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Journal of Controlled Release 233 (2016) 19–28

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

A novel approach to administration of peptides in women: Systemic absorption of a GnRH agonist via transvaginal ring delivery system☆

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ARTICLE INFO

Article history:
Received 29 October 2015
Received in revised form 11 April 2016
Accepted 24 April 2016
Available online 26 April 2016

Keywords:
Intravaginal Ring
EVA
GnRH Agonist
Peptide
Phase 1
Transepithelial

ABSTRACT

trans-Epithelial delivery of medication across the vagina has proven successful for administration of small, lipophilic molecules such as sex steroids. However, little information is available regarding the vaginal delivery of larger and more polar molecules that currently require parenteral administration because the vaginal epithelium is perceived as a barrier to absorption of larger molecular weight (MW) molecules. Six healthy women underwent administration of 18 or 36 mg of leuprolide, a GnRH agonist and a larger MW peptide, via a novel ethylene vinyl acetate (EVA) ring transvaginal drug delivery system (TVDS). Serum levels rose within 8 h following insertion: low dose at 310 pg/ml and high dose at 1220 pg/ml, i.e. levels typically following parenteral injections of leuprolide. GnRHa biological activity was validated by secretion of gonadotropins and sex steroids. These results demonstrate that the non-keratinized vaginal epithelium permits a rapid absorption of a biologically active peptide and that there is significant potential for a novel TVDS to deliver peptides and possibly other macromolecules therapeutically.

Significance statement: Current routes of administration of medications can include oral, subcutaneous, intravenous, intramuscular, transcutaneous, etc. Many of these approaches have limitations, including pain, poor tolerability, lack of adherence, and inadequate delivery. Peptides, in particular, cannot typically be given orally because they are broken down in the intestinal tract before they are absorbed. While the skin is an attractive way to deliver medications, its superb intrinsic barrier function often makes this route untenable at times. The vaginal epithelium, in contrast, is not keratinized and can allow absorption of other molecules. In this study, we demonstrate that a novel transvaginal drug delivery system (TVDS) is capable of delivering peptide therapeutics to women in a non-parenteral fashion as demonstrated by both blood levels and biologic effects of its delivery.

1. Introduction

A major therapeutic limitation of peptide and protein treatments has been their requirement for intramuscular or parenteral administration. Consequently, transepithelial delivery of medications has been an area of substantial interest and research. Among the various cutaneous sites for such medication delivery, the vagina is a potentially favorable one not only for local but also for systemically acting drugs for several reasons. These include: a) dense vasculature; b) lack of squamous keratinization (a property shared only with the buccal mucosa, which is a site incapable of supporting long term administration); c) direct access to the systemic circulation; d) ability to circumvent the 'hepatic first pass phenomenon' inherent in oral administration; e) demonstrated permeability to sex steroids [1]; f) potential for high drug absorption capacity in a sustained release fashion for weeks to months [1]; g) minimization of daily medication administration compliance issues; and h) ease of use [2,3].

Despite these advantages, conventional wisdom is that vaginal administration is limited by the molecular size of the therapeutic agent(s) and their polarity due to size barriers inherent in skin epithelium [4]. Additionally, physiological variations of the vaginal epithelium throughout the menstrual cycle can affect both drug solubility and vaginal membrane penetration [1,5]. Despite these largely theoretical

☆ This study was funded by Combinent Biomedical Systems and the Swensrud Foundation

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[1] A consultant to Combinent.
[2] No disclosures.
[3] A co-Founder of Combinent and the Chief Technology Officer at Combinent.
[4] A co-Founder of Combinent and acquired equity in Combinent, after which he had no further involvement in the design or conduct of the research.
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http://dx.doi.org/10.1016/j.jconrel.2016.04.035

limitations, vaginal rings have proven quite successful in delivering small lipophilic molecules such as the naturally occurring sex steroids estradiol and progesterone for hormone replacement therapy and synthetic estrogens and progestins for contraception with substantial market success in these two areas [6,7].

The normal functioning of the hypothalamic-pituitary-ovarian axis critically depends upon the pulsatile secretion of Gonadotropin-Releasing Hormone (GnRH) from the hypothalamus [8]. In contrast, continuous GnRH administration or long acting Gonadotropin-Releasing Hormone agonists (GnRHa) pharmacologically desensitize pituitary gonadotropin secretion and silence all downstream gonadal function in both sexes [9,10,11]. Thus, parenteral administration of a wide variety of GnRH analogues have been used in women and men to induce a biochemical castration over the past thirty-five years with therapeutic indications including the treatment of precocious puberty, endometriosis, uterine fibroids, and prostate cancer [12]. As restoration of normal reproductive function in women promptly ensues following the discontinuation of GnRHa administration, this biochemically-induced 'medical castration' has provided a remarkably safe and reversible suppression of reproductive function in all of these medical conditions over the past three decades and now accounts for a several billion dollar annual pharmaceutical market. However, all of these indications require periodic injections since administration of a peptide is required.

In comparison with sex steroids, all GnRHa are peptides, larger and more polar molecules. Systemic vaginal drug delivery of leuprolide, the prototypical GnRHa, has been possible in experimental studies in the rat as evidenced by their ability to stimulate luteinizing hormone (LH) and follicle secreting hormone (FSH) levels following transvaginal administration [13]. However, current vaginal ring technologies are incapable of continuously releasing molecules over a few hundred molecular weight including peptides and proteins. To our knowledge, transvaginal delivery of GnRHa, or for that matter any macromolecule, has never been shown in higher animals or humans.

The present study was thus undertaken to design, develop, and test a novel transvaginal delivery polymer system that could release larger molecular weight substances such as a peptide in the form of a prototypical GnRHa, leuprolide (synthetic, man-made 9 amino acid sequence: p-Glu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt) hormone that is similar to the natural hormone, GnRH, that is produced in the brain. Importantly, these tests were conducted in humans and measured both serum GnRHa levels and physiologic responses of gonadotropin and sex steroid responses. Leuprolide was selected as it is representative of the class of GnRHa; is approximately 40× more potent than the natural sequence GnRH; is commercially available; is much less polar that natural sequence GnRH; and is capable of inducing pituitary gonadotropin desensitization at far lower doses than the natural sequence GnRH.

All of the five commercially available transvaginal rings designed to release small steroidal molecules (NuvaRing®, Femring®, Estring®, Fertiring®, and Progering®) utilize a rate controlling membrane. However, these particular constructs enable the controlled release of only low molecular weight molecules capable of passive diffusion through the outer membrane. This particular design has the potential risk of 'dumping,' or rapid increases in serum levels of their contents, if a break in the ring and/or the membrane occurs, as there is not a matrix system underneath. Consequently, in order to release a larger peptide such as leuprolide acetate via this route, a new intravaginal ring design without a membrane, i.e. a homogenous cross-section ring, was developed. This vaginal ring system went through several critical design steps in order to create formulation parameters that achieved desired release kinetics for peptides. These included iterative optimization for the appropriate size, mechanical properties, and stiffness that provided desired release kinetics for a peptide alone or in combination with sex steroids. This included a careful selection of the appropriate mixture of medical grade ethylene-vinyl acetate (EVA) polymers and excipients to control for stiffness/rigidity of the ring, minimization of the knit-line formation, and to effectively control the release kinetics of the ring. Finally, a scaled-up manufacturing approach to create rings that could be approved for use in humans was developed.

2. Methods

2.1. Ring design, construction, and loading

Selection of polymers for the ring was limited to medical grade EVA (Evatane® by Arkema, France). This provides superior biocompatibility over silicone-based polymers and also exhibits good mechanical properties. Evatane is a random copolymer of Ethylene and Vinyl Acetate made by a high-pressure radical polymerization process.[6] These specialty thermoplastic copolymers are inherently flexible, resilient, tough, and show excellent resistance to environmental stress cracking.[7] Although Evatane is available in various grades (varying ratio of ethylene to vinyl acetate), they all possess similar general properties. The following table (Table 1) demonstrates typical physical properties of three Evatane polymers that were used[8]:

A scalable process of melt extrusion was developed to produce the intravaginal rings under current good manufacturing practices (cGMP) guidelines. (See Fig. 1).

EVA copolymer granules were cryogenically milled to reduce the EVA bead size to a fine powder.

Initial attempts to use Evatane showed that the some of the lots had a high amount of vinyl acetate residues. In order to reduce the level of monomeric vinyl acetate in EVA copolymer, the beads were washed with water.[9]

150 g of Evatane 40–55 was weighed into a weighing pan. One liter of de-ionized water was placed in a 2-L beaker and stirred using an Arrow Engineering Mixer with a setting of 2.5. The polymer was charged into the beaker while mixing. The copolymeric beads were stirred in water for 20 min. After stirring, the material was filtered and collected. Evatane beads were washed nine times by repeating the washing and filtration cycle. Following the wash-filter cycle the washed-filtered beads were collected and further drained by passing the copolymeric material through a 1700-μm sieve. Finally, Evatane, along with the sieve, was placed in a vacuum oven for 48–72 h.[10]

The presence of residual (unreacted) monomeric vinyl acetate present in EVA beads was determined using head-space gas chromatography (HS-GC). The remaining vinyl acetate monomer of the raw and the washed EVA beads were determined to be 1458 ppm and 0.9 ppm, respectively.[5]

The intravaginal leuprolide acetate rings were prepared from the washed polymer (EVA). 18 mg, 36 mg, or 54 mg leuprolide acetate was dissolved or dispersed in approximately 10 mL of ethanol in scintillation vials, followed by the addition of 1400 mg of Evatane to the ethanolic solution while mixing using a rotary shaker. The resulting mixtures were solvent-casted in dry ice using ethanol as the solvent (Pharmco; Cat. No. 111 USP 200 CSGL; Lot No. M8241). The solvent

[6] http://www.evatane.com Accessed 8 October 2015

[7] Elvax® technical literature. Du Pont. http://www.dupont.com//content/dam/dupont/products-and-services/packaging-materials-and-solutions/packaging-materials-and-solutions-landing/documents/elvax_molding_compounding_extrusion.pdf Accessed 8 October 2015.

[8] Evatane® technical datasheets. Arkema. http://www.evatane.com/export/shared/.content/media/downloads/products-documentations/altuglas-international/pof/evatane/tds-evatane-18-150.pdf; http://www.evatane.com/export/shared/.content/media/downloads/products-documentations/altuglas-international/pof/evatane/tds-evatane-28-40.pdf; http://www.evatane.com/export/shared/.content/media/downloads/products-documentations/altuglas-international/pof/evatane/tds-evatane-40-55.pdf Accessed 8 October 2015

[9] Ron, Eyal S. and Tan, Hock, inventors; 2009 Oct. 22. Devices that include ethylene-vinyl acetate copolymers and methods of making and using same. WO 2009129459 A1.

[10] Evatane® technical datasheet. Arkema. http://www.evatane.com/export/sites/evatane/.content/medias/downloads/tds/tds_33_45_PV.pdf DOI May 2012. Accessed 8 October 2015.

Table 1
Evatane grades that were used and their main typical properties[1].

Grades	Specified properties*		Typical properties						
	Vinyl acetate content (%)	Melt index (g/10mn)	Melting point (°C)	Vicat point (°C)	Ring & Ball (°C)	Tensile strength at break (MPa)	Elongation at break (%)	Hardness shore A	Density (g/cm^3)
18–150	17–19	135–175	88	47	102	10	300–500	85	0.94
28–40	27–29	35–45	70	<40	106	10	800–1000	73	0.95
40–55	38–41	48–62	54	<40	97	7	900–1100	50	0.96
Test method	FTIR	ASTM D1238	D.S.C.	ASTM D1525	ASTM E28	ASTM D638		ASTM D2240	ASTM D1505
ISO standard		1133	11,357	306	NF EN1238	527		868	1183

Melt index done at 190 °C and under 2.16 kg.
(*) Properties routinely measured during the standard quality control procedure.

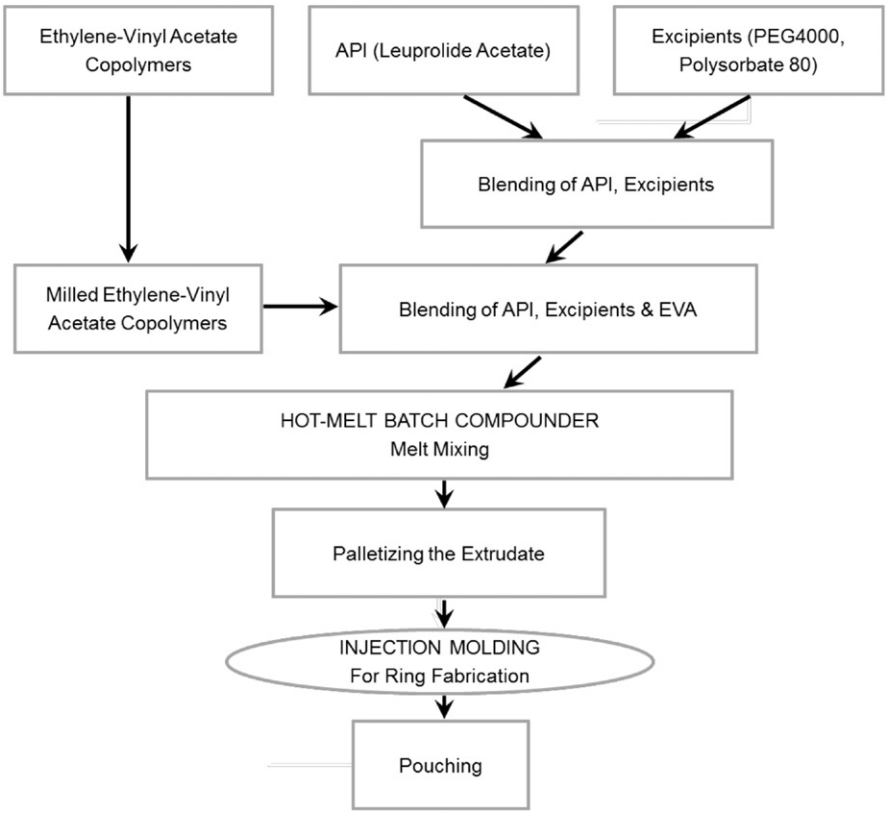


Fig. 1. Flow diagram of scalable process of melt extrusion producing the intravaginal ring.

was allowed to evaporate overnight, and the dry EVA/drug mixtures were then ground into powders.[11]

The milled EVA polymers were combined with the initial blend or granulation of leuprolide acetate (PolyPeptide Laboratories Inc.), PEG4000 (Union Carbide Corp/Dow Chemical Co.), and Polysorbate 80 (Uniqema/Croda PLC) to produce leuprolide acetate/EVA granulation that was fed to the extruder, after which the extrudate was pelletized and rings were formed by injection molding. The resulting rings were individually wrapped in moisture- and light- protective packaging to protect the GnRHa peptide.

The EVA/drug powders were placed in an injection molding unit (DSM, Geleen, Holland). The injector was heated to approximately 80 °C. The molten EVA/drug compositions were extruded into stainless steel mold at 10 °C, creating a ring with an outer diameter of 50 mm and a cross section of 4 mm.[12]

The formed intravaginal EVA rings were tested for their properties (physical appearance, identification, potency, related substances/impurities, leuprolide acetate release rate, see Table 2) and supplied to the clinic.

A typical process was conducted as follows:

A 750 g batch of leuprolide acetate extruded pellets, 2.4% w/w, was manufactured using the components described in Table 3 below[13]:

2.1.1. Primary mixing

30 g of polyethylene glycol, NF was placed in the bowl of a GMX-Lab Micro High Shear Mixing System with a 1 l bowl and 1 l blades. The GMX-Lab Micro was closed with the chopper set to OFF. The polyethylene glycol, NF was then mixed with a plow speed of 155 rpm for approximately 30 s. 18 g of the leuprolide acetate was then added into the 1 l bowl of the GMX-Lab Micro. The remaining 30 g of the polyethylene glycol, NF was used to rinse the liner from the leuprolide acetate into the 1 l bowl of the GMX-Lab Micro. The GMX-Lab Micro was then closed with

[11] Ron, Eyal S. et al., inventors; 2011 Nov. 17. Devices and methods for treating and/or preventing diseases. United States patent US 20110280922 A1.
[12] Ron, Eyal S. and Tan, Hock, inventors; 2009 Oct. 22. Devices that include ethylene-vinyl acetate copolymers and methods of making and using same. WO 2009129459 A1.
[13] Ron, Eyal S., inventor; 2013 Jun. 20. Vaginal Drug Delivery Devices and Manufacturing Methods. WO/2013/090871.

Table 2
Release properties of the intravaginal EVA rings.

Attribute	Acceptance criteria		
Physical appearance	Translucent whitish ring		
Identification	EVA by FTIR, conformed to typical IR peaks		
	Leuprolide by RP-HPLC, conformed to the retention time		
Assay	90.0–110.0% of label claims as free base		
Content uniformity	USP <905> (AV < 15)		
Related substances/impurities	Individual: NMT 1.0% Total: NMT 5.0%		
Compression force	10 mm		Report results
	20 mm		
	30 mm		
	33 mm		
Dissolution (pH 4)	- Report results of cumulative release over 28 days (%)		
	- Report results of sampling and assay at: 1 h, 4 h, 1, 2, 3, 5, 7, 10, 14,18, 21, 25 and 28 days		
Microbial limit test	Aerobic plate count:	<100 cfu/g	- Absence of *Pseudomonas aeruginosa*
	Yeast & mold plate count:	<10 cfu/g	- Absence of *Staphylococcus aureus*
			- Absence of *Candida albicans*

Further optimization of the ring manufacturing process took place while clinical trials were being conducted. The goal was to create a scalable process that will form intravaginal rings that meet the performance criteria as it was set out above.

the chopper set to OFF and the Polysorbate 80, NF was slowly charged from the syringe into the GMX-Lab Micro bowl while mixing with a plow speed of 155 rpm. The combination was mixed for approximately 3 min.

2.1.2. Secondary mixing:

332.25 g of ethylene vinyl acetate copolymer, milled (Evatane EVA 18–150, milled) was then added into the bowl of a GMX-Lab Micro High Shear Mixing System with a 4 l bowl and 4 l blades (1st Layer). The polyethylene glycol, NF, leuprolide acetate, and Polysorbate 80, NF mixture was then collected from the GMX-Lab Micro 1 l bowl and transferred to the 4 l bowl of the GMX-Lab Micro (2nd Layer). 332.25 g of ethylene vinyl acetate copolymer, milled (Evatane EVA 28–40, milled) was then added into the 4 l bowl of the GMX-Lab Micro (3rd Layer). The GMX-Lab Micro was closed and the combination was mixed for approximately 5 min at 425 rpm with the chopper set to ON, low speed range. The blend was then transferred into a container double lined with poly bags with 2 desiccant, 4 unit silica gel between the inner and outer liner.

2.1.3. Hot melt extrusion procedure

A Leistritz ZSE 18 HP Extruder System with a 25:1 extruder barrel was arranged with the following barrel configuration: Open Barrel (Feed); Closed Barrel; Closed Barrel; Open Barrel (Vent); Closed Barrel; Final Melt Plate. The 25:1 length/diameter ratio twin screws were assembled as shown in Fig. 2 and installed into the extruder. A 3.0 mm single bore round die and spacer was installed onto the final melt plate. Supply and return connections were made between a Tempered Water Generator (TWG) and the extruder. In particular, tempered water lines were connected to the extrusion barrel manifold, one set of cooling water lines was connected to the feeding barrel, one set of cooling lines was connected to the gear box, and all supply and return valves were placed in the open position.

Table 3
Components of leuprolide acetate extruded pellets, 2.4% w/w.

Component	% w/w of extrudate	Theoretical amount for 750 g	Amount dispensed
Evatane 28–40	44.3	332.25	332.25
Evatane 18–150	44.3	332.25	332.25
PEG 4000	8.0	60.0	60.0
Polysorbate 80, NF	1.0	7.5	*7.575
Leuprolide Acetate	2.4	18.0	18.0
Total extrusion blend	100.0	750	750.075

* The listed components were dispensed according to the mass listed in the far right column. 7.575 g of Polysorbate 80, National Formulary (USP-NF) (NF) was dispensed into a 60 mL syringe. A1% excess of the Polysorbate 80, NF over the theoretical amount of 7.5 g was dispensed to allow for residual losses in the syringe.

The TWG and pump were turned on, the chilled water set point was adjusted to 13.0 °C, and the tempered water set point was adjusted to 30.0 °C. A K-Tron KCL24T20 Feeder w/ 12 mm diameter 20 pitch screws was connected to, and positioned behind the extruder. The K-Tron was bonded/grounded to the extruder and the impeller inside of the feeder hopper was installed so as not to touch the wall of the hopper. The extruder was turned on and the temperature set points for each heating zone were set according to Table 4 below:

A 30-min wait time was observed to allow the extruder to reach thermal equilibrium. The K-Tron feeding method was set to normal and the K-Tron feeder hopper was filled with the blend from the GMX-Lab Micro described above. The feed rate was set to 0.75 kg/h and the K-Tron was run until the material began to flow to prime the feed screws. The K-Tron's auto calibration routine was then executed and the K-Tron was aligned with the extruder feed opening. The K-Tron feeder was set to Gravimetric Dosing mode and the feed rate set point for the extruder was set at 100% for feeder 1 and 0.0% for feeder 2. A Dorner Cooling Conveyer was aligned with the extruder die with cooling fan 1 OFF and fans 2, 3, and 4 ON.

The feed opening of a Scheer Bay BT-25 Pelletizer was aligned at the end of the cooling conveyor. A container double lined with poly bags with 2 desiccant, 4 unit silica gel between the inner and outer liner was placed beneath the pelletizer discharge chute to collect the pelletized material.

The screw drive was started at a rate of 10.0 rpm and the screws were allowed to turn a few revolutions to ensure proper installation. The screw speed was then increased to 50 rpm (+10 rpm) and feeder 1 was started at 100% of the feed rate total for an effective feed rate of 0.75 kg/h (+0.2) kg/h. The cooling conveyer was bypassed and the extruder was run into a waste container for 5 min, or until the extrudate was translucent with no dark spots. The conveyer and pelletizer speeds were adjusted to draw the extrudate in such a way as to produce pellets approximately 5 mm or smaller. The product funnel on the extruder was monitored to ensure buildup of material did not occur. If excessive build-up was observed, excess material was vacuumed from the product funnel area. During the batch, the heated zone set points were adjusted within the ranges noted in Table 4 above to maintain the target temperature.

The extrudate was visually examined to verify that the extrudate was translucent with no dark spots. If extrudate appearance became opaque or output decreased, the melt plate was heated to 80 °C to 90 °C using a torch. The K-Tron feeder hopper was refilled as necessary and set to the Gravimetric Dosing mode after each re-fill. The pelletized extrudate was added into an 8 quart V-Shell blender and blended for

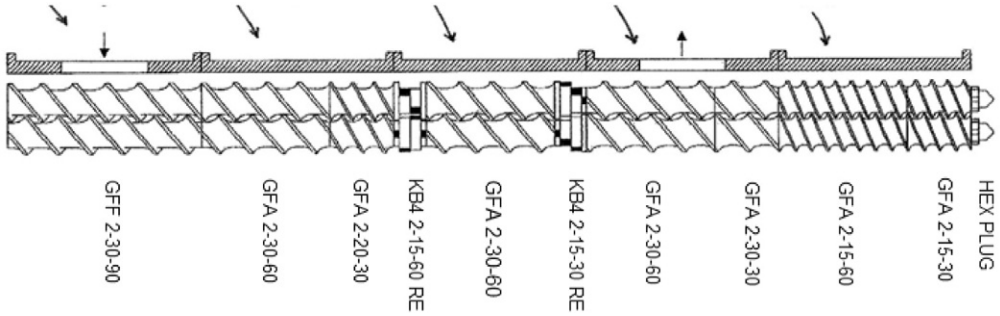


Fig. 2. A schematic diagram of the barrel and the elements configuration of the extrusion system.
Ron, Eyal S., inventor; 2015 Jan. 1. Vaginal Drug Delivery Devices and Manufacturing Methods. United States patent US 20150004213.

5 min (±1 min). The blended extrudate was then transferred into a container double lined with poly bags with 2 desiccant, 4 unit silica gel between the inner and outer liners.

3. Injection molding procedure

The pelletized and blended extrudate was then transferred to the hopper of a Sesame Nano-Molder Injection Molding Machine configured with the mold plates of Fig. 3, which defined a mold cavity with a 4 mm minor ring diameter and a 54 mm major ring diameter. A ring-shaped drug delivery device was injection molded using a 2.0 mm circular nozzle, a molding pressure of 1550 bar, a speed setting of 100 mm per second, a mold temperature of 55 °C, and a barrel temperature of 85 °C. The finished drug delivery device was then allowed to cool and removed from the mold.

To determine the ring's mechanical performance, a model was developed which mimics the intravaginal mechanical forces that a TVDS may typically experience due to increased intra-abdominal pressure (e.g. coughing, running, or defecating) [15]. This method measures the ring compressibility (elastic modulus) by determining the mechanical force required to reach a fixed distance at four compression points: 10, 20, 30 and 33 mm. NuvaRing® was used as a standard comparator due to its high level of patient acceptability [16]. A summary of the different formulations, including the different EVA weights and excipients that were tested for their mechanical properties is shown in Tables 5 and 6.

Higher molecular weight blends of EVA (e.g. Batch A) provided higher ring rigidity similar to the rigidity of NuvaRing®. It was found that a composition of EVA Copolymer with 28% vinyl acetate (VA) content, (Evatane® 28-40) blended with EVA Copolymer with 18% VA content (Evatane® 18-150) provided the optimum mechanical properties balancing rigidity and flexibility.

The ring had an acceptable compression force that supported easy insertion by the woman, yet once in place allowed it to re-expand to its original round shape and resist the mechanical stress inside the vaginal cavity, thus increasing the intravaginal retention of the rings. The addition of vaginally-accepted excipients [14] allowed us to modify the release kinetics iteratively while acting as plasticizers to reduce the mechanical stiffness of the neat polymers to an acceptable compression force.

Table 4
Temperature Set Points for Extruder Heating Zones.

Zone	Feeding zone	1	2	3	4	Melt plate
Set point (°C)	N/A	80	80	80	80	Record actual
Set point range (°C)	N/A	±10	±10	±10	±10	N/A
On/off	Off	On	On	On	On	Off

3.1. Subjects

This study was reviewed and approved by the Institutional Review Board of the Partners Healthcare system and conducted at Brigham and Women's Hospital. Written informed consent was obtained and documented in accordance with the Declaration of Helsinki. Healthy normal volunteers were recruited to participate in a three-day outpatient study beginning between days 1–7 of their menstrual cycle (i.e., the early follicular phase), during which there is a nadir of their endogenous sex steroid hormone levels. A negative urinary human gonadotropin test was obtained prior to study initiation to assure their non-gravid state. Twenty-two female subjects aged 18–40 years were screened for the study; ten screen-failed and the remainder were lost to follow-up. Reasons for screen failure included laboratory abnormalities, such as mildly elevated baseline LFTs and prolactin levels, increased BMI, and irregular menstruation. Six subjects were lost to follow up before their baseline visit, and six subjects participated. The demographics of the six participants are included in Table 7.

3.2. Trial design

On the first study day, four baseline blood samples of 5 mL were drawn at 20-min intervals over an hour (Time = 0, +20, +40, +60)

Fig. 3. One of the two mold plates.

Table 5
Component formulations of rings.

Sample name	Batch A	Batch B	Batch C	Placebo (neat EVA)	Placebo (with excipients)
EVA 18–150	44.3%	44.3%	44.3%	50%	45.5%
EVA 28–40		44.3%	44.3%	50%	45.5%
EVA 28–25/25	44.3%				
PEG 4000	8%	8%	8%		8%
Tween 80	1%	1%	1%		1%
Leuprolide	24 mg/g	24 mg/g	24 mg/g		

Table 6
Formulation of an intravaginal ring containing leuprolide acetate.

Sample name	2010-024-09	2010-024-11	2010-024-16	2010-024-36	NF1
EVA 18-150	44.3%	44.3%	44.3%	44.3%	88.6%
EVA 28-40	44.3%	44.3%	44.3%	44.3%	–
PEG 4000	8%	8%	8%	8%	8%
Tween 80	1%	1%	1%	1%	1%
Leuprolide	24 mg/g	24 mg/g	24 mg/g	24 mg/g	24 mg/g
Comments	Pilot equipment 0.5 kg/h	Pilot equipment 0.1 kg/h	Pilot equipment 0.1 kg/h	Pilot equipment 0.1 kg/h	Bench top preparation

and pooled to determine pretreatment levels of leuprolide, gonadotropins, and sex steroids. The vaginal rings were then inserted on the morning of Day 1. The first cohort of three women received vaginal rings containing 18 mg of leuprolide acetate, and the second cohort of three subjects received 36 mg. Subsequent labs were drawn at noon and 4–5 pm on Day 1, and at 8–9 am, noon, and 4–5 pm on the two following days. The rings were withdrawn on Day 3. Serum levels of leuprolide, LH, FSH, estradiol, and progesterone were determined on all samples.

3.3. Safety and tolerability

A visual examination of the vaginal epithelium was conducted at baseline and daily thereafter and findings recorded in a standard manner. A colposcopy was conducted at baseline before ring insertion and on Day 3 after ring removal to ensure no changes (e.g. evidence of inflammation) in the vaginal epithelium occurred.

3.4. Assay methodologies

All reproductive hormone levels were determined by the Reproductive Endocrine Unit Reference Core Assay Laboratory of the Massachusetts General Hospital, which is both a GLP-compliant and CLIA-certified site. All serum samples were coded and blinded by the clinical research team prior to submission to the laboratory. The hormone levels from the experimental subjects were compared to normative data from 120 normal ovulatory menstrual cycles in which daily levels of gonadotropins and sex steroids were determined [19].

Serum leuprolide levels were measured by a commercial enzyme-linked immunosorbent assay (ELISA) method using a commercial kit (Catalog # S-1144, Peninsula Laboratories, LLC). This immuno-assay is widely used for pharmacokinetic research studies of leuprolide, but it does not distinguish between intact leuprolide and its breakdown fragments. Results therefore represent a combination of intact and metabolic products and thus serve as a composite index of the changes in relative drug concentrations. All specimens from each participant's study were tested in the same assay whose minimum detectable concentration was <50 pg/ml with a reportable range of 50–2000 pg/ml. Seven of the total of 53 specimens from all study subjects contained <50 pg/ml, i.e. the limit of assay sensitivity. These seven samples were constituted of mostly baseline and early sampling determinations from the low dose group.

4. Results

4.1. Controlling release kinetics

The in vitro release of leuprolide over time was determined in pH 4 buffer media. This is representative of the local pH of a normal healthy vagina, and is also a pH that is typically employed to measure the in vitro drug release characteristics from intra-vaginal rings. The pKa of leuprolide acetate is reported as 9.6.[14] Leuprolide acetate has three ionizable sites: the imidazaloyl nitrogen of histadine (pKa = 6), the phenolic hydroxyl of tyrosine (pKa = 10), and the guanidine nitrogen of arginine (pKa = 13). The resultant pKa value is attributed to both the tyrosine moiety and the extremely basic guanidine nitrogen. In consequence, leuprolide is predominantly ionized over the pH range that is of physiological relevance, so these test conditions were considered to be appropriate. Similarly, in vivo, we could also expect only minor differences in vaginal absorption of the drug to occur within the known pH variations that exist between different ethnicities and population age groups and also in women with lowered vaginal lactobacillus levels where the pH also increases towards neutrality.

We previously demonstrated that the drug particle size affects the release kinetics from a homogenous matrix with larger particles increasing the contact area between adjacent particles to create a less tortuous path resulting in faster release kinetics [17,18]. Thus, incorporating drugs with different drug particle sizes and loading could be used to adjust the desired release kinetics. Batches B and C had the same formulations (Table 5), but Batch C was processed at a lower extruding rate (0.1 vs 0.5 kg/h). The extruding rate did not affect the mechanical properties of the formed rings. Similarly, adding various excipients to the EVA mixture did not noticeably affect the mechanical properties of the rings. However, Batch A was made with EVA that had a lower melt flow index (i.e. higher MW), and as a result the mechanical properties of Batch A in comparison to Batches B and C (Table 5) displayed ca. 50% higher compression force of 400 g at 33 mm (Fig. 4).

To enable the release of larger molecular weight molecules such as leuprolide acetate, which require low drug loading, excipients needed to be added to allow continuous zero order kinetic drug release to be achieved. This pattern of drug release was one of our goals in designing this system. These excipients could be porosigens, i.e. excipients added to the drug to create the physical contact between the different particles that allow the drug to continue to be released from the matrix. As the excipients diffuse out of the ring matrix, the porosigens create tortuous paths for diffusion of the entrapped drugs should the drug particles be too small and not in contact with each other. Alternatively, some excipients can act as plasticizers that affect the diffusion of the drug through the EVA matrix for drugs that are soluble in this matrix, such as estradiol. The effect of different plasticizing excipients (such as polysorbate or polyethylene glycols) on the release kinetics is presented in Fig. 5.

Formulation 1 includes only estradiol with no excipients, while Formulation 2 has additional plasticizers that accelerate the release kinetics by improving the diffusion of the estradiol through the EVA. Considering all these variables, we investigated different formulations that would allow us to create an intravaginal ring containing leuprolide acetate (Table 6).

Table 7
Subject demographics.

Subject	Race	BMI	LMP	Menstrual cycle (days)	Date of insertion
1	White	19.8	5/22/2008	30	6/24/2008
2	White	22.8	6/16/2008	28–31	7/15/2008
3	White	23.7	8/4/2008	28	8/5/2008
4	White	28.9	7/5/2008	25–28	9/23/2008
5	White	23.4	8/29/2008	30	10/21/2008
6	Hispanic	22.8	10/15/2008	30	12/9/2008

[14] CDER Application Number 21–379 Clinical Pharmacology and BioPharmaceutics Review(s): Eligard 22.5 mg (LA-2550). Atrix Laboratories, Inc. http://www.accessdata.fda.gov/drugsatfda_docs/nda/2002/21-379_Eligard_biopharmr.pdf. Accessed 30 March 2016.

The preparation of the rings in Fig. 6 employed similar conditions. However, formulation NF1, with less vinyl acetate (without EVA 28–40), provided prolonged release with slower drug release rates.

Ultimately, EVA rings were loaded with either 18 mg of leuprolide acetate, which was anticipated to release ~50 μg/day, or 36 mg which was estimated to release ~100 μg/day. These levels were calculated to be sufficiently high to induce a relatively complete desensitization of pituitary gonadotropin secretion and consequent ovarian quiescence given good vaginal absorption.

4.2. Human studies

Six women met the eligibility criteria that included: normal healthy females aged 18–40, a BMI between 19 and 29, normal prolactin levels and a negative pregnancy test at the time of enrollment. Their average age was 26 years (range 18–40 years) and no leuprolide was detected in their baseline bloods. There were nine patients screened who did not meet exclusion criteria and the reasons were as follows: abnormal laboratory results, Body Mass Indexes (BMIs) greater than allowed in the study, and irregular menses. Following insertion of the GnRHa rings, serum leuprolide concentrations rose in all six females to a mean of 310 pg/ml in the low dose and 1220 pg/ml in the high dose group that peaked on Day 1. Fig. 7 demonstrates these results graphically along with known published data on expected serum levels of leuprolide parenteral depot injection.

Mean serum leuprolide levels rose from 100 pg/mL levels to a peaks of 310 pg/ml in the 18 mg dose group and from 90 pg/mL to a peak of 1220 ng/ml in the 36 mg group within 8 h of vaginal ring insertion and remained elevated compared to baseline over the full three day course of study in the 36 mg group. By Day 3, mean leuprolide levels remained detectable with a mean of 160 pg/ml in the 18 mg group and 310 pg/ml in the 36 mg group.

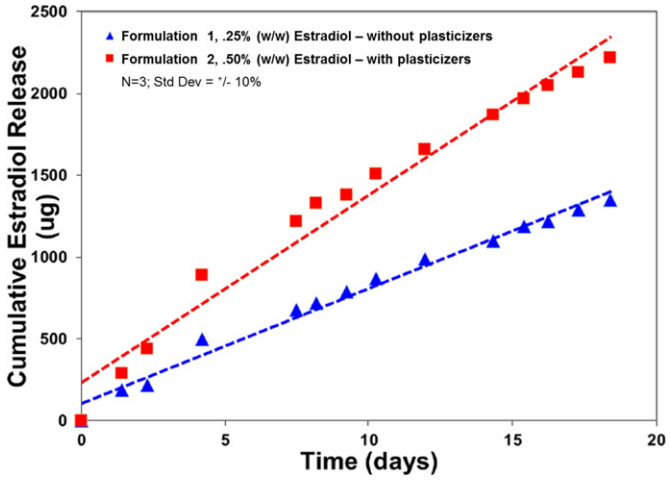
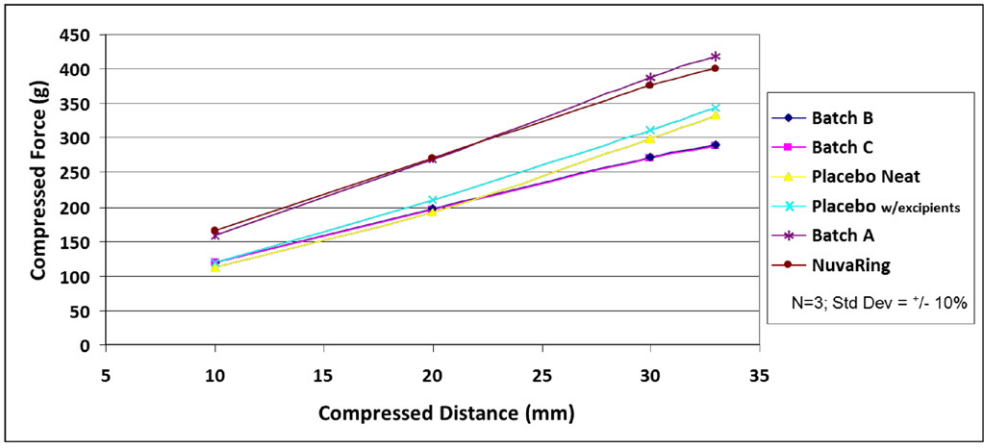


Fig. 5. Effect of inclusion of plasticizing excipients on estradiol release.

Parallel increases of serum FSH (Fig. 8a), LH (Fig. 8b), and estradiol (Fig. 8c) occurred, confirming the biological activity of the leuprolide detected by ELISA. Serum FSH levels peaked at 11.2 mIU/ml in the 18 mg group and 24.4 mIU/ml in the high dose group. Similarly, LH levels peaked at 20.8 mIU/ml in the low dose group and at 46.2 mIU/ml in the high dose group relative to mean of the normative early follicular phase values of anticipated for this stage of the menstrual cycle (5.4 mIU/mL). These elevations of LH and FSH levels were rapid, with the most dramatic increase observed 8 h post-ring insertion for both gonadotropins. Compared to the daily normative data from 120 normal female early follicular phases indicating an average FSH (7.3 mIU/mL), these FSH levels were also clearly elevated in the study subjects. Although the sample size was small, all subjects in



Batch	Vinyl Acetate Composition (%w/w)	Melt Flow Index (190°C / 2.16 kg)	Processing
A	Composite blend of (i) 17-19 % and (ii) 27-29%	Composite blend of (i) 135-175 g/10min and (ii) 22-29 g/10min	
B	Composite blend of (i) 17-19 % and (ii) 27-29%	Composite blend of (i) 135-175 g/10min and (ii) 35-45 g/10min	Extrusion rate (kg/hr) = 0.5
C	Composite blend of (i) 17-19 % and (ii) 27-29%	Composite blend of (i) 135-175 g/10min and (ii) 35-45 g/10min	Extrusion rate (kg/hr) = 0.1

Fig. 4. Effect of polymer melt flow index on ring compression force.

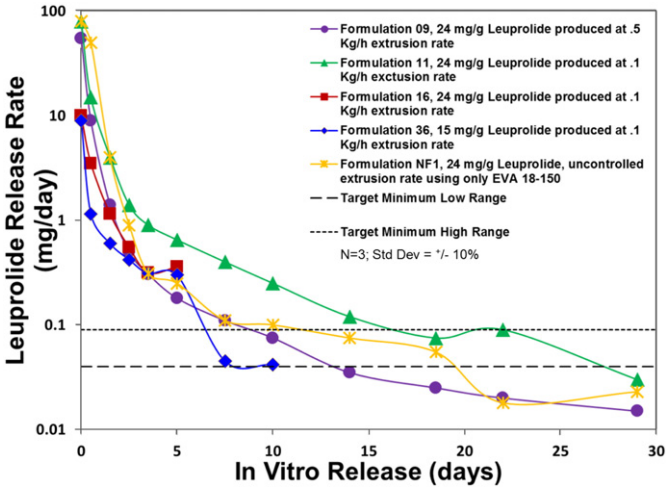


Fig. 6. Effect of extrusion rate on leuprolide release rate.

the high dose group exhibited peak serum levels of LH and FSH greater than one standard deviation outside of the maximal reference value. Two of the three subjects in the low dose group also demonstrated peak LH and FSH levels greater than one standard deviation outside of this reference range.

Consistent with the biologic activity representative of the leuprolide entering the circulation, elevated estradiol and progesterone levels occurred, lagging behind the gonadotropin secretion in a typical fashion. While FSH and LH secretion peaked on Day 1, subsequent increases in estradiol were observed on Day 2 with the time to peak estradiol concentrations varying between subjects. The low dose group peaked at 122 pg/ml (56–239 pg/ml), whereas the high dose peaked at 138 pg/ml (88-208 pg/mL) relative to the normative early follicular phase values anticipated of 61 pg/ml. One subject in the low dose group had a dramatic increase in her serum progesterone, suggesting some residual functioning of her corpus luteum from the previous cycle that is often seen during GnRHa administration during the early follicular phase of the menstrual cycle. However, serum progesterone levels in all the remaining subjects demonstrated only mild increases in progesterone secretion in both low and high dose GnRHa groups.

One subject in the high dose GnRHa group had peak levels of serum leuprolide of 2000 pg/mL. This same subject also demonstrated the most dramatic increases in peak FSH (37 mIU/ml) and peak LH

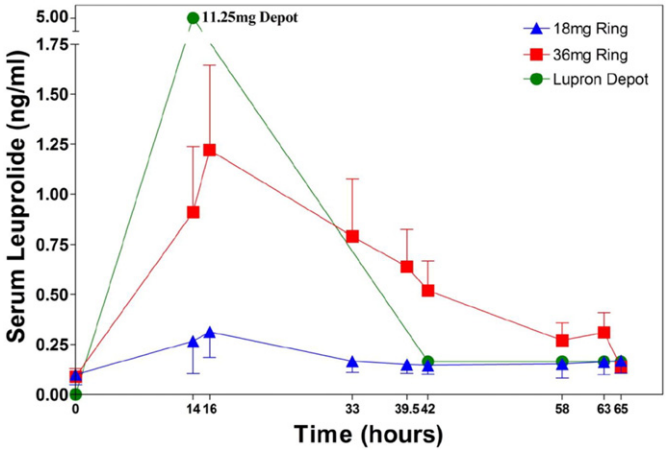


Fig. 7. Mean serum leuprolide levels as measured by GnRH ELISA assay in low dose (18 mg) and high dose (36 mg) vaginal rings compared to published data on parenteral depot injection.

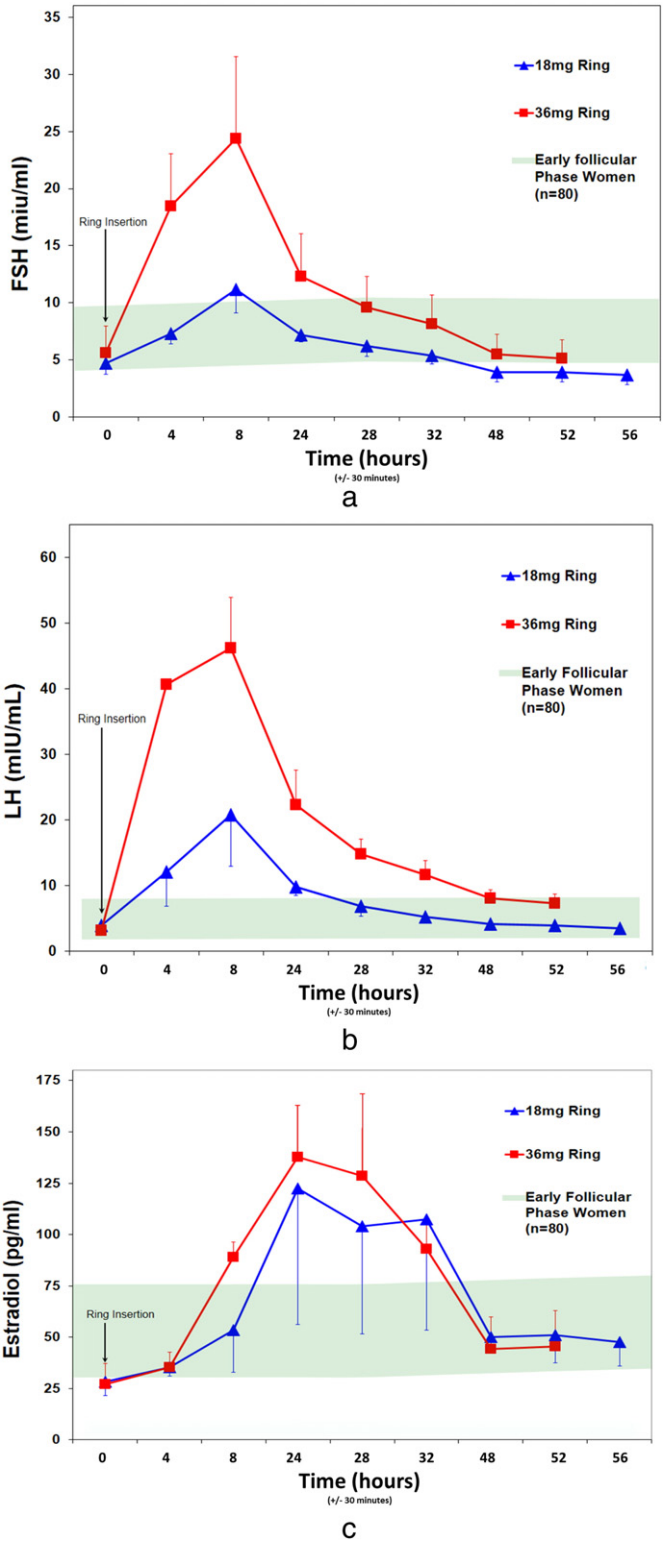


Fig. 8. a: Mean serum FSH concentration post-EVA ring insertion in low dose (18 mg) and high dose (36 mg) cohorts compared to normative data of early follicular phase b: Mean serum LH concentration post-EVA ring insertion compared to normative data of early follicular phase c: Mean serum estradiol concentration post-EVA ring insertion compared to normative data of early follicular phase.

(60.6 mIU/ml). By three days post-GnRHa administration, elevation in her serum concentrations of leuprolide (430 pg/ml), FSH (8.3 mIU/ml), and LH (10.2 mIU/ml) persisted. This elevated serum LH remained >1SD above the maximal reference value at three days post-insertion.

4.3. Safety parameters

All study subjects tolerated the rings well and without any evidence of vaginal irritation by visual inspection or colposcopy. Vaginal ring displacement occurred in three of the six subjects; in subjects 2 and 3, the ring dislodged overnight between Day 1 and Day 2 associated with Valsalva maneuvers but was easily replaced by the subjects themselves and validated as to their location by the primary investigator on Day 2. The rings that were tested in this study were designed primarily for their drug elution and delivery properties. They were not optimized for their 'fit and feel' and patient comfort. As a result the rings were, at times, ejected from the vagina when a few individuals exhibited any internal pressure, through defecating, coughing etc. Interestingly, these temporary dislocations did not appear to affect absorption of leuprolide significantly in subject 2 but may have in subject 3.

5. Discussion

This study demonstrates that effective, non-parenteral absorption of a polar peptide of considerably larger size than sex steroids could be achieved via delivery from a novel vaginal ring delivery system with resulting therapeutic serum levels of GnRHa. The agonist biological activity of the circulating GnRHa was confirmed by subsequent elevations of serum LH and FSH and ovarian sex steroid secretion. To provide some context for these results, a single intramuscular injection of the three month Leuprolide® Depot injection containing 11.25 mg produces a mean plasma leuprolide concentration 36.3 ng/mL 4 h post-intramuscular injection. After reaching steady state by weeks 3–12, however, mean circulating leuprolide concentrations subsequently plateau to levels of 230 = /− 90 pg/ml (SS) at 12 weeks, i.e. levels sufficient to maintain the desired therapeutic suppression of the pituitary-gonadal axis for 3 months with these levels. Although the present study was limited to a 56 h period per FDA requirements, the resulting serum levels of leuprolide achieved in both the low and high dose groups achieved this target range within hours of vaginal application of our ring.

Vaginal rings are well established to deliver relatively physiological levels of natural estradiol and progesterone systemically for hormone replacement treatment in menopausal women, as well as pharmacologic levels of synthetic steroids for contraception in reproductively active women, to circumvent the known hepatic first pass phenomenon of synthetic sex steroids that occurs when administered orally. The current studies now demonstrate the potential feasibility of developing a vaginal ring drug delivery system that could extend the therapeutic window of transvaginal delivery to combine the established systemic delivery of sex steroids with that of a larger peptide such as a GnRHa. Such a combined 'GnRHa + sex steroid' TVDS ring could potentially be used to administer GnRHa to induce a complete quiescence of the pituitary-ovarian axis that would completely block ovarian folliculogenesis and steroidogenesis but also permit coincidental administration of physiologic levels of natural estradiol and progesterone (i.e. 'add back' regimens). The advantage of this combination would be to maintain physiologic levels of sex steroid to support responsive target organs of bone, vaginal epithelium, and abolish the CNS effects of GnRHa-induced biochemical castration with hot flashes.

Such combination rings containing GnRHa + natural sex steroids would be useful in two clinical settings. In women with polycystic ovarian disease (PCOD), one therapeutic goal is to suppress ovarian androgen secretion that drives their hirsutism and potentially contributes to their insulin resistance while avoiding the castrational side effects of GnRHa administration alone [20]. In addition, it would also be possible to add insulin-sensitizing agents to these rings to treat their insulin resistance in theory. Similarly, GnRHa's ability to completely suppress gonadotropins and hence ovulation to provide contraception has traditionally been limited by the side effect of leaving women with hot flashes, decreased bone density, and vaginal atrophy. Combining physiologic 'add-back' of natural sex steroids with GnRHa administration would thus open the possibility of using such a combined vaginal ring in a contraceptive mode.

In addition, a current standard therapy for women with endometriosis is to combine intramuscular leuprolide injections with oral norethindrone acetate to control the castrational symptoms that typically accompany isolated GnRHa therapy. However, oral norethindrone acetate is subject to both first hepatic first pass metabolism and the vagaries of patient compliance. A transvaginal drug delivery system that could co-administer controlled release of a GnRHa and simultaneously add back sex steroid hormones would thus open a considerably wider range of more convenient methods of drug delivery in several important clinical settings. It could also support the co-administration of other medications such as anti-HIV treatments that have been shown to reduce infectivity when administered transvaginally but which often become limited by compliance [21].

Several limitations of this study exist. The number of subjects and duration relate to FDA imposed IND restrictions. Also the population size was too small to detect statistically significant differences between the low and high dose groups; however, the trends are consistent with a dose-response relationship between ring leuprolide concentrations in addition to their physiologic effects. The low dose ring was inserted immediately at the end of subject 1′s menses as opposed to subjects 2 and 3 in whom the ring was inserted at the beginning of their menses. Insertion of the ring at the end of their menses, i.e. in the middle of the follicular phase, likely explains the more exuberant rise in estradiol seen in subject 1.

Future research should examine longer time courses in larger numbers of subjects to induce complete reproductive quiescence investigate and titrating GnRHa dosing to achieve target serum concentrations. Longer-term studies will also help to determine the stability of hormone release which, according to our in vitro studies, follow near zero order kinetics and are stable for weeks. The results of this study suggest that the use of a vaginal ring for controlled and constant delivery of a large peptide, such as GnRH agonists, is not only feasible but represents a potentially novel therapeutic platform that will enable several potentially interesting treatments or combinations of treatments in a variety of clinical settings. Currently, there are other FDA-approved combinations of a contraceptive vaginal ring containing two synthetic steroids, etonogestrel and ethinyl estradiol (NuvaRing®). Although delivery of proteins has been previously demonstrated from vaginal rings, such studies have employed IVR's of more complex designs which comprise a ring body into which a drug-loaded inserts is placed [22]. Our study utilizes a simple matrix design IVR without incorporating a rate controlling membrane. The biological and therapeutic implications of this study extend beyond the clinical applications of leuprolide, and this study opens the door for broader investigations into the transvaginal delivery of peptides and proteins. Future research will determine what other larger molecules can be absorbed transvaginally in addition to refining the dosing and formulations of our current delivery systems.

Authorship

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Contributed to writing of the paper: all.

Acknowledgements

Funding for this study was supplied in part by The Swensrud Foundation and Combinent Biomedical Systems. An exclusive worldwide license to the novel vaginal ring delivery system was acquired by Juniper Pharmaceuticals, Inc., in April 2015. Final editing of the manuscript and the submission thereof was funded by Juniper Pharmaceuticals.

Paul Woodcock and Amy Raskopf (Juniper Pharmaceuticals) provided editorial and proof reading assistance.

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