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Formulation and characterization of nanoparticle-based ethinyl estradiol transdermal drug delivery system for contraception and menopausal disorders

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Abstract: Nanoparticles based TDDS was employed to overcome the adverse effects of oral contraceptives. A transdermal patch of Ethinyl Estradiol (EE) nanoparticles was aimed to provide sustained release of the drug and lower dosage frequency. The patch was designed with Eudragit-based polymeric films or EE-loaded chitosan nanoparticles poured onto a polyvinyl alcohol backing membrane, with a non-ionic surfactant (span-20) and a plasticizer (n-butyl phthalate) using solvent evaporation method. Nanoparticles were analyzed for their size, morphology, yield and entrapment efficiency. The patches were analyzed for their folding endurance, thickness, weight, drug content, *in vitro* release pattern, FTIR and DSC. All patches were transparent, having a uniform, smooth surface. The folding endurance of all the patches indicated optimum flexibility. *In vitro*, release and *Ex-Vivo* permeation studies showed that F1 containing nanoparticles exhibited the most optimum drug release in 72h (97.6%). The release pattern demonstrated was diffusion controlled. FTIR, DSC studies indicated no interaction between drug and excipients. The accelerated stability studies were performed at 40°C and 70% relative humidity for six months. The product was found stable. The developed patches of EE nanoparticles were expected to improve patient compliance by reducing dose frequency and provide optimum therapy by sustained drug release for contraception.

Keywords: Nanoparticles, ethinyl estradiol, biopolymers, chitosan, eudragit, patches.

INTRODUCTION

The female reproductive system comprises ovaries, uterus, fallopian tubes and vagina. The distress and complications in the normal physiological function of the system result in various problems like premenstrual syndrome, bleeding disorders and menstrual cycle-related disorders. Contraceptive therapy is considered the best option for these problems (Thiyagarajan *et al*., 2021, Casado-Espada *et al*., 2019).

Oral contraceptive like Ethinyl Estradiol (EE) is considered safe, effective and well-tolerated. It reduces bleeding by promoting the shedding of endometrium and dysmenorrhea (Jaber *et al*., 2022). The contraceptive function of EE is induced by thickening the cervical mucus, making sperm transport difficult (Both *et al*., 2019, Casado-Espada *et al*., 2019).

Although using EE as an Oral Contraceptive (OC) is conventional, it shows many complications like headache, mood changes/depression, nausea, vomiting and weight gain (Jaber *et al*., 2022). Oral EE undergoes first-pass metabolism affecting the drug's bioavailability, dosing frequency and efficacy (Agarwal *et al*., 2021). The effective doses of OC are relatively high, leading to vascular complications like myocardial infarction and ischemic stroke (O'Kelly *et al*., 2022). The daily dose for 21 days also leads to poor compliance and reduced efficacy of OC (Casado-Espada *et al*., 2019).

The transdermal route is effective in overcoming the challenges rendered by the oral route. Transdermal patches of EE contain a considerably low drug dose with reduced frequency compared to OC, thus reducing the risk of vascular events (O'Kelly *et al*., 2022). The transdermal patches of EE have more efficacy and improved patient compliance than other routes, bypassing the first-pass effect and providing sustained and slow systemic drug distribution into the plasma to provide a long-term impact. TDDS contraceptives have the advantage of lower and sustained peak serum concentration after application (Nelson *et al*., 2021).

A novel transdermal contraceptive patch loaded with EE nanoparticles is recommended to overcome the non-compliant dosage regimen of combined oral contraceptives. The EE-loaded nanoparticles incorporated into the transdermal patch will allow slow and sustained release of the drug into systemic circulation and tissues. The EE in nanoparticles will diffuse through the skin and get absorbed into the bloodstream (Khezri *et al*., 2020). The choice of biopolymers and the nature of excipients in nanoparticles are very important in determining their safety, efficacy and stability. The present study used two biodegradable, biocompatible polymers, Chitosan and

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sodium tripolyphosphate (1:1), for coating EE. Studies confirmed that biopolymer-coated EE nanoparticles are safe, effective, stable and less toxic. The combined oral contraceptive shows 0.3-9% pill failure due to improper adherence and occasional nausea and vomiting (Sivasankaran and Jonnalagadda, 2021).

The lesser dose (50ug/day) of this novel dosage form will complete the treatment course of 3 days with minimum adverse reactions of EE and improved patient compliance. Chitosan is a cationic polymer and sodium tripolyphosphate, an anionic polymer, will cross-linkage through ionic gelation to form polymeric nanoparticles to achieve maximum stability and minimum toxicity (Talib *et al*., 2021).

The structure of the mentioned drug, Ethinyl Estradiol, drawn by ChemDraw Professional 2016, is as follows:

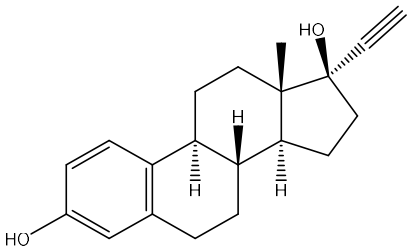

Fig. 1: Structure of Ethinyl Estradiol.

The research aimed to formulate and characterize the nanoparticle-based EE transdermal drug delivery system to bypass the GI adverse effects, provide a weekly dosing schedule, reduce dose frequency and ensure better drug delivery.

MATERIALS AND METHODS

Materials

Ethinyl Estradiol was gifted by MEDIPHARM Pvt. Ltd., Lahore, Pakistan. Eudragit RL 100, Eudragit RS 100, Hydroxy Propyl Methyl Cellulose (HPMC) (K-100), Chitosan, Sodium hydroxide, Mono basic potassium phosphate, Ethyl Cellulose, Tween-80, Span-20, PEG 400, Sodium, Tripolyphosphate, Propylene Glycol, DMSO 10%, Di-butyl phthalate (DBP) and Polyvinyl alcohol (PVA) were purchased from Sigma Aldrich (Merck, Pakistan). Methanol, Isopropanol, Dichloromethane, Ethanolic Solution, diethyl ether and Chloroform used were of analytical grade purchased from Merck Pakistan. Double distilled water was prepared and used throughout the study.

Preparation of transdermal patch

Backing layer

A solution of 2% PVA, 1 % HPMC K-15 and DBP (20% weight of HPMC) was prepared in 100mL of water. The solution was then poured onto Petri dishes and dried to obtain a backing layer.

Drug formulation layer

Method 1

ERL 100 and ERS 100 (3:2) or HPMC K100 and ethyl cellulose (2:1) were added solvent system (Isopropanol-dichloromethane, equal volumes). Plasticizer, di-butyl phthalate along with permeation enhancer, Span 20 were dissolved in the solvent system. The EE organic solution separately prepared was mixed with solvent system. The drug solution was poured onto the backing layer and dried for 24h. The dried patch was removed from the petri dish and cut into the desired size.

Method 2

The nanoparticles of EE were made using the ionic gelation method. A clear solution of cationic polymer chitosan in 1% acetic acid and EE in 1% ethanol were mixed and surfactant Tween 80 was added. A solution of anionic polymer sodium Tripolyphosphate was added dropwise into the mixture of EE and Chitosan with continuous stirring. The particle cloud at the bottom of the flask was separated by centrifugation at 4500 rpm for 30 minutes. The pellets formed were then washed with the diethyl ether to get the nano-suspension. After adding DMSO and PEG-400 (as plasticizers) into nano-suspension, it was blended with backing layer solution and poured on a petri dish of 80cm^2 and dried at 40^{o}C for 24h. The dried patch was removed from the petri dish and stored in aluminium foil at 25^{o}C

Pre formulation studies

Hygroscopicity testing

A glass vessel was weighed (W1). The drug was placed in the vessel, weighed (W2) and placed in a desiccator containing saturated solutions of ammonium chloride. After 24 hours, the drug was weighed again (W3). The difference in weight of the drug before and after exposure to moisture was calculated using the formula:

$$\text{Difference in weight} = \frac{W3 - W2}{W2 - W1} \times 100$$

Size distribution analysis

A stack of test sieves was aligned in order, with the largest pore size at the top, the smallest at the bottom and a receiver placed under all the sieves to collect the sample. The side-taping method of motion was used to pass the sample through sieves. The sample retained on each sieve was weighed and the % retained was calculated:

$$\% \text{ retained} = \frac{\text{retained sample on sieve}}{\text{total weight of sample}}$$

Melting point analysis

The sample was placed on a hot Fisher Johns apparatus (MP-1/02) stage and observed using an eyepiece and stage light. The temperature was noted when the sample started melting and when it completely melted.

Flow properties

Flow properties were determined using three methods, i.e., Angle of Repose, Compressibility Index (Car's index) and Hausner ratio. The angle of Repose was determined by the flow-through funnel method and the diameter of the circle

and height of the heap were calculated to analyze the flow properties using the formula:

$$\theta = \tan -1\left(\frac{2h}{d}\right)$$

Crystallinity

A small amount of EE was added to the insoluble solvent to make dispersion. The drop of the dispersion was applied on the dry glass slide. The crystals of EE were observed under a 100X objective lens of a Compound microscope (LABOMED).

Solubility analysis

A dried test tube was weighed. EE was dissolved in 10mL buffer/organic solvents in the test tube until a drug dispersion was formed. The dispersion was filtered and subjected to Thermogravimetric analysis until the solvent evaporated. The test tube was weighed again and the weight difference was calculated to estimate the amount of drug dissolved in buffers of pH 1-8 separately.

Calibration curve of EE

A stock solution was prepared by adding 50 mg of EE in 50mL (1mg/mL) of phosphate buffer pH 6.8. The working solution (20µg/mL) was prepared by adding 1mL of stock solution to 49mL of buffer. Dilutions of 2µg/mL, 4µg/mL, 6µg/mL, 8µg/mL and 10µg/mL were prepared using the working solution with buffer. All the dilutions were analyzed at 281nm by UV visible spectrophotometer (AQUARIUS CECIL INSTRUMENTS, CE7400S) and a calibration curve of EE was plotted between concentration and absorbance.

Dose calculations

The standard daily dose of Ethinyl estradiol is 10µg to 50 µg. 3mg of ethinyl estradiol was added to organic solution for casting 80cm^2 patch. Each 4cm^2 patch contains 150µg of Ethinyl estradiol to provide 50 µg of the mentioned drug daily for continuous three days application.

$$\text{Total Drug in a patch} = \text{Daily Standard Dose} \times \text{Number of Release Days}$$

Post formulation studies

Characterization of EE nanoparticles

Particle size analysis

The dried nanoparticles were suspended in 10mL water (10µg/mL) to prepare a sample which was then used to determine particle size and polydispersity index by the principle of laser light scattering using a zeta sizer (Malvern Nano ZS-90, UK), with a scattering angle of 90°.

Percentage yield

The dried nanoparticles were weighed by sensitive analytical balance (Shimadzu ATY224) and their weight was compared to the total weight of the drug and polymers used in the formulation. The % yield was calculated using the formula:

$$\text{Percentage yield} = \frac{\text{Quantity of Dried nanoparticles (mg)}}{\text{Quantity of Ethinyl Estradiol + Polymers (mg)}} \times 100$$

Encapsulation efficiency (%)

Nanoparticles (n=3) were centrifuged (Hettich, MIKRO185) and 1ml of supernatant was taken and diluted 10 times with phosphate buffer (9mL). The dilution was examined under a UV-visible spectrophotometer (CECIL CE7400S) at λ_{max} 281nm. Encapsulated drug amount was calculated using the formula:

$$\text{Encapsulated drug amount} = \frac{\text{(Total Ethinyl Estradiol Used – Free Ethinl Estradiol)}}{\text{Total Ethinyl Estradiol Used}} \times 100$$

In-vitro release study of EE-loaded bio polymeric particles

The *in-vitro* drug release studies (n=6) were performed using a modified USP type II dissolution apparatus using 450mL of phosphate buffer (6.8 pH) as a dissolution medium maintained at 37±0.5°C. Nanoparticles having 3mg of the loaded drug were packed in empty tea bags and attached to the paddle of dissolution apparatus. The paddle was rotated at 50rpm at maintained sink conditions for dissolution through tea bags. Samples were withdrawn at different time intervals of 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72h, diluted 10 times twice using buffer and analyzed via UV-Visible Spectrophotometer (CECIL CE7400S) at 281nm.

Characterization of EE transdermal patch

Physical characterizations

The patch was cut into the desired area (2×2cm^2) and examined for clarity, color, texture and flexibility. The individual and average weight of 20 randomly selected patches was determined using an electronic balance (WA2003N) and contents uniformity (weight variation) was determined. The thickness of three randomly selected patches from each formulation was determined using a Vernier Caliper. The average thickness was calculated. Folding endurance was performed by repeatedly folding the randomly selected patches in a single place until they cracked.

Content uniformity

The patch of 4cm^2 was cut to multiple parts (n=3) and were crushed to be dissolved in a buffer of pH 6.8 under magnetic stirrer for 24 h. Represented samples were drawn from each solution and diluted with buffer (100 times). Samples were then analyzed using a UV spectrophotometer (CECIL CE7400S) at 281nm wavelength (Chavan *et al*., 2022).

In-vitro release studies of transdermal patch

The patches of 4cm^2 from each formulation were analyzed using the same procedure performed earlier on nanoparticles. Here the patch was tied with the paddle of dissolution apparatus with drug layer on the outer side. The samples were withdrawn at various time intervals and analyzed for EE using UV-Visible Spectrophotometer. The contents were determined using standard curve drawn earlier.

Kinetics of drug release

Different kinetic models were applied on the release data using Drug Dissolution Solver Analysis Add-in Software Version 1.3 (DDSolver) to all the formulations and the best one was selected based on release properties. Additionally, the Korsmeyer-Peppas model was applied to assess the mechanism of drug release ($n<0.43$ = Fickian diffusion release, $n\geq0.43$ to ≤0.85 = Anomalous (non-Fickian) release, $n=0.85$ Case II release and $n>0.85$ Super Case II release) from the patch (Hussain *et al*., 2020).

Ex-vivo Skin permeation studies

Franz diffusion cell was used to perform the *ex-vivo* skin permeability of a nanoparticle-loaded transdermal patch of EE. The freshly slaughtered sheep mucosa was collected from local slaughter house and rinsed with normal saline. The epithelial and fat cells were removed to obtain dermal layer intact. The both types of patches, i.e. prepared with DMSO as permeation enhancer and without permeation enhancer (DMSO) with drug layer side were attached to the epidermal side of mucous membrane. The treated mucous membrane with patches was fixed between a receptor and donor cell of 10mL capacity Franz Diffusion Cell, while the epidermis attached to patch was faced towards the donor cell and dermis side to receptor cell both containing appropriate volumes of buffer solution pH 6.8 to provide sink conditions. The apparatus was operated at 37±0.5 °C with continuous stirring at a magnetic stirrer. The whole donor and junction of donor with receptor compartment were wrapped with Parafilm to avoid evaporation and drying. The samples (1mL) were withdrawn at 0.5, 1, 2, 4, 6, 8, 12, 18, 24, 36, 48 and 72h from the receptor cells and the equivalent volume of the solution (pH 6.8) was replaced. The samples were analyzed using UV-Visible Spectrophotometer at 281nm. The obtained results were used to plot the graphs on Microsoft Excel with time versus cumulative drug permeation per unit area.

Differential scanning calorimetric analysis

A DSC calorimeter (TA Instruments, D2000) was used to obtain the DSC curve. Before using the cell, DSC was calibrated against indium as standard using a temperature-controlled DSC cell. A small amount (5mg) of the testing sample was enclosed in a crucible and placed in a temperature-controlled DSC cell on a hermetic pan of aluminium and heated (10^{o}C per minute) under an inert nitrogen atmosphere (Aljabali *et al*., 2022).

Fourier-transform infrared spectroscopic studies

FTIR spectrophotometer was used to take FTIR spectroscopy of pure drug (EE), drug loaded nanoparticles and optimized patch. The data for samples was created against a spectral range of 4000-400cm^{-1} (Simu *et al*., 2022).

The sample was placed in an FTIR spectrophotometer that directed beams of IR at the sample and measured how much of the beam and at which frequencies the sample absorbed the infrared light.

Accelerated stability studies

Accelerated studies for EE nanoparticles loaded patches were performed at 40^{o}C and 75% relative humidity for 6 months. The samples were evaluated for drug content at 1, 2, 3 and 6-month intervals. The physicochemical characteristics of patches were also evaluated.

Patch test for skin irritation

Patch test was performed on the inner forearm of five healthy volunteers of the research group after ensuring intact skin free from any underlaying conditions. The optimized patch was applied on the skin by little force and adhesive tape was placed on its back. The test patch was left on the volunteer's skin for 72h. The test patch was then removed to check for any signs of erythema or erythema leading to edema on the skin and scores were generated between 0 to 7 depending upon severity of erythema as well as any of known effects (glazed appearance) appeared on the skin using a scale with score 0 to 6 (Robinson, 2001). The monitoring was based on appearance of any signs of irritation, such as erythema (redness), oedema (swelling), vesicles (blisters), or pruritus (itching).

Ethical approval

Office of Research Innovation and Commercialization (ORIC), University of Veterinary and Animal Sciences, Lahore issued Certificate of Ethical Handling of Experimental Animals numbered DR/614 Dated 21/12/2021 for handling and procedures applied.

STATISTICAL ANALYSIS

The formulation testing does not have a sample size to be tested statistically only dissolution kinetics require analysis and that have a tool of DDSolver for analysis. The manuscript contains all the analytical methods sufficient to describe state involved.

RESULTS

Pre formulation studies

Pre-formulation analysis of the EE was performed to find the characteristics suitable for transdermal delivery of drug. The identity and purity analysis were also performed to load the accurate dose of drug in the patch. The summary of all the results is tabulated in table 2.

Calibration curve of EE

Calibration curve of ethinyl estradiol was drawn to estimate the response of instrument against the fixed incremental concentrations (fig. 2). The curve was also used to estimate the drug contents from the content uniformity and the *in-vitro* drug release samples of nanoparticles as well as transdermal patches.

Post formulation studies

Characterization of EE nanoparticles

The drug loaded nanoparticles were analyzed for size using zeta sizer, morphology by electron microscope and

content analysis by UV-Visible Spectrophotometer. The results of the test performed are summarized in the table 3.

Physicochemical characterization of transdermal patches

The patch of the desired area (4cm^2) was examined for appearance, thickness (vernier caliper), folding endurance (physical folds), contents uniformity (weight variation) and content uniformity (UV-Visible Spectrophotometer). The results of the above tests are concise in the table 4.

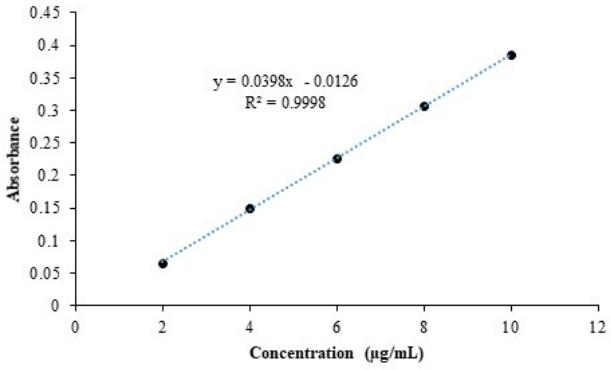

Fig. 2: Standard calibration curve of EE in 6.8 pH PBS

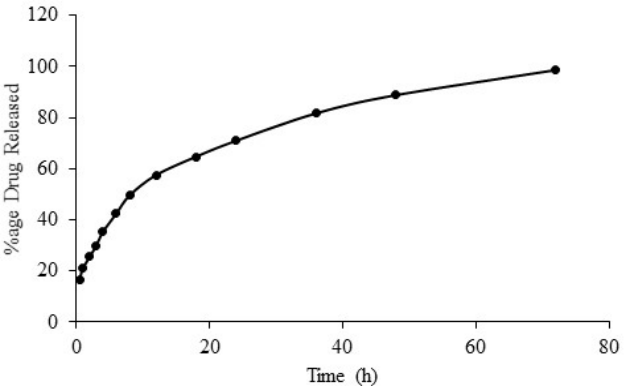

Fig. 3: Drug release profile of EE loaded nanoparticles at 6.8 pH PBS

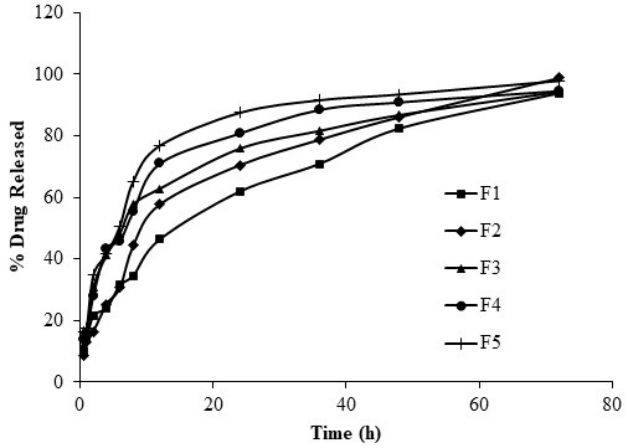

Fig. 4: *In-vitro* ethinyl estradiol release from TDDS patches at 6.8 pH PBS

In-Vitro drug release studies

In-vitro drug release studies were performed on the nanoparticle's formulation (without loading to patch) to establish a drug release relationship between time and amount of EE released as a function of combined diffusion and dissolution-controlled release matrices (fig. 3). The transdermal patch loaded with nanoparticles as well as prepared by solvent casting method was studied in phosphate buffer pH 6.8 and curves of time verses concentration were drawn to estimate the amount of drug released as a function of time (fig. 4).

Kinetics of drug release

The *in-vitro* drug release studies were further estimated by applying various kinetic models i.e. zero order, First order, Higuchi and Korsmeyer-Peppas using a freeware DDSolver Add-in program (table 5). The best fit model was selected on the basis of three parameters e.g. regression coefficient R^2, Akaike Information Constant (AIC) and Model Selection Criterion (MSC). These models were then used to interpret the mechanism of drug release from the TDDS patch formulations.

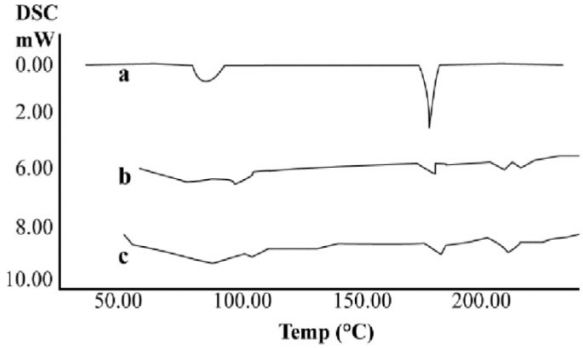

Fig. 5: Thermograms of pure EE (a), Nanoparticles of EE (b), Transdermal patch of EE nanoparticles

Compatibility analysis

The drug matrix with polymers, ionic gelation agents and permeation enhancers was studied for compatibility among ingredients by two tests i.e., Differential Scanning Calorimetry and Fourier Transform Infrared Spectroscopy. The results are depicted in figures as under (fig. 5 and 6).

Accelerated stability studies

The optimized formulation on the basis of desired drug release and better physicochemical properties was selected for short term stability studies in stability chamber. The formulation was assessed after 1, 2, 3, 6 months to set the achieved parameters (table 6).

DISCUSSION

Pre formulation studies

The melting point is a significant determinant of compound's identity and purity. The melting point of EE i.e., 173°C, indicated that the sample was pure ethinyl

estradiol. Hygroscopicity is the determinant of the moisture content of the compound and its stability in the moist environment. The difference between the weight of the EE before and after heating was found less than 1 %, indicating that the EE is slightly hygroscopic as per the European Pharmacopoeia Guidelines. Crystal morphology was studied using optical compound microscope and the sample showed hemihydrates of Estradiol (Du *et al*., 2019).

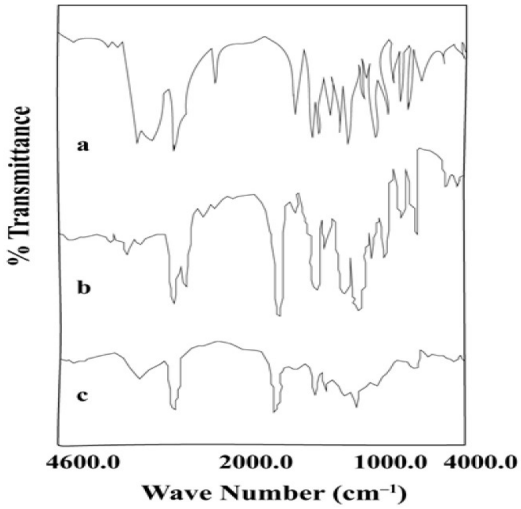

Fig. 6: FTIR transmission/absorption spectra of (a) Ethinyl Estradiol, (b) EE nanoparticles, (c) Optimized Patch

The flow properties of the EE were determined by the angle of repose, the Car's compressibility index and the Hausner's ratio. The angle of Repose for EE was estimated to be 43°, Hausner's ratio was commuted as 1.22, which indicated poor flow properties. The side tap sieving method was used to determine the particle size distribution of Estradiol. The sample exhibited very fine textured coarse particles ranging in size from 5-7 µm. Estradiol was insoluble in water and slightly soluble in 6.8 pH PBS, freely soluble in alcohol and soluble in acetone. The log P value of EE was 3.7, demonstrating the drug's lipophilic nature. The calibration curve of EE was linear with R2 found to be 0.999 that indicated instrument's ability to analyte the set of concentrations accurately within 2-20µg/mL. This standard calibration curve was used for approximation of the sample EE concentration of LogP values as well as laterally for drug release analysis values.

Post formulation studies

Characterization of EE nanoparticles

The particle size and polydispersity index were evaluated by a zeta sizer. The particle size for nanoparticles loaded with EE was 236nm and for blank nanoparticles as 210nm. The disparity of nanoparticles was assessed by the Polydispersity index values (PDI value ≤ 0.1 = good monodisperse system, PDI > 0.3 = nanoparticles polydispersity). The PDI for EE-loaded nanoparticles was found to be from 0.160 to 0.185. For blank nanoparticles, PDI was 0.117 to 0. The lesser the PDI value least is the size of particles and greater uniformity.

The nanoparticles were spherical with a narrow particle size distribution and uniform scattering. The biopolymers chitosan and sodium tripolyphosphate in the optimum combination resulted in high entrapment of the drug in nanoparticles (93%) (table 3).

In-vitro release study of EE loaded bio polymeric particles

The drug release of EE loaded nanoparticles showed continuous slow parabolic release for almost 72h representing that biopolymers have efficiently controlled the drug release from the nanoparticles (fig. 3). The texture and surface characterization of nanoparticles observed under compound microscope showed swell and porous surface. The swollen porous texture could release drug from particles by diffusion-controlled method.

Physicochemical characterization of transdermal patches

The physicochemical parameters of patches were assessed to justify the suitability of the patch for transdermal use (Lengert *et al*., 2020). All the patches formed were colourless, transparent and smooth except for a patch of F1 formulation, which was cloudy due to the presence of nanoparticles. Polymeric films with Chitosan and Sodium Tripolyphosphate were adherent in nature and had compatible physicochemical properties. The polymer ratio (Chitosan: Sodium Tripolyphosphate=1:1) was the main determinant of optimum formulations. Despite the lipophilic characteristics of EE, the NPs showed improved hydrophilicity due to the added surfactant (Tween 80) (Lengert *et al*., 2020). Due to maximum solubility in an aqueous acidic solution, Chitosan can control the drug's release. The cationic nature of Chitosan allowed crosslinking with other various anionic polymers. The polymeric film of the patches was flexible and non-brittle. The plasticizer PEG made the film elastic, flexible and uniform.

Eudragit RL 100 and Eudragit RS 100 were used as polymers in F2 formulation and Span 20 as surfactant (gelling agent) that made the film elastic in nature. F2 was designed based on varying polymer percentages, solvent volume and setting time.

F1 resulted in smooth, semi-transparent and wrinkle-free film. F1 presented a uniform patch weight, while patches of other formulations had a non-uniform weight. The drug content of all the formulations was relatively low and unacceptable except for F1 >97%. The permeation enhancer DMSO (0.03mL) was found to formulate patch film with uniform weight (Aggarwal *et al*., 2013). The folding endurance value of F1, F2 and F3 was found to be optimum, which showed that the patches had sufficient flexibility and were non-brittle. All formulations passed the folding endurance test except F4 and F5.

Table 1: Different formulations of EE Transdermal patch

Ingredients	F1	F2	F3	F4	F5
Eudragit RL 100 (mg)		300		100	150
Eudragit RS 100 (mg)		200		100	100
Chitosan (mg)	200				
Sodium Tripolyphosphate (mg)	200				
HPMC (K-100) (mg)			300		
Ethyl Cellulose (mg)			150		
DMSO 10% (mL)	0.03		0.04		
PEG 400 (mL)	0.1				
Span-20 (mL)		0.1			0.2
Tween-80 (mL)	0.014				
di-n-butyl phthalate (mL)		0.4	0.6	0.04	0.04
Solvents[1] (mL)	2	10	20	8	20
Backing membrane solution[2](mL)	20	20	20	20	20

[1]Isopropanol-dichloromethane for Eudragits, [2] double distilled water

Table 2: Pre-formulation studies of EE Transdermal patch

Parameter	Instruments/Apparatus/ Method	Results
Melting point	Fisher Johns Apparatus	173 °C
Hygroscopicity	Desiccator	Slightly hygroscopic
Crystallinity	Compound Microscope	Orthorhombic Crystal System
Flow properties	Hausner's Ratio/Angle of Repose	Poor
Particle size distribution	Side Tap Sieving Method	Very fine particles
Log P	Partitioning Funnel	3.7
Aqueous solubility (6.8 pH phosphate buffer)	Thermogravimetry	0.01 parts per liter

Table 3: Characteristics of EE-Loaded Nanoparticles

Parameters	Results
Size	Loaded 236nm, Blank 210nm
Morphology	Spherical shaped
Yield (%)	92
Encapsulation efficiency (%)	93
Drug content (%)	90

Table 4: Physiochemical properties of EE patch

Formulation	Physical Appearance	Thickness (µm)	Folding endurance (no. of times)	Weight (mg) 2×2cm^2	Drug content (%)
F1	C, S, NU	500±0.39	200±0.90	45.52±0.92	97.59±0.67
F2	C, S, U	611±0.65	210 ± 0.52	49.67±2.28	93.65±0.85
F3	C, S, NU	752±0.93	240± 0.36	57±0.92	80.16±0.39
F4	C, S, U	760±0.54	96± 0.98	58.2±0.36	70.59±0.73
F5	C, S, NU	721±0.74	65± 0.67	56.81±0.48	90.13±0.67

C= Crystalline, S=Smooth, NU=Non-Uniform, U=Uniform

Table 5: Kinetic Modeling of TDDS formulations F1-F5

Formulations	Zero-order			First Order			Higuchi			Korsmeyer-Peppas			
	R^2	AIC	MSC	R^2	AIC	MSC	R^2	AIC	MSC	R^2	AIC	MSC	N
F1	0.54	92.57	0.60	0.92	72.72	2.40	0.98	55.13	4.00	0.99	44.65	4.96	0.45
F2	0.49	96.04	0.50	0.96	67.93	3.05	0.96	68.38	3.01	0.97	65.98	3.23	0.43
F3	-0.22	103.06	-0.38	0.87	78.36	1.87	0.78	84.26	1.33	0.95	68.94	2.72	0.32
F4	-0.19	104.12	-0.36	0.93	72.15	2.55	0.78	85.58	1.33	0.93	73.18	2.46	0.32
F5	-0.42	106.47	-0.53	0.95	69.18	2.86	0.70	89.55	1.01	0.91	76.55	2.19	0.30

Table 6: Accelerated stability studies of Transdermal patch of EE-loaded Nanoparticles at 40°C and 75% RH.

Time (month)	1	2	3	6
Physicochemical properties	Unchanged	Unchanged	Unchanged	Slight Color Change
Drug content	≥ 85%	≥ 85%	≥ 85%	≥ 85%

In-vitro release studies of EE patches

The release profile of the drug in all five formulations was examined for 72h. The results showed that F1 released the drug in better way in the given time. While formulations F2, F3, F4 and F5 released abruptly during the first 12h of analysis and showed steady release till 72h. The formulation F1 of EE-loaded nanoparticles with chitosan and sodium tripolyphosphate in a 1:1 ratio demonstrated the maximum drug release (98%) in 72h (fig. 4) while F2 and F5 showed 100% release but more rapid first phase and then declining.

Kinetics of drug release

Percentage release data of five formulations (F1-F5) was fitted to four different kinetic models i.e., zero order, first order, Higuchi and Korsmeyer-Peppas to explain the mechanism of release and concentration dependency or independency of Ethinyl Estradiol from the patch. DDSolver Add-in software was used to estimate the optimized model-based mechanism of drug release from patches. The parameters of each model were estimated i.e., regression coefficient (R^2), its value >0.97, AIC values lesser than 50 and MSC values more than 3 (Hussain *et al*., 2020). The formulation F1 followed the Higuchi as well as Korsmeyer-Peppas kinetic model that depicted drug release independent of concentration and was evaluated as swelling controlled drug delivery system. Higuchi dependance proved the release from the patch matrix dependent on diffusion of drug through porous texture patch surface so a slow sustained drug release. The Korsmeyer-Peppas model described the n value as 0.45 that correspond to anomalous release (combined diffusion and dissolution controlled) for slab (patch) formulations. So, the release from the nanoparticle-loaded patch (F1) was found superior to all the release mechanism of other formulations. F2 formulation was the next closer formulation with both first order and Higuchi partial dependance due to MSC value greater than 3.0, that also correspond to partially dependence on concentration and partially concentration independent release. Similarly better dependence on Korsmeyer-Peppas model with n value of 0.433 correspond to swelling controlled combined diffusion and dissolution drug release of non-Fickian mechanism. All other formulations didn't follow any of the model specifically that represented complete dependence on concentration (Fickian release) and major burst release during first 12h of analysis (Hussain *et al*., 2015). So, based on above assumptions formulation F1 patch was further selected for permeability, patch test and compatibility analysis.

Ex-vivo skin permeability studies

The values of permeation flux calculated from the permeation data were found substantially higher for permeation of the patch when loaded with permeation enhancers as the quantity of enhancer increased. The patch without a permeation enhancer showed a permeation flux value of 1.1 ± 0.2 $\mu g/cm^{-2}h^{-1,}$ while the patch with DMSO as a permeation enhancer showed a permeation flux value of 4.12 $\mu g/cm^{-2}h^{-1}$. Studies have been reported that showed nanoparticles efficiently cross skin by using DMSO (Luo *et al*., 2021).

Differential scanning calorimetric analysis

EE's characteristic endothermic peak (fig. 5a) was observed at 183.61°C, which indicates its melting point. Ethinyl estradiol showed stability up to 78°C, then it showed water loss from its structure that decreased its residual mass till 101°C. The EE nanoparticle (fig. 5b) exhibited an endothermic peak at 179.24°C. However, the optimized patch showed this peak at 209.20°C (fig. 5c). In DSC analysis, all the excipients were found compatible based on the sharpness and less than 15% shift in the melting peak of EE. So, it had been observed that both alone nanoparticles and optimized patches contain EE in its original form. There were decreased weight peaks (mW) of drug observed in the patch formulation that suggested complete solubility and better blending efficiency of the matrix.

Fourier-transform infrared spectroscopic studies

The FTIR analysis demonstrated no significant peak shift in the spectra of the drug (fig. 6a) and formulation matrix (fig. 6c). However, some drug peaks were found to overlap in the same polymer region (fig. 6a, b). FTIR studies revealed no chemical interaction between the drug and polymers due to nonappearance of any new peaks and disappearance of existing peaks. The FTIR spectra of EE showed a large band from 3436 to 3227cm^{-1} due to OH stretching from the intermolecular H-bonds; intense bands at 2970, 2935 and 2866cm^{-1} from CH_3 and CH_2 asymmetric stretching and CH_2 symmetric stretching, respectively; medium-intense bands from C=C stretching of the aromatic ring at 1616, 1581, 1500 and 1469cm^{-1}; and a band at 1253cm^{-1} from phenolic C–O stretching (Kiemle *et al*., 2016).

However, the characteristic functional group from the IR spectrum of nanoparticles exhibited vibrational frequency in correspondence with that of the drug spectrum (3435cm^{-1} due to OH stretching, 2953cm^{-1} from alkane stretching, 2822- 2772cm^{-1} from dimethyl amino

stretching and C=O ester stretching at 1734cm^{-1}). Likewise, the same vibrational frequencies of characteristic functional groups in the patch formulation revealed the correspondence to that of drug-coated nanoparticles spectra (3452cm^{-1} from OH stretching, 2959-2828cm^{-1}due to C-H stretching and 1460cm^{-1} from C–H bending, alkane, –CH2 and –CH3). Overall, no significant shift in absorption spectra was observed, confirming no physical and chemical interaction among the drug and polymers of the optimized patch.

Accelerated stability studies

There was slight to no change in the physicochemical characteristics (Color, Uniformity, Texture) of the patch even after six months. The patch drug content was found more than 85% at 40^{o}C and 75% RH, as shown in table 6. The result showed that the EE nanoparticles loaded patches were stable throughout the months, which ensured the successful development of the transdermal patch.

Skin patch test

The severity of the reaction after application of the patch to the selected area of skin was recorded and evaluated using a standardized grading scale as described by Robinson in 2001. The severity of the skin reaction of the transdermal drug delivery system was classified as non-irritating, mildly irritating, moderately irritating, or severely irritating along with skin appearance (0-7).

In this study, the patch test showed that the nanoparticles patch was non-irritating (no erythema), as well as there was no or very least known effects were observed. The volunteers developed no significant skin reactions or irritation except for the glazed appearance of one volunteer was reported having score of 1. One volunteer was also reported with erythema (redness) of skin with score of 0. This finding provided strong evidence for the safety of the EE nanoparticles patch and supported its further development and clinical evaluation. (Nelson *et al*., 2021).

CONCLUSION

EE-loaded nanoparticles were successfully prepared and loaded into the transdermal patch. The use of permeation enhancers (DMSO) successfully increased the permeation of the patch. According to physicochemical characterization, release profile and drug contents, F1 has declared an optimized formulation. Patch improved bioavailability and reduced dose frequency of EE for achieving contraception. The patch test for skin irritation ensured the safety of the formulation.

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