Development of formulations for uterine targeting of some drugs via vaginal route -a non -invasive method for treatment of Endometrial Cancer

A brief synopsis submitted to

The Maharaja Sayajirao University of Baroda

In partial fulfilment for the award of degree of

DOCTOR OF PHILOSOPHY

in

PHARMACY

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FEBRUARY 2018

INTRODUCTION

Uterine Disorders

Poor hormonal control over uterus and/or malfunctioning of endometrial linings of the uterus may result in abnormal uterine conditions - termed as uterine disorders. Abnormal vaginal bleeding, dysmenorrhea and menorrhagia are the key signs and symptoms of the uterine disorders.

The most common types of uterine disorders are: (1, 2)

Non-cancerous uterine conditions

- Uterine fibroids
- Endometriosis
- Adenomyosis
- Uterine polyps
- Endometrial hyperplasia

Uterine cancers

- Endometrial cancer
- Cervical cancer
- Uterine sarcomas

Endometrial cancer (EC)

Statistics

Endometrial cancer is the sixth most common type of cancer of the female reproductive organs worldwide, with 320,000 new cases diagnosed in 2012. About 53 percent of EC cases occurred in more developed countries. The peak prevalence of endometrial cancer was in Northern America and Europe; and the lesser incidence in Africa and Asia. The American Cancer Society has estimated 63,230 new cases and 11,350 deaths in the United States in the year 2018. (3, 4).

Risk factors

Prolonged unopposed estrogen exposure is associated with most endometrial cancers. Estrogen replacement therapy prescribed to control menopausal symptoms increases the risk of developing endometrial cancer by 2 to 20 fold, with an increasing risk correlating with the duration of use (5). Long-term exposure to the endogenous estrogen, as happens in estrogen-producing tumors, and with enhanced peripheral conversion of androgens to estrone in adipose tissue, is also linked with an increased risk for developing endometrial hyperplasia

and cancer. Apart from these factors, high blood pressure and diabetes mellitus may increase the risk of endometrial cancer (6).

Tamoxifen (a selective estrogen receptor modulator) is extensively used for the treatment of breast cancer. It works as an estrogen antagonist in breast tissues and as an agonist in bone and endometrial tissues(7). The use of tamoxifen is associated with a 6 to 8 fold surge in the occurrence of endometrial cancer (8). Age also represents a significant risk factor for developing endometrial cancer. Most women are diagnosed after menopause, with only 15% diagnosed before the age of 50 years and only 5% before 40 years of age (9). Nulliparous and obese younger women are more likely to develop EC and have well differentiated endometrioid histology than older women (10, 11).

Treatment Modalities

Current therapies available for EC management are medical, surgical, radiological, and genetic modalities. Surgical procedure includes hysterectomy (removal of the uterus). The surgical procedures include removal of the uterus, fallopian tubes, and ovaries. Pelvic lymph nodes may also be removed and/or sampled to be examined for the spread of the cancer. Depending on the spread of the cancer, other treatments, such as radiation and/or chemotherapy are recommended. The invasive surgical procedure has its own complications. Hysterectomy is not an appropriate option for women who wants to have pregnancy or wants to avoid surgery. If the cancer is not benign, then surgery may not be helpful, and so chemotherapy or other treatments may be used (12).

Chemotherapy

The International Federation of Gynecology and Obstetrics (FIGO) staging systems, classified endometrial cancer in four classes. Chemotherapy is used as an effective treatment option at all the stages of EC, either as an adjuvant or main therapy (13-16). Different treatment regimens containing various combinations of chemotherapeutic agents to treat EC are in practice. The combination therapy of Paclitaxel and Carboplatin is most widely used treatment considering its advantages over other combination chemotherapies. The results of clinical studies suggest that Paclitaxel and Carboplatin has significant activity in advanced endometrial cancer with minimal side effects. 3 year progression free survival and overall survival rates were 50% and 75%, respectively, in Paclitaxel and Carboplatin receiving group and 37.5% and 50%, respectively, in the cisplatin, doxorubicin, and cyclophosphamide receiving group (17-20).

Mechanism of Action

Paclitaxel - It binds specifically to β-tubulin present in the microtubules and stabilizes it. Paclitaxel causes mitotic arrest since, the microtubules appear in the M phase of the cell cycle. Thus, chromosomes are not able to attain a metaphase spindle configuration. This blocks advancement of mitosis, and prolonged triggering of the mitotic checkpoint activates apoptosis or return to the G-phase of the cell cycle without cell division (21).

Carboplatin – Inside the cell, carboplatin undergoes hydrolysis of 1,1-cyclobutane dicarboxylate ring, becoming positively charged. This allows carboplatin to interact with nucleophilic molecules within the cell, including DNA, RNA and protein, which results into the formation of platinum adducts. This process occurs through covalent binding of carboplatin to the N7 site of purine bases, forming DNA-protein or DNA-DNA complexes. These complexes prevent DNA replication and transcription, results in breaks and miscoding, and if identified by p53 and other checkpoint proteins, induces apoptosis (22).

Presently available forms of Paclitaxel and carboplatin and limitations thereof

Paclitaxel- It is available in the market in the form of Paclitaxel Injections. As Paclitaxel is highly hydrophobic molecule, lipid-based solvents are used as a vehicle. Solubility of paclitaxel is enhanced using a mixture of 50:50 Cremophor EL® (CrEL, a non-ionic surfactant polyoxyethylated castor oil; BASF, Florham Park, NJ, USA) and ethanol. Before administration via intravenous infusion, it must be further diluted 5 to 20 fold with normal saline or 5% dextrose solutions.

Cremophor EL is biologically and pharmacologically active and leaches plasticizers from standard intravenous tubing, releasing di-(2-ethylhexyl) phthalate (DEHP). Its infusion produces histamine release with consequent well-described hypersensitivity reactions, including anaphylaxis. Moreover it has been also associated with hyperlipidemia, abnormal lipoprotein patterns, aggregation of erythrocytes, and prolonged, sometimes irreversible sensory neuropathy which may be associated with demyelination and axonal degeneration. CrEL can also cause neutropenia (23).

A different type of marketed formulation is Albumin bound paclitaxel (Abraxane®; AbraxisBioScience and AstraZeneca). It works on the principle of EPR assisted uptake of nanovectors. Various nanotechnologies are also studied *viz* liposomes, dendrimers, super paramagnetic nanoparticulates, polymer-based platforms, gold nanoshells, silicon- and silica-based nanoparticles, carbon-60 fullerenes, and nanocrystals. All these preparations are IV administered and thus associated with diverse chemotherapeutic side effects such as Low

blood counts, Alopecia, Peripheral neuropathy, Arthralgias and myalgias, Abnormal ECG, Weakness and fatigue etc.

Carboplatin-

Carboplatin possess good solubility in water (14mg/ml). On account of its water solubility, various IV injectable formulations available in the market. Various nano-carrier based systems i.e. Nanoparticles (24), Liposomes (25), Nanocapsules (26) etc, are reported in literatures in order to achieve tumor targeting and to reduce systemic side effects. However, due to IV administration of these formulations, non-specific distribution of the drug and nano-carrier system occurs, which ultimately leads to more systemic side effects.

Paclitaxel and carboplatin intravenous injections are administered as combination chemotherapy. Carboplatin dose is calculated as per the Calvert formula to achieve desired AUC and administered via IV route with paclitaxel 175 mg/m^{2}over 3 hours. Cycle is repeated every 3 weeks for 6 to 9 cycles (27). Intravenous chemotherapy causes phlebitis at the site of injection that leads to poor patient compliance and more typical chemotherapeutic systemic side effects related with the drug and/or excipients.

Importance of Tumor Targeting

The long-standing problem of chemotherapy is the lack of tumor-specific treatments. Conventional chemotherapy depend on the premise that quickly proliferating cancer cells are more likely to be killed by a cytotoxic agent. In reality, however, cytotoxic agents have very less or no specificity, which leads to systemic toxicity, causing severe side effects. Therefore, various “molecularly targeted cancer therapies” have been developed for use in specific cancers.

Approaches for targeting

Passive targeting

Administered nanocarriers accumulate in tumors mostly because of the enhanced permeability and retention effect (EPR effect), which is due to angiogenic processes that produce highly permeable blood vessels in tumors and their characteristic abnormal lymphatic drainage that leads to the accumulation of the nano-scale particles in them, allowing the release of the cytotoxic drugs close to the tumor cells. Nanocarriers of size range 100-200 nm with prolonged blood circulation time, leads to effective targeting via EPR effect (28, 29).

Active targeting

Active targeting is achieved by attaching specific ligands to the nanocarrier structures, allowing a selective recognition of different receptors or antigens overexpressed in the tumor cell surfaces, increasing the cytotoxicity of the anticancer agents in tumors and avoiding most of their side effects, since the exposure of healthy cells to the drug is minimized (30). Various methods are used to target cancer cells e.g. Folate conjugated systems (31), Albumin bound systems (32), Hyaluronic acid based targeting systems (33), Transferrin based targeting systems (34), mAbs and Peptidic targeting systems (35, 36) etc.

Uterine targeting

Endometrial canceris associated with a 50% relapse rate and had a 5-year survival of 18–27%. Moreover, recent studies shows that, the survival of women with stage I–II EC is 35–50% and for stage III–IV EC is 0–15%(37). These figures illustrate the dire need for a deeper understanding of the methods for uterine targeting for the treatment of EC, as well as the necessity to develop novel and more effective therapeutic modalities against recurrent disease. Uterine targeting may represent a reasonable and innovative approach for the treatment of Endometrial Cancer refractory to standard treatment modalities (38).

Vaginal Route for drug administration

The vaginal route for drug administration have been well established through its long and well-studied history of safety and efficacy. Drugs are easily and rapidly absorbed through the vaginal epithelium, and there are no adipose tissue or other cell layers with metabolic enzymes to traverse as with the transdermal or oral routes. The GI tract and hepatic first-pass effects are avoided. Vaginal administration allows low, continuous dosing, which results in stable drug levels and may, in turn, achieve a lower incidence of side effects and improve patient compliance. Various vaginal formulations such as pessaries, vaginal films, foams and gels have been used to administer drugs using this route. However, anatomical structure of vaginal canal does not allow these formulations to stay longer in its place. The most common problem is leakiness of the vaginal route. However, vaginal ring technology makes drug administration easy and discreet for patients, giving them complete control over the treatment method and its reversibility (39).

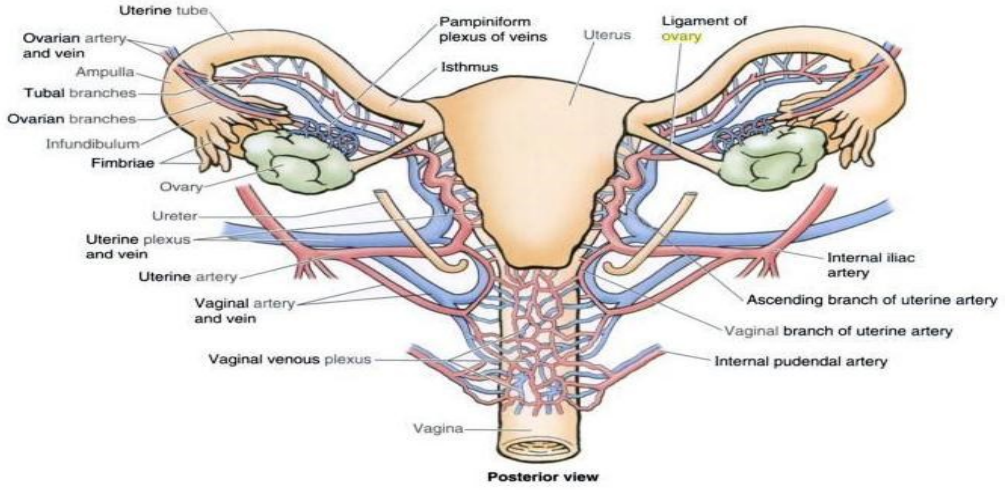
First Uterine Pass Effect

In recent years, the existence of direct transport mechanisms between the vagina and uterus has been demonstrated, resulting in preferential uterine delivery of hormones that are administered vaginally (40). By analogy to hepatic effects seen after oral administration, this phenomenon is termed as the first uterine pass effect (41).

This locally functional "portal" system occurs through four proposed mechanisms by which drug can pass from vagina to uterus (42).

- Diffusion through tissues
- Passage through the cervical lumen from vagina to the uterus
- Transport via venous or lymphatic circulatory system
- Countercurrent vascular exchange with diffusion between utero-vaginal veins and/or lymph vessels and arteries.

The passive targeting of the drug could also be assisted by the EPR effect.



Advantages of First Uterine Pass Effect (41)

- Targeting the drugs directly to the site of action and hence achieving maximum therapeutic effect by lowest possible dose
- Reduction in side effects of drugs by avoiding the systemic absorption
- Increasing the patient compliance by avoiding painful injectable route and also self-medication being made possible by vaginal administration
- Passive targeting to uterus can be possible

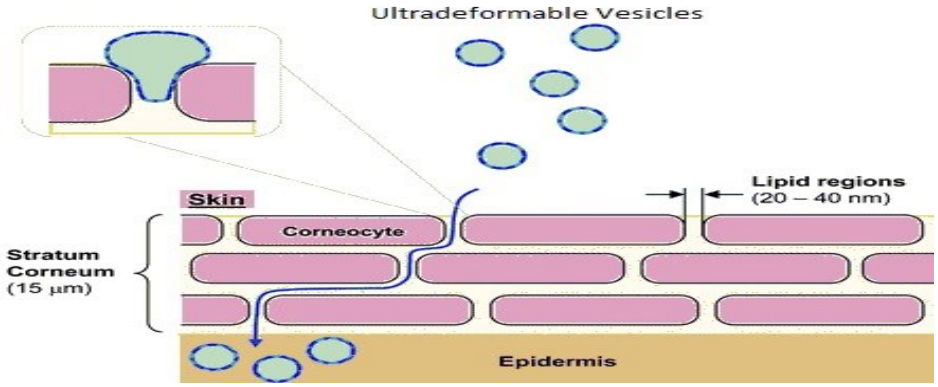
Intra-vaginal drug delivery leads to drug targeting to the uterus by the means of functional mechanism called First Uterine Pass Effect. The drug can be targeted to uterus when administered intra vaginally.

Formulation Development

Ultra deformable vesicles (UDVs)

Ultradeformable vesicles comprising phospholipids and an edge activator (EA) is a novel formulation in vesicular drug delivery. UDVs, by the virtue of their enhanced elasticity compared to the conventional liposomes, are more amenable to the transport of therapeutic agents across the biological membranes (43). Elasticity in these vesicles is attributed to the

presence of an EA, which is generally a single chain surfactant with a high radius of curvature, capable of weakening the lipid bilayers of the vesicles and increasing their deformability. During destabilization and owing to their affinity for curved configurations, surfactants attempt to gather at the location of enhanced pressure and consequently lead to a decrease in the energy needed for the change of shape. The maximum deformability is obtained when the vesicular membrane attempts to optimize its local composition in response to an external, spatially anisotropic stress (44).



Properties and applications of UDVs (45)

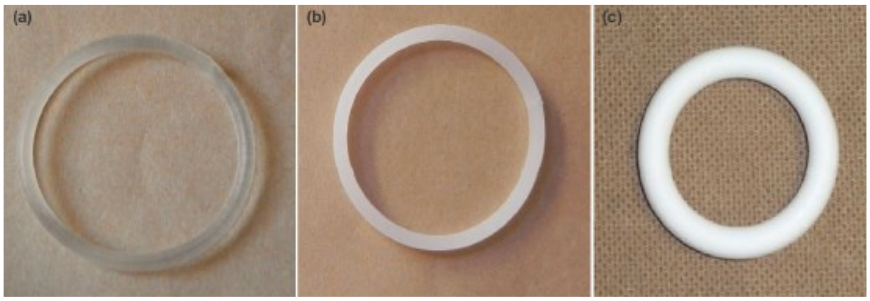
- They are able to transport therapeutic agents through very narrow pathways between most cells in the biological membranes (e.g. Stratum Corneum).
- Unlike Nanoparticles, Liposomes and other nano-carrier systems, UDVs are able to penetrate vaginal mucus through low viscosity channels.
- UDVs are biocompatible, biodegradable and are capable of protecting the encapsulated drug from metabolic degradation.
- UDVs have also been used as transporters for various macromolecules, including peptides, proteins (insulin, albumin), DNA, antigens, corticosteroids etc, and have been proven to significantly augment amount of drug permeated.
- With their great penetration abilities, UDVs offer a non-invasive means of drug delivery.

Intra Vaginal Rings (IVRs)

The vaginal route has been extensively studied as a site for drug administration. As a consequence, a novel controlled release system known as intra vaginal ring was developed. Vaginal rings are torus shaped devices formed by a polymeric material (e.g. poly-dimethylsiloxane/silicone, ethylene vinyl acetate copolymer (EVA) etc.) which holds the active ingredient (46). The ring is simply inserted into the vagina and it releases the drug in a controlled manner. The rate of release is controlled by several factors like the relationship

between the characteristic dimensions, the initial load of active ingredient, the presence of excipients, the design of the IVRs etc (47). The use of vaginal rings presents numerous advantages (46) like,

- It permits the controlled release of drug
- Avoids daily administration
- Allows the use of low drug dose and the simultaneous administration of several drugs by the same device
- It is user controlled



Intra Vaginal Rings (Matrix type)

Intra Vaginal Rings (Reservoir type)

Aim and Objectives

The aim of the study is to target the drugs available for Endometrial Cancer by vaginal route to achieve the following:

- Targeting the drugs directly to the site of action and hence achieving maximum therapeutic effect by lowest possible dose.
- Reduction in side effects of drugs by targeting it directly to the site of action.
- Increasing the patient compliance by avoiding painful injectable route and self-medication being made possible by intra vaginal rings.

EXPERIMENTAL

Preformulation Studies

Identification of procured drugs

Fourier transform infrared spectroscopy (FT-IR) and differential scanning calorimetry (DSC) of the obtained sample were performed and the generated spectra were compared with those available in literatures to confirm the authenticity of the drug.

Analytical method development

HPLC methods for quantitative estimation of Paclitaxel and Carboplatin were developed and validated. Supelco® C18 [25cm (L) × 4.6mm (ID) with 5µm (PS)] was used for both the drugs. The mobile phase system Acetonitrile: Water (60:40) was used for Paclitaxel and the detection wavelength was set to 227 nm. The runtime was set to 10 minutes. The estimation of Carboplatin was done using Acetonitrile: Water (20:80) as a mobile phase. The detection wavelength for UV detector was set to 230 nm for Carboplatin. The runtime was set to 10 min.

Analytical interference study was also performed to ensure that the selected formulation excipients were not interfering with drug estimation methods otherwise it may result in falsely elevated or lowered concentration values. The interference studies were performed by spiking standard drug aliquots with all formulation excipients at their maximum fraction which are expected to be present in analytical samples of respective studies.

Drug-excipients compatibility studies

The compatibility among drug and other formulation components were analyzed. Herein, a blend of drug and other excipients, at their maximum anticipated level in the formulations, was prepared and its FT-IR spectrum as well as DSC thermogram was compared with that of individual formulation components.

Preparation of Paclitaxel loaded ultradeformable vesicles (UDVs)

The UDVs were prepared by Thin Film Hydration method. Drug, Lipids and Surfactant in different proportions were taken in Round Bottom Flask and dissolved in Methanol: Chloroform (1:9) mixture.

The organic solvents were evaporated using rotary flask evaporator under vacuum on a thermostatic water bath at 45± 2°C at the speed of 100 RPM.

After evaporation of all the organic solvents, the RBF was kept in desiccator overnight to ensure complete removal of organic solvent.

The thin film was hydrated by 2.5ml aliquots of triple distilled water. Hydration media, previously heated at 50°C was added followed by shaking RBF manually for 2-3 minutes after each addition of aliquot into RBF, maintained at same temperature on thermostatic water bath. Hydration of film was done for 10min.

Probe sonication method was used for size reduction of MLVs prepared by thin film hydration method. Sonication was performed at 80% amplitude with 0.8 second pulse rate for 20 second exposure time to obtain desired size range and visually clear dispersion.

Formulation optimization

Preliminary experiments for screening of several process as well as formulation variables were performed to identify the factors significantly affecting the desired formulation characteristics (selected as response variables).Amount of Drug (mg) (X1), Lipid:Surfactant Ratio (X2) and DSPC:EggPC Ratio (X3) were selected as independent variables and they were set at low, medium and high levels on the basis of the results of initial trials. Box–Behnken statistical design with 3-factors, 3- levels, and 17 runs was specifically employed for the optimization study using Design-Expert software (Design-Expert 7, State-Ease Inc., Minneapolis, USA).

Preparation of Carboplatin loaded ultradeformable vesicles (UDVs)

Lipids and Edge Activator were accurately weighed and dissolved in 10 ml of $CHCl_3$: MeOH mixture 9:1. The clear solution was transferred to the 100 ml round bottom flask (RBF) and thin film was obtained. The film was hydrated using 10 ml distilled water containing carboplatin for 20 min at 55 °C. The nano vesicular dispersion was sonicated to reduce size of the vesicles to about 300 nm. The vesicular dispersion was then subjected to gradient assisted temperature controlled passive equilibrium process for enhancement of the drug encapsulation into the formed vesicular dispersion.

Formulation optimization

The parameter affecting the entrapment and loading of carboplatin in ultradeformable nano vesicles were identified and categorized in formulation and process parameters. Plackett–Burman study was used to screen various factors affecting the formulation. Box–Behnken statistical design with 3-factors, 3- levels, and 17 runs was specifically employed for the optimization study using Design-Expert software (Design-Expert 7, State-Ease Inc., Minneapolis, USA). Drug:Lipid ratio (D:L) (X1), Lipid:Edge activator Ratio (L:EA) (X2) and HSPC:SPC Ratio (X3) were selected as independent variables and they were set at low, medium and high levels on the basis of the results of initial trials.

Methods for estimation of response variables of UDVs of Paclitaxel and Carboplatin

Entrapment Efficiency (%EE)

PTX loaded UDVs formulation was centrifuged at 5000 RPM for 10 min to separate un-entrapped drug in sediment. The supernatant was separated and methanol was added to it in order to dissolve the lipids and to extract drug in solution. The amount of paclitaxel was estimated by high performance liquid chromatography technique at 227 nm.

CBP loaded UDVs was filled into the pre-activated dialysis tube (MWCO 12,000 Da). The tube was placed in the beaker containing distilled water. The vesicular dispersion was dialyzed against distilled water for 2h. The amount of the drug present in the vesicular dispersion was then measured using the developed HPLC method after suitable dilutions using methanol.

The entrapment efficiency was calculated using following formula-

% Entrapment Efficiency = Estimated Entrapped drug/Total drug added to formulation ×100

Vesicular size and size distribution

50 µl of the vesicular formulation was added to 2 ml of distilled water in order to obtain proper vesicle density in the final dispersion for measurement of size of the vesicles. The dispersion thus prepared was filled in clear disposable sizing cuvettes and the globule size was measured using ZetaSizer (Nano ZS, Malvern Instruments, UK) equipped with a He-Ne laser at 633 nm and scattered light detector at an angle of 90°.

Zeta Potential

50 µL of liposomal dispersion was added to 2 ml of distilled water. The diluted sample was transferred to the disposable zeta cell and zeta potential was recorded using ZetaSizer (Nano ZS, Malvern Instruments, UK).

Deformability evaluation

For the measurement of deformability of the nano-vesicles, formulations were extruded at constant pressure through the polycarbonate filter membrane having a pore diameter of 100 nm using a stainless steel pressure holder for 25 mm diameter filters. The amount of vesicle dispersion that was extruded during 5 min was measured, and the vesicle was monitored before and after extrusion. The deformability of the nano-vesicles was measured using following equation (48):

$$E = J \times \left(\frac{R_v}{R_p}\right)^2$$

Where, E= elasticity of vesicle membrane;

J= amount of dispersion extruded;

R_v= vesicles size (after extrusion); and

R_p= pore size of the barrier

In-vitro characterization of UDVs of Paclitaxel

Drug Loading (% w/w)

Drug loading efficiency shows the amount of drug entrapped with respect to the total solid content taken for the preparation of the formulation. The drug loading was calculated using following formula-

% drug loading = $(W_{drug}/W_{total}) \times 100$

Where W_{drug} is the weight of drug entrapped and W_{total} is the total weight of drug, EA, and lipids.

In vitro drug release study

In vitro release of drug from UDVs were evaluated by the dialysis bag diffusion technique in phosphate buffered saline (PBS) pH 4.0 with 1% SLS. The aqueous UDVs dispersion was placed in activated dialysis membrane (MWCO- 12000, Hi media, India) closed at one end. The other end was closed with the clip and whole bag was immersed in beaker containing PBS pH 4.0 with 1% SLS which was stirred at 100 rpm and maintained at 37 ± 1 °C throughout the experiment. At predetermined time intervals, samples were withdrawn from the receptor compartment (media) and sink conditions were maintained by addition of fresh media. The samples were analysed for amount of drug released using previously described HPLC method. All the experiments were performed in triplicate, and the average values were taken.

Ex vivo permeation study

The ex vivo permeation of PTX loaded UDVs was determined across Porcine Vaginal Membrane. Tissue was mounted into Franz's diffusion chamber between donor and receptor compartment. The receptor compartment was filled with 1% SLS in PBS (pH 7.4) with constant stirring at 100 rpm. The vaginal membrane was carefully mounted onto Franz's diffusion cell so that the mucosal membrane faces donor compartment and other side bathed in receptor compartment media. An aliquot of PTX UDVs dispersion was added to the donor compartment. The cell assembly was maintained at 37 ± 1 °C and the contents of the receptor compartment were constantly stirred at 100 rpm. Samples were withdrawn (1 ml) via the sampling port at predefined time intervals and replenished with equal volume of fresh medium to maintain sink condition throughout the experiment.

Vaginal membrane deposition study

Vaginal membrane deposition study was carried out at the end of the permeation experiment. The surface of the vaginal membrane was washed five times with 50% ethanol to remove excess drug from the surface. The vaginal membrane was then cut into small pieces. The tissue was further homogenized with 70% methanol and left for 24 h at room temperature. After shaking and centrifuging for 5 min at 3500 rpm, the paclitaxel content in the supernatant was determined by HPLC method.

Haemolytic toxicity study

Haemolytic toxicity of the formulation was determined using red blood cell (RBC) lysis assay as per the method described in literature (49). Paclitaxel marketed formulation (GROSS^{TM}-100, Emcure® Pharmaceuticals Ltd.) and UDVs formulation were diluted with 0.9% normal saline. Human blood sample was obtained from the nearby blood bank and centrifuged at 3500 rpm for 10 min and the supernatant was discarded. The sediment pellet containing RBCs was diluted with normal saline solution to obtain 5% w/v suspension. The RBC suspension was mixed with distilled water, normal saline, paclitaxel marketed formulation and paclitaxel UDVs. The suspension with distilled water was considered as producing 100% haemolysis whereas normal saline producing no haemolysis, hence considered as blank. The samples were incubated for 1 h at 37°C. After incubation, all the mixtures were centrifuged at 3500 rpm for 10 min to separate non-lysed RBC. The supernatant was collected and diluted with an equal volume of PBS (pH 7.4) and absorbance was taken at 540 nm against supernatant of normal saline containing sample. The absorbance value of distilled water containing sample was considered for 100 % haemolysis and the percent haemolysis for other samples was determined using the following equation

%Haemolysis = (Absorbance of sample/ Absorbance of distilled water containing sample)*100

In Vivo Biodistribution Study of PTX loaded UDVs

In order to examine the uterine drug targeting efficiency of the UDVs formulation, in vivo biodistribution studies were carried out using 99mTc labelled PTX. Paclitaxel was labelled using 99mTc-pertechnetate as per the method available in literatures (50). The labeling efficiency was checked using instant TLC method. The bio distribution after vaginal administration of the UDVs formulation loaded with radio labelled PTX was compared with the plain drug solution of the radiolabelled PTX in Female New Zealand white rabbits. At predetermined time intervals, the scintigraphic images were captured using a Single Photon

Emission Computerized Tomography (SPECT, LC 75–005, Diacam, Siemens AG, Earlangan, Germany) gamma camera.

In-vitro characterization of UDVs of Carboplatin

Drug Loading

Drug loading efficiency of Carboplatin loaded UDVs was determined using the procedure mentioned for paclitaxel loaded UDVs.

In vitro drug release study

Drug release from the carboplatin loaded UDVs was studied by using dialysis bag technique in phosphate buffered saline (PBS) pH 4.0. The aqueous UDVs dispersion equivalent to 10 mg of drug was placed in activated dialysis membrane (MWCO- 12000, Hi media, India) closed as one end. The other end was closed and whole bag was immersed in a beaker containing PBS pH 4.0 which was stirred at 100 rpm and maintained at 37 ± 1 °C throughout the experiment. At predetermined time intervals, samples were withdrawn from the receptor compartment and sink conditions were maintained by addition of fresh media. The samples were analysed for amount of drug released using HPLC method. All the experiments were performed in triplicate, and the average values were taken.

Ex vivo permeation study

The ex vivo permeation of carboplatin loaded UDNVs was determined across Porcine Vaginal Membrane. Tissue was rinsed three times with saline solution, whipped, stripped from the underneath connective tissues and muscles, and mounted onto Franz's diffusion chamber between donor and receptor compartment. The receptor compartment was filled with PBS (pH 7.4) with constant stirring at 100 rpm. The vaginal membrane was carefully mounted into Franz's diffusion cell so that the mucosal membrane facing donor compartment and other side bathed in receptor compartment media. An aliquot of carboplatin loaded UDVs dispersion was added to the donor compartment. The cell assembly was maintained at 37 ± 1 °C and the contents of the receptor compartment were constantly stirred at 100 rpm. Samples (1 ml) were withdrawn via the sampling port at predetermined time intervals and replenished with equal volume of fresh medium to maintain sink condition throughout the experiment.

Vaginal membrane deposition study

Vaginal membrane deposition study was carried out at the end of the permeation experiment. The surface of the vaginal membrane was thoroughly washed five times with distilled water to remove excess drug from the surface. The vaginal membrane was then cut into small pieces. The tissue was further homogenized with 10% methanol and left for 24 h at room

temperature. After shaking and centrifuging for 5 min at 3500 rpm, the carboplatin content in the supernatant was determined by HPLC method.

Haemolytic toxicity study

Haemolytic toxicity of the formulation was determined using red blood cell (RBC) lysis assay as per the method described in literature (49). Carboplatin marketed formulation (Carbokem Nova 150mg/15ml Inj, Alkem Laboratories Ltd) and UDVs formulation were diluted with 0.9% normal saline. Human blood sample was obtained from nearby blood bank and centrifuged at 3500 rpm for 10 min and the supernatant was discarded. The sediment pellet containing RBCs was diluted with normal saline solution to obtain 5% w/v suspension. The RBC suspension (0.5 ml) was mixed with 1 ml each of distilled water, normal saline, carboplatin marketed formulation and carboplatin UDVs. The suspension with distilled water was considered as producing 100% haemolysis whereas normal saline producing no haemolysis, hence considered as blank. The samples were incubated for 1 h at 37°C. After incubation, all the mixtures were centrifuged at 3500 rpm for 10 min to separate non-lysed RBC. The supernatant was collected and diluted with an equal volume of PBS (pH 7.4) and absorbance was taken at 540 nm against supernatant of normal saline containing sample. The absorbance value of distilled water containing sample was considered for 100 % haemolysis and the percent haemolysis for other samples was determined using the following equation

%Haemolysis = (Absorbance of sample/ Absorbance of distilled water containing sample)*100

RESULTS AND DISCUSSION

Preformulation studies

Preformulation studies are necessary to establish identity, purity and compatibility of the drug with excipients. FTIR spectrum of paclitaxel was recorded and compared with the reference spectrum to establish its identity. Experimental melting point obtained was in the range of 213-215°C which was comparable with the reported range of 213-216°C. Analytical interference study was performed to ensure the non-interference of the excipients in the analysis of the drug. FTIR study was performed with individual component and that of the mixture to ensure drug excipient compatibility.

The HPLC analytical method was developed for the estimation of both the drugs. The calibration plot for paclitaxel has shown linearity in the range of 2 to 10 ppm with R^2 value of 0.9995. The values for LOD and LOQ were obtained 0.311ppm and 0.944ppm respectively. The calibration plot for carboplatin has shown linearity in the range of 2 to 18 ppm with R^2

value of 0.9996. The values for LOD and LOQ were obtained 0.407ppm and 1.234ppm respectively.

Formulation and optimization of PTX loaded UDVs

Plackett–Burman design was used to screen the factors which are significantly affecting the dependent variables. Box-Behnken design was selected to optimize the independent variables. Analysis of variance suggested the fitness of the model and 3D response surface plots were utilized to examine the influence of independent variables on selected responses. The DSPC and Egg-PC with different Tg values were selected to maximize the entrapment and stabilize the vesicular system.

Good entrapment efficiency (> 90 %) was achieved with a vesicular size (< 300 nm) and polydispersity index (< 0.3) in desired range for optimized batch. All the UDVs formulation loaded with PTX showed zeta potential in the range of-25 to -40 mV which is large enough to represent colloidal stability of the UDVs formulation. The negligible difference in size, after passing through membrane filters of smaller pore diameter indicated that these vesicles could deform or change their shape.

Formulation and optimization of CBP loaded UDVs

Plackett–Burman design was used to screen the factors which are significantly affecting the dependent variables. Box-Behnken design was selected to optimize the independent variables. Analysis of variance suggested the fitness of the model and 3D response surface plots were utilized to examine the influence of independent variables on selected responses. The HSPC and SPC with different Tg values were selected to maximize the entrapment and stabilize the vesicular system.

The entrapment efficiency of carboplatin, a hydrophilic molecule, achieved was in the range of 65 to 68 % with vesicular size (~ 300 nm) and polydispersity index (< 0.3) in desired range for optimized batch. All the UDVs formulation loaded with CBP showed zeta potential in the range of -20 to -30 mV which is large enough to represent colloidal stability of the UDVs formulation. The negligible difference in size, after passing through membrane filters of smaller pore diameter indicated that these vesicles could deform or change their shape.

In-vitro characterization of PTX loaded UDVs

Drug Loading (%w/w)

Drug loading of PTX in UDVs was found in the range of 9 to 10 %w/w which is significantly higher when the drug loading is >90% in UDVs. The optimum concentrations of the lipids

and surfactants in the final optimized batch might have contributed to its higher loading efficiency.

In vitro drug release study

The drug release of paclitaxel from the UDVs in 24 h was obtained in the range of 10 to 12% while the plain drug suspension was showing 97 to 99 % drug release in 24 h. The nanovesicular system is capable of holding the drug for longer period of time than the plain drug suspension because of its entrapment in the vesicles phospholipid bilayers.

Ex vivo permeation study

The permeation of Paclitaxel through porcine vaginal membrane was found to be in the range of 54 to 56 % in case of Paclitaxel loaded UDVs formulation while 9 to 11 % in case of plain drug suspension of Paclitaxel in 24 h. The transmembrane flux was found to be in the range of 59 to 61μg/cm^2/h in case of Paclitaxel loaded UDVs formulation while 10 to 11 μg/cm^2/h in case of plain drug suspension of Paclitaxel in 24 h. The tissue permeation was achieved five times higher in the case of PTX loaded UDVs than plain drug suspension of PTX. The deformability of the developed formulation might contribute to the higher permeation of the drug through biological membrane.

Vaginal membrane deposition study

The paclitaxel loaded UDVs formulation has shown tissue deposition of around 5 % while plain drug suspension exhibited around 1.25 % of tissue deposition. The values were in good agreement with their tissue penetration capabilities.

Haemolytic toxicity study

The UDVs formulation of Paclitaxel exhibited significantly less % haemolysis as compared to the marketed product. The comparatively higher toxicity of the marketed formulation may be due to its excipient cremophor EL based solvent system to solubilize water insoluble paclitaxel.

In Vivo Biodistribution Study of PTX loaded UDVs

The mean labeling efficiency of PTX was >98 % at pH 6.5. Incubation of 99mTc-PTX in human serum and 0.9 % saline at 37℃ revealed that the labeling of PTX was extremely stable. When 99mTc-PTX loaded UDVs were administered via vaginal route, maximum amount of drug was found in uterus even after 48 hours with no distribution in any other organs of the body. In the case of 99mTc-PTX plain drug administration only 4 % activity was detected in uterus after 24 hours.

In-vitro characterization of CBP loaded UDVs

Drug Loading (%w/w)

Drug loading of CBP in UDVs was found 4 to 5 %w/w which is significantly higher when the drug entrapment is as higher as 65 to 68 % in UDVs. The optimum concentrations of the lipids and surfactants in the final optimized batch might have contributed to its higher loading efficiency.

In vitro drug release study

The drug release of carboplatin from the UDVs in 24 h was obtained in the range of 12 to 16% while the plain drug suspension was showing >99 % drug release in 24 h. The nanovesicular system is capable of holding the drug for longer period of time than the plain drug suspension because of its entrapment inside the hydrophilic compartment of the nano-vesicles.

Ex vivo permeation study

The permeation of carboplatin through porcine vaginal membrane was found to be in the range of 40 to 45 % in case of carboplatin loaded UDVs formulation while 5 to 7 % in case of plain drug solution of carboplatin in 24 h. The transmembrane flux was found to be 45 to 47 $\mu g/cm^2/h$ in case of carboplatin loaded UDVs formulation while 6 to 7 $\mu g/cm^2/h$ in case of plain drug suspension of carboplatin in 24 h. The tissue permeation was achieved seven times higher in the case of CBP loaded UDVs than plain drug solution of CBP. The deformability of the developed formulation might contribute to the higher permeation of the drug through biological membrane.

Vaginal membrane deposition study

The carboplatin loaded UDVs formulation has shown tissue deposition of around 2 % while plain drug solution of carboplatin showed around 1 %. The results were in good agreement with the tissue penetration capability of UDVs formulation.

Haemolytic toxicity study

The carboplatin loaded UDVs formulation has shown significantly less haemolysis than the marketed formulation. The higher haemolytic toxicity of the marketed formulation might be due to direct exposure of the drug to the cells. The carboplatin molecule is encapsulated in the UDVs formulation hence showing lesser haemolytic toxicity.

ONGOING WORK

Histopathology study

Histopathology study using goat vaginal mucosa will be performed to observe the pathological changes in the vaginal mucosa in contact with the drugs and drug loaded formulations, administered intra vaginally to target the uterus. The vaginal tissue will be analyzed at suitable magnification using optical microscope and will be compared with the vaginal tissue treated with phosphate-buffered saline (pH 7.4) and isopropyl alcohol, as negative and positive control, respectively.

Biodistribution of CBP loaded UDVs

The radiolabeling of CBP using 99mTc was studied. The results of ITLC performed to evaluate the labeling efficiency of the CBP molecule showed that colloids formation occurs while labeling using 99mTc with labeling efficiency less than 50%. Hence, the radiolabeling method was not suitable to study biodistribution of CBP. The alternative method would be explored to study biodistribution of the CBP.

Stability study

The stability testing is being carried out to evaluate the influence of a variety of environmental factors, such as temperature and humidity, and to establish a shelf life for the drug product at recommended storage conditions. The study protocol was prepared as per the ICH Q1A (R2) guidelines for stability testing of new drug substances and products. The UDVs dispersions of both the drugs were stored in transparent glass vials at 2-8 °C (refrigerated) and 25 -30 °C (room temperature) for 3 months. At specific time intervals of 1, 2, and 3 months, the samples will be taken and their physical appearance will be examined. In addition, the mean vesicle size and entrapment efficiency will be measured. Stability study of the intravaginal rings as a final dosage form will also be performed.

Development of Intravaginal Ring (IVR)

The developed UDVs formulations of both the drugs will be incorporated into reservoir type intravaginal ring using medical grade silicon as a final dosage form. The reservoir type hollow IVR has been developed in the lab using stainless-steel molds. The UDVs loaded with drugs will be converted to a formulation which is suitable to be retained in the reservoir of the IVR and also provide sustained release as desired

FUTURE STUDIES

Cell line studies

In vitro cytotoxicity studies (MTT Assay), intracellular uptake studies will be performed on MCF-7 cell line at different concentrations of drugs and drug loaded UDVs formulations at

two different incubation times (24 and 48 hours). The intracellular uptake study will be performed by both qualitative i.e. using confocal microscopy and using Fluorescence-activated cell sorting (FACS) to measure quantitative cellular uptake. Mean fluorescence intensities will be compared with the plain drug solution and UDVs loaded formulations.

In vivo pharmacodynamics studies

Pharmacodynamic study will be performed to observe the effect of UDVs formulations on the endometrial cancer associated hyperplasia induced in female New Zealand white rabbits using suitable chemical induction method. The analysis of the hyperplasia caused by chemical induction of endometrial cancer in rabbits will be assessed using ultrasound. Three different rabbits, will be induced the disease. The developed formulations of CBP and PTX will be administered intravaginally using rod insert or gel of the formulation. The effect of the treatment with the formulations will be compared with the third rabbit (untreated).

CONCLUSION

The aim of the present study was to develop the drug delivery system which enables uterus targeting via non-invasive intravaginal route for the treatment of endometrial cancer. The UDVs formulations of Paclitaxel and Carboplatin showed excellent results for the %EE and Loading of the drugs. Moreover, the drug release from the paclitaxel and carboplatin loaded UDVs formulation was found to be in controlled manner as compared to the plain drug solutions. The formulations have also exhibited promising results in ex-vivo and in-vitro studies. The in-vivo biodistribution studies using gamma scintigraphy has revealed the preferential uptake and retention of UDVs formulation by the uterus when the formulation was administered by vaginal route. Hence, uterine targeting by vaginal route seems to be a promising approach for the treatment of disorders related to uterus where the tissue concentration of the drug exceeds the systemic absorption anticipating a dose reduction needed to elicit the therapeutic action and avoidance of the side effects.

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