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Some factors affecting the vaginal absorption of human calcitonin in rats

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Summary

We studied the absorption of human calcitonin (HCT) from rat vaginal mucosa. The absorption of HCT was affected by pH, but scarcely by the osmotic pressure of the HCT solution. The pharmacological availability of HCT after vaginal administration without any additives and at pH 7.4 was low. However, vaginal absorption of HCT was promoted by coadministration of sodium deoxycholate, and by the peptidase inhibitors bestatin, leupeptin and pepstatin A. The effects of peptidase inhibitors on the rate of degradation of HCT in a 9000 × *g* supernatant of vaginal mucosa homogenate were determined in order to clarify why peptidase inhibitors promoted the absorption of HCT. The promotion of vaginal absorption of HCT was correlated with the inhibition of HCT degradation by peptidase inhibitors.

Introduction

Human calcitonin (HCT), a polypeptide composed of 32 amino acids, lowers blood calcium levels and inhibits bone resorption. HCT has been used to treat Paget's disease, osteoporosis and hypercalcemia. It has been reported that absorption of HCT from rat oral mucosa can be promoted by bile salts and sugar esters (Nakada et al., 1988, 1989a). Like the oral mucosa, vaginal mucosa is not covered with a stratum corneum, and the epithelium is composed of stratified squamous cells. There have been few studies of peptide absorption from vaginal mucosa as compared with absorption from nasal and rectal mucosa. A previous study (Okada et al., 1983) showed that vaginal absorption of insulin and phenol sulfonphthalein in rats depends on the stage in the estrus cycle, i.e., absorption was poor during proestrus, slightly better during estrus, and good during metestrus and diestrus. These results could be explained by changes in the thickness of the vaginal epithelium. This cyclic change in vaginal absorption is a disadvantage of vaginal administration of drugs. However, many patients with

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osteroporosis are postmenopausal women (Albright et al., 1941) who have no estrous cycles and in whom the vaginal epithelium is extremely thin (Okada, 1991). Both the lack of estrous cycles and the thinness of the vaginal epithelium might make it convenient to deliver HCT via the vagina. Furthermore, we are aware of no detailed studies on the vaginal absorption of calcitonin. Thus, the objectives of the present study were (a) to investigate the effect of pH, osmotic pressure and additives on vaginal absorption of HCT in rats, and (b) to study the mechanisms by which peptidase inhibitors promote absorption of HCT.

Materials and Methods

Materials

HCT was obtained from the Peptides Institute (Minou, Japan). Quillajasaponin (Quillayanin P-20®) was a gift from Maruzen Kasei Co., Ltd (Onomichi, Japan). Sugar ester (Ryoto Sugar Ester® P-1670) was supplied by Mitsubishi-Kasei Food Corp. (Tokyo, Japan). It mainly consisted of sucrose palmitate and was also a mixture of mono-, di- and triesters. The ratio of the constituent fatty acids, myristic, palmitic and stearic acids, was 2:80:18. Bestatin, amastatin, leupeptin and pepstatin A were obtained from Sigma Chemical Co. (St Louis, U.S.A.). Sodium deoxycholate was purchased from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). Leucine aminopeptidase (from pig kidney) was obtained from Worthington Biochemical Co. (Freehold, U.S.A.). Other reagents used were of reagent grade and were obtained commercially.

HCT preparations

HCT solutions with or without additives were prepared by dissolving 0.51 mg of HCT in 1 ml of distilled water. HCT was prepared by mixing equal volumes of HCT solution and 0.263 M citric buffer (pH 3 or 5), 0.2 M Tris-HCl buffer (pH 7.4) or 0.171 M sodium phosphate buffer (pH 7.4). In vivo, sodium phosphate buffer (pH 7.4) was used to study the effects of osmotic pressure on the vaginal absorption of HCT. The osmotic pressure was adjusted with sodium chloride.

Animals

Immature female Wistar rats (4 weeks old) were selected in view of the sensitivity of the bioassay (Harper and Tovernd, 1973), their lack of estrous cycle (Critchlow and Bar-Sela, 1967), and the thinness of their vaginal mucosa, being comparable with that of postmenopausal women (Sezaki, 1986). The animals were fasted for 20 h prior to experiments. During experiments, they were anaesthetized with sodium pentobarbital (40 mg/kg i.p.) and kept on a warm surface (38 °C).

Preparation of supernatant from homogenates of rat vaginal mucosa

Rats were killed with diethyl ether and the vaginas were removed. The mucosal surface was wiped with absorbent cotton to remove the mucus gel layer. Each vagina was put between slide glasses in a hexane vat cooled with dry ice-acetone. 10 min later, one glass was separated from the other. Thus, the vaginal mucosal layer was separated from the muscular layer containing the serosa. The mucosal layer was rinsed twice in isotonic KCl, and then homogenized in 5 volumes of isotonic KCl in a Potter homogenizer at 4 °C. The homogenate was centrifuged at $9000 \times g$ in a refrigerated (4 °C) centrifuge for 10 min. The protein concentration in the resulting supernatant was determined using the Lowry method with serum bovine albumin as a standard.

Determination of HCT by high-performance liquid chromatography (HPLC)

HPLC analysis was carried out according to a previously reported method (Nakada et al., 1987). The HPLC system consisted of Waters model 510 HPLC pumps, a model U6K sample injector and a Unisil Pack 5C18 reverse-phase ODS column (150 × 4.6 mm; GL Science, Tokyo, Japan). The mobile phase was a mixture of methanol and water (70:30, v/v) containing 3 ml of the ion-pairing reagent (PIC®-A LOW UV, Waters, U.S.A.) per l. The flow rate was 1.0 ml/min at 30 °C. HCT in the eluate was monitored spec-

trophotometrically at 214 nm using a Waters Model 481 UV detector.

Absorption study

HCT preparations were administered to the vagina in vivo using a sonde (Natsume Seisakusho Co., Ltd, Tokyo, Japan) for the mouse. After administration, the vagina was closed with Aron Alpha gel® (Toa-Gosei Co., Nagoya, Japan) in order to prevent leakage of HCT. Blood samples (about 0.2 ml) were withdrawn periodically from the jugular vein. Plasma was obtained by centrifugation at $15000 \times g$ for 3 min.

Analytical method

The determination of HCT in rat plasma by radioimmunoassay suffered from interference by rat calcitonin in plasma (Burford et al., 1975). Therefore, a bioassay measuring calcium levels in plasma was used instead of the radioimmunoassay. Plasma calcium levels were determined by the Wako® Calcium C Test (Wako Pure Chemical Industries, Ltd, Osaka, Japan) (Nakada et al., 1987). The percent decrease in plasma calcium (ΔA, total decrease) from 0 to 4 h after administration of HCT was calculated according to the following equation in line with the method of Hirai et al. (1981):

$$\Delta A = \frac{AUC_c - AUC_H}{AUC_c} \times 100$$

where AUC_c denotes the area under the plasma calcium level vs time curve from 0 to 4 h after administration of additives only, and AUC_H represents administration of HCT with or without additives.

The ΔA (up to 33%) after intramuscular administration of HCT increased in proportion to the dose up to 2 μg per animal, as reported previously (Nakada et al., 1989a).

Degradation of HCT by leucine aminopeptidase and the effects of peptidase inhibitors

The kinetics of degradation of HCT by leucine aminopeptidase was studied by incubating, in triplicate, 25 μl of HCT preparation (510 μg/ml), 25 μl of leucine aminopeptidase solution (including 0.5 units in pH 7.4 phosphate buffer solution) and 50 μl of pH 7.4 phosphate buffer solution at 37 °C. The kinetics of degradation of HCT in the supernatant of the homogenate with peptidase inhibitors was examined by incubating, in triplicate, 25 μl of HCT preparation (510 μg/ml), 25 μl of homogenate supernatant and 50 μl of peptidase inhibitors solution (prepared in pH 7.4 phosphate buffer solution) at 37 °C. Residual HCT was determined by HPLC analysis at regular intervals.

Vaginal tract pH

The pH of the vaginal tract was measured with a small glass electrode containing a reference electrode and connected to a standard pH meter (Model FL-50, Fuji Kagaku Keisoku, Tokyo, Japan).

Treatment of glass vessel surfaces

The surfaces of all glass materials were treated with 3% (w/v) trimethylchlorosilane in benzene to prevent adsorption of HCT. After such treatment, each glass surface was washed five times with 5 ml of methanol and air-dried at room temperature.

Statistics

Results are expressed as means ± S.E. Student's *t*-test or Bartlett and Duncan's test was used to analyze the results.

Results

Effects of pH

The effects of pH on changes in plasma calcium levels after vaginal administration of HCT at a dose of 6.4 μg per animal under isotonic conditions are shown in Fig. 1. The decrease in plasma calcium level at pH 3.0 was greater than that at pH 5.0 and 7.4. The ΔA values at pH 3.0, 5.0 and 7.4 were 30.6, 27.0 and 17.7%, respectively. There was a significant difference in the ΔA at pH 3.0 and 7.4. (Bartlett and Duncan's test; $0.01 < p < 0.05$).

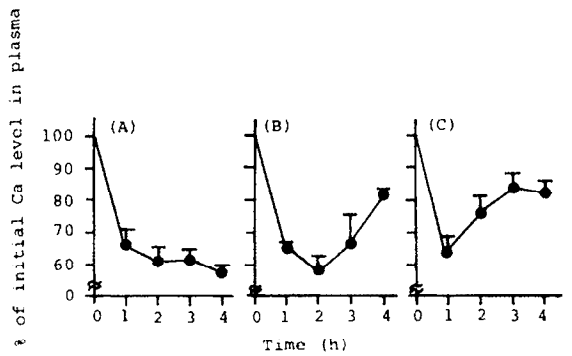

Fig. 1. Effect of pH on changes in plasma calcium levels after vaginal administration of HCT in rats. Dose of HCT: 6.4 μg. pH of HCT preparation: (A) pH 3.0, (B) pH 5.0, (C) pH 7.4. Each point and bar represents the mean $\pm$ S.E. of three animals.

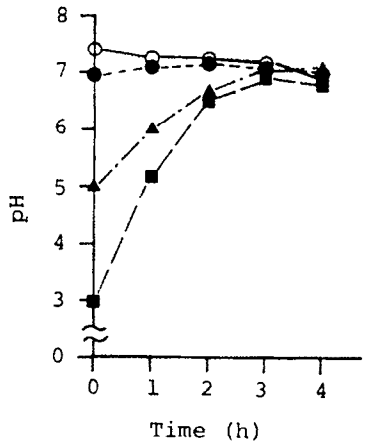

Fig. 2. Vaginal pH after vaginal administration of HCT in rats. pH of HCT preparation: (●) normal control vagina, (■) pH 3.0, (▲) pH 5.0, (○) pH 7.4. Each point represents the mean of three animals.

pH of vaginal tract

The changes in pH after administration of HCT are illustrated in Fig. 2. The control vaginal pH was 6.8–7.1. The pH in the vaginal tract gradually returned to normal after vaginal administration of the various HCT preparations.

Effects of osmotic pressure

Fig. 3 depicts the effects of osmotic pressure on changes in the plasma calcium levels after administration of HCT. Irrespective of whether the pH was 3.0 or 7.4, no significant differences were found in the ΔA among 120 mOsm/kg, 280 mOsm/kg (isotonic) and 590 mOsm/kg (Bartlett and Duncan's, $0.05 < p$).

Effects of various additives

Table 1 lists the effects of various additives on the decreases in plasma calcium levels after vaginal administration of HCT. Plasma calcium levels remained unaltered in the case of additives being given without HCT. The ΔA was found to be $12.3 \pm 3.1\%$ when HCT was given without additives. When HCT and sodium deoxycholate were administered together, a significant decrease occurred in the plasma calcium level. However, sucrose palmitate and quillajasaponin, which have been demonstrated to promote the absorption of HCT from the oral mucosa (Nakada et al., 1989a), had no such effect. Citric acid was also ineffective.

TABLE 1

Effects of various additives on the decreases in plasma calcium levels after vaginal administration of HCT in rats

Additive	Additive concentration	HCT dose (μg)	pH	ΔA [a]
Control		6.4	7.4	12.3 ± 3.1
Sodium deoxycholate	22.5 mg/ml	1.6	7.4	7.0 ± 1.6
	22.5 mg/ml	6.4	7.4	21.1 ± 1.9 [b]
Sucrose palmitate (Ryoto Sugar Ester® P-1670)	22.5 mg/ml	6.4	7.4	0.1 ± 1.1 [c]
Quillajasaponin (Quillayanin P-20®)	22.5 mg/ml	6.4	7.4	12.0 ± 1.7
Citric acid	10.0% w/v	6.4	1.8	17.0 ± 1.9

[a] Each value is the mean ± S.E. for three or four animals. Significantly different from control (no additive) by Bartlett and Duncan's test; [b] $0.01 < p < 0.05$. [c] $p < 0.01$.

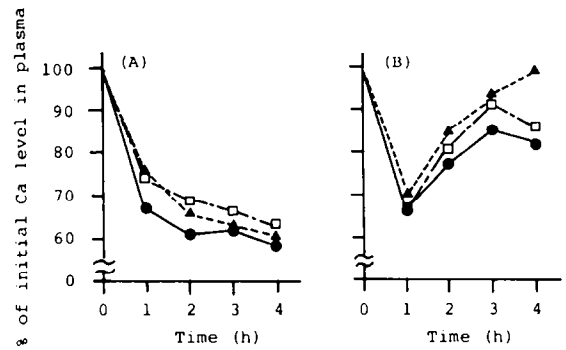

Fig. 3. Effect of osmotic pressure on changes in plasma calcium levels after vaginal administration of HCT in rats. Dose of HCT: 6.4 μg. pH of HCT preparation: (A) pH 3.0, (B) pH 7.4. Osmotic pressure of HCT preparation: (□) 120 mOsm/kg, (●) 280 mOsm/kg (isotonic), (▲) 590 mOsm/kg. Each point represents the mean of three animals.

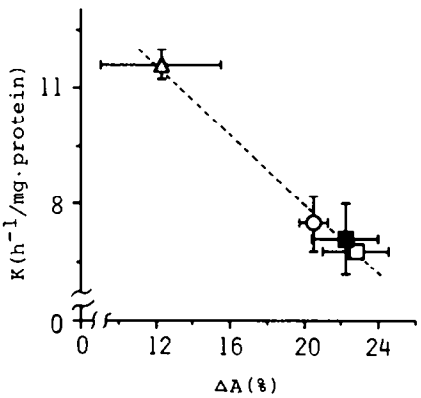

Fig. 4. Relationship between the decreases in plasma calcium levels after vaginal administration of HCT with various peptidase inhibitors in rats at pH 7.4 (ΔA) and the degradation rate constants of HCT in the supernatants of rat vaginal mucosa homogenates with various peptidase inhibitors at 37 °C (pH 7.4). Dose of HCT: 6.4 μg. Peptidase inhibitors were: (■) pepstatin (5 μg/ml), (□) bestatin (50 μg/ml), (○) leupeptin (500 μg/ml), (△) control (no peptidase inhibitor). Each point and bar represents the mean ± S.E. of three or four experiments. $Y = -0.46X + 17.22$; $r = -0.997$, $p < 0.01$.

Effects of peptidase inhibitors

Table 2 details the effects of peptidase inhibitors on ΔA. ΔA tended to increase on addition of pepstatin A, leupeptin, amastatin and bestatin. Optimal concentrations of peptidase inhibitors were observed for the enhancement of vaginal absorption. Significant differences from the control were found with bestatin at 50 μg/ml, pepstatin A at 5 μg/ml and leupeptin at 500 μg/ml.

Degradation of HCT by leucine aminopeptidase

The degradation of HCT by leucine aminopeptidase obeyed pseudo first-order kinetics and the degradation rate constant of HCT was determined as 0.26 ± 0.02 nmol/min per unit.

Relationship between effects of peptidase inhibitors on absorption and stability of HCT

The relationship between the effect of peptidase inhibitors on the degradation rate constants of HCT in the supernatant of the vaginal mucosa homogenate and the enhancement of vaginal ab-

TABLE 2

Effects of peptidase inhibitors after vaginal administration of HCT [a] in rats at pH 7.4

	Concentration of peptidase inhibitor (μg/ml)			
	0	5	50	500
Control	12.3 ± 3.2			
Exopeptidases				
Bestatin		19.1 ± 4.6	22.8 ± 1.8 [b]	15.2 ± 2.7
Amastatin		15.9 ± 1.3	16.7 ± 2.2	16.4 ± 4.9
Endopeptidases				
Leupeptin		14.1 ± 2.7	16.6 ± 1.9	20.5 ± 0.8 [b]
Pepstatin A		22.2 ± 1.8 [b]	16.3 ± 1.5	13.9 ± 2.4 [c]

Values are expressed as ΔA (%) (mean ± S.E.; $n = 3$ or 4).

[a] Dose of HCT; 6.4 μg.

[b] Significant differences from control (no peptidase inhibitor) by Bartlett and Duncan's test ($0.01 < p < 0.05$).

[c] Significant difference between ΔA at 500 μg/ml and at 5 μg/ml by Bartlett and Duncan's test ($0.01 < p < 0.05$).

sorption of HCT is shown in Fig. 4. The degradation rate constant of control was 11.6 ± 0.5 h^{-1} mg protein^{-1} in the absence of peptidase inhibitors. Addition of the peptidase inhibitors decreased the degradation rate constants of HCT. Furthermore, the effect of peptidase inhibitors on the vaginal absorption of HCT resulted in an increase in ΔA with lowering of the degradation rate constants. There was a significant correlation between the degradation rate constants and the ΔA ($r = -0.997$, $n = 4$, $p < 0.01$).

Discussion

The effects of the pH of HCT solutions on vaginal absorption of HCT under isotonic conditions were investigated. Fig. 1 shows that the ΔA at pH 3.0 is greater than those at pH 5.0 and 7.4. The ΔA at pH 3.0 amounted to 30.6%. These results indicate that HCT can readily be absorbed from the vagina under acidic conditions without any additives. It appears, that the effect of pH on the absorption of HCT is due to a conformational change in HCT, and to variations in the charge of HCT and in the epithelial surface of the vaginal mucosa. Over 2 h after administration of HCT preparations, the pH in the vaginal tract returned to about 6.5–7.2 (Fig. 2), however, the plasma calcium level at pH 3.0 remained as low as about 60% of the initial level for 4 h after dosing (Fig. 1). The reasons why the effects of pH on changes in plasma calcium levels are different from the changes in vaginal pH are not known. The pH of the vaginal tract in rats is about 7.0, as shown in Fig. 2. In postmenopausal women, the pH of the vagina increases from 4.5–5.5 to 7.0–7.4, owing to a decrease in cellular glycogen content (Okada, 1991). Therefore, neutral HCT preparations may be most suitable for clinical use. Moreover, vaginal absorption of HCT was not significantly influenced by the osmotic pressure of the preparation. Additives which have been reported to promote the absorption of HCT from the buccal gingiva (Nakada et al., 1989a) were also tested. Of those examined, sodium deoxycholate was found to be the only effective promoter of vaginal absorption. We have reported that sodium deoxycholate and sodium tauroglycocholate inhibit the degradation of HCT in buccal mucosa supernatants (Nakada et al., 1987). It has been reported that sodium salicylate, Na_2EDTA and aprotinin promote absorption to different degrees in the rectal, nasal and buccal membranes (Aungst and Rogers, 1988). The vaginal mucosa consists of stratified squamous epithelium, as does the oral mucosa. However, sucrose palmitate and quillajasaponin, which promote the oral absorption of HCT (Nakada et al., 1989a), did not enhance vaginal absorption. In addition, vaginal absorption is enhanced only poorly by fatty acids and surfactants, including sodium oleate and polyoxyethylene 9-lauryl ether, which are known to enhance rectal and nasal absorption of hydrophilic drugs (Okada, 1991). These results demonstrate that promotion depends on the absorption site, and that a substance which is a promoter at one site may have no effect at another, even when both sites are histologically similar. Hirai et al. (1981) reported that sodium glycocholate acts as a protease inhibitor and promotes absorption of insulin from nasal mucosa in rats. We have reported that dihydroxy bile salts, including sodium glycocholate and sodium deoxycholate, can promote absorption of HCT from the oral mucosa, and that this effect is related to the inhibition of HCT degradation in the supernatant of oral mucosa (Nakada et al., 1989b). In the present work, the promoting effect of sodium deoxycholate on the absorption of HCT after vaginal administration may be correlated to the inhibition of protease. As shown in Table 2, bestatin, leupeptin and pepstatin A promoted vaginal absorption. Optimal concentrations of peptidase inhibitors were observed for the enhancement of vaginal absorption. In order to determine whether a reduction in proteolytic enzyme activity in vaginal mucosa is important for vaginal absorption of HCT, as it is for absorption from oral mucosa, we investigated the relationship between the promotion of absorption and the reduction of proteolytic enzyme activity. A significant correlation was found to exist between the degradation rate constant and the ΔA, as shown in Fig. 4. This indicates that the activities of peptidase inhibitors are related to the promotion of vaginal absorption of HCT.

Two of the inhibitors which were effective as promoters of HCT absorption, leupeptin and pepstatin A, are peptidase inhibitors for trypsins. The vaginal mucosa is deficient in pepsin, trypsin and chymotrypsin, which are present in gastric and intestinal secretions (Stratford and Lee, 1986). The reasons why leupeptin and pepstatin A are effective promoters remain to be clarified. However, leucine aminopeptidase, which is inhibited by bestatin, is present in the vaginal mucosa (Stratford and Lee, 1986), and HCT is rapidly degraded by leucine aminopeptidase. Thus, one of the enzymes inhibited by bestatin would be leucine aminopeptidase, hence, such inhibition may be partly responsible for promoting the absorption of HCT.

In conclusion, vaginal absorption of HCT was markedly influenced by pH, whereas scarcely any effect of osmotic pressure was seen. Peptidase inhibitors promoted the absorption of HCT from the vagina. They may have acted by inhibiting the degradation of HCT in the vaginal mucosa. On consideration of how these results might be applied to humans, we believe that it is now necessary to investigate (1) how the histological differences in vaginal mucosa between immature and old rats affect the absorption of HCT, (2) the bioavailability of vaginally administered HCT, as determined by HPLC or using a radioactive isotope, and (3) vaginal administration of HCT suppositories.

References

Albright, F., Smith, P.H. and Richardson, A.M., Postmenopausal osteoporosis. *J. Am. Med. Assoc.*, 116 (1941) 2465–2474.

Aungst, B.J. and Rogers, N.J., Site dependence of absorption-promoting actions of laureth-9, Na salicylate, Na_2EDTA, and aprotinin on rectal, nasal, and buccal insulin delivery. *Pharm. Res.*, 5 (1988) 305–308.

Burford, H.J., Ontjes, D.A., Cooper, C.W., Palow, A.F. and Hirsch, P.F., Purification, characterization and radioimmunoassay of thyrocalcitonin from rat thyroid glands. *Endocrinology*, 96 (1975) 340–348.

Critchlow, V. and Bar-Sela, M.F., Control of the onset of puberty. In Martini, L. and Ganong, W.F. (Eds), *Neuroendocrinology*, Vol. 2, Academic Press, New York, 1967, pp. 101–162.

Haper, C. and Tovernd, S.U., Ability of thyrocalcitonin to protect against hypercalcemia in adult rats. *Endocrinology*, 93 (1973) 1354–1359.

Hirai, S., Yoshiki, T. and Mima, H., Effect of surfactants on the nasal absorption of insulin in rats. *Int. J. Pharm.*, 9 (1981) 165–172.

Nakada, Y., Awata, N., Nakamichi, C. and Sugimoto, I., Stability of human calcitonin in the supernatant of the rat's oral mucosa homogenate. *Yakuzaigaku*, 47 (1987) 217–223.

Nakada, Y., Awata, N., Nakamichi, C. and Sugimoto, I., The effect of additives on the oral mucosal absorption of human calcitonin in rats. *J. Pharmacobio-Dyn.*, 11 (1988) 395–401.

Nakada, Y., Ikuta, Y. and Awata, N., Absorption of human calcitonin through the rat's gingiva. *Yakuzaigaku*, 49 (1989a) 166–173.

Nakada, Y., Awata, N., Ikuta, Y. and Goto, S., The effect of bile salts on the oral mucosal absorption of human calcitonin in rats. *J. Pharmacobio-Dyn.*, 12 (1989b) 736–743.

Okada, H., Yashiki, T. and Mima, H., Vaginal absorption of a potent luteinising hormone-releasing hormone analog (Leuprolide) in rats. II. Effect of estrous cycle on the vaginal absorption of hydrophilic model compounds. *J. Pharm. Sci.*, 72 (1983) 173–176.

Okada, H., Vaginal route of peptide and protein drug delivery. In Lee, V.H.L. (Ed.), *Peptide and Protein Drug Delivery*, Dekker, New York, 1991, pp. 633–666.

Sezaki, H., *Drug Delivery System*, Nankoudou, Tokyo, 1986, p. 105.

Stratford, R.E., and Lee, V.H.L., Aminopeptidase activity in homogenates of various absorptive mucosa in the albino rabbit: implications in peptide delivery. *Int. J. Pharm.*, 30 (1986) 73–82.