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Rectal Absorption of [Asu^{1,7}]-Eel Calcitonin in Rats

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[Asu^{1,7}]-eel calcitonin ([Asu^{1,7}]-ECT) was not absorbed well from rat rectum. Although an unstirred layer may exist as a diffusion barrier for [Asu^{1,7}]-ECT absorption, transport through the epithelial cell membrane is the limiting step for [Asu^{1,7}]-ECT absorption. Coadministration of 0.33 M phenylalanine enamine of ethylacetoacetate (PheEtAA), 0.17 M diethylethoxymethylenemalonate (DEEMM), 0.6 M sodium salicylate (SA), or 0.05 M sodium *p*-chloromercurylphenyl sulfate (*p*-CMP) increased the rectal [Asu^{1,7}]-ECT absorption significantly. In particular, PheEtAA and DEEMM resulted in a more than 180 times greater *AUC* of [Asu^{1,7}]-ECT compared to that when [Asu^{1,7}]-ECT alone was administered rectally, and these two adjuvants were more effective than SA and *p*-CMP on the rectal [Asu^{1,7}]-ECT absorption, though all four adjuvants coadministered at the above concentration with sodium cefmetazole caused similar increases of rectal cefmetazole absorption.

Keywords——rat rectal absorption; mucin layer; [Asu^{1,7}]-ECT bioavailability; plasma drug concentration; adjuvant dose

Calcitonin, a polypeptide composed of 32 amino acids, exists widely in mammals and fish and is used clinically for the treatment of Paget's disease, osteoporosis and hypercalcemia, because it decreases the blood calcium concentration by decreasing the outflux of calcium from bone. [Asu^{1,7}]-eel calcitonin ([Asu^{1,7}]-ECT) was synthesized by Morikawa *et al.*[1] and is also used for clinical purposes. Since [Asu^{1,7}]-ECT is more stable in aqueous solution than natural calcitonin, it may be considered that [Asu^{1,7}]-ECT is more suitable for pharmaceutical preparations.

Although [Asu^{1,7}]-ECT is administered intramuscularly or subcutaneously in clinical use, another administration route may be desirable for long term therapy with [Asu^{1,7}]-ECT. In this paper, rectal absorption of [Asu^{1,7}]-ECT was studied in rats, and enhancement of the rectal [Asu^{1,7}]-ECT absorption was attempted by using several nonsurfactant adjuvants.

Materials and Methods

Materials——[Asu^{1,7}]-eel calcitonin was supplied by Toyo Jozo Co., Ltd. (Tokyo, Japan). Sodium cefmetazole was supplied by Sankyo Co., Ltd. (Tokyo, Japan), and sodium alginate (m.w. 48000 to 185000) and sodium pectate (m.w. 20000 to 40000) were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan) and Sigma Co., Ltd. (Mo, U.S.A.), respectively. Diethylethoxymethylenemalonate was purchased from Sigma Co., Ltd. Sodium salicylate (SA) and sodium *p*-chloromercuryphenyl sulfate were purchased from Nakarai Chemical Co., Ltd. (Kyoto, Japan). Sodium phenylalanine enamine of ethylacetoacetate was prepared according to the method described by Dane *et al.*[2] Other reagents used were of analytical grade.

Animals——Male Wistar rats, 225 to 275 g, were fasted for 16 h prior to experiments. During the experiment, rats were anesthetized with sodium pentobarbital (40 mg/kg, *i.p.*) and kept on a hot surface at 38 °C.

Preparation and Dosage Volume of Microenema——Microenema containing drug and/or adjuvant was prepared with distilled water, and was administered at a dosage volume of 0.5 ml/kg for *in vivo* experiments or at a dosage volume of 0.2 ml/loop for the *in situ* rat rectal loop study.

Animal Studies——Microenema containing [Asu[1,7]]-ECT was administered at 1 cm depth from the anus in rats with polyethylene tubing (PE 50) and the anus was ligated with thread to avoid leakage of the microenema. Blood was collected from the jugular vein and was centrifuged to obtain the plasma. An *in situ* rectal loop study was carried out to determine the absorption of cefmetazole from the rectum. The rectal loop was prepared by ligation at the anus and at a position 4 cm distance from the anus. Absorption of cefmetazole was determined by measuring the amount of cefmetazole remaining in the loop 15 and 60 min after administration according to the method described in a previous paper.[3]

Assay——Assay of [Asu[1,7]]-ECT was carried out using the enzyme immunoassay described in a previous paper.[4] Therefore, plasma concentration of [Asu[1,7]]-ECT was represented as immunoreactive [Asu[1,7]]-ECT (IR-[Asu[1,7]]-ECT). It has been reported[5] that the immunologically active site of [Asu[1,7]]-ECT is located at the C-terminal amino acid of the molecule. The minimum detectable concentration of plasma IR-[Asu[1,7]]-ECT was 1 mU/ml in this method. Therefore, assay of plasma IR-[Asu[1,7]]-ECT at lower concentrations was carried out after concentrating a large volume of plasma by evaporation under nitrogen gas. Assay of cefmetazole was carried out by using the high performance liquid chromatography (HPLC) method described in a previous paper.[6]

Results and Discussion

Rat rectal absorption of [Asu[1,7]]-eel calcitonin ([Asu[1,7]]-ECT) administered alone at a dose of 800 U/kg or 1600 U/kg in microenema was poor, as can be seen from the plasma [Asu[1,7]]-ECT concentrations in Fig. 1.

To estimate the absorption of [Asu[1,7]]-ECT after rectal administration, the area under the curve of plasma [Asu[1,7]]-ECT concentration (*AUC*) after rectal administration was compared to that after intramuscular injection. The *AUC* of [Asu[1,7]]-ECT administered intramuscularly at a dose of 5 U/kg was 108.5 ± 29.8 mU min/ml. The corresponding values after rectal administration at 800 U/kg and 1600 U/kg were 82.5 ± 14.8 mU min/ml ($n=4$) and 209.7 ± 45.3 mU min/ml ($n=4$), respectively, so a 160 times greater dose of [Asu[1,7]]-ECT given rectally was required to obtaine the same *AUC* value to that after intramuscular administration at a dose of 5 U/kg.

Poor rectal absorption of [Asu[1,7]]-ECT seems to be due to its high molecular weight of 3363 daltond. Passage through the unstirred layer including mucin layer is one of the steps of drug absorption. It has been suggested that the activity of several nonsurfactant adjuvants such as diethylethoxymethylenemalonate (DEEMM) (unpublished data) and enamine[7] to enhance rectal drug absorption is related to their chelating ability with calcium ion, and it has also been reported[8] that removing the calcium ion from mucin causes the sedimentation of mucin.

Thus, in order to examine the role of the mucin layer in the rectal absorption of [Asu[1,7]]-ECT, we investigated firstly whether coadministration of sodium alginate or sodium pectate influences the rectal absorption of [Asu[1,7]]-ECT (alginate and pectate can chelate with calcium ion,[9] so both additives may cause sedimentation of mucin by removing calcium ion, and they

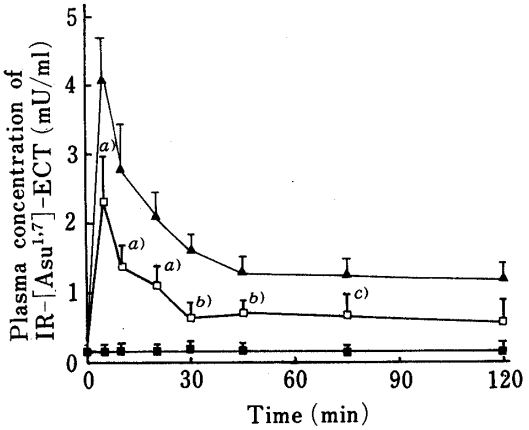

Fig. 1. Plasma IR-[Asu[1,7]]-ECT Concentration in Rats with (□, ▲) or without (■) Rectal Administration of [Asu[1,7]]-ECT at a Dose of 800 U/kg (□) or 1600 U/kg (▲)

Each value represents the mean ± S.D. ($n=5$). *a*) $p<0.001$ against ■. *b*) $p<0.05$ against ■. *c*) $p<0.1$ against ■.

are not absorbed due to their high molecular weights) and secondly whether pretreatment of rat rectal lumen with 2 M NaCl solution influences the [Asu[1,7]]-ECT absorption (it has been reported[10] that treatment with 2 M NaCl disrupts the unstirred layer).

As can be seen in Fig. 2, each treatment increased the plasma [Asu[1,7]]-ECT concentration compared to that without treatment (Fig. 1). The area under the blood concentration curve (*AUC*) values obtained on coadministration with sodium alginate or pectate were 263.5 ± 38.7 mU min/ml and 238.4 ± 41.6 mU min/ml, respectively, so the *AUC* value was increased only three times compared to that with no treatment. Even pretreatment with 2 M NaCl only gave an *AUC* value of 405.8 ± 52.6 mU min/ml which is five times the no treatment (Fig. 1).

The above findings suggest that although the mucin layer does act as a diffusion barrier for rectal [Asu[1,7]]-ECT absorption, the limiting step in the absorption may be in the passage of [Asu[1,7]]-ECT through the surface membrane of epithelial cells.

It has been reported that several nonsurfactant adjuvants such as enamines[7,11,12] and salicylate analogs[13-15] enhance the rectal absorption of polar compounds and insulin. In this study, enhancing action of phenylalanine enamine of ethylacetoacetate (PheEtAA), DEEMM, sodium salicylate (SA) and *p*-chloromercuryphenyl sulfate (*p*-CMP) on the rat rectal absorption of [Asu[1,7]]-ECT was examined.

As shown in Fig. 3, the plasma [Asu[1,7]]-ECT concentration after rectal administration at a dose of 800 U/kg in microenema containing each adjuvant was increased significantly compared to those without any adjuvant (Fig. 1).

The coadministered dose of each adjuvant was determined on the basis of the enhancing efficacy on the rectal absorption of cefmetazole, which is a relatively small molecular weight

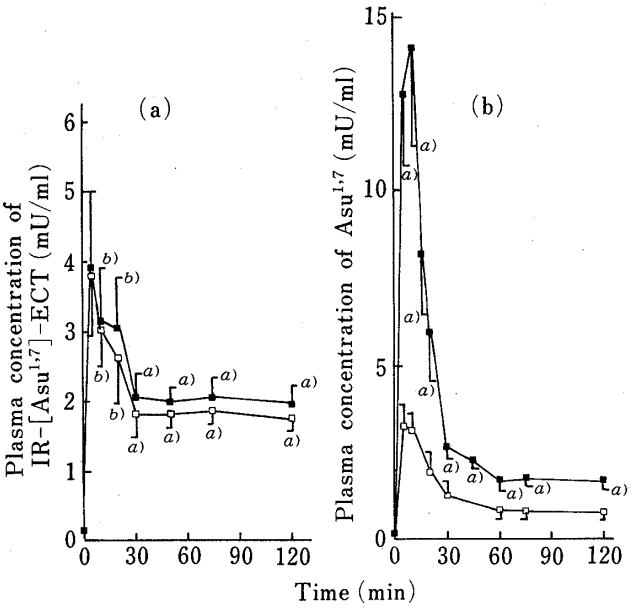

Fig. 2. (a) Effect of Sodium Alginate (■, 2%) or Sodium Pectate (□, 2%) in Microenema on the Plasma Concentration of IR-[Asu[1,7]]-ECT after Rectal Administration at a Dose of 800 U/kg, (b) Effect of Pretreatment with 2 M NaCl (■) or 0.15 M NaCl (□) for 5 min before Administration of [Asu[1,7]]-ECT on the Plasma IR-[Asu[1,7]]-ECT Concentration

Each value represents the mean ± S.D. ($n=4$).
a) $p<0.001$ against □ in Fig. 1. *b*) $p<0.05$ against □ in Fig. 1.

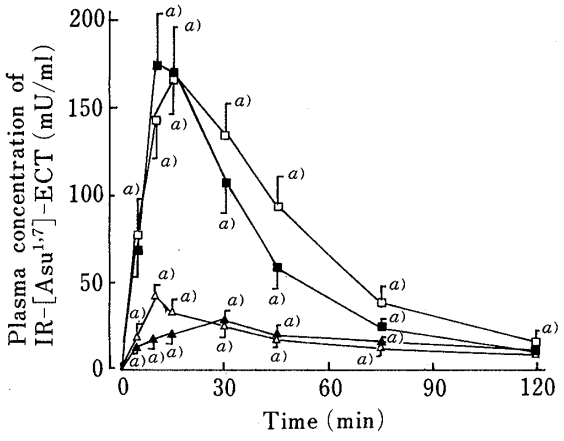

Fig. 3. Plasma Concentration of IR-[Asu[1,7]]-ECT after Rat Rectal Administration at a Dose of 800 U/kg in Microenema Containing 0.17 M DEEMM (■), 0.33 M PheEtAA (□) or 0.6 M SA (△), or 0.05 M *p*-CMP (▲)

p-CMP was administered 15 min before the administration of [Asu[1,7]]-ECT. Each value represents the mean ± S.D. ($n=4$).
a) $p<0.001$ against □ in Fig. 1.

TABLE I. Effect of Each Adjuvant on the Rectal Absorption (Determined by Disappearance) of Sodium Cefmetazole when Administered at a Dose of 2.5 mg/kg in Rat Rectal Loop (Absorption was Determined 15 min and 60 min after Administration)

Adjuvant[a] (concentration in microenema)		Percent absorption of cefmetazole[b]	
		15 min	60 min
Control (no adjuvant)		5.2±1.8	6.8±1.1
Diethylethoxymethylenemalonate	(0.17 M)	42.5±6.3	78.4±5.8
Phenylalanine enemine of ethylacetoacetate	(0.3 M)	44.8±8.1	72.7±8.4
Sodium salicylate	(0.6 M)	43.8±7.5	71.8±6.3
p-Chloromercuriphenyl sulfate	(0.05 M)	39.4±6.7	75.6±8.3

a) Adjuvant was coadministered with cefmetazole except for *p*-chloromercuriphenyl sulfate, which was administered 15 min before the administration of cefmetazole.
b) Each value represents the mean±S.D. ($n=4$).

drug compared to [Asu[1,7]]-ECT, because the efficacy of each adjuvant may depend on the molecular size of the main drug, and cefmetazole is not absorbed well (Table I) due to its low lipophilicity. For this study, the *in situ* loop method was employed and the absorption of cefmetazole was determined by measuring the amount remaining 60 min after administration of cefmetazole with adjuvant. This method was used because adsorption and degradation of cefmetazole in the rectal lumen is negligible; recovery of cefmetazole at 60 min after administration without any adjuvant into the loop was more than 90% (Table I). The concentration of each adjuvant in the microenema required to cause about 75% absorption of cefmetazole was 0.33 M for PheEtAA, 0.17 M for DEEMM, 0.6 M for SA and 0.05 M for *p*-PCM, as shown in Table I. Therefore, for the study of [Asu[1,7]]-ECT rectal absorption, these concentrations of adjuvant in the microenema were used.

The *AUC* value of [Asu[1,7]]-ECT when coadministered rectally with each adjuvant are summarized in Table II, DEEMM and PheEtAA showed stronger adjuvant action than SA and *p*-CMP did, though all four adjuvants showed similar enhancing efficacy on the rectal absorption of cefmetazole when administered at the concentrations described above. The *AUC* of [Asu[1,7]]-ECT increased more than 180 times when the drug was coadministered with either PheEtAA or DEEMM compared to that when it was administered alone.

We found recently that the adjuvant action of DEEMM may be similar in part to that of diethylmaleate, which enhances rectal cefmetazole absorption by decreasing the reduced nonprotein sulfhydryls in rectal tissue.[16] However, since it has also been reported that adjuvant action of DEEMM was partly suppressed by the coadministration of calcium ion,[16] as was found for PheEtAA,[12] even though coadministered calcium ion had no effect on the action of diethylmaleate, it may be speculated that the strong adjuvant action of DEEMM and PheEtAA on [Asu[1,7]]-ECT absorption involves the removal of calcium ion from the rectal mucosal area. Therefore, since the calcium-chelating action of DEEMM and PheEtAA may result in the sedimentation of mucin by removing calcium ion from the mucin layer, the effect of coadministration of alginate on the ability of SA to enhance the rectal [Asu[1,7]]-ECT absorption was examined. However, coadministration of alginate caused some increase (but not statistically significant) in the *AUC* of [Asu[1,7]]-ECT compared to the presence of SA alone as an adjuvant in the microenema (Table II), suggesting that the stronger adjuvant action of PheEtAA and DEEMM may occur at epithelial cells rather than in the mucin layer.

Since the rectal absorption of [Asu[1,7]]-ECT was evaluated by the *AUC* method and

TABLE II. Comparison of *AUC* of [Asu[1,7]]-ECT After Rectal Administration at a Dose of 800 U/kg (and 400 U/kg for Salicylate Study) in Microenema when Coadministered with Adjuvant or when Pretreated with Sodium Chloride or *p*-Chloromercuriphenyl Sulfate (*p*-CMP)

Adjuvant (concentration in microenema)	*AUC* for 120 min after rectal administration, mU·min/ml[b]	$\frac{AUC_{adjuvant}}{AUC_{control}}$
Control (no adjuvant)	82.5±14.8	1
Sodium alginate (2%)	263.5±38.7	3.2
Sodium pectate (2%)	238.4±41.6	2.9
Sodium chloride[a] (0.15 M)	127.3±24.6	1.5
(2.0 M)	405.8±52.6	4.9
Diethylethoxymethylenemalonate (0.17 M)	15265.4±1275.3	185.6
Phenylalanine enamine of ethylacetoacetate (0.3 M)	17652.7±1418.6	214.0
Sodium salicylate (0.6 M)		
800 U/kg	2108.6±325.4	25.0
400 U/kg	721.3±102.8	17.5
Sodium salicylate (0.6 M) +sodium alginate (2%)	2675.3±364.2	32.4
p-CMP[a] (0.05 M)	1826.4±162.8	22.1

More than four rats were used for each experiment.
a) Sodium chloride and *p*-CMP were administered 5 and 15 min, respectively, before the administration of [Asu[1,7]]-ECT.
b) Each value represents the mean±S.D. ($n \geq 4$).

TABLE III. Effect of Each Adjuvant on the Plasma IR-[Asu[1,7]]-ECT Concentration After Intramuscular Injection at a Dose of 40 U/kg

Adjuvant (concentration in microenema)	Plasma concentration of IR-[Asu[1,7]]-ECT, mU/ml after administration (the mean±S.D., $n=3$)		
	10 min	45 min	75 min
Control (no adjuvant)	30.1±3.6	22.2±3.4	16.2±2.4
Diethylethoxymethylenemalonate (0.17 M)	31.5±4.1	19.8±4.1	16.7±2.8
Phenylalanine enamine of ethylacetoacetate (0.3 M)	29.5±3.1	21.8±3.5	17.2±1.9
Sodium salicylate (0.6 M)	30.7±4.3	21.6±4.2	16.6±3.8
p-Chloromercuriphenyl sulfate (0.05 M)	32.5±2.8	21.7±4.6	16.9±2.4

Each adjuvant was administered rectally 5 min before the *i.m.* injection of [Asu[1,7]]-ECT.

coadministered adjuvant could influence the behavior of [Asu[1,7]]-ECT in plasma, the plasma [Asu[1,7]]-ECT profile was studied after intramuscular injection of [Asu[1,7]]-ECT at a dose of 40 U/kg 1 min after rectal administration of a microenema containing only an adjuvant. As shown in Table III, none of the adjuvants affected on the plasma [Asu[1,7]]-ECT profile, suggesting that the *AUC* of [Asu[1,7]]-ECT is not affected by the coadministration of adjuvant.

The enhancement of rectal absorption of [Asu[1,7]]-ECT has been discussed in terms of the plasma IR-[Asu[1,7]]-ECT concentration, but it should be considered whether each adjuvant influences the stability of [Asu[1,7]]-ECT in the rat rectal compartment after the administration.

When microenema containing 800 U of [Asu[1,7]]-ECT/ml was administered in the absence of adjuvant into the rat rectal loop, the amount of [Asu[1,7]]-ECT remaining in the loop was $85.1 \pm 6.2\%$ ($n=4$) at 15 min and $83.7 \pm 5.4\%$ ($n=4$) at 45 min after administration. Thus, [Asu[1,7]]-ECT appears to be stable in the rat rectal compartment. Therefore, the absorption-enhancing action of each adjuvant is not related to the stability of [Asu[1,7]]-ECT in the rat rectal compartment. Furthermore, the observed disappearance of [Asu[1,7]]-ECT from the rat rectal loop may be largely related to the binding of [Asu[1,7]]-ECT to the rectal membrane.

The dose of [Asu[1,7]]-ECT used in this study was rather high to give an effective plasma IR-[Asu[1,7]]-ECT concentration for clinical purposes (see in Figs. 2 and 4). The high dose was used so that the rectal [Asu[1,7]]-ECT absorption could be determined by using the immunoreactive assay method. Further study for clinical application should be carried out using a bioassay method based on the pharmacological response, *e.g.*, by monitoring the serum calcium concentration.

In conclusion, although rectal mucosal mucin layer does act as a diffusion barrier for [Asu[1,7]]-ECT absorption, it is suggested that the limiting step is poor transport through the rectal surface cell membrane due to the high molecular weight of [Asu[1,7]]-ECT. Rectal absorption of [Asu[1,7]]-ECT was increased significantly by the coadministration of either DEEMM or PheEtAA with more than 180 times greater *AUC* as compared to that after rectal administration of [Asu[1,7]]-ECT alone. Rectal absorption of [Asu[1,7]]-ECT was also enhanced by the coadministration of either SA or *p*-CMP but their adjuvant action was weaker than that of DEEMM or PheEtAA, though all four adjuvants the rectal absorption of cefmetazole (which has a relatively small molecular weight) to similar extents when administered at the doses used in this study.

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