Percutaneous Absorption of Elcatonin and Hypocalcemic Effect in Rat

Taro OGISO,* Masahiro IWAKI, Isako YONEDA, Mina HORINOCHI and Katsuaki YAMASHITA

Faculty of Pharmaceutical Sciences, Kinki University, Kowakae 3-4-1, Higashi-Osaka 577, Japan. Received June 27, 1990

The percutaneous absorption of elcatonin (EC), a hypocalcemic peptide, was investigated. A transdermal dosage form of EC was produced using a gel base, absorption enhancer and protease inhibitor, and applied to rats for 24 h. The combination of bile salt such as taurocholate and glycocholate, and *n*-octyl- β -D-glucoside or *n*-octyl- β -D-thioglucoside (OTG) exerted the potent enhancing effect on the absorption of EC, and a potent hypocalcemic effect was shown for 24 h or longer. The least level of plasma calcium was obtained 6 h or longer after application, suggesting the relatively rapid absorption of EC. The apparent bioavailability of EC in system 5 was 4.6%, this value being noteworthy in the percutaneous absorption of peptides. When the enhancing effect of taurocholate and OTG was separately measured, both agents acted additively on the absorption of EC. An EC ointment maintained the hypocalcemic effect after storage for 15 d at 40°C. The transdermal dosage form has the potential to be an efficient drug delivery system for Paget's disease and osteoporosis.

Keywords elcatonin; percutaneous absorption; hypocalcemic effect; absorption enhancer; bile salt; octyl glucoside; octyl thioglucoside; rat

Calcitonin, a hypocalcemic peptide hormone, has been used for the treatment of Paget's disease as well as certain types of osteoporosis.¹⁾ Elcatonin (EC), synthetic [Asu^{1,7}]-eel calcitonin, also inhibits osteoclastic bone resorption stimulated by bone resorptive factors.²⁾ Recently, there is growing evidence that EC action can stimulate osteoblastic bone formation.³⁾ Peptides, such as EC, are given effectively only by injection, because, when taken orally, they are digested by the proteolytic enzymes in the gastrointestinal tract and metabolized by the liver. In addition, it has been generally considered difficult for a peptide hormone to be absorbed through the intestinal mucosa. Furthermore, calcitonin therapy is inconvenient because the drug must be injected subcutaneously or intramuscularly. More simple or convenient administration may be favorable as a dosage route for EC and calcitonin. This is an important problem for patients who are unable to have injections or are in need of long-term therapy.

The rectal administration of EC is reported as a dosage route.^{4,5)} However, the bioavailability and the ability to lower serum calcium levels after rectal administration are relatively small.^{4,5)}

The transdermal route of administration is useful for drugs with low bioavailability after oral dosing. Drug administration *via* skin has the advantage of bypassing the hepatogastrointestinal "first-pass" metabolism associated with oral administration. It may also be useful for short-acting drugs since transdermal absorption tends to be slow, and prolonged effects may be realized.

It is reported that the absorption of human calcitonin through rat's gingiva is enhanced by the coadministration of sodium cholate,⁶⁾ and that bile salts will inhibit the degradation of the peptide in oral mucosa supernatant.⁷⁾ In addition, the absorption of thyrotropin-releasing hormone through rat skin is shown to be enhanced by *n*-octyl- β -D-glucoside (OG).⁸⁾ These observations led to our interest in the percutaneous (p.c.) absorption of EC based on the combined use of the enhancers.

The present study was designed to evaluate the p.c. absorption of EC and the effect of various absorption promoters and protease inhibitors, in addition to maintaining the prolonged effect after the transdermal application.

Experimental

Materials [Asu^{1,7}]-eel calcitonin (6400 U/mg, Toyo Jozo Co., Ltd.), a synthetic analogue of eel calcitonin in which the disulfide bridge between the first and seventh amino acids in the eel calcitonin molecule is replaced by a $-\text{CH}_2\text{CH}_2-$ bridge⁹⁾ was used throughout this study. Hiviswako 104 and Carbopol 934 (Carbopol), gel bases, were obtained from Wako Pure Chemical Industries and Kishida Chemical Co., Ltd., respectively. Gabexate mesilate and bestatin were obtained from Ono Pharmaceutical Industry Co. and Sigma Chemical Co. (St. Louis, MO, U.S.A.), respectively. Bile salts, *n*-octyl- β -D-thioglucoside (OTG) and OG were reagent grade commercial products. Gentamycin solution (5 mg/ml) was purchased from Boehringer Mannheim Co. Male Wistar rats (Japan SLC Inc.), weighing 270–300 g, were used throughout this experiment.

Preparation of Gel Ointments The pH value of Carbopol was adjusted to 6.5 with 10% NaOH. EC dissolved in a mixture of water and propylene glycol was mixed with the gel base and was followed by the addition of other additives. In one ointment (Rp. 10), Hiviswako 104 was vigorously

TABLE I. Composition of Elcatonin Gel Ointment and Absorption Enhancer and Protease Inhibitor Used

Rp. 1–9		Rp.	Additive I		Additive II	Rp. 10	
Carbopol 934	2.0 g	1	OG	Bestatin	5.0 mg	Glycerin	93.1 g
Elcatonin	25.0 mg	2	OG	Taurocholate	1.0 g	Hiviswako 104	1.0 g
Propylene glycol	10.0 g	3	OTG	Bestatin	5.0 mg	Elcatonin	25.0 mg
Gentamycin sol.	2.0 g	4	OTG	Gabexate	20.0 mg	Triethanolamine	1.37 g
Additive I	1.5 g	5	OTG	Taurocholate	1.0 g	Gentamycin sol.	2.0 g
Additive II	—	6	OTG	Deoxycholate	1.0 g	OTG	1.5 g
Purified water ad.	100.0 g	7	OTG	Glycocholate	1.0 g	Taurocholate	1.0 g
		8	OTG	—			
		9	—	Taurocholate	1.0 g		

OG, *n*-octyl- β -D-glucoside; OTG, *n*-octyl- β -D-thioglucoside.

mixed with glycerin heated at 65°C and, after cooling, EC dissolved in gentamycin solution was added to the base. Details of the ointment composition are listed in Table I.

Intravenous (i.v.) Administration On the day before the experiment, the rat jugular vein was cannulated with a silicon tubing^{10,11} and rats were fasted for 16 h prior to the experiment. EC dissolved in saline was administered intravenously at 40 or 80 U/rat. After administration, blood samples were collected periodically through the tubing. The plasma was separated immediately by centrifugation and stored frozen until assay.

In Vivo p.c. Absorption Experiment On the day before the experiment, the rat jugular vein was cannulated with a silicon tubing^{10,11} and the hair of the abdominal area was carefully removed with an electric clipper and an electric razor to prevent damage to the stratum corneum. The animals were fasted for 16 h prior to the experiment, but water was given freely. The transdermal therapeutic system (TTS) which was prepared using a corresponding gel ointment described in Table I, as depicted in Fig. 1, was fixed on the shaved abdominal skin (0.5 g ointment/4 cm²/rat) with an adhesive (Aronalpa, Konishi Co.) and immediately occluded with an adhesive tape. The application of system to rat was done for 24 h and then the remaining ointment was wiped off with absorbent cotton soaked in warm water. The fast was broken at this time. Blood samples from the cannulated jugular vein were collected periodically for 48 h after dosing.

Determination of Plasma Calcium Concentration The plasma calcium concentration was determined by the *o*-cresolphthalein complexone method using the Calcium C-Test Wako (Wako Pure Chemical Industries) and 0.01 ml plasma.

Pharmacokinetic and Statistical Analyses The area under the plasma calcium concentration-time curve (the difference in the area between the EC-dosing group and control group, *AUC*) after i.v. administration and topical application of EC was calculated by the trapezoidal method up to 24 h. The area under the first moment curve (*AUMC*) and the mean residence time (*MRT*) were calculated by means of the following equations¹²:

$$AUMC = \int_0^{24} t \cdot C_p dt$$

$$MRT = AUMC/AUC$$

where C_p is the difference in plasma calcium concentration between EC-dosing and control rats at time, t .

The apparent bioavailability was calculated by the following equation:

$$\text{apparent bioavailability} = \frac{AUC_{p.c.} \cdot \text{Dose}_{i.v.}}{AUC_{i.v.} \cdot \text{Dose}_{p.c.}} \times 100$$

where $AUC_{p.c.}$ and $AUC_{i.v.}$ are *AUC* after p.c. and i.v. (40 U/rat) administrations, respectively. The bioavailability was calculated from AUC_{0-24} , because the ingestion of diet enhanced the calcium level in the plasma.

The means of all data are presented with their standard deviation (mean $\pm$ S.D.). One-way analysis of variance (ANOVA) with Scheffe's multiple comparison procedure was used to compare each data, and a p value of 0.05 or less was considered to be significant.

Results

Plasma Calcium Concentration after Single i.v. Administration The plasma calcium concentrations after i.v. administration of EC (40 or 80 U/rat) are shown in Fig. 2. The plasma calcium levels decreased rapidly in both dosing and a significant hypocalcemic effect was obtained 3–24 h after administration, although the effect declined 30 h after dosing. The AUC_{0-24} values were 57.89 ± 7.48 and 73.87 ± 6.19 mg·h/dl for the 40 U and 80 U dosing, respectively.

Plasma Calcium Concentration after Single p.c. Administration The plasma calcium concentrations during a single p.c. application of TTS are shown in Figs. 3 and 4. The pharmacokinetic parameters calculated are listed in Table

Fig. 1. Sectional View of Transdermal Therapeutic System

A, ointment; B, aluminum foil; C, silicon rubber (2.26 cm i.d., 1.0 mm thickness).

Fig. 2. Changes in Plasma Calcium Concentrations Following i.v. Administration of EC

○, 40 U/rat; △, 80 U/rat; ●, saline.

Fig. 3. Changes in Plasma Calcium Concentrations Following p.c. Application of Systems 2 and 5

Each point represents the mean $\pm$ S.D. ($n=4-9$). A, system 2; B, system 5. ●, placebo (control) system; ○, EC system.

Fig. 4. Changes in Plasma Calcium Concentrations Following p.c. Application of Systems 6 and 7

Each point represents the mean $\pm$ S.D. ($n=4-7$). A, system 6; B, system 7. $\bullet$, placebo system; $\circ$, EC system.

TABLE II. Pharmacokinetic Parameters Calculated by the Moment Method after Percutaneous Application of Elcatonin Systems

System No.	AUC (mg $\cdot$ h/dl)	AUMC (mg $\cdot$ h ² /dl)	MRT (h)	Apparent bioavailability (%)
1	30.99 $\pm$ 11.75	371.41 $\pm$ 159.80	12.06 $\pm$ 1.99	2.68
2	46.25 $\pm$ 19.25 ^{a)}	623.71 $\pm$ 233.74	13.81 $\pm$ 2.36	3.99
3	36.13 $\pm$ 21.93	445.49 $\pm$ 249.06	12.83 $\pm$ 1.68	3.12
4	17.25 $\pm$ 10.07	271.52 $\pm$ 106.34	18.16 $\pm$ 6.19	1.49
5	53.50 $\pm$ 14.96 ^{b)}	752.04 $\pm$ 298.40	13.66 $\pm$ 3.46	4.62
6	31.97 $\pm$ 17.89	505.37 $\pm$ 238.24	16.32 $\pm$ 2.35	2.76
7	40.85 $\pm$ 20.01	513.32 $\pm$ 292.53	12.34 $\pm$ 2.37	3.53
8	26.40 $\pm$ 20.66	341.64 $\pm$ 271.20	13.91 $\pm$ 3.02	2.28
9	35.02 $\pm$ 16.72	433.42 $\pm$ 114.39	13.37 $\pm$ 3.60	3.02
10	35.23 $\pm$ 33.53	490.35 $\pm$ 444.31	16.31 $\pm$ 6.88	3.04

Each value represents the mean $\pm$ S.D. of 4–9 experiments. The system was prepared using the corresponding gel ointment (0.5 g) described in Table I. The applied dose of elcatonin was 800 U/4 cm². a) Significantly different from No. 1, 3, 4 and 6–10, $p < 0.05$. b) Significantly different from No. 1–4 and 6–10, $p < 0.05$.

II. The calcium levels decreased relatively rapidly and the least level was obtained 6 h or longer after application. The calcium levels were gradually increased after the cessation of application, probably due to the ingestion of calcium (diet).

The application of the preparation without the enhancer and/or inhibitor listed in Table I failed to reduce the plasma calcium level (data not shown). Therefore, it is suggested that the enhancers and inhibitors used would significantly promote the absorption of EC through rat skin. System 5 showed an excellent hypocalcemic effect, giving the highest bioavailability among the systems applied ($p < 0.05$).

Systems 2 and 7 also showed higher hypocalcemic effects than those of other preparations, although the difference in the values of the latter system did not reach statistical significance. These preparations contained taurocholate or glycocholate and OG or OTG as an enhancer, suggesting that they are effective enhancers. The system containing gabexate, a protease inhibitor, however did not exert a significantly hypocalcemic effect. There was not a significant difference in MRT values between all systems.

Enhancing Effect of Taurocholate and OTG Individually on p.c. Absorption In order to clarify whether or not taurocholate and OTG exert a synergetic or additive effect, the system (No. 8 and 9) containing each agent alone was prepared and applied to rat. The AUC value was shown in Table II. The sum of AUC for No. 8 and No. 9 roughly approximated that for system 5 which contained both taurocholate and OTG. Thus, it appears that both agents

Fig. 5. Changes in EC Activity in Ointment in the Course of Time

Rp. 5 ointment was stored at 40°C for 15 (Δ) and 30 d ($\times$). $\bullet$, placebo ointment; $\circ$, immediately after preparation.

might not act synergetically on the enhancing effect.

Stability of EC in Ointment Rp. 5 ointment was stored in a polystyrene microtube with a cap at 40°C for 15 or 30 d and then a system prepared was applied to rat. The hypocalcemic effect is shown in Fig. 5. The hypocalcemic action was still as exerted after storage for 15 d as immediately after preparation, but the effect was extremely decreased after 30 days' storage.

Discussion

The p.c. absorption of peptides including calcitonin is

reported by Muranishi *et al.*⁸⁾ They estimated the effect of Azone on the *in vitro* penetration, but not *in vivo*, and show that the enhancer increases the absorption of calcitonin.⁸⁾ To date, however no quantitative data are available concerning the p.c. absorption of EC.

The lower limit for molecular weight for which p.c. absorption becomes a limiting factor is assumed to be 5000.¹³⁾ But the quantities of compounds in the skin expressed as moles exhibit a disproportionate decrease with increasing molecular weight.¹³⁾ Since the molecular weight of EC is 3363.8,⁹⁾ it is assumed that EC may be partly absorbed through the skin. Consequently, the assumption was clearly demonstrated by our experiments using absorption promoters.

It now seems that the dominant path for polar molecules is the polar region of the intercellular lipid (the intercellular route) in the stratum corneum and the appendages, *e.g.* the hair follicles and the sweat ducts (the shunt route).¹⁴⁾ At short times, the flux through the shunts is much larger than that through the stratum corneum.¹⁴⁾ The relatively shorter lag time, observed in this experiment (Figs. 3 and 4), suggests the transport of EC, at least partly, through the routes. The diffusional resistance of the stratum corneum is reduced when the skin is occluded, due to the existence of free water¹⁵⁾ and pathways develop in the stratum corneum in which water molecules diffuse more easily.¹⁶⁾ Many absorption enhancers will interact with the polar head groups of the lipid *via* hydrogen bonding and ionic interactions.^{17,18)} The consequent disturbance of the lipids and alterations in head group interactions will upset the packing at the head region.¹⁷⁾ OG, OTG and bile salts might also interact with the head groups. These enhancers used thus might increase the volume of the aqueous layer so that more water enters the tissue. This swelling provides a greater cross-sectional area for polar diffusion and a larger fractional volume of free water. Consequently, the major absorption pathway of EC would be *via* shunt routes and intercellular channels.

Below the stratum corneum is the viable epidermal layer, the most metabolically active layer in skin.^{19,20)} Metabolism, being mainly hydrolysis, of EC may reduce the amount of drug systemically available and pharmacologic activity. The high hypocalcemic activity after p.c. administration of EC indicated that the additives such as bile salts might largely inhibit the hydrolytic enzyme activity in the viable tissues. It is shown that bile salts significantly inhibit the degradation of human calcitonin in oral mucosa homogenate, thus suggesting the inhibition of peptidases in the mucosa.⁷⁾ Therefore, bile salts used would act as the absorption enhancer and protease inhibitor in this study.

When taurocholate was used in combination with OTG, they showed an apparently additive effect on p.c. absorption of EC (Table II), although the conclusive evidence must await further studies, *e.g.* the direct determination of the peptide in serum. Our results suggest that taurocholate probably increased skin permeability by a different mechanism than that for OTG.

The bioavailability, calculated based on the *AUC* of 40 U/rat (*i.v.*), of EC following p.c. application of system 5 was 4.62%. This value was much more than that (0.8%) after rectal administration in rats, and the hypocalcemic effect was maintained much longer than in the rectal

administration.⁴⁾ As a result, the transdermal system of EC has the potential to be an efficient drug delivery system. System 4, containing gabexate, did not produce a significant effect. A possible mechanism involved in the decreased effect is unclear. However, a likely explanation is that the protease inhibitor might not penetrate through the cell membrane, owing to the highly water-soluble property, and therefore EC penetrated into viable skin might be partly hydrolyzed by the proteases.

The reason why the ability to lower serum calcium levels was not proportional to the doses (40 and 80 U/rat) may be that the doses used were much larger than the dose for the adequate response (*e.g.* 10 or 40 U/man, twice weekly), and thus the ability to lower the levels attained nearly a maximum at these doses. Yamaguchi *et al.* report that bone calcium content is increased significantly by the presence of 10 and 100 ng EC/ml in the *in vitro* experiment, but the increased extent is not proportional to the doses.²¹⁾

When the hypocalcemic effect was compared with that after the *i.v.* injection of EC (40 or 80 U/rat), the administration by p.c. route showed an equivalent effect to the *i.v.* injection, although a much higher dose of EC was required in the p.c. dosing. The hypocalcemic effect after p.c. administration may be maintained over a more prolonged period, if the system is applied more than a day.

The study on changes of EC activity in the system in the course of time revealed a significant decrease in the hypocalcemic effect after 30 days' storage at 40 °C, but not after 15 d. This may be mainly due to the decline in the antibacterial activity of gentamycin (10 mg/100 g ointment) added as an antiseptic. If a more effective antiseptic is used, the changes would probably be prevented.

These systems produced no erythema and no edema during application for 24 h. Therefore, these systems appear not to have irritant effects on the skin. The negligible irritant effect may make the formulation acceptable for clinical use.

In conclusion, the present results lead us to postulate that the drug delivery system of EC gave a constant decrease in plasma calcium levels over a prolonged period. The transdermal system has the potential to be an efficient drug delivery system for Paget's disease and osteoporosis. Our data present an evidence of p.c. absorption of peptide, EC.

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