Vaginal Absorption of a Potent Luteinizing Hormone-Releasing Hormone Analogue (Leuprolide) in Rats II: Mechanism of Absorption Enhancement with Organic Acids

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Abstract □ Organic acids such as citric acid enhance the vaginal absorption of luteinizing hormone-releasing hormone, the potent analogue (leuprolide), and insulin. The mechanism of this absorption enhancement was investigated with leuprolide and hydrophilic markers such as phenol red and Evan's blue. The absorption of the analogue was increased by lowering the pH of the solution and increased more by adding citric, succinic, tartaric, and malonic acids. The absolute bioavailability after vaginal administration of the 5% citric acid solution was 16.7% at pH 3.5 and 38.4% at pH 1.8. The enhancing potency of the absorption correlated well with the chelating ability of the organic acids. The vaginal absorption of phenol red was also enhanced with citric acid and edetic acid, but the enhancing effect of edetic acid was eliminated by adding equimolar calcium ion. These results suggest that the acidifying and chelating abilities of the acids may result in a potent enhancement of the vaginal absorption of leuprolide. A leakage experiment using Evan's blue on the vaginal membrane indicated that the blood–vaginal epithelium barrier was loosened with the administration of citric acid; this change was overcome rapidly.

Keyphrases □ Leuprolide—vaginal absorption, potent luteinizing hormone-releasing hormone analogue in rats, mechanism of absorption enhancement with organic acids □ Absorption, vaginal—potent luteinizing hormone-releasing hormone analogue in rats, mechanism of absorption enhancement with organic acids □ Hormones—potent luteinizing hormone-releasing hormone analogue (leuprolide) in rats, vaginal absorption, enhancement with organic acids

In a previous study (1), an investigation on the administration routes of leuprolide (I), a potent luteinizing hormone-releasing hormone (II) analogue, was carried out in rats to establish a self-administration method for antitumor therapy in humans:

(Pyro)Glu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NH-CH_2CH_3
I

(Pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
II

Good absorption of the analogue was observed with vaginal suppositories containing organic acids such as citric acid and succinic acid, and the vaginal application was proposed as a rational dosage method of the analogue for long-term therapy.

A series of investigations on the vaginal absorption of alcohols and alkanoic acids have been reported (2–6), but a few studies on the hydrophilic compounds and their absorption enhancement have been reported (7).

Most of the organic acids, which enhanced the vaginal absorption in the previous study, have a chelating ability. On the other hand, compounds possessing strong chelating ability, such as edetic acid (8–10) and tetracyclines (11), have been known to enhance the intestinal absorption of hydrophilic compounds, and the enhancement was supposedly due to the direct interaction with calcium or magnesium ions in the intestinal membrane.

In the present study, the mechanism of the vaginal absorption enhancement by the organic acids was investigated with leuprolide and hydrophilic marker compounds (phenol red and Evan's blue). The vaginal absorption-enhancing effect of citric acid was also confirmed on other hydrophilic, high-molecular compounds, II and insulin.

EXPERIMENTAL

Animals and Materials—Sexually mature female Sprague-Dawley rats[1] aged 120–150 days and weighing 250–330 g were used. Animals exhibiting two or more consecutive 4-day estrous cycles on daily morning examination of vaginal smears were used at diestrus.

Leuprolide [2]and II[2] were used after dehydration (1). Chemicals of reagent quality were used without further purification.

Vaginal Absorption of Leuprolide—The vaginal absorption of leuprolide was evaluated by determining the ovulation-inducing activity (1). The analogue was dissolved in 0.1 *M* glycine buffer (pH 2.02 and 3.47), 0.2 *M* phthalate buffer (pH 4.76), 0.2 *M* phosphate buffer (pH 6.70), 0.01 *N* HCl (pH 2.08), and 0.9% NaCl solution (pH 5.13) and administered vaginally with cotton balls at doses of 0.2–2 μg/kg/0.2 ml. Tonicity of the solution was measured by an osmometer[3] and adjusted to be isotonic with sodium chloride. Bovine serum albumin[4] and aprotinin[5] were added to each solution of leuprolide to prevent the loss by adsorption or proteolysis (1). After administration of the solution, the vaginal orifice was closed with a surgical adhesive.

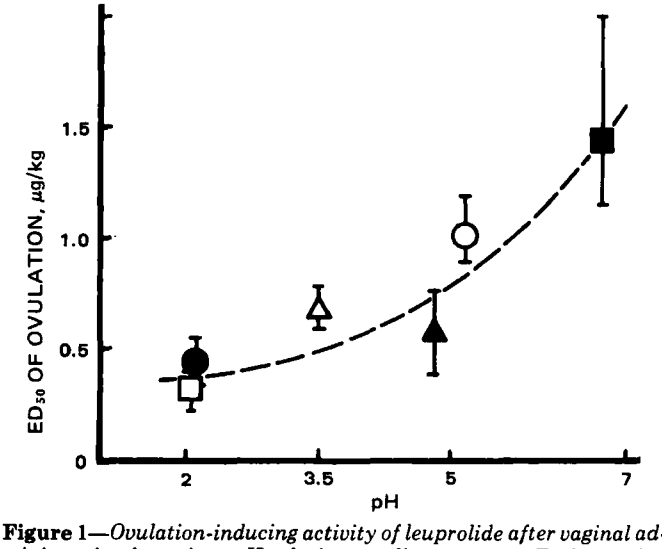

Figure 1—*Ovulation-inducing activity of leuprolide after vaginal administration in various pH solutions to diestrous rats. Each solution was adjusted to be isotonic with sodium chloride. Bars represent the 95% fiducial limits. Key: (○) 0.9% NaCl; (●) 0.012 N HCl; (□) 0.1 M glycine buffer (pH 2.02); (△) 0.1 M glycine buffer (pH 3.47); (▲) 0.2 M phthalate buffer (pH 4.76); (■) 0.2 M phosphate buffer (pH 6.70).*

[1] Clea Japan, Inc., Tokyo, Japan.
[2] These peptides were synthesized in Central Research Division of Takeda Chemical Ind., Ltd., Osaka, Japan.
[3] Fiske Osmometer, Model G-66, Fiske Associates, Inc., Uxbridge, Mass.
[4] Wako Pure Chemical, Ind., Ltd., Osaka, Japan.
[5] Trasylol, Bayer A. G., Leverkusen-Bayerwerk, West Germany.

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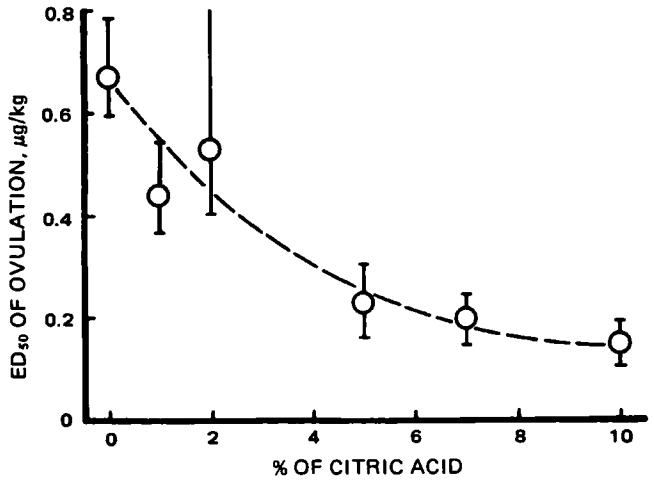

Figure 2—*Ovulation-inducing activity of leuprolide after vaginal administration in the aqueous solutions containing different concentrations of citric acid to diestrous rats. Each solution was adjusted to pH 3.5 with 10* N *NaOH or 2* N *HCl and to be isotonic with sodium chloride. Bars represent the 95% fiducial limits.*

Citric, succinic, tartaric, malonic, malic, acetic, lactic, and ascorbic acids were used at a concentration of 0.238 *M* to enhance the vaginal absorption of the analogue. The analogue dose was 400 ng/kg/0.2 ml. Each solution was adjusted to pH 3.5 with 10 *N* NaOH or 2 *N* HCl to be isotonic with sodium chloride.

The concentration effect of citric acid on the absorption enhancement was determined at 1, 2, 5 (0.238 *M*), 7, and 10% of the acid at pH 3.5. The enhancing effect of 5% dipotassium edetate solution (pH 3.5) and 5% citric acid solution (pH 1.8) were also examined.

Vaginal Absorption of Phenol Red—Phenol red was administered vaginally at a dose of 2 mg/kg/0.2 ml in the buffer (pH 2.0, 3.5, 5.0, and 6.7), 5 and10% citric acid (pH 3.5), 5% citric acid (pH 6.6), 0.12 *M* edetic acid (pH 6.7), and 0.12 *M* edetic acid and calcium chloride (pH 6.7) solutions under pentobarbital (50 mg/kg) and phenobarbital (100 mg/kg) anesthesia. Urine was collected at 3-hr intervals by a polyethylene tube[6] cannula and the collection was completed by washing the bladder with 1 ml of 0.9% NaCl solution through the catheter. Phenol red in the urine was determined by a modified method (12). Statistical evaluation of the data was performed using Student *t* test.

Effect of Citric Acid on the Leakage of Evan's Blue—To examine the time-course of the local reaction with citric acid, leakage of Evan's blue from the blood capillary on the vaginal membrane was evaluated. Evan's blue (10 mg/kg/ml) was intravenously injected immediately, or 0.5, 1.5, and 3.5 hr after vaginal administration of 5 or 10% citric acid solution, and the rats were sacrificed by decapitation 30 min later to examine the vaginal membrane. For the recovery examination, the vaginal membrane was treated with 10% citric acid solution for 1 hr, then washed with 10 ml of 0.9% NaCl solution. Evan's blue was injected immediately, 0.5, and 1.5 hr after washing.

Vaginal Absorption of II and Insulin—The ovulation-inducing activity of II after vaginal administration of a suppository consisting of an oleaginous base[7] with or without 10% citric acid was estimated in rats as described previously (1).

For evaluation of vaginal absorption of insulin, plasma glucose levels were determined using *o*-toluidine–boric acid (13). Insulin at doses of 0.5–20 U/kg iv was administered in 0.9% NaCl solution and vaginally by means of a cotton ball (~12 mg) soaked with 0.05 *M* KCl–HCl buffer solution (pH 1.70) or 10% citric acid solution (pH 1.72) to rats under anesthesia. Blood was taken from the tail vein 0.5, 1, 2, 3, 4, and 6 hr after the administration. The decrease of plasma glucose level (percent × hour) was expressed by integrating the decrease to the initial level from 0 to 6 hr.

RESULTS

Vaginal Absorption of Leuprolide—The ovulation-inducing activity of leuprolide after vaginal administration in the various pH solutions is shown in Fig. 1. The activity was increased by acidification to a level ~4 times greater at pH 2.02 than at pH 6.70.

[6] PE-50, Clay Adams Co., Parsippany, N.J.
[7] WITEPSOL S55, Dynamit Nobel Aktiengesellshaft, West Germany.

Table I—Ovulation-Inducing Activity of Leuprolide after Vaginal Administration in Aqueous Solution Containing Organic Acids to Diestrous Rats

Organic Acid	Ovulation[a]	Chelating Ability[b]
None	0/10	—
Citric	9/10	1.21
Succinic	10/10	0.80
Tartaric	2/10	0.37
Malonic	7/10	0.25
Malic	3/10	0.13
Acetic	5/10	—
Lactic	1/10	0.06
Ascorbic	0/10	—

[a] Number of rats induced ovulation per number of rats examined. Each solution containing 0.238 *M* of the organic acid was adjusted to pH 3.5 with 10 *N* NaOH or 2 *N* HCl to be isotonic with sodium chloride. Leuprolide was administered at a dose of 400 ng/kg/0.2 ml. [b] Gram ions of calcium ion sequestered by 1 *M* of organic acids at pH 10 and 30° (Ref. 7). The value of edetic acid was 1.75.

The activity of the analogue after vaginal administration in a pH 3.5 aqueous solution containing 0.238 *M* organic acids is shown in Table I. Polybasic carboxylic acids such as citric, succinic, malic, and malonic acids markedly enhanced the absorption. The enhancing effect was also recognized with acetic acid but not with lactic and ascorbic acids.

The concentration effect of citric acid on the ovulation-inducing activity of the analogue is shown in Fig. 2. The activity was potentiated with increasing concentration and approached a maximum level at >7% of the acid.

In addition, marked enhancing effects were obtained with 5% citric acid solution (pH 3.5 and 1.8) or 5% dipotassium edetate solution (pH 3.5) (Table II). Acidification of the solution from pH 3.5 to 2.0 doubled the activity of the analogue, and a further addition of 5% citric acid increased the activity by 3 times at both pH 3.5 and 1.8. Edetic acid showed a slightly stronger enhancing effect.

Vaginal Absorption of Phenol Red—Vaginal absorption of phenol red was evaluated by the urinary excretion (Fig. 3). After intramuscular injection, phenol red was rapidly excreted into the urine at 90.1 ± 3.0% (mean ±*SE*) of dose in 3 hr, and 96.3 ± 3.2% in 6 hr. After vaginal administration, excretion was sustained during 6 hr. Urinary excretion after vaginal administration in pH 2.0–6.7 buffer solutions was 28.9–38.4% of the dose in 6 hr and tended to decrease slightly with reduced pH.

Vaginal absorption of phenol red was also increased by the addition of 5 or 10% citric acid in pH 3.5 solution but was not affected by the addition of 5% citric acid in pH 6.6 solution. Although edetic acid significantly enhanced the absorption ($p < 0.01$), further addition of equimolar calcium chloride cancelled the enhancement effect.

Effect of Citric Acid on the Leakage of Evan's Blue—The time-course of the local reaction of citric acid on the vaginal membrane and its recovery from the reaction were evaluated by a leakage experiment of Evan's blue. Five percent citric acid solution induced faint staining after only 30 min, whereas 10% citric acid solution induced deep staining after 30 min and 1 hr, which gradually faded 2 and 4 hr after administration. Change of the vaginal membrane induced by 1-hr treatment with 10% citric acid solution was rapidly recovered by washing the lumen with 0.9% NaCl solution; there was a faint staining at 1 hr but none 2 hr after washing.

Vaginal Absorption of II and Insulin—Ten percent citric acid enhanced the ovulation-inducing activity of II by 30 times after the vaginal administration (Table III).

The decreased plasma levels of glucose (percent × hour) after intravenous and vaginal administration of insulin are shown in Fig. 4. A 1.1-U/kg intravenous dose of insulin caused a 125% × hr plasma glucose level decrease in 6 hr. The comparably effective doses for the vaginal administration were 30.0 U/kg in the buffer solution (pH 1.70) and 6.0 U/kg in 10% citric acid solution (pH 1.72). Absolute bioavailability, estimated by the effective doses of the latter, was 18%.

DISCUSSION

Our previous study (1) revealed that good absorption of leuprolide occurred after vaginal administration, and this absorption was enhanced by the addition of some carboxylic acids. In the present study, to establish the mechanism of absorption enhancement, the effects of pH and organic acids on the vaginal absorption of the analogue as well as a few hydrophilic compounds were determined.

The vaginal absorption of leuprolide was enhanced by a pH reduction in the solution, whereas that of phenol red tended to decrease with

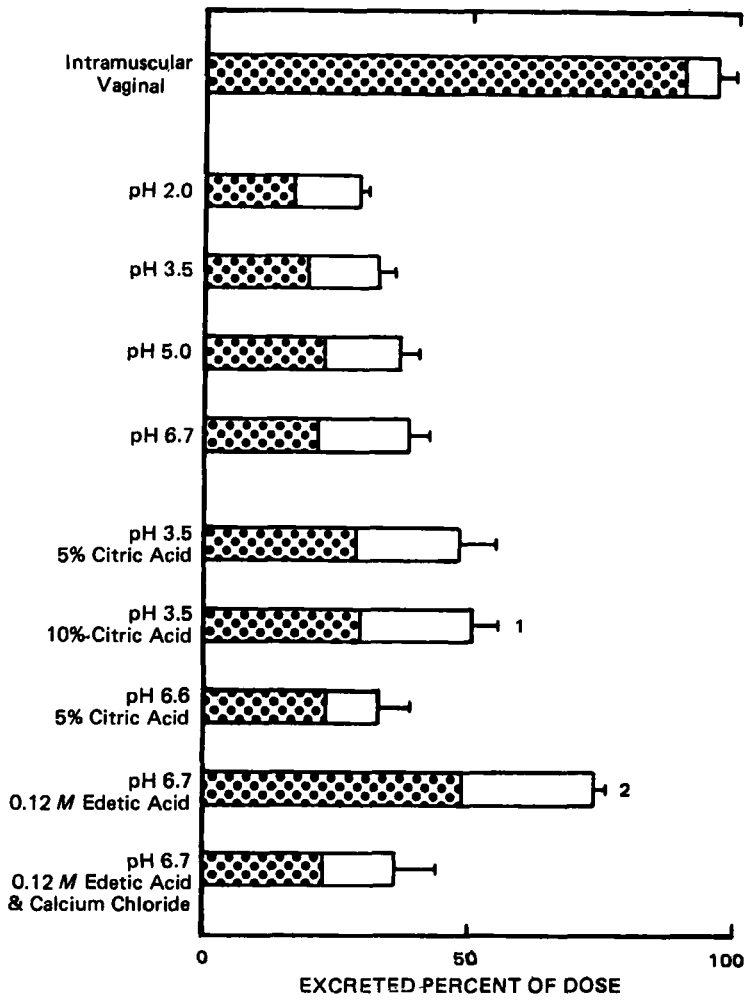

Figure 3—*Effects of pH, citric acid, and edetic acid on vaginal absorption of phenol red in diestrous rats. Each solution was adjusted to be isotonic with sodium chloride. Bars represent the mean ±SE of five rats. Significant in comparison with the same pH buffer solution: (1)* $p < 0.05$; *(2)* $p < 0.01$. *Key: (▨) 0–3 hr; (□) 3–6 hr.*

acidification. Leakage of Evan's blue on the vaginal epithelium was not induced by a moderate pH change, but it was slightly induced by a strong acid or alkali solution[8]. It can be assumed that the effect of pH on the vaginal absorption of leuprolide is due mainly to structural changes of the epithelial membrane but is also due to the self-association and conformational change of the peptide or to the change of electric charge on the epithelial surface. It is known that the pH of the vaginal tract of normal women is 3.5–3.9 (14) and that of the vaginal fluid is 4.0–4.7 (15). Therefore, an acid preparation of pH 3–4 should not disturb the normal condition of the vaginal tract. Surface of the vaginal membrane of rats exhibited a pH close to that of the applied bulk solution[8] [this is also true in rabbits (5)].

The polybasic carboxylic acids enhanced the vaginal absorption of leuprolide from aqueous solution (adjusted to pH 3.5) as well as from an oleaginous suppository, whereas lactic and ascorbic acids did not elicit the effect. The enhancing effect with 5% citric acid solution was of the same degree at both pH 1.8 and 3.5 (Table II). Bioavailability of the analogue after vaginal administration of the 5% citric acid solution (pH 1.8) was 38.5 and 58.8% of that after intravenous and subcutaneous injection, respectively.

The chelating ability of the organic acids given by gram ions of calcium ion sequestered is summarized in Table I (16). Edetic acid with the strongest ability (1.75) is followed successively by citric, succinic, tartaric, malonic, and malic acids. Lactic acid has the weakest ability. It is likely that the enhancing effect of the carboxylic acids on vaginal absorption of leuprolide, which has a good correlation with the chelating ability of the acids, is due to chelating ability as well as to the acidification of the solution. Acetic acid, which has negligible chelating ability, also enhanced vaginal absorption. This may be due to the fact that acetic acid is known to corrode mucous membranes and to have a strong interaction with peptides. The 0.12 *M* edetic acid solution enhanced the absorption of phenol red by 2 times ($p < 0.01$); the enhancement was cancelled by the addition of equimolar calcium ion. This result also supports the assumption that the chelating ability of carboxylic acids causes the enhancement of vaginal absorption. The vaginal absorption of phenol red

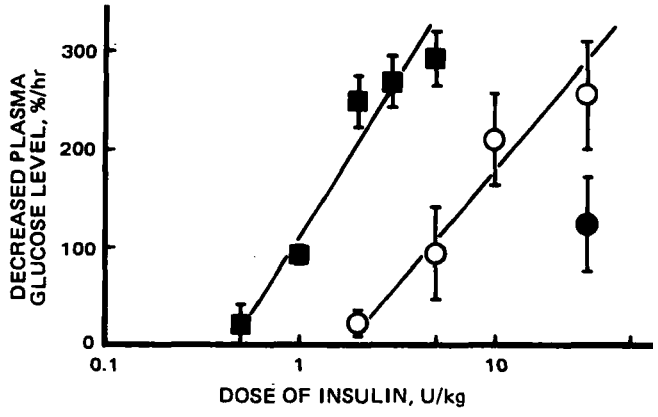

Figure 4—*Decreased plasma levels of glucose after subcutaneous and vaginal administration of insulin to diestrous rats. The decreased plasma level of glucose (percent per hour) was exhibited by integrating the decreased percent against the initial level for 6 hr. Each point represents the mean ±SE of five rats. Key: (■) subcutaneous; (○) vaginal in 10% citric acid (pH 1.72); (●) vaginal in 0.05 M KCl–hydrochloric acid buffer (pH 1.70).*

[8] Unpublished results.

Table II—Ovulation-Inducing Activity of Leuprolide after Vaginal Administration in Buffered Solutions Containing Citric Acid or Edetic Acid to Diestrous Rats

Buffered Solution	Dose of Leuprolide, ng/kg										ED_{50}, ng/kg
	60	80	100	200	300	400	500	600	800	1000	
0.1 *M* Glycine buffer (pH 3.47)						0/10	2/10	3/13	8/10	5/5	669 (596–785)[b]
5% Citric acid (pH 3.5)			1/10[a]	4/10	6/10	9/10					227 (161–305)
5% Dipotassium edetate (pH 3.5)			1/10	6/10	9/10						176 (128–229)
0.1 *M* Glycine buffer (pH 2.02)				2/10	5/10	6/10	7/10	10/10			320 (232–395)
5% Citric acid (pH 1.8)	1/10	0/10	7/10	10/10							99 (85–132)

[a] Number of rats induced ovulation per number of rats examined. [b] 95% fiducial limits.

Table III—Ovulation-Inducing Activity of Luteinizing Hormone-Releasing Hormone (II) after Vaginal Administration to Diestrous Rats

Additives	Dose of II, μg/rat	Ovulation[a]	ED_{50}, μg/rat
None	10	1/10	24.4 (17.3–32.1)[b]
	20	2/10	
	40	10/10	
	60	9/10	
	80	10/10	
Citric acid (10%)	0.4	1/10	0.82 (0.68–1.14)
	0.6	1/10	
	0.8	4/10	
	1.0	8/10	
	10.0	4/4	

[a] Number of rats induced ovulation per number of rats examined. [b] 95% fiducial limits.

was also enhanced with citric acid at pH 3.5 but only slightly enhanced at pH 6.6. This was also observed with leuprolide (1).

The vaginal absorption of leuprolide was enhanced by an increase in citric acid concentration but reached a maximum level at >7% of the acid. The vaginal absorption of phenol red was also enhanced with 5% citric acid but increased less with 10% citric acid. The hypertonic solution with sodium chloride showed a tendency to decrease the absorption[8]. The leakage of Evan's blue on the vaginal epithelium was obviously enhanced by an increase in acid concentration. These results indicate that the enhancement of permeability of the membrane for the hydrophilic compounds is caused by an increase in the acid concentration, but the apparent absorption most likely is suppressed by exudation of body fluid from the vaginal membrane due to hypertonicity of the acid solutions.

A time-course study on the change of vaginal epithelial membrane with citric acid revealed that faint staining by Evan's blue injected intravenously was observed in the early stage after treatment with 5% citric acid solution and deep staining 30 min and 1 hr after treatment with 10% citric acid solution; even in the latter, the stain gradually faded. The change in vaginal membrane treated with 10% citric acid solution recovered rapidly following washing with physiological saline solution, and the stain was barely visible 1 hr later. It is suggested that the 5% acid concentration in aqueous solution or jelly, sufficient to exert an enhancement on vaginal absorption of the analogue, will produce only a slight change and, hence, a rapid recovery of the vaginal epithelium.

The vaginal absorption of II and insulin, which are hydrophilic and high molecular compounds, was also enhanced markedly by adding citric acid.

It appears that the acidification and chelating abilities of organic acids result in their potent absorption-enhancing activity on the vaginal absorption of leuprolide. Changes in the vaginal epithelial membrane induced with citric acid are slight and recovery of the epithelium is relatively rapid.

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