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Development of Luteinizing Hormone Releasing Hormone (LHRH) Delivery Systems for Vaginal Mucosal Route

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The objective of this study was to find a rational dosage form for vaginal mucosal delivery of LHRH. Vaginal absorption of LHRH was estimated by measuring its ovulation inducing effect in rat and in vitro vaginal membrane permeation study in rabbit. The effects of different hydrogel bases, such as Polycarbophil and Pemulen compared with solutions on vaginal membrane permeation of LHRH were investigated. Sodium laurate, disodium ethylenediamine tetraacetate (EDTA) and sodium tauro-24,25-dihydrofusidate (STDHF), which are effective peptidase inhibitors were chosen as additives to a LHRH hydrogel delivery system and LHRH solutions. A Polycarbophil hydrogel formulation showed 3.4 times increase in LHRH vaginal membrane permeability compared with a solution formulation. Vaginal membrane permeability from the Polycarbophil was greater than that from Pemulen hydrogels. This may be due to the larger bioadhesive values. LHRH solution with EDTA(2%), STDHF(1%) and sod. laurate(0.5%) showed 4.1 times, 4.8 times and 6.0 times of ovulation inducing activity compared with control. These results suggest that enzyme inhibition effect of EDTA, STDHF and sod. laurate may result in substantial enhancement of vaginal absorption. By administration of Polycarbophil hydrogels containing LHRH the ovulation inducing activity was 3.3 times greater than the solutions. This result indicates the bioadhesive hydrogels as well as peptidase inhibition significantly improved absorption of LHRH. By coadministration with these inhibitors the ovulation inducing activity of Polycarbophil hydrogel containing LHRH was comparable with subcutaneous administration in ovulation inducing activity.

Key words : LHRH, Ovulation inducing effect, Vaginal membrane permeability, Bioadhesive hydrogel, Enzyme inhibitors

INTRODUCTION

Peptides are difficult to be absorbed through mucosal membranes because of their relatively large molecular size and susceptibility to proteolytic breakdown. Nevertheless these routes have received increasing attention as an alternative to parenteral injection because of convenience and efficacy for patients who are unable to have injections or are in need of long term therapy. However, the efficiency of mucosal absorption is quite low without absorption enhancers.

Approaches to alleviate this problem have been to use penetration enhancers to enhance membrane permeability and enzyme inhibitors to inhibit proteolytic degradation. Many investigators have reported that various absorption promoters such as chelators (Nishihata *et al.*, 1985; Yamashita *et al.*, 1987) , surfactants (Sakaki *et al.*, 1986; Hirai *et al.*, 1981), bile salts (Moses *et al.*, 1983; Murakami *et al.*, 1984; Aungst *et al.*, 1988), fatty acids (Mishima *et al.*, 1987; Muranishi *et al.*, 1977) and nonsurfactant (Miyake *et al.*, 1984 ; Peters *et al.*, 1987) can enhance the absorption of poorly absorbed drugs and peptides from the rectum, nasal cavity and vagina.

Besides penetration enhancement and peptidase inhibition, the use of a mucoadhesive hydrogels, which was reported to promote rectal absorption of insulin and Calcitonin analogue (Morimoto *et al.*, 1980 & 1984), might be useful. The development of bioadhesive dosage forms for controlled drug delivery via mucous membranes is interest with regard to local drug therapy and the systemic administration of peptides and other drugs poorly absorbed from the gastrointestinal tract.

Luteinizing hormone releasing hormone (LHRH) stimulates the gonadotropin of the anterior pituitary

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to secrete both luteinizing hormone (LH) and follicle-stimulating hormone (FSH). According to recent reports (Brogden *et al.*, 1990), a single dose of LHRH and its analogue stimulates the release of pituitary gonadotropins, multiple dose produce reversible pituitary desensitization, and this specific blockade of gonadotropin provides possibility as a new therapeutic agent in treatment of variety of disorders dependent on sex hormone secretion, such as hormone sensitive prostate cancer, premenopausal breast cancer, endometriosis and uterine leiomyoma. The secretion of LHRH is known to be pulsatile and LHRH has a relatively short circulatory half-life. A bioavailability of 1-1.5% (relative to intravenous) has been reported for nasally administered LHRH in humans (Anik *et al.*, 1984). Okada *et al.*, (1982) reported that the absolute bioavailability of leuprolide, an LHRH analog, was 3.8% in comparison to intravenous injection. Also they reported that vaginal absorption was enhanced by organic acids and the absolute bioavailability was increased to 20%.

In a previous paper (Han *et al.*, 1994; Park *et al.*, 1994), we reported on the proteolysis profile of LHRH/[D-Ala^6]LHRH in rectal, nasal and vaginal mucosal homogenates. LHRH/ [D-Ala^6]LHRH was subject to degradation by numerous enzymes in mucosal homogenates. The half life of LHRH/[D-Ala^6]LHRH in vaginal homogenates was much greater than in rectal and nasal homogenates. Therefore, we selected vaginal route for LHRH mucosal delivery.

The dosage forms for rectal and vaginal delivery of peptides and proteins are the conventional ones such as solutions, gels and suppositories (Morimoto *et al.*, 1984 & 1987). Of these, gels are the most efficient because they offer a proper balance between retention at the site of administration and the rate of peptide release from them. It has been reported (Lehr *et al.*, 1990) that Polycarbophil has the highest binding affinity to human mucosal epithelial cells. The force of detachment as determined by the tensiometer method for Polycarbophil was reported as 9.19±1.15 mN/cm^2. Pemulen is a acrylates/C_{10-30} alkylacrylate crosspolymer using as a polyelectrolyte oil/water emulsifier for the manufacture of cosmetic products. This novel primary emulsifier has a small lipophilic portion in addition to a large hydrophilic portion. In the present study, we investigated these polyacrylic acid polymers as a vaginal hydrogel dosage form for improved bioavailability of LHRH.

MATERIALS AND METHODS

Animals and materials

Female New Zealand White rabbits, 2-3 kg and mature Sprague-Dawley rats, 120-150 d of age, 200-240 g, which exhibited two regular 4-day estrous cycles, were used. LHRH and EDTA (Sigma Chemical Co. St Louis, MO, USA), sodium laurate (Tokyo Chemical Industries Co. Tokyo, Japan), STDHF (California Biotechnology Inc. Mountain View, CA, USA), Polycarbophil and Pemulen (Goodrich Chemical, Clevaland, OH, USA) were used as received. All other materials were reagent or analytical grade.

Table I. pH and viscosity of Pemulen and Polycarbophil hydrogel

Formulation (NaOH solution)	Pemulen		Polycarbophil	
	pH	viscosity(cP)	pH	viscosity(cP)
R I (0.1 N)	4.58	186445	4.85	22715
R II (0.3 N)	5.65	155834	5.73	35921
R III (0.5 N)	7.15	120441	8.47	29582

Preparation of Polycarbophil/Pemulen hydrogel

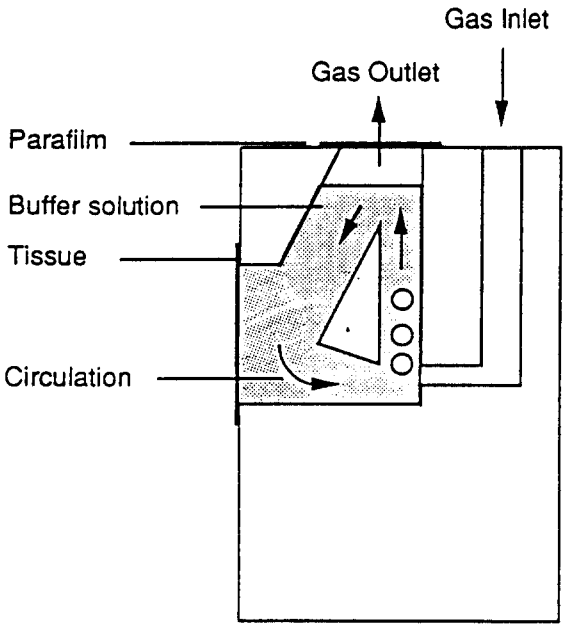
By adding 25 ml of NaOH solution (0.1, 0.3 or 0.5 N) into 1 g of Pemulen/Polycarbophil, three kinds of formulation (Rx I, II, III) were prepared for the LHRH release study (Table I). LHRH was dissolved in each gel base. If the mixing process trapped the air in the gel preparation, the air was removed by centrifugation for 10 min at 3000 rpm. The viscosity of the gel preparation was measured with a coaxial cylinder viscometer (Haake Viscometer) at 37°C. The pH of the gel preparations was also measured after 2-3 days when gels reached a steady state pH. The gel preparations were stored in the dark at 6°C.

Diffusion study

LHRH diffusion studies were performed using side-by-side diffusion cells (Precision Instrument Design, Los Altos, CA, USA) (Scheme 1). For the diffusion study of hydrogel, donor chamber's volume was adjusted by mounting parafilm between donor chamber and vaginal membrane. In case of solutions, both donor and receiver chambers had the same buffer solution except that 0.1 mg/ml of LHRH was present on the donor side only. The donor and receiver solutions were stirred by oxygen gas bubbles, which also provided aeration for the tissues. The diffusion cells were kept in jacketed aluminum blocks to maintain the temperature at 37±1°C. The area of the tisssue to be studied was 1.767 cm^2. The volume of the donor and receiver chambers was 3.5ml. Samples were taken periodically from the receiver chamber and replaced by fresh buffer solution maintained at 37±1°C. The diffusion study lasted for about four hours.

Release study

LHRH release studies were performed using the



Scheme 1. Schematic diagram of side by side diffusion cell (receptor chamber)

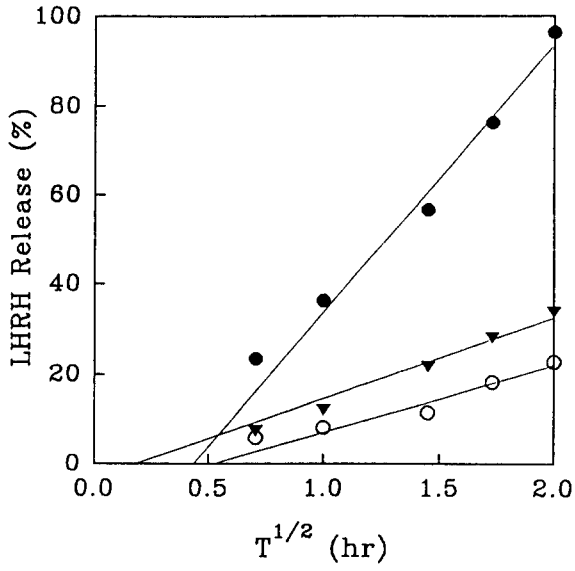


Fig. 1. LHRH release from Polycarbophil (●), Pemulen (○) and Polycarbophil & Pemulen (1 : 1) Mixture (▼). Linear equation for Polycarbophil, y=53.248x-17.950 (r^2=0.999); for Pemulen, y=11.450x-1.374 (r^2=0.992); for Mixture, y=20.683x-7.542 (r^2=0.997)

same diffusion cells as diffusion studies. Cellulose acetate membrane filters (pore size; 0.8 μm, diameter; 25 mm) was used instead of vaginal membrane between donor and receiver chamber.

Estimation of vaginal absorption of LHRH

The absorption of LHRH from the rat vagina was estimated by ovulation inducing activity according to the method of Yamazaki *et al.* (1977). Vaginal smears of rats were obtained daily before 9:00 AM to check the estrous cycle. Only rats having two regular 4-day cycles immediately prior to experimentation were selected. After vaginal administration of LHRH to diestrus rat, in a solution or hydrogel dosage form, rats were killed by cervical dislocation and oviducts were detached approximately 18 hours post-dosing. The oviducts were individually compressed between two slides and were inspected under a microscope (Phillip Harris Co., Ltd.) for the presence of ova.

RESULTS AND DISCUSSION

LHRH release from Pemulen/Polycarbophil hydrogel

The ability of a drug to exert its therapeutic action depends primarily on two consecutive physical events. First the drug must be able to diffuse from the vehicle to the membrane surface and second, the drug must be able to penetrate the membrane. Both those processes can influence the transmucosal absorption rate, the slowest step being rate-limiting. In order to evaluate which of these two processes was the rate-limiting step in LHRH transmucosal absorption, we determined the rate of LHRH release from Pemulen and Polycarbophil hydrogels and its flux through excised rabbit vaginal membrane.

As can be seen in Fig. 1, plotting the amount of LHRH released through cellulose acatate membranes from each vehicle as a function of the square root of time, a linear relationship was obtained. The excellent linearities (r>0.99) obtained indicated that the kinetics of LHRH release from these gels followed the simplified Higuchi diffusion model.

Diffusion study

It was observed that the additives increased the release rate of LHRH from Polycarbophil and Pemulen hydrogel (data is not shown here). However the rate-limiting step is considered as penetration through the vaginal membrane. The percent flux of LHRH versus time for vaginal membrane from Pemulen, Polycarbophil hydrogels and solutions are shown in Fig. 2.

The permeability of LHRH from Polycarbophil hydrogel was 3.4 times greater than that of solution. The permeability coefficient (P) of LHRH is calculated from the slope, the donor chamber volume and the surface area of the tissue, using equation(1).

$$P = \frac{\text{Amount transported}}{\text{Total amount}} \times \frac{\text{Donor chamber volume}}{\text{Time} \times \text{Area}}$$

$$= (\text{slope} \times 100)\frac{\text{Donor chamber volume}}{\text{Area}} \qquad (1)$$

Fig. 3 shows the vaginal permeability coefficient of

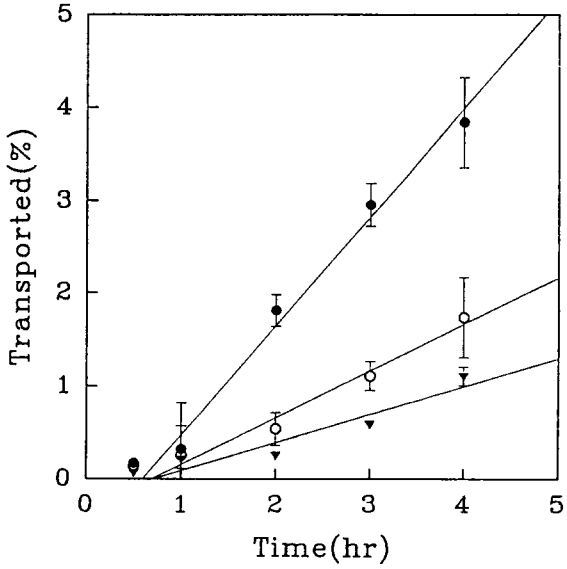


Fig. 2. Permeability study of LHRH through the vaginal membrane tissue from Polycarbophil hydrogel (●), Pemulen hydrogel (○) and Solution (▼). The error bar represents the standard deviation of four replicates. Linear equation for Polycarbophil, y = 1.172x-0.679 (r^2=0.987); for Pemulen, y=0.502x-0.345 (r^2=0.974): for Solution, y = 0.301x-0.210 (r^2=0.888)

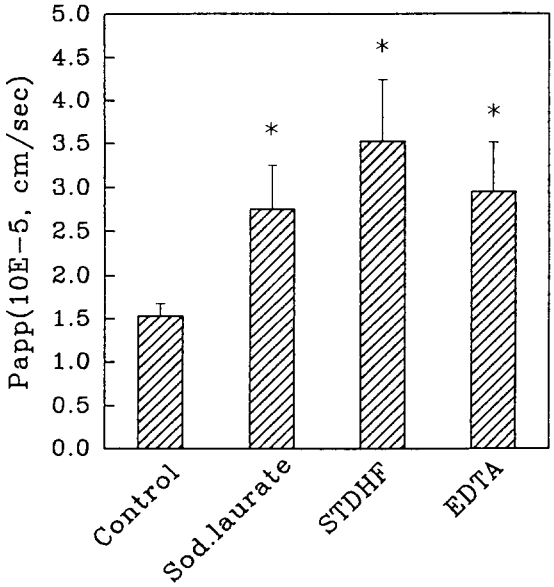


Fig. 3. Vaginal permeation coefficient of LHRH solution (control) with sod. laurate, STDHF or EDTA. Error bars represent standard errors on the mean for n=4. Asterisk denote significant difference compared with the control (P<0.05).

LHRH solution with EDTA, STDHF and sod.laurate. EDTA, STDHF and Sod. laurate increased the permeability coefficient significantly by 2-3 times compared with solution.

Ovulation inducing activity of LHRH after its subcutaneous or vaginal administration

Table II. Microscopic characteristics of the vaginal smear as a function of the sexual cycle

Phase	Duration days	Microscopic characteristics of vaginal smears
Diestrus	1-3	Exclusively leukocytes
Proestrus	~1	Leukocytes and nucleated epithelial cells
Early estrus	0.5-1	Epithelial cells, may have some cornified cells
Estrus	0.5-1	Exclusively cornified cells
Metestrus	~1	Leukocytes and cornified cells

Table III. Ovulation following subcutaneous administration of LHRH at various estrous stages of the rat

Stages of estrous cycle at the administration	"Fresh" ova (in the ampulla)		"Shed" and "Cleft" ova (in the lower part than ampulla)	
	No. Ovu/Ex[a]	No.Ova[b]	No. Ovu/Ex[a]	No.Ova[b]
Proestrus	5/5	14.6	0/5	—
Estrus	0/5	—	5/5	9.4
Metestrus	0/5	—	5/5	7.4
Diestrus	5/5	6.2	0/5	—

[a]No. Ovu/Ex: Number of rats ovulating/Number of rats examined. [b]No. Ova: Number of Ova in ovulating rats (mean).
*The subcutaneous dose of LHRH, 16.92 nmole/220 g rat, was given at 14 : 30.

The estrous cycle of the rat was completed in four to five days and was confirmed by Rugh's method (Table II; Rugh, 1968). Ovulation induction by administration of LHRH was induced one day prior to natural ovulation. After subcutaneous administration of LHRH in each stage of estrous, the extent of ovulation is shown in Table III. All animals that were given LHRH in proestrus and diestrus stages were examined for fresh ova in the ampulla, but in esturus and metestrus stages were not examined. This result indicates that induction of ova in proestrus is by natural or administered LHRH, whereas induction of ova in diestrus results from externally administered LHRH. Consequently, the ovulation inducing activity of LHRH could be evaluated by the administration in diestrus rat.

The ovulation inducing activities of LHRH by subcutaneous administration to diestrus rats are shown in Table IV. The ED50's of the activity evaluated by means of Litchfield & Wilcoxon I (Confidence Limits of ED50 of pharmacological calculation program; Tallarida *et al.*, 1987) were 12.84 nmole/220 g rat. We have reported that the ovulation inducing effect of [D-Ala^6]LHRH by subcutaneous administration and ED50 were found to be 0.51 nmole/220 g rat (Han *et al.*, 1994). The activity of LHRH was 25 times less than that of [D-Ala^6]LHRH.

The activities of LHRH/ [D-Ala^6]LHRH solution by vaginal administration were 5.2 and 8.3 times less

Table IV. Dose-response relationship in induction of ovulation by subcutaneous (s.c.) or vaginal administration of LHRH solution in the diestrus rat

	Dose of LHRH for S.C. (nmole/220 g rat)					Dose of LHRH for Vaginal (nmole/220 g rat)			
	4.23	8.46	12.69	16.92	33.83	42.3	63.0	84.6	126.9
No. Ovu/Ex[a]	0/5	1/5	3/5	3/5	5/5	1/5	2/5	4/5	4/5
No. Ova[b]	0.0	0.6	4.5	6.2	10.8	1.0	2.0	2.8	4.2
ED50	12.84 (9.09-18.13) nmole/220 g rat					67.10 (43.91-102.53) nmole/rat			

a) No. Ovu/Ex: Number of rats ovulating/Number of rats examined. b) No. Ova: Number of Ova in ovulating rats (mean).

Table V. Ovulating-inducing activity of LHRH solution after vaginal administration with additives to diestrus rats

Additives	Dose of LHRH (nmole/220g rat)					ED50[a] (nmole/rat)	Relative potency
	4.23	8.46	12.69	16.92	21.15		
None[b]	—	—	—	—	—	67.10	1.0
EDTA(2%)	—	0/5[c]	2/5	3/5	3/5	16.33	4.1
STDHF(1%)	0/5	1/5	2/5	3/5	4/5	14.12	4.8
sod.laurate(0.5%)	1/5	2/5	3/5	4/5	—	11.13	6.0
EDTA(2%)+STDHF(1%)	1/5	2/5	2/5	4/5	—	10.59	6.3
sod.laurate(0.5%)+STDHF(1%)	1/5	1/5	3/5		—	10.56	6.4

[a]Fiducial limits (95%).
[b]None: No additives for LHRH solution (presented in Table IV).
[c]Number of rats with induced ovulation per number of rats examined.
*LHRH was administered at 14:30 on the day of diestrus.

Table VI. Ovulating-inducing activity of LHRH after vaginal administration of Polycarbophil hydrogel with additives to diestrous rats

Additives	Dose of LHRH (nmole/220g rat)								ED50[a]	Relative potency
	2.5	5.0	7.5	10.0	12.5	20.0	30.0	40.0		
None	—	—	—	0/4	—	2/4[b]	3/4—	4/4	20.67	1.0
EDTA (2%)	—	1/4	2/4	2/4	3/4	—	—	—	8.27	2.5
STDHF (1%)	0/4	1/4	2/4	3/4	—	—	—	—	7.29	2.8
sod.laurate (0.5%)	—	1/4	2/4	3/4	4/4	—		—	6.97	3.0

[a]Fiducial limits (95%).
[b]Number of rats with induced ovulation per number of rats examined.
*LHRH was administered at 14:30 on the day of diestrus.

than that by subcutaneous injection, respectively.

Enhancement of vaginal absorption of LHRH by the coadministration of peptidase inhibitors

It has been known that the proteases constituting the enzymatic barrier at each mucosal route are different and the proteolytic activities against small peptides are most active in the rectal route, followed by the buccal, nasal and vaginal (Lee *et al.*, 1991). In our previous study (Han *et al.*, 1994; Park *et al.*, 1994), the proteolytic activities of individual mucosal homogenates against the LHRH/[D-Ala^6]LHRH were significantly different from one another. LHRH/[D-Ala^6]LHRH were most rapidly degraded in rectal mucosal homogenates followed by nasal and vaginal. And we also report that EDTA, STDHF and sodium laurate significantly inhibited the proteolysis of LHRH/[D-Ala^6]LHRH in mucosal homogenates of rabbit. The ovulation inducing activity of LHRH after vaginal administration in a solution form with peptidase inhibitors is shown in Table V. The ED50 were 67.10 nmole/220 g rat without enzyme inhibitors, 16.33 nmole/220 g rat with EDTA (2%), 14.12 nmole/220 g rat with STDHF (1%) and 11.13 nmole/220 g rat with sodium laurate (0.5%). EDTA which produces alteration in cell membrane structures by removing Ca^{2+} ions, causes an increase in membrane permeability of some hydrophilic drugs in rat intestine (Yamashida *et al.*, 1987). STDHF, a derivative of fusidic acid has physico-chemical properties which are similar to those of free and conjugated bile salts. Longenecker *et al.* (1987) suggested that STDHF increases the nasal absorption of insulin by formation of mixed micelles of insulin and STDHF. Hoogdalem *et al.* (1989) suggested that as an absorption enhancing mechanism, STDHF may enhance paracellular or transcellular mucosal permeability comparable with that

of bile salts or resistance to metabolic degradation. Three peptidase inhibitors employed as absorption enhancers markedly increased the potency of LHRH applied vaginally. EDTA(2%) and STDHF (1%) increased 4.1 and 4.8 times the ovulation inducing activity of LHRH (Table V). Coadministration with sodium laurate exhibited the strongest ovulation inducing activity with six times the potency of control. Mishima *et al.* (1987) reported the leucine aminopeptidase activity was decreased with increasing alkyl chain length of fatty acid salts. Moreover, they reported that fatty acid salts have chelating ability for Ca^{2+} ions. They suggest that the absorption promoting mechanism on nasal absorption of insulin in rat is not only enhanced permeability through the intercellular space by eliminating Ca^{2+} ions but also inhibition of leucine aminopeptidase activity. Combined administration of both STDHF and EDTA increased the ovulation inducing activity of LHRH compared with only STDHF or EDTA. The ovulation enhancing effect of LHRH with three inhibitors corresponds to the proteolysis inhibition effect in mucosal homogenates (Han *et al.*, 1994). These results suggest that the enzyme inhibition effect of EDTA, STDHF and sodium laurate may result in a potent enhancement of vaginal absorption of LHRH.

Vaginal absorption of LHRH from Polycarbophil hydrogel

As a research of mucosal delivery system, William *et al.* (1982) developed a soluble hydroxypropylcellulose cartridge impregnated with drug for vaginal drug delivery. The cartridge hydrates to a high viscosity gel and releases drug over an extended period of time. We chose Polycarbophil as a vaginal LHRH Delivery System, because Polycarbophil shows markedly better mucoadhesive properties compared with Pemulen (data is not shown here.) and LHRH release and vaginal membrane permeability from Polycarbophil hydrogel is considered to be better than from Pemulen. The ovulation inducing activity of LHRH after vaginal administration of Polycarbophil bioadhesive hydrogel is shown in Table VI. By administration of LHRH using this hydrogel, ovulation inducing activity was increased 3.2 times compared to a solution. Polycarbophil hydrogels containing LHRH with EDTA (2%), STDHF (1%) or sodium laurate increased the potency of LHRH by 2.5, 2.8 and 3.0 times control (Table VI). The ovulation inducing activity of LHRH after vaginal administration using Polycarbophil hydrogel with sodium laurate (0.5%) was greater than that of a subcutaneous injection.

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