DEVELOPMENT OF INTRAVAGINAL LIPOSOMAL GEL OF CLOMIPHENE CITRATE FOR TREATMENT OF POLYCYSTIC OVARY SYNDROME

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7. SUMMARY AND CONCLUSION

7.1 Summary

Currently, there is huge interest in the scientific community, physician and drug industry to exploit polycystic ovary syndrome. PCOS is one of the most common hormonal disorders among women of reproductive age. “PCOS is a challenging experience for women, many remain undiagnosed and experience delays in diagnosis”. Polycystic ovary syndrome is diagnosed easily in women presenting with infertility. PCOS is highly variable ranging from 2.2% to 26% globally. So far as, no effective single drug is identified for PCOS treatment, the treatment of PCOS is a challenging problem worldwide at present. Due to its complex pathogenesis and unrecognized etiology, there is no effective preventive measure available for PCOS till now. In spite of the tremendous progress made in the development of drug delivery system, there is an urgent need to find out alternate therapeutic regimen to control PCOS syndrome.

Now a days new site specific formulation are prepared to get direct therapeutic effect. These site specific and novel drug delivery systems provide more therapeutic effect as compare to oral drug delivery. Oral drug delivery system is easy for administration but due to its some of draw-back, it is not preferred for all drug administrations. Whereas parenteral drug delivery system provide higher therapeutic effect as compared to other routes of administration because of its avoidance of first pass metabolism. But parental drug delivery also having some of draw-back related to drug stability and solubility.

The aim of present work was to prepare liposomal gel formulation to delivery drug to specific site to enhance the permeability of drug. The rational of this study is to design and evaluate an intra-vaginal site specific, liposomal gel containing clomiphene citrate, which can be targeted to uterus (vagina) in time dependent manner, to modulate the effective drug level in blood for poly cystic ovary syndrome. Human vagina remains to be a relatively unexplored route of drug delivery despite of its potential as a non-invasive route of drug administration. The presence of dense network of blood vessels & avoidance of by-pass first pass metabolism has made the vagina an excellent route of drug delivery over conventional drug delivery.

The vagina has been explored as a favorable site for the local and systemic delivery of drug used for the treatment of female specific conditions. Vaginal administration of drug is mainly used for the treatment of local infection such as vaginitis, candidiasis, vaginosis and other UTI infections. In addition, vaginal formulations have a great potential for the systemic

absorption of drugs because of the surface area, rich blood supply and permeability to a wide range of compound, including proteins and peptides. Vaginally administered agent and formulations are mainly used and are being developed to provide protection against microbial infection, AIDS, PCOS and other sexually transmitted diseases. In the present research work, we have to attempt to develop a noble vaginal drug delivery system for clomiphene citrate.

7.1.1 Pre-formulation of Clomiphene citrate

Pre-formulation is an integral part of the entire development process. It is the study of the physical and chemical properties of the drug prior compounding process. These studies focus on those physicochemical properties of the drug that could affect its performance and development of an efficacious dosage form. These studies are indispensable protocol for development of safe, effective and stable dosage form. The obtained drug sample was identified by various analytical techniques such as IR spectroscopy, UV spectroscopy, melting point, partition coefficient, DSC, XRD. Preformulation study of Clomiphene citrate was performed for the vaginal drug delivery system. Clomiphene citrate was yellowish-white in color, odorless, tasteless, and solid crystalline powder. Melting point determined by capillary tube method and was found 142.60±0.282°C similar to the literature (McLeish, 1998), (Patent US 2016/0152551A1). The absorption maxima of the drug were found to be at 294nm, 235nm and 204nm similar to literature (McLeish, 1998), (Patent US 2016/0152551A1). Solubility of Clomiphene citrate is determined with different solvents as per result it is freely soluble in Glacial acetic acid, sparingly soluble in methanol, ethanol, SVF, DCM & chloroform and slightly solubility in ethyl acetate, water & diethyl ether. The solubility of the drug was checked at different pH 2, 4, 5.8, 6.8 and 7.4 (because pH of vaginal fluid found in the ranges from pH 3.5 to pH 4.5). As per solubility results the drug was sparingly soluble at pH 2 & pH 4, sparingly soluble at pH 5.8, very slightly soluble at pH 6.8 & pH 7.4. The calibration curve of Clomiphene citrate as shows in graph indicated the regression equation Y=0.0229x-0.0025 & R^2 value 0.999 for λ_{max}-294 and Y=0.0406x - 0.00231 & R^2 value 0.999 for λ_{max}-235, which shows good linearity. The partition coefficient of Clomiphene citrate in n- Octanol: Water was found to be 1.645±0.009, this indicated that the drug was lipophilic in nature. The principle FT IR absorption peaks of Clomiphene citrate at 1740 cm^{-1} (C=O), was observed in the spectra of Clomiphene citrate. The observed sharp endothermic peak of the DSC study represented melting point at 142.34°C (Patent US 2016/0152551A1). XRD study of Clomiphene Citrate showed its angle of reflection 2θ at

6.3267, 9.66, 10.78, 11.48, 12.61, 16.40, 17.02, 18.71, 20.40, 21.28, 23.57, and 24.66. These values represented the crystalline nature of the drug. This above observations confirmed the purity, free from any type of impurities and authenticity of the Clomiphene citrate.

7.1.2 Analytical method development for Clomiphene citrate

Analytical methods were developed and validated as per ICH guidelines concerning linearity, accuracy, precision, and ruggedness, limit of detection, limit of quantitation, robustness and forced degradation studies of the drug through UV-Visible spectroscopy and HPLC. The first analytical method was developed for the drug through UV-Visible spectroscopy by using SVF (Simulated Vaginal Fluid) as solvent, shows its absorption maxima at 290nm, 233, and 213nm similar to literature (acceptance limits ±5nm), (McLeish, 1998). The calibration curve for the method was linear over the concentration range 6-60µg/ml and 3-30µg/ml for drug at 290nm and 233nm respectively. The determination of coefficients (R^2) was 0.9994 and 0.999 respectively, which shows good linearity. The method was found to be précised as the percentage RSD value for repeatability and intermediate day was found to be less than 2%. The accepted limits of accuracy (recovery) was found for 80%, 100% and 120%, as observed data were within the required range (%RSD less than 2%), which indicates good recovery value of the drug. The result from robustness indicted the small change in the condition but not shows a significant effect for the determination of the drug. The LOQ and LOD were found to be 0.315µg/ml & 0.954µg/ml at 290nm and 0.240µg/ml, & 0.726µg/ml, at 233nm. Minor change on absorbance was studied by keeping all other parameters constant in robustness but %RSD was in a limit. The force degradation study for different processes as acid hydrolysis, oxidation, photodegradation, heat-induced degradation, the spectra of Clomiphene citrate was not shown any significant degradation or no additional peak of sample after 1hr, 2hr, and 4hr for the different process but in base degradation of drug showed degradation and no peak was observed at 290nm and at 233nm peak was shifted which shows that drug was susceptive in basic condition.

Another analytical method was developed through UV-Visible Spectroscopy by using methanol as solvent. Clomiphene citrate shows absorption maxima of at 294nm, 235nm and 204 nm similar to literature (acceptance limits ±5nm), (McLeish, 1998). The calibration curve for the method was linear over concentration range 3-60µg/ml for clomiphene citrate at 294nm, 3-24µg/ml for clomiphene citrate at 235nm. The determination of coefficients (R^2) was 0.9991, 0.9989, 0.9988 and 0.999, 0.9994, 0.9994 respectively. The methods were found

to be precise as % RSD value for repeatability and intermediate day was found to be less than 2%. The accepted limits of accuracy (recovery) were found for 80%, 100%, and 120%, all observed data was within the required range, which indicated its good recovery value. The result of, robustness indicted the small change in the condition, but not significantly affect the determination of Clomiphene citrate. The LOD and LOQ were found to be 0.229µg/ml, 0.694µg/ml and 0.126µg/ml, 0.382µg/ml respectively. In force degradation studies for different processes as acid/base hydrolysis, oxidation, photodegradation, heat-induced degradation the drug did not show any significant degradation or no additional peak of sample after 1hr, 2hr, and 4hr for the different process. But in oxidation degradation study at 235nm did not show any peak which indicated that drug was susceptive for oxidation conditions.

Another analytical method was developed through RP-HPLC by using different chromatographic conditions as shown below.

Stationary phase: C18 column (10µm particle size, length 250, Internal Diameter 4.6 mm)

Elution mode: Linear pressure gradient [A: B (35:65)v/v]

Mobile phase A:	0.02% Trifluoroacetic acid	**Mobile phase B**:	Methanol
Column temperature:	30^0C	**Flow rate:**	1.0ml/min
Run time:	15 min	**Diluent:**	Methanol
Wavelength:	290nm	**Injection volume:**	5 µl
Detector:	UV-Visible		

The retention time for E-clomiphene citrate and Z-clomiphene citrate was found to be 9.984 and, 11.181 respectively. A linear curve was obtained in the range of 1-6µg/ml with an equation of y= 3410x-265.4 and R^2=0.9996 for E-isomer and y= 4162x-166.04 and R^2=0.9995 for Z-isomer. The solution was reanalyzed by the proposed method, results indicate that the recoveries were well within the acceptance range of 50%-125%, indicating a good degree of sensitivity of the method towards the detection of analytes in the sample. The precision of the developed method was expressed in repeatability, Inter-day, and Intraday. The method was found to be precise due to low values of the %RSD, as 1.216, 1.458 and 1.372 for E-isomer and 0.496, 0.937 and 1.663 for Z-isomer respectively. The limit of detection and limit of quantitation of the method were calculated basing on the standard deviation of the response and the slope (s) of the calibration curve at approximate levels of the LOD and LOQ. The %RSD obtained for change of flow rate, change in column temperature and mobile phase ratio were found to be below 2, which were within the acceptance criteria.

Ruggedness was determined by carrying out analysis by two different analysts and the respective percentage recovery was noted and the % RSD obtained for change of analyst was found to be below 2. In specificity, the % interference of blank was found to be below 1, which was within the acceptance criteria (Percentage Interference of Blank or placebo should not be more than 1), hence the method was specific for the drug. Chromatogram was not shows any significant degradation or no additional peak of sample after 1hr, 2hr, and 4hr at the different processes of acid/base hydrolysis, oxidation, photodegradation, and thermal degradation. From above results we successfully developed analytical methods of analysis of clomiphene citrate in different solvents

7.1.3 Compatibility study

Drug-excipient compatibility is an important phase in the pre-formulation stage of drug development. The potential interactions between drugs and excipients have effects on the physicochemical properties, bioavailability, and stability of the dosage form. Drug-excipient interaction can further be helpful in the selection of excipient for the development of stable dosage form. Compatibility study between drug and excipients was performed by FTIR, DSC, and XRD. In the FTIR study for compatibility of pure drug, excipients and physical mixture studies were carried out to eliminate the possibility of interaction between drug and excipients. All the spectrum peaks revealed that corresponding peaks of drug were present at C=O at 173.92cm^{-1}, antisymmetric and symmetric stretching of acyl chains (CH_2) at 2933.83cm^{-1}, asymmetric stretching vibrations of CH_2 and CH_3 groups at 148.17cm-1, P=O stretching band at 1282.71cm^{-1}, C-C stretching at 1170.83cm^{-1}, $N+(CH3)^3$ stretching at 964.44cm^{-1}, C-C-C stretching at 906.57cm^{-1} (Patent US 2016/0152551A1). Hence no interaction was observed in this mixture through FTIR study. In DSC study for compatibility thermogram of the physical mixture of drug and excipients, characteristic peaks of the drug was appeared at 112.95°C to 155.33°C. All the spectrum peaks revealed that corresponding peaks of drugs were present in the above spectra along with some excipients peaks. Hence no interaction was observed in this mixture. From the XRD diffractogram of physical mixture, we found that, there was no incompatibility between drug and excipients as a physical mixture. The characteristic peaks of Clomiphene citrate at 3.8654, 5.3284, 9.7040, 10.6557, 11.6804, 11.6804, 12.7094, 16.9355, 17.4061 & 18.8393 were observed in the spectra of the physical mixture. Hence no interaction was observed in this mixture through XRD study. From the above studies for compatibility, we found that there was no incompatibility between

the drug and excipients. The drug and all excipient were compatible with each other. So the physical mixtures were considered for formulation.

7.1.4 Development, design and characterization of liposomes

7.1.4.1 Development and characterization of liposomes

Based on the literature reviewed by us, liposomes were prepared by thin film hydration method. In preparation of liposomes different parameters were used. Liposomes were optimized for different lipids, pH, organic solvent, temperature, molar ratio, effect of molar ratio in excipient. Optimizations of liposomes for phospholids were performed using Phospholipid S100 or Phospholipid P100 and Phospholipid S75 both Cholesterol in a ratio of Phospholipid: Cholesterol (0.02:0.01). On the bases of visual appearance, stability, microscopic study, pH (4.214±0.009), and percentage drug entrapment efficiency (80.303±0.199%) Phospholipid S75 (Lipoid S75) was selected for further study. Afterwards, pH, molar ratio, temperature, solvent selections were also studied that LS75 as choice of phospholipid, Chloroform as solvent, 60^0C as optimum which confirmed range, 0.02:0.01 ratio of Phospholipid: cholesterol were best for liposome formation. On selective conditions liposomes were prepared and spectroscopic study was performed to check incorporation of drug. The results from FTIR, DSC and, XRD shows that drug was fully incorporate in liposomes.

7.1.4.2 Optimization of liposome using 3 level factorial designs

Liposomes were optimized by using 3 level factorial designs. A full factorial design experiment was designed to study the effect of variables on liposome performance and characteristics different batches were prepared using 3 level factorial design. The statistical validity of the polynomials was established based on ANOVA provision in the design expert software. Response data of all formulations were fitted to the quadratic model. According to design expert software, the best-fitted model was quadratic for response Y. All the responses were fitted to model to establish full model (FM) polynomial equation and Response (entrapment efficiency) checked by ANOVA and provides significant results. The effect of independent factors on the response, three-dimensional (3D) plots & contour plot by observed response surfaces formed hillsides with large curvatures confirms that they were typically influenced by the interaction effect of concentrations of both dependent factors. Checkpoint analysis was gave the comparisons of the predicted and experimental results, which shows very close agreement, indicating the success of the design combined with a desirability

function for the evaluation and optimization of liposome formulations which provided LS75 & Cholesterol ratio (2:1) and predicted the response 79.699%. Whereas, the observed response was found to be 80.121% with percentage error 0.299.

Particle sizes, zeta potential, spectroscopic study (FTIR, XRD, and DSC) of liposomes were performed and show significant results. Liposomes were freeze-drier and %drug entrapment efficiency has been determined and the result shows 79.197±0.146% drug entrapment efficiency.

7.1.5 Development and characterization of Intra-vaginal liposomal gel.

Carbopol 934 was taken in different concentration of 1%, 1.5%, 2%, 2.5% & 3% and added in 10 ml lyophilized liposome dispersion (50 mg of Clomiphene citrate) and kept overnight until Carbopol get swelled. The pH was adjusted to 4.2 using 0.1N NaOH and stirred slowly till a gel was obtained. Similarly other gel formulations were prepared using chitosan. Chitosan was taken in different concentration of 1%, 1.5%, 2%, 2.5% & 3% and dissolved in 10ml dispersion of liposome (50 mg of Clomiphene citrate) and kept overnight until chitosan get swelled. The pH of solutions was adjusted to 4.2 with dilute acetic acid.

Prepared liposomal gel of clomiphene citrate were optimized for best formulation by evaluating gel on different parameters as visual appearance, texture, pH of gel, drug content, rheological measurements, spreadability. Prepared liposomal gel formulation of clomiphene citrate shows , pH of formulations CCG-3, CC75-4, CCG-5, CCG-9, and CCG-10, were found to be 4.231±0.002, 4.275±0.002, 4.246±0.001, 4.238±0.001 and 4.238±0.001 respectively. Drug content of formulations CCG-3, CC75-4, CCG-5, CCG-9, and, CCG-10, were concluded to be 97.500±0.454, 94.091±0.347, 93.030±0.378, 91.061±0.378, and 89.470±0.330 respectively. The viscosity of formulations CCG-3, CC75-4, CCG-5, CCG-9, and CCG-10, were found to be 8777±1.527, 8734±0.881, 9738±1.154, 7647±1.154 and 9653±1.452cp respectively. Spreadability of formulations CCG-3, CC75-4, CCG-5, CCG-9, and CCG-10, were found by 7.1±0.033, 6.9±0.152, 6.8±0.057, 6.6±0.088 and 6.7±0.033 cm respectively. Texture or strength of formulations CCG-3, CC75-4, CCG-5, CCG-9, and CCG-10, were found to be, 5.42±0.389, 5.38±0.376, 5.38±0.917, 5.37±0.814 and 5.36±0.450gm mm^{-1} sec^{-1} respectively. Cumulative drug release of formulations control gel, CCG-3, CC75-4, CCG-5, CCG-9 and CCG-10, were found as 45.695±0.249%, 76.991±0.404%, 74.045±0.236, 68.959±0.138%, 61.636±0.272% and 57.286±0.248% within 72hrs. respectively. Drug loaded Clomiphene citrate liposomal gel CCG-3 demonstrated significant

maximum drug release up to 76.991± 0.404% within 72hrs followed by a sustained manner. In intra-vaginal liposomal gel formulation permeation of clomiphene citrate was increased as compared with control gel. Based on the above results CCG-3 formulation was having pH 4.231±0.002, drug content 97.500±0.454%, spreadability 7.1±0.033cm, viscosity 8777±1.527cp, gel strength 5.42±0.389gm mm^{-1} sec^{-1} and cumulative drug release 76.991± 0.404 %. CCG-3 was showed to be best formulation & selected for further evaluation. Drug release kinetic studies were performed for the zero-order, first-order, Higuchi's and Kormeyer-Peppas model. For the optimized formulations, the % drug release vs. time (zero-order), log percent drug remaining vs. time (first-order), log percent drug release vs. square root of time (Higuchi plot), and log of log % drug release vs. log time (Korsmeyer and peppas Exponential Equation) were plotted. In each case, the R^2 value was calculated from the graph and reported. Considering the determination coefficients, the Higuchi model was found (R^2=0.926) to fit the release data best. It was concluded from all the findings, that the formulation following sustain released mechanism from intravaginal liposomal gel.

7.1.6 Stability of intra-vaginal liposomal gel of Clomiphene citrate

The physical stability of the Clomiphene citrate loaded liposomal gel was evaluated for 180 days, at 30 days intervals. The stability study was carried out mainly to evaluate the effect of storage on organoleptic property (color, odor & visual appearance), pH, drug content, viscosity and spreadability. Liposomal gel were stored in closed amber-colored glass vials at control (room temperature), refrigerator(4±2°C) away from direct light and also at 25±2°C/60±5% RH, and 40±2°C/75±5% RH for 180 days. The effects of storage on the gel were determined by UV-Visible Spectrometer. The results obtained from stability studies protocol, there were no significant change in organoleptic property (color, odor & visual appearance), pH, drug content and spreadability at control (room temperature), refrigerator (4±2°C), 25±2°C/60±5% RH, and 40±2°C/75±5% RH for 180 days. Hence on the bases of obtained results, the formulated intravaginal liposomal gel was stable for 6 months.

7.1.7 *IN-VIVO* evaluation of intra-vaginal liposomal gel of Clomiphene citrate.

The letrozole induces PCOS in Wistar female rats. It acts by inhibition of aromatase, leading high level of Luteinizing hormone and a decrease in Follicle-stimulating hormone. An imbalance in hormones level produces alteration in steroid hormones (Testosterone, Estrogen, and Progesterone) which regulate ovarian function. This imbalance level in hormones

produces cysts in the ovary. PCOS is secondarily associated with Type-2 diabetes (hyperglycemia), hypertension and dyslipidemia etc.

For *IN-VIVO* study, Institutional Animal Ethics Committee (IAEC), Panjab University Chandigarh has approved the experimental animal protocol *(Protocol approved number: PU/45/99/CPCSEA/IAEC/2019/362)*. Thirty-five female Wistar rats weighing 180-220gm were issued by the Central Animal House of Panjab University, Chandigarh. Animals were caged in standard polypropylene cages and acclimatized for one week in the animal house at a controlled temperature of 23^0C, humidity 60 ± 5% under 12h light and dark cycle. All animal protocol experiments were carried out according to the guidance of the committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines on the ethical use of animals.

In the present study, PCOS rats had irregular estrus cycle (more diestrus phase) whereas control rats exhibited a regular estrus cycle. As evident in our results marked significant decrease in luteinizing hormone, testosterone & estradiol, and significant increase in follicle stimulating hormone, and progesterone level. Histological evaluation of ovaries of PCOS group showed the absence of corpora lutea, cysts formation appeared. Fewer atretic follicles with fluid antrum and higher incidence of pyknotic granulosa cells were observed as compared to control group, and ovaries of different treated groups were showed normal follicles, blood vessels, the disappearance of cysts and healthy corpora lutea, as compare to PCOS group. Significant increase in body weight of PCOS group was observed as compare to control groups and on treatment a significant decrease in body weight as compare to PCOS group. Hyperglycaemia was observed in PCOS group, a significant increase was observed in PCOS group as compare to control group and a significant decrease in treated groups as compare to PCOS group. PCOS has been linked to dyslipidaemia. In PCOS groups, a significant increase in S-cholesterol and triglyceride were observed as compare to control group and a significant decrease in treated groups as compare to PCOS group. HDL level shows a significant decrease in PCOS group as compare to control group and a significant increase as compare to PCOS group. GSH evaluation for its antioxidant effect shows a significant decrease in PCOS group as compare to control group and a significant increase in treated groups as compare to PCOS group. Nitric Oxide (Nitrite/Nitrate) level shows a significant increase in PCOS group as compare to control group and a significant decrease in treated groups as compare to PCOS rat group. Hormonal levels were measured, as LH level show a significant increase in PCOS rats

as compared with control group and a significant decrease in treated groups as compare to PCOS group. FSH level shows a significant decrease in PCOS group as compare to control group and a significant increase in treated groups as compare to PCOS group. Progesterone level shows a significant decrease in PCOS group as compared to control group and a significant increase in treated groups as compare to PCOS group. Testosterone level shows a significant increase in PCOS group as compare to control group and a significant decrease as compare to treated groups as compare to PCOS group, and estradiol level shows a significant decrease in PCOS group as compare control group and a significant increase in treated groups as compare to PCOS group

During the experimental protocol, some behavioral change was observed in Letrozole (1mg/day) treated rats. During PCOS induction duration depressed behavior was observed in all female Wistar rats. This depressed behavior in rats was investigated. This behavioral change occurred due to imbalance hormonal level in PCOS rats. The low level of estrogen or estrogen deficiency may cause a decline in serotonin that contributes to mood swings or depression. After treatment of PCOS, change in behavior was observed. This study clearly showed that Clomiphene citrate intravaginal liposomal gel formulation recovered or balanced the reproductive hormonal level and recovered secondary associated diseases as hypertension, diabetes and dyslipidemia after seven days treatment in PCOS Wistar female rats.

7.2 Conclusion: In conclusion, we have demonstrated that the chronic treatment with intravaginal liposomal gel of Clomiphene citrate has the potential to prevent the development and progression of PCOS. The Cumulative drug release study of intravaginal liposomal gel shows a higher percentage of drug release, stable gel properties and recovered or balanced the reproductive hormonal activity level in *IN-VIVO* study. These studies demonstrate that intravaginal liposomal gel of Clomiphene citrate has a beneficial action on hormonal activity level and potential to selectively improve PCOS and its secondary associated complications, like hypertension, diabetes, and dyslipidemia.