibility of incomplete hydration of the lipid/surfactant film on the walls during hydration process.

Niosomal drug delivery system perhaps an useful strategy towards targeted drug delivery in cancer chemotherapy. The potential for niosomes in cancer drug delivery is infinite with novel applications constantly being explored. Niosomes play a significant role in cancer drug delivery. In the past, cancer patients were using various anticancer drug formulations, but they were less successful and had major side effects. Niosomes have attracted the attention of pharmaceutical formulators.

Formulation of Niosomes

The preparation methods should be chosen according to the use of the niosomes, since the preparation methods influence the number of bilayers, size, size

distribution, and entrapment efficiency of the aqueous phase and the membrane permeability of the vesicles.

A. Ether injection method:

This method provides a means of making niosomes by slowly introducing a solution of surfactant dissolved in diethyl ether into warm water maintained at 60°C. The surfactant mixture in ether is injected through 14-gauge needle into an aqueous solution of material. Vaporization of ether leads to formation of single layered vesicles. Depending upon the conditions used the diameter of the vesicle range from 50 to 1000 nm¹¹

B. Hand shaking method (Thin film hydration technique):

The mixture of vesicles forming ingredients like surfactant and cholesterol are dissolved in a volatile organic solvent (diethyl ether, chloroform or methanol) in a round bottom flask. The organic solvent is removed at room temperature (20°C) using rotary evaporator leaving a thin layer of solid mixture deposited on the wall of the flask. The dried surfactant film can be rehydrated with aqueous phase at 0-60°C with gentle agitation. This process forms typical multilamellar niosomes¹²

C. Sonication:

A typical method of production of the vesicles is by sonication of solution as described by Cable. In this method an aliquot of drug solution in buffer is added to the surfactant/cholesterol mixture in a 10-ml glass vial. The mixture is probe sonicated at 60°C for 3 minutes using a sonicator with a titanium probe to yield niosomes¹²

D. Micro fluidization:

Micro fluidization is a recent technique used to prepare unilamellar vesicles of defined size distribution. This method is based on submerged jet principle in which two fluidized streams interact at ultra high velocities, in precisely defined micro channels within the interaction chamber. The impingement of thin liquid sheet along a common front is arranged such that the energy supplied to the system

remains within the area of niosomes formation. The result is a greater uniformity, smaller size and better reproducibility of niosomes formed¹¹

E. Multiple membrane extrusion method:

Mixture of surfactant, cholesterol and dicetyl phosphate in chloroform is made into thin film by evaporation. The film is hydrated with aqueous drug polycarbonate membranes, solution and the resultant suspension extruded through which are placed in series for upto 8 passages. It is a good method for controlling niosome size¹¹

F. Reverse Phase Evaporation Technique (REV):

Cholesterol and surfactant (1:1) are dissolved in a mixture of ether and chloroform. An aqueous phase containing drug is added to this and the resulting two phases are sonicated at 4-5°C. The clear gel formed is further sonicated after the addition of a small amount of phosphate buffered saline (PBS). The organic phase is removed at 40°C under low pressure. The resulting viscous niosome suspension is diluted with PBS and heated on a water bath at 60°C for 10 min to yield niosomes¹³

G. Trans membrane pH gradient (inside acidic) Drug Uptake Process (Remote Loading):

Surfactant and cholesterol are dissolved in chloroform. The solvent is then evaporated under reduced pressure to get a thin film on the wall of the round bottom flask. The film is hydrated with 300 mM citric acid (pH 4.0) by vortex mixing. The multilamellar vesicles are frozen and thawed 3 times and later sonicated. To this niosomal suspension, aqueous solution containing 10mg/ml of drug is added and vortexed. The pH of the sample is then raised to 7.0-7.2 with 1M disodium phosphate. This mixture is later heated at 60°C for 10 minutes to give niosomes¹⁴

H. The "Bubble" Method:

It is novel technique for the one step preparation of liposomes and niosomes without the use of organic solvents. The bubbling unit consists of round-bottomed flask with three necks positioned in water bath to control the temperature. Water-

cooled reflux and thermometer is positioned in the first and second neck and nitrogen supply through the third neck. Cholesterol and surfactant are dispersed together in this buffer (pH 7.4) at 70°C, the dispersion mixed for 15 seconds with high shear homogenizer and immediately afterwards “bubbled” at 70°C using nitrogen gas¹⁵

Proniosomes:

Proniosomes are dry, free-flowing formulations of surfactant-coated carrier, which can be rehydrated by brief agitation in hot water to form a multi-lamellar niosome suspension suitable for administration by oral or other routes.^{16,17}

Proniosomes minimize of niosomes physical instability such as aggregation, fusion and leaking. Proniosome-derived niosomes are superior to conventional niosomes in convenience of storage, transport, distribution and dosing. Stability of dry proniosomes is expected to be more stable than a pre-manufactured niosomal formulation. In release studies proniosomes appear to be equivalent to conventional niosomes. Size distributions of proniosome-derived niosomes are somewhat better than those of conventional niosomes. so the release performance in more critical cases turns out to be superior.^{15,16,18,19}

Proniosomes are dry powder, which makes further processing and packaging possible. The powder form provides optimal flexibility, unit dosing, in which the proniosome powder is provided in capsule could be beneficial. A proniosome formulation based on maltodextrin was recently developed that has potential applications in deliver of hydrophobic or amphiphilic drugs. The better of these formulations used a hollow particle with exceptionally high surface area. The principal advantage with this formulation was the amount of carrier required to support the surfactant could be easily adjusted and proniosomes with very high mass ratios of surfactant to carrier could be prepared. Because of the ease of production of proniosomes using the maltodextrin by slurry method, hydration of surfactant from proniosomes of a wide range of compositions can be studied.^{15,19,20}

In the preparation of proniosomes non-ionic surfactants, coating carriers and membrane stabilizers are commonly used. The non-ionic surfactants used are Span

(20, 40, 60, 80, 85), Tween (20, 60, 80). The coating carriers used are sucrose stearate, sorbitol, maltodextrin (MaltrinM500, M700), glucose monohydrate, lactose monohydrate, spray dried lactose and membrane stabilizers like cholesterol and lecithin are also used.^{18,19,21,22,23,24,25}

Advantage of Proniosome^{15,19,23}

- a) Drug targeting agent and provide controlled release.
- b) Entrapping both hydrophilic and hydrophobic drugs
- c) Greater physical and chemical stability
- d) Easy of transport, distribution, measuring and storage
- e) Proniosome powder can further be processed to make beads, tablets or capsule
- f) Low toxicity due to nonionic nature
- g) Simple method
- h) Low cost
- i) More uniform in size
- j) It is a versatile delivery system with potential use with wide range of active compounds

Methods of Preparation of Proniosomes

Spraying method:

Proniosomes were prepared by spraying the surfactant in an organic solvent into sorbitol powder and then evaporating the solvent. Because the sorbitol carrier is soluble in the organic solvent, it is necessary to repeat the process until the desired surfactant load has been achieved²⁶. The surfactant coating on the carrier comes out to be very thin and hydration of this coating allows multilamellar vesicles to form.

Slurry method:

Proniosomes were produced by slurry method using maltodextrin as a carrier. The time required to produce proniosomes by this is independent of the ratio of surfactant solution to carrier material. In slurry method, the entire volume of surfactant solution is added to maltodextrin powder in a rotary evaporator and vacuum is applied until the powder appears to be dry and free flowing.²⁷ Drug containing proniosomes-derived niosomes can be prepared in manner analogous to that used for the conventional niosomes, by adding drug to the surfactant mixture prior to spraying the solution onto the carrier (sorbitol, maltodextrin) or by addition of drug to the aqueous solution used to dissolve hydrate the proniosomes.

Coacervation phase separation method:

In this method, accurately weighed amount of surfactant, carrier (lecithin), cholesterol and drug are taken in a clean and dry wide mouthed glass vial (5 ml) and solvent is added to it followed by simple mixing. To prevent the loss of solvent, the open end of the glass vial can be covered with a lid and heated over water bath at 60-70°C for 5 minutes until the surfactant dissolved completely. The mixture should be allowed to cool down at room temperature till the dispersion gets converted to a proniosomes.

Carriers used in the preparation of proniosomes

Maltodextrin:

It is a mixture of glucose, disaccharides and polysaccharides, obtained by the partial hydrolysis of starch. Maltodextrin is a flavourless, easily digested carbohydrate made from cornstarch. A maltodextrin is a short chain of molecularly linked dextrose (glucose) molecules, and is manufactured by regulating the hydrolysis of starch. A white or almost white, slightly hygroscopic powder or granules, freely soluble in water.

Sorbitol:

It is also known as glucitol, Sorbogem and Sorbo, is a sugar alcohol that the human body metabolizes slowly. It can be obtained by reduction of glucose,

changing the aldehyde group to a hydroxyl group. Sorbitol is found in apples, pears, peaches, and prunes. It is synthesized by sorbitol-6-phosphate dehydrogenase, and converted to fructose by succinate dehydrogenase and sorbitol dehydrogenase. Succinate dehydrogenase is an enzyme complex that participates in the citric acid cycle. Sorbitol is a sugar substitute.

Mannitol:

It is a white, crystalline organic compound. This polyol is used as an osmotic diuretic agent and a weak renal vasodilator. It was originally isolated from the secretions of the flowering ash, called manna after their resemblance to the Biblical food, and it is also referred to as mannite and manna sugar.²⁸

Formation of niosomes from proniosomes

Another method of producing niosomes is to coat a water-soluble carrier such as sorbitol with surfactant. The result of the coating process is a dry formulation in which each water-soluble particle is covered with a thin film of dry surfactant. This preparation is termed "Proniosomes". The niosomes are recognized by the addition of aqueous phase at $T > T_m$ and brief agitation.¹⁵

T = Temperature.

T_m = mean phase transition temperature

Maltodextrin-based proniosome provides rapid reconstitution of niosomes with minimal residual carrier. Slurry of maltodextrin and surfactant was dried to form a free-flowing powder, which could be rehydrated by addition of warm water.

Separation of Untrapped Drug

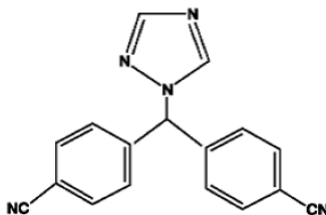
The removal of untrapped solute from the vesicles can be accomplished by various techniques, which include:^{11,15}

1. Dialysis
2. Gel Filtration
3. Centrifugation

DRUG PROFILE

1. LETRAZOLE²⁹

Letrozole is a Non Steroidal aromatase inhibitor (inhibitor of estrogens synthesis). It is chemically described as 4,4'-(1H-1,2,4-Triazol-1ylmethylene) dibenzonitrile. The structural formula is



Empirical formula : C₁₇H₁₁N₅

Description: White to yellowish crystalline powder, practically odourless.

Solubility: Freely soluble in dichloromethane, slightly soluble in ethanol, and practically insoluble in water.

Molecular weight : 285.31g/mol

Melting point : 184°C to 185°C

Indication

For the extended adjuvant treatment of early breast cancer in postmenopausal women who have received 5 years of adjuvant tamoxifen therapy. Also for first-line treatment of postmenopausal women with hormone receptor positive or hormone receptor unknown locally advanced or metastatic breast cancer. Also indicated for the treatment of advanced breast cancer in postmenopausal women with disease progression following antiestrogen therapy.

Pharmacodynamics

Letrozole is an aromatase inhibitors used in the treatment of breast cancer. Aromatase inhibitors work by inhibiting the action of enzyme aromatase which converts androgens to estrogens by a process called aromatization. As breast tissue

is stimulated by estrogens, decreasing their production is a way of suppressing recurrence of breast tumour tissue.

Mechanism of action:

Letrozole is a nonsteroidal competitive inhibitor of aromatase and thus, in postmenopausal women, inhibits conversion of adrenal androgens (primarily androstenedione and testosterone) to estrogens (estrone and estradiol) in peripheral tissues and cancer tissue. As a result, Letrozole interferes with estrogens-induced stimulation or maintenance of growth of hormonally responsive (estrogens and/or progesterone receptor positive or receptor unknown) breast cancers.

Pharmacokinetics

Absorption : Rapidly and completely absorbed. Absorption is not affected by food.

Distribution : Letrozole is weakly protein bound hence the volume of distribution ($V_{ol D}$) is approximately 1.9 liters per kg of body weight (high volume of distribution)

Biotransformation: Hepatic, by the CYP isoenzymes 3A4 and 2A6 (CYP 3A4 and CYP 2A6), to an inactive carbinol metabolite and its ketone analog.

Half-life : 2 days

Time to steady-state concentration : Plasma 2 to 6 weeks.

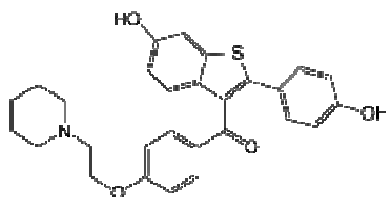
Note: Steady-state plasma concentrations are 1.5 to 2 times higher than would be predicted on the basis of single-dose measurements, indicating some nonlinearity in Letrozole pharmacokinetics with daily administration. However, steady-state concentrations are maintained for extended periods, without further accumulation of Letrozole.

Elimination: Renal, approximately 90% of a dose (approximately 75% as the glucuronide conjugate of the inactive metabolite, 9% as two unidentified metabolites, and 6% unchanged).

Dose: The recommended dose 2.5 mg administered once a day, without regard to meals.

2. RALOXIFENE³⁰

Raloxifene is a Selective estrogen-receptor modulator (SERM); high affinity for estrogen receptor. It is chemically described as (2-(4-Hydroxyphenyl)-6-hydroxybenzo(b)thien-3-yl) (4-(2-(1-piperidinyloxy)phenyl) methanone. The structural formula is



Empirical formula : C₂₈H₂₇NO₄S

Description : Crystalline powder, odourless.

Solubility: Soluble in organic solvents like ethanol, DMSO and dimethyl formamide (DMF)

Molecular weight : 473.583g/mol

Melting point : 143-147 °C

Category

Antineoplastic Agent

Endocrine-Metabolic Agent

Indication

For the prevention and treatment of osteoporosis in post-menopausal women, as well as prevention and treatment of corticosteroid-induced bone loss. Also for the reduction in the incidence of invasive breast cancer in postmenopausal women with osteoporosis or have a high risk for developing breast cancer.

Pharmacodynamics

Raloxifene, a selective estrogen receptor modulator (SERM) of the benzothiophene class, is similar to tamoxifen in that it produces estrogen-like effects on bone and lipid metabolism, while antagonizing the effects of estrogen on breast and uterine tissue. Raloxifene differs chemically and pharmacologically from naturally occurring estrogens, synthetic steroidal and nonsteroidal compounds with estrogenic activity, and antiestrogens. Estrogens play an important role in the reproductive, skeletal, cardiovascular, and central nervous systems in women, and act principally by regulating gene expression. When estrogen binds to a ligand-binding domain of the estrogen receptor, biologic response is initiated as a result of a conformational change of the estrogen receptor, which leads to gene transcription through specific estrogen response elements of target gene promoters. The subsequent activation or repression of the target gene is mediated through 2 distinct transactivation domains of the receptor: AF-1 and AF-2. The estrogen receptor also mediates gene transcription using different response elements and other signalling pathways. The role of estrogen as a regulator of bone mass is well established. In postmenopausal women, the progressive loss of bone mass is related to decreased ovarian function and a reduction in the level of circulation estrogens. Estrogen also has favourable effects on blood cholesterol.

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, thus slowing the rate of bone loss. The maintenance of bone mass by raloxifene and estrogens is, in part, through the regulation of the gene-encoding transforming growth factor- β 3 (TGF- β 3), which is a bone matrix protein with antiosteoclastic properties. Raloxifene activates TGF- β 3 through pathways that are estrogen receptor-mediated but involve DNA sequences distinct from the estrogen response element. The drug also binds to the estrogen receptor and acts as an estrogen agonist in preosteoclastic cells, which results in the inhibition of their proliferative capacity. This inhibition is thought to contribute to

the drug's effect on bone resorption. Other mechanisms include the suppression of activity of the bone-resorbing cytokine interleukin-6 promoter activity. Raloxifene also antagonizes the effects of estrogen on mammary tissue and blocks uterotrophic responses to estrogen. By competing with estrogens for the estrogen receptors in reproductive tissue, raloxifene prevents the transcriptional activation of genes containing the estrogen response element. As well, raloxifene inhibits the estradiol-dependent proliferation of MCF-7 human mammary tumor cells *in vitro*. The mechanism of action of raloxifene has not been fully determined, but evidence suggests that the drug's tissue-specific estrogen agonist or antagonist activity is related to the structural differences between the raloxifene-estrogen receptor complex (specifically the surface topography of AF-2) and the estrogen-estrogen receptor complex. Also, the existence of at least 2 estrogen receptors (ER α , ER β) may contribute to the tissue specificity of raloxifene.

Pharmacokinetics

Absorption : Approximately 60% of an oral dose is absorbed, but presystemic glucuronide conjugation is extensive. Absolute bioavailability of raloxifene is 2.0%.

Distribution : 2348 L/kg [oral administration of single doses ranging from 30 to 150 mg]

Biotransformation : Hepatic, by the CYP isoenzymes 3A4 and 2A6 (CYP 3A4 and CYP 2A6), to an inactive carbinol metabolite and its ketone analog.

Half-life : 27.7hrs

Metabolism:Hepatic, 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, providing strong evidence that raloxifene is not metabolized by cytochrome P450 pathways.

Elimination : Raloxifene is primarily excreted in feces, and less than 0.2% is excreted unchanged in urine.

Recommended Dose : The recommended dose 60 mg orally daily.

LITERATURE REVIEW

Carlotta Marianeciet al.,³¹ explained about the use of nanotechnology and during the last decades how the interest increased among the scientists regarding the formulation of surfactant vesicles, as a tool to improve drug delivery. Niosomes are self assembled vesicular nanocarriers obtained by hydration of synthetic surfactants and appropriate amounts of cholesterol or other amphiphilic molecules. In this review they updated composition, preparation, characterization/evaluation, advantages, disadvantages and application of niosomes.

S. Biswal et al.,³² presented an overview of theoretical concept of factors affecting niosome formation, techniques of niosome preparation, characterization of niosome, applications, limitations and market status of such delivery system. Niosomes exhibit more chemical stability than liposomes (a phospholipids vesicle) as non-ionic surfactants are more stable than phospholipids. Non-ionic surfactants used in formation of niosomes are polyglyceryl alkyl ether, glucosyl dialkyl ether, crown ether, polyoxyethylene alkyl ether, ester-linked surfactants, and steroid-linked surfactants and a spans, and tweens series.

N.B. Mahale et al.,³³ described history, all factors affecting niosome formulation, manufacturing conditions, characterization, stability, administration routes and also their comparison with liposome and given relevant information regarding various applications of niosomes in gene delivery, vaccine delivery, anticancer drug delivery. They explained how vesicular systems delivering drug in controlled manner to enhance bioavailability and get therapeutic effect over a longer period of time.

Hamdy Abdelkader et al.,³⁴ reviewed the recent advances in non-ionic surfactant vesicles, self-assembly, fabrication, characterization, drug delivery, applications and limitations.

Toshimitsu Yoshioka et al.,³⁵ formulated multilamellar vesicles of a series of sorbitan monoesters (Span 20,40, 60 and 80) and a sorbitan trioleate (Span 85)

using a mechanical shaking technique without sonication. 5(6)-Carboxyfluorescein(CF) was used as a model solute to investigate entrapment efficiency and release. Entrapment efficiency increased linearly with increasing concentration of lipid and most efficient entrapment of CF occurred with Span 60 (HLB 4.7).

Joseph V.M. et al.,³⁶ formulated the niosomes with a versatile anticancer drug such as Etoposide by ether injection method surfactant (tween 40 or 80), cholesterol and drug in 4 different ratios by weight, 1:1:1, 2:1:1, 3:1:1, & 4:1:1. The niosomes characterized for size, shape, entrapment efficiency, stability and *in vivo* release profile (by exhaustive dialysis).

V sankar et al.,³⁷ reviewed out different aspects related to proniosomes preparation, characterization, entrapment efficiency, *in vitro* drug release, applications and merits.

Ruckmani et al.,³⁸ encapsulated Zidovudine in proniosomes which reduced drug leakage from vesicles stored at room temperature and given targeted delivery of ZDV to macrophages in spleen and liver.

Hanan, El-Laithy et al.,³⁹ designed a novel sustained release proniosomal system for Vinpocetine using sugar esters (SEs) as non-ionic surfactants to avoid marked first-pass effect and improved its absorption and dissolution.

Sanjoy Kumar Dey et al.,⁴⁰ prepared and evaluated a biodegradable nanoparticulate system of Letrozole (LTZ) intended for breast cancer therapy. LTZ loaded poly (lactide-co-glycolide) nanoparticles (LTZ-PLGA-NPs) were prepared by emulsion-solvent evaporation method using methylene chloride and polyvinyl alcohol. LTZ-PLGA-NPs were characterized by particle size, zeta potential, infrared spectra, drug entrapment efficiency and *in vitro* release.

Nita Mondal et al.,⁴¹ incorporated Letrozole in nanoparticles which were prepared by solvent displacement technique and characterized by transmission electron microscopy, poly-dispersity index and zeta potential measurement.

Tuan HiepTrana et al.,⁴² tried to improve the physicochemical properties and bioavailability of a poorly water-soluble drug, raloxifene by solid dispersion (SD) nanoparticles using the spray-drying technique. These spray-dried SD nanoparticles were prepared with raloxifene (RXF), polyvinyl pyrrolidone (PVP) and Tween 20 in water. The improved dissolution of raloxifene from spray-dried SD nanoparticles appeared to be well correlated with enhanced oral bioavailability of raloxifene in rats.

Tara Pritchard et al.,⁴³ validated SMA-raloxifene for the management of CRPC using a mouse xenograft model. Biodistribution of raloxifene was 69% higher in tumours following SMA raloxifene compared with free raloxifene.

AIM OF THE WORK

The aim of the present research work is to develop stable Letrozole loaded proniosome formulation and stable Raloxifene loaded proniosome formulation and to evaluate *in vitro* characteristics and *in vivo* pharmacokinetic parameters of prepared formulations.

OBJECTIVES OF THE WORK

Cancer is a leading cause of death worldwide. Worldwide in 2012, an estimated 14.1 million new cases of cancer occurred and an estimated 8.2 million people died from cancer. Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030. The current focus in development of cancer therapies is on targeted drug delivery to provide therapeutic concentrations of anticancer agents at the site of action and spare the normal tissues. Worldwide, breast cancer is the second most common type of cancer after lung cancer. It is the primary cause of cancer death among women globally, responsible for about 40000 US women deaths in 2001.

Aromatase inhibitors are used for the treatment of breast cancer in postmenopausal women. Letrozole is the hormonal anticancer drug which can be employed to treat aromatase dependent breast cancer. Aromatase is an enzyme that catalyses biosynthesis of estrogen from testosterone (androgen). Letrozole is potent & selective inhibitor of aromatase. It inhibits the production of estrogens in postmenopausal women. It works by blocking cytochrome P-450 (CYP) which turns the hormone androgen to small amount of estrogen in the body. This means that less estrogen is available to stimulate the growth of hormone receptor positive breast cancer cells. It does not stop the ovaries from making estrogen, therefore, aromatase inhibitors affects only on postmenopausal females.

Raloxifene hydrochloride is a non-steroidal benzothiophene, is a second-generation selective oestrogen receptor modulator that binds to oestrogen receptors. It has mixed pharmacological action. It shows oestrogen agonist effects on bone and the cardiovascular system and oestrogen antagonist effects on endometrial and breast tissue. Raloxifene hydrochloride is poorly soluble drug as it belongs to class

II category according to BCS classification. Raloxifene hydrochloride has oral bioavailability of only 2% owing to extensive first pass metabolism. Therefore, it is necessary to increase the solubility and dissolution rate of Raloxifene hydrochloride which leads to improvement in oral bioavailability. Enhancement in oral bioavailability can be achieved by reducing the hepatic first pass metabolism. Such problem with conventional dosage form can be minimized by any suitable novel drug delivery system.

The objective of present work was to utilize potential of novel drug delivery system for improvement in oral bioavailability of Raloxifene hydrochloride and the most important goal of cancer chemotherapy is to minimize the exposure of normal tissues to drugs while maintaining their therapeutic concentration in tumors. Now the pharmaceutical formulators are looking for vehicles through which drugs can be delivered to the specific target. One of the vehicles which can be employed to deliver the drug to specific site is niosome.

- i. Niosomes can be used to vesiculate both hydrophilic and lipophilic drugs.
- ii. Niosomal vesicles are composed of non-ionic surfactant with/without cholesterol or other lipids.
- iii. Niosomes have lower toxicity due to non-ionic nature of the surfactant and act to improve the therapeutic index of the drug

A number of vesicular drug delivery systems such as liposomes, niosomes, transferosomes, and ethosomes and proniosomes were used to target the drug. Among these vesicular systems proniosomes gains more attention because of their advantages such as targeting the drugs to the specific sites, greater physical and chemical stability during sterilization and storage, entrap both hydrophilic and hydrophobic drugs, ease of transfer, distribution and dosing. The main objective of the present study was to formulate & evaluate letrozole proniosome and raloxifene proniosome.

SCOPE OF THE PRESENT WORK

To provide an ideal drug delivery system of anticancer drugs in order to maintaining the therapeutic plasma concentration for a required period of time.

To provide the proniosomal drug delivery system

- a) For the patient compliance
- b) Effectiveness of anticancer therapy
- c) Reduction of adverse effect.

This is achieved by maintaining the plasma drug concentration at the level with in therapeutic range for the required period of time.Hence it is absolute necessity to develop effective drug delivery system with minimum dose for reducing undesired side effects.

MATERIALS AND INSTRUMENTS USED

Letrozole was obtained from Sun pharmaceuticals Advanced Research Centre, Vadodara, India;Raloxifene was obtained from Cipla Ltd, India, Cholesterol, Span 20, Span 60, Maltodextrin was purchased from S.D. Fine Chem Ltd, Mumbai. All other chemicals and solvents are analytical grade.

The instruments which used for this experiment were Rotary flash evaporator (Super fit, India), Electronic digital balance (Shimadzu, Japan), Dialysis membrane 50 (Hi media, India), Digital pH meter (ELICO, India), Double beam UV/ Visible spectrophotometer (Lab India), Zetasizer (Malvern, England), Probe sonicator (Electro sonic Industries, India), Trinocular Optical microscope (Olympus, Japan), Scanning electron microscope (Hitachi, Japan), Refrigerated Centrifuge (Plasto Crafts Industries Private Limited, India), FTIR Spectrophotometer (Bruker Alpha-E), Differential Scanning Calorimeter (Shimadzu, Japan) and High Performance Liquid Chromatography (Shimadzu, Japan).

EXPERIMENTAL PROTOCOL

- a) Procurement of drugs, surfactants, cholesterol and other excipients for formulation development
- b) Preformulation study
 - i. Solubility
 - ii. Characterization of the drug, excipients and its mixture using melting point determination, UV spectroscopy, Infrared spectroscopy and differential scanning calorimetry.
 - iii. Preparation of calibration curve of drug
- c) Primary development of trial batches to establish the required profiles
- d) Physico chemical evaluation of prepared formulations
- e) Selection of best formulation in each category based on the evaluation
- f) *In vitro* drug release study

- g) Stability study of best formulation
- h) Pharmacokinetic study of best formulation

Preformulation study

Solubility is one of the most important physicochemical properties studied during pharmaceutical preformulation. For liquid dosage form development, accurate solubility data are essential to ensure the robustness of the finished product. For solid dosage forms, solubility data are important in determining if an adequate amount of drug is available for absorption *in vivo*. If a compound has a low aqueous solubility, it may be subject to dissolution rate-limited or solubility-limited absorption within the gastrointestinal (GI) residence time.

The drug-excipient compatibility is holding the major role for a formulator to prepare therapeutically effective formulations. This pre formulation study was carried out using FTIR spectroscopic determination of drug molecule alone and combination of drug molecule with all excipient used in the formulation process.

Differential Scanning Calorimetry was performed to study the thermal behavior of drug alone, Span 60, Span 20, cholesterol, maltodextrin, and two selected proniosome powder formulations.

Calibration Curve of Letrozole

UV/Visible spectrophotometry:

The weighed quantity 50 mg of Letrozole was placed in 50ml standard measuring flask. Letrozole was dissolved by using 10ml of ethanol and the volume was made up to the mark. The aliquots were prepared to obtain the concentrations of 2, 4, 6, 8, 10 and 12mcg/ml using the above solution. The samples were analysed using UV/Visible spectrophotometer at 240 nm.

HPLC method:

The analysis was carried out on a reversed-phase C18 (250 mm × 4.6 mm, 5 µm) column with an isocratic mobile phase of methanol-water (70:30,v/v),

at a flow rate of 1.0 mL min⁻¹. Detection was carried out at 239 nm with a UV-visible spectrophotometric detector.

Calibration Curve of Raloxifene hydrochloride

UV/Visible spectrophotometry:

The weighed quantity 50 mg of Raloxifene hydrochloride was placed in 50ml standard measuring flask. Raloxifene hydrochloride was dissolved by using 10ml of methanol and the volume was made up to the mark. The aliquots were prepared to obtain the concentrations of 2, 4, 6, 8, 10 and 12mcg/ml using the above solution. The samples were analysed using UV/Visible spectrophotometer at 289 nm.

HPLC method:

The mobile phase consisted of a mixture of buffer (pH7.4)-acetonitrile (60:40 v/v). The flow rate was set to 0.8 ml /min, Injection volume 20µl, The Column used is C18 (150mm). The detection wavelength was set to be at 287 nm. RP-HPLC analysis was performed isocratically at room temperature.

Formulation of Letrozole/Raloxifene loaded proniosomes

Proniosomes were prepared by the slurry method. The required quantity of span-20/span-60, cholesterol and drug were dissolved in chloroform in a 100ml round bottom flask containing the required quantity of maltodextrin carrier. Additional chloroform was added to form slurry in the case of lower surfactant loading. The flask was attached to a rotary flash evaporator to evaporate solvent at 60 rpm, a temperature of 45 ± 2°C, and a reduced pressure of 600mmHg until the mass in the flask had become a dry, free flowing product. These materials were further dried overnight in a desiccator under vacuum at room temperature. This dry preparation is referred to as 'proniosomes' and was used for preparations and for further study on powder properties. These proniosomes were stored in a tightly closed container at refrigerator temperature until further evaluated.

Evaluation of proniosomes

Angle of repose:

The angle of repose of dry proniosomes powder was measured by a funnel method⁴⁴. The proniosomes free flowing powder was poured into a funnel which was fixed at a position so that the 13mm outlet orifice of the funnel is 5cm above a level black surface. The powder flows down from the funnel to form a cone on the surface and the angle of repose was then calculated by measuring the height of the cone and the diameter of its base with the help of calibrated scale.

Optical Microscopy:

The proniosomes derived niosomes were mounted on glass slides and viewed under a microscope for morphological observation after suitable dilution. The photomicrograph of the preparation was obtained from the microscope by using a digital camera.

Vesicle size:

The vesicle dispersions were diluted about 100 times in the same solvent medium used for their preparation. Vesicle size was measured on a particle size analyzer.

Zeta Potential:

The particle charge was one of the most important parameter in assessing the physical stability of any colloidal dispersion. The large number of particles was equally charged, then electrostatic repulsion between the particles was increased and thereby physical stability of the formulation was also increased. Zeta potential analysis was done for determining the colloidal properties of the prepared formulations. The diluted proniosome derived niosome dispersion was determined using zeta potential analyzer based on electrophoretic light scattering and laser Doppler velocimetry method. The temperature was set at 25°C. Charge on vesicles and their mean zeta potential values were obtained directly from the measurement.

Entrapment efficiency:

Entrapment efficiency of the proniosomes derived niosomal dispersion was be done by separating the untrapped drug by dialysis method and the drug remained entrapped in niosomes was determined by complete vesicle disruption using 0.1% Triton X-100 and analyzed spectro photometrically for the drug content after suitable dilution with pH7.4 phosphate buffer and filtered through what mann filter paper. The percentage of drug encapsulation (EE (%)) was calculated by the following equation:

$$EE \% = [(C_t - C_r) / C_t] \times 100\%$$

Where C_t is the concentration of total drug

C_r is the concentration of free drug

Scanning electron microscopy (SEM):

The scanning electron microscopy (SEM) is one of the most important instrument used for analysis of surface morphology. The particle size of proniosomes is a very important characteristic. The surface morphology such as roundness, smoothness, and formation of aggregates and the size distribution of proniosomes were studied by Scanning Electron Microscopy (SEM). Proniosomes were sprinkled onto the double- sided tape that was affixed on aluminum stubs. The aluminum stub was placed in the vacuum chamber of a scanning electron microscope.

In vitro drug release study:

This study was carried out using open end cylinder method. One end of the tube is tightly covered with a Himedia dialysis membrane. The proniosome powder was placed over the membrane in the donar chamber. The donar chamber is then lowered to the vessels of the glass beaker containing 100 ml of phosphate buffer (pH 7.4) which act as a receptor compartment so that the dissolution medium outside and the vesicles preparation inside were adjusted at the same level. The release study was carried out at $37 \pm 0.5^\circ\text{C}$, and the stirring shafts were rotated at a speed of 50 rpm. Five millilitre samples were withdrawn periodically at predetermined time

intervals. Every withdrawal was followed by replacement with fresh medium to maintain the sink condition. The samples were analysed spectro photometrically.

Drug release kinetic data analysis:

The release data obtained from various formulations were studied further for their fitness of data in different kinetic models like Zero order, first order, Higuchi's and korsmeyer-peppas's. In order to understand the kinetic and mechanism of drug release, the result of *in vitro* drug release study of the prepared proniosome was fitted with various kinetic equation like zero order (cumulative % release vs. time), first order (log cumulative % remain vs time) and higuchi's model (cumulative % drug release vs. square root of time).

To understand the release mechanism *in vitro* data was analyzed by korsmeyer-peppas's model (log cumulative % drug release vs. log time) and the exponent n was calculated through the slope of the straight line. $M_t / M_\infty = b t^n$, Where M_t is amount of drug release at time t , M_∞ is the overall amount of the drug, b is constant, and n is the release exponent indicative of the drug release mechanism. If the exponent $n = 0.5$ or near, then the drug release mechanism is Fickian diffusion, and if n have value near 1.0 then it is non-Fickian diffusion. R^2 values were calculated for the curves obtained by regression analysis of the above plots.

Stability of proniosomes:

The optimized batch was stored in airtight sealed glass vials at different temperatures. Surface characteristics and percentage drug retained in proniosomes and parameters for evaluation of the stability, since instability of the formulation would reflect in drug leakage and a decrease in the percentage drug retained. The proniosomes were sample at regular intervals of time (0, 1, 2 and 3months), observed for colour change, surface characteristics and tested for the percentage drug retained after being hydrated to form niosomes and analyzed by spectrophotometer.

Formulation size, shape and number of vesicles per cubic mm were measured before and after storing for 30 days. Light microscope is used for the determination of size of vesicles and the numbers of vesicles per cubic mm was

measured by haemocytometer. Number of niosomes per cubic mm = Total number of niosomes x dilution factor x 400/Total number of small squares counted.⁴⁵

Pharmacokinetic study

Male rabbits (weighing 1.5–2 kg) were used for the bioavailability study. Animals were housed in the standardized conditions at the animal house of the JKK Nattraja College of Pharmacy. All animals were acclimatized and kept under constant temperature ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$). All animal procedures were performed in accordance to the approved protocol for use of experimental animals set by the standing committee on animal care of the JKK Nattraja College of Pharmacy. Animals were divided into three groups of three rabbits in each group. The study was designed as a single oral dose.

Group 1 not received any drug (Control)

Group 2 received an equivalent of 1mg Letrozol/kg body weight of rabbits.

(Or)

Group 2 received an equivalent of 30mg of Raloxifene Hcl/kg body weight of rabbits.

Group 3 received drug loaded proniosomal powders (the best formulation that exhibited the maximum EE% and the slowest release rate).

Blood samples (about 1 ml) were withdrawn from the sinus orbital into heparinized tubes at 0, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24h after each administration. The blood samples were centrifuged immediately at 3000 rpm for 10 min to obtain the plasma samples and were stored at -20°C for subsequent assay using HPLC.

Pharmacokinetic analysis

Maximum plasma drug concentration (C_{max}), area under the plasma drug concentration-time profile (AUC), the area under first moment curve (AUMC), the elimination half life ($t_{1/2}$), the mean residence time (MRT) and other pharmacokinetic parameters were evaluated using PK solver MS Excel Add-in programme[®].

OBSERVATION AND INFERENCE

In the present study, Letrozole/Raloxifene loaded proniosomes prepared by slurry method with different ratio of surfactant to cholesterol. In the FT-IR study all characteristic peaks obtained from pure drug were appeared in Cholesterol, maltodextrin physical mixture and maltodextrin based proniosome spectra, which indicates no remarkable change in their position after successful method of preparation. This revealed that there is no chemical interaction and stability of drug during whole method of preparation. The DSC thermograms showed there were no physical or chemical interaction

The angle of repose of proniosome powder and the physical mixture of drug along with maltodextrin powder were compared which showed that the proniosome powder had smaller angle of repose than physical mixture of drug along with maltodextrin powder. This is due to the smooth surface of proniosome powder which is consistent with the scanning electron microscopic observation. By increasing the concentration of surfactant the angle of repose decreases. Scanning electron microscopy shows the porous surface of the pure maltodextrin particles, this makes them effective carrier and provides more surface area for the coating of the surfactant mixture. Coating of the surfactant mixture on the carrier particles could be shown by the SEM images of proniosome. SEM shows the particle size of various formulations varied due to variation in the composition of formulations and the mean particle size in the range of $59 \pm 0.64\mu\text{m}$ to $106 \pm 0.72\mu\text{m}$ in case of letrozole and $46 \pm 0.32\mu\text{m}$ to $89 \pm 0.62\mu\text{m}$ in case of raloxifene.

From optical microscopy it was observed that niosomes formed was multilamellar spherical with a few being slightly elongated. The smaller size may result from efficient hydration of a uniform and thin film of surfactant mixture at low surfactant loading, compared with higher surfactant loading.

The entrapment efficiency was found to be in the range of 42.26 ± 0.02 to $83.64 \pm 0.12\%$ in case of letrozole and $40.41 \pm 0.06\mu\text{m}$ to $82.44 \pm 0.28\mu\text{m}$ in case of raloxifene. Higher surfactant concentration shows the higher entrapment efficiency which might be due to the high fluidity of the vesicles. Cholesterol content also hit the drug entrapment efficiency. The higher entrapment may be explained by high

cholesterol content. There are reports that entrapment efficiency was increased, with increasing cholesterol content and by the usage of span-60 which has higher phase transition temperature. The larger vesicle size may also contribute to the higher entrapment efficiency. Cholesterol is known to abolish the gel-to-liquid phase transition of niosomes which makes the niosome less leaky.

The zeta potential analysis was performed to get information about the surface properties of the niosome derived from proniosomes. Zetapotential is an important parameter to maintain stability of niosomes. Colloidal particle with zeta potential around ± 30 mv is physically stable. The zeta potential of best formulation was found to be +36.32 in case of Letrozole loaded niosomes and +22.3 in case of raloxifene loaded niosomes derived from proniosome. It indicates that the excellent stability of the formulation.

In vitro release of the proniosomes loaded with Letrozole/raloxifene were studied in the 0.1 N HCl (pH 1.2) and phosphate buffer pH 7.4 respectively. Result shows that drug release decreases by increasing the cholesterol content. Letrozole niosomes of formulation LS207 prepared by Span20 shows 99.34% of the drug release at 18th hour, formulation LS604 prepared by Span 60 shows 98.05% of drug release at 24th hour. Raloxifene niosomes of formulation RS210 prepared by Span20 shows 98.68% of the drug release at 18th hour, formulation RS606 prepared by Span 60 shows 97.42% of drug release at 24th hour. The niosomal system is remain for further drug release.

The *in vitro* release data was applied to various kinetic models like zero order kinetics, Higuchi's plot and Peppas's plot to predict the drug release kinetic mechanism. The zero order plots of formulations were found to be fairly linear as indicated by their high correlation values. Therefore, it was ascertained that the drug release from all the formulation followed either near zero or zero order kinetics. Correlation values of Higuchi's plot revealed that the mechanism of drug release is diffusion. Korsmeyer-Peppas plot slope values (release exponent) revealed the fact that the drug release follows super case I transport diffusion. Based on the entrapment efficiency, release study and kinetic modeling formulation LS604 and RS606 were subjected for stability study and *in vivo* pharmacokinetic study. In

stability study there was no remarkable change in the drug content. The test of significance applied for stability data which shows that there is no significance difference ($P>0.05$) between the stability data of formulation. The pharmacokinetic data obtained from *in vivo* study shows better bioavailability when compared with pure Letrozole or Raloxifene.

SUMMARY AND CONCLUSION

Currently proniosomes have been studied by researcher as a choice of oral drug delivery system for anticancer drugs to provide a better oral bioavailability considering, high penetration property of the niosome encapsulated agents through biological membrane and the stability of them.

Cancer is a leading cause of death worldwide. The four most common cancers occurring worldwide are lung, female breast, bowel and prostate cancer. Breast cancer is the most frequently diagnosed cancer and the primary cause of cancer-related death in women worldwide. Aromatase inhibitors and selective oestrogen receptor modulator are used for the treatment of breast cancer in postmenopausal women. The most important goal of cancer chemotherapy is to minimize the exposure of normal tissues to drugs while maintaining their therapeutic concentration in tumors.

Proniosomes proved to be the potential carriers for efficient oral delivery of lipophilic or amphiphilic drugs. Henceforth an attempt was made to improve the oral delivery of Letrozole and Raloxifene by loading into maltodextrin based proniosome powders separately.

Letrozole loaded maltodextrin based proniosomes and Raloxifene loaded maltodextrin based proniosomes were prepared by slurry method with different ratio of span20,span60 and cholesterol and evaluated for micromeritic properties and the results indicate acceptable flow properties.

The formation of niosomes and surface morphology of optimized proniosome formulations were studied by optical and scanning electron microscopy, respectively which has showed smooth surface of proniosome. FT-IR, differential scanning calorimetry studies performed to understand the solid state properties of the drug reveal the absence of chemical interaction, drug transformation from crystalline to amorphous and molecular state. Further evaluated for entrapment efficiency, *in vitro* release, kinetic data analysis, stability study and pharmacokinetic analysis. The formulation LS604, RS606 which showed higher entrapment efficiency and *in vitro* releases of 98.05% and 97.42% respectively at the end of

24 hours was found to be best among all formulations. The drug release was explained by zero order kinetics. The stability study results showed that the prepared proniosome formulations were stable. The pharmacokinetic data obtained from *in vivo* study shows better bioavailability when compared with pure Letrozole and Raloxifene.

In conclusion, we can state that, besides providing the controlled systemic delivery of Letrozole and Raloxifene, an attempt was made to prepare proniosomal drug delivery system and evaluate its performance. Proniosome provides an effective means of delivering the drug through the oral route. The stable proniosome formulation was prepared and it is highly successful in enhancing oral bioavailability of the drug. Thus a dry free flowing product like proniosomes will be a promising industrial product.

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