The Metabolic Inhibition Model Which Predicts the Intestinal Absorbability and Metabolizability of Drug: Theory and Experiment

Takashi Mizuma, Shoji Awazu*

Department of Biopharmaceutics and Drug Rational Research Center, School of Pharmacy, Tokyo University of Pharmacy and Life Science. * To whom correspondence should be addressed: 1432-1 Horinouchi, Hachioji, Tokyo 192-03. E-mail: awazu@ps.toyaku.ac.jp. Fax: +81-426-76-3126.

ABSTRACT

The intestinal absorption of analgesic peptides (leucine enkephalin and kyotorphin) and modified peptides in rat were studied. Although these peptides were not absorbed, the absorbability (absorption clearance) of
these peptides were increased in the presence of peptidase inhibitors. In order to kinetically analyze these phenomena, we proposed the metabolic inhibition model, which incorporated the metabolic clearance (metabolizability) with the absorption clearance. Metabolic activity was determined with intestinal homogenates. The higher the metabolic clearance was, the lower was the absorption clearance. The relationships between the absorption clearance and the metabolic clearance of the experimental data as well as of the theoretical values were hyperbolic. This model predicted the maximum absorption clearances of cellobiose-coupled leucine enkephalin (0.654 μl/min/cm) and kyotorphin (0.247 μl/min/cm). Details of the experimental methods are described.

INTRODUCTION

The small intestine is the organ for nutrient absorption, which consists of digestion/metabolism and transport. Although the digestion/metabolism of dietary protein or peptides or oligo- or polysaccharides is required for absorption, medicinal drugs must be transported to systemic circulation without metabolic degradation to perform their pharmacological action. Membrane transportability is also expected to be low, since these compounds are hydrophilic and their molecular sizes are large. Therefore, the estimation of each process (metabolism and transport) is required for the evaluation of intestinal absorption. For the kinetic analysis of intestinal absorption, we proposed the metabolic inhibition model (1, 2), which can evaluate the intestinal absorption of analgesic peptides, leucine enkephalin (Tyr-Gly-Gly-Phe-Leu, LE) and kyotorphin (L-tyrosyl-L-arginine, KTP). In this report, we summarize the experimental and analytical procedures for the evaluation of intestinal absorption based on the metabolic inhibition model. KTP is an endogenous compound, which releases methionine enkephalin, possessing analgesic activity (3), from the striatum (4), and is hydrolyzed by peptidases such as aminopeptidase (5-8). Leucine enkephalin (LE), an opioid peptide, is easily degraded to destyrosyl LE by aminopeptidase in the intestine (9,10). Thereby, we clarified the rate-limiting process in the peptide absorption, and predicted the absorbability to discuss the oral delivery of KTP.

THEORY AND EXPERIMENT

Metabolic inhibition model

Kinetic analysis of the correlation between absorption clearance and metabolic clearance were performed based on Eq. 1 according to the metabolic inhibition model for absorption (1,2) shown in Figure 1.

$$Clabs = \frac{CLovt}{1 + RCmet \times Ra} \quad (1)$$,where CLovt is overall transport clearance of peptide from the mucosal to the serosal sides, where no metabolic degradation of peptide in intestinal tissue occurs during absorption process. This value means the maximum of the absorbability and is defined as

$$Clovt = \frac{CLm\text{-}i \times CLi\text{-}s}{CLi\text{-}m + CLi\text{-}s} \quad (2)$$. Here, CLm-i, CLi-m and CLi-s were transport clearances of peptide from the mucosal side to the intestinal tissue, from the intestinal tissue to the mucosal side, from the intestinal tissue to the serosal side, respectively. RCmet is the ratio of clearances expressed by Eq. 3.

$$RCmet = \frac{CLmet,int}{CLi\text{-}m + CLi\text{-}s} \quad (3)$$.

Ra, which is the remaining activity of the metabolism in intestinal tissue, is obtained by dividing CLmet in the presence of peptidase inhibitor with CLmet in its absence. CLmet,int is the intrinsic clearance of metabolism in intestinal tissue, and is CLmet in the absence of any inhibitors. The theoretical relationship between the absorption clearance and the metabolic clearance according to Eq. 1 is shown in Fig. 2. The hybrid parameters, CLovt and RCmet, are obtained by fitting data to Eq. 1, using a nonlinear least squares fitting program, MULTI (11). The effect of complete inhibition of peptidase activity on absorption rate (Ei), which is defined by dividing CLovt with the CLabs in the absence of inhibitors, is expressed by Eq.4.

$$Ei = 1 + RCmet \quad (4)$$

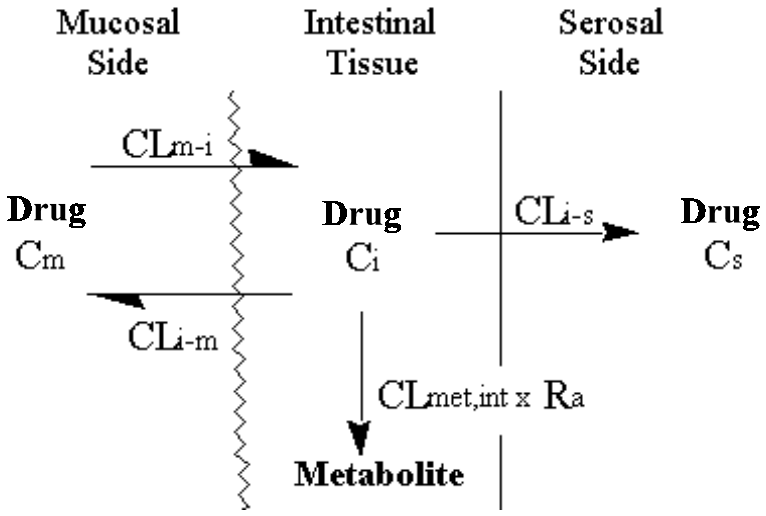

Figure 1: Metabolic inhibition model for intestinal absorption of drug. Ci, Cs and Cm are the drug concentrations in the intestinal tissue, on the serosal side and on the mucosal side, respectively. CLm-i, CLi-m and CLi-s are the transport clearances from the mucosal side into the intestinal tissue, and from the intestinal tissue to the mucosal side and from the intestinal tissue to the serosal side, respectively. LCmet,int is the intrinsic clearance of metabolism by peptidase in the intestinal tissue. Ra is a remaining activity in the intestinal tissue in the presence of peptidase inhibitors.

Estimation of absorption clearance

Absorption clearance, CLabs, was calculated by Eq. 5, which is the integrated form of Eq. 6 based on definition.

$$CLabs = \frac{Xabs}{AUCmuc_{,0-T}} \quad (5)$$

Xabs is the amount of peptide absorbed from the mucosal to the serosal sides for the time from 0 to T.

$$Vabs = CLabs \times Cmuc \quad (6)$$

Vabs and Cmuc represent the absorption rate and mucosal concentration respectively. Xabs and AUCmuc represent the amount of drug absorbed to the serosal side and the area under the curve of peptide concentration on the mucosal side respectively. AUCmuc was calculated by the trapezoidal rule (12).

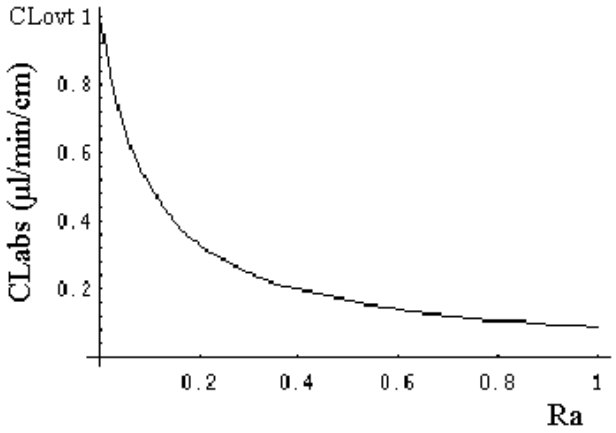

Figure 2: Relationship between absorption clearance remaining activity (Ra) based on the metabolic inhibition. The line represents the simulated values based on the cast that CLovt and RCmet are 1 µl/min/cm and 10 respectively.

Estimation of metabolic clearance and/or remaining activity of metabolic enzyme

The remaining activity (Ra) was calculated by Eq. 7, when the metabolism follows first-order kinetics (1).

$Ra = \frac{kmet,inhi}{kmet,cont}$ **(7)** , where kmet is rate constant of metabolism, and kmet,inhi and kmet,cont are kmet in the presence of peptidase inhibitor and kmet of control, respectively. Kmet is calculated using Chom = Co exp(- kmet × t) **(8)**. T, Co and Chom represent t and the concentrations at time 0 and t, respectively. The kmet is calculated by fitting data to Eq. 8 using MULTI.

Ra is also calculated as follows (2): $Ra = \frac{CLmet,inhi}{CLmet,cont}$ **(9)** , considering that

$CLmet = \frac{Xmet}{AUChom_{,0-T}} \times Ww$ **(10)** , and $Xmet = (C_T - C_0) \times \frac{100}{H}$ **(11)** , where CLmet,inhi and CLmet,cont are CLmet in the presence of peptidase inhibitors and CLmet of control, respectively. Xmet is the amount metabolized per one gram of wet weight of intestine in intestinal homogenate, and is calculated by Eq. 5. $AUChom_{0-T}$ is the area under the concentration curve of peptide in the reaction mixture for the time from 0 to T, and is calculated by the trapezoidal rule. Ww is wet weight per length of everted intestine (g/cm). C_T and C_0 are peptide concentrations in the reaction mixture at time T and 0, respectively. H is the concentration of homogenate (%, W/V) of the reaction mixture.

Estimation of elimination clearance from luminal side

Elimination clearance (CLeli) from the mucosal side was obtained by fitting the data to Eq. 12 using the nonlinear least squares fitting program MULTI when the elimination from the mucosal side follows first-order kinetics (1).

$$Cmuc = Co \exp\left(\frac{-CLeli \times t}{Vmuc}\right) \quad \textbf{(12)}$$, where t, Co and Vmuc represent the time, the mucosal side concentration at time 0 and the medium volume in the mucosal side, respectively.

CLeli was also calculated by Eq. 13 (2): $$CLeli = \frac{Xeli}{AUCmuc_{,0,T}} \quad \textbf{(13)}$$. Xeli is the amount eliminated from the mucosal side, and is calculated by $Xeli = (C_T - C_0) \times Vmuc$ **(14)**. $AUCmuc_{,0\text{-}T}$ is the area under the concentration curve of peptide on the mucosal side from the time 0 to T, and is calculated by the trapezoidal rule (12). C_T and C_0 are peptide concentration on the mucosal side at time T and 0, respectively.

MATERIALS AND METHODS

Leucine enkephalin (LE), gentiobiose, kyotorphin (KTP) and L-tyrosyl-D-arginine ([D-Arg^2]kyotorphin, D-KTP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cellobiose and Sephadex G-25 superfine were purchased from Nacalai Tesque (Kyoto, Japan) and Pharmacia LKB Biotechnology (Tokyo, Japan), respectively. Ng-nitro-L-arginine benzyl ester (Arg(NO2)-OBzyl) tosylate and N, N'-dicyclohexylcarbodiimide (DCC) were from Peptide Institute, Inc. (Osaka, Japan). N-t-Butyloxycarbonyl-L-tyrosine (Boc-Tyr) was from Wako Pure Chemical Industries. (Osaka, Japan). L-Arginyl-L-tyrosine (retro-isomer of KTP, Ret-KTP) was purchased from Bachem Feinchemikalien AG (Switzerland).

Synthesis of Cellobiose-coupled LE (CcpLE). CcpLE was synthesized according to the method reported in the previous study (13), which basically followed Gray's method (14). Briefly, LE, cellobiose and sodium borohydride (1:10:10 mole ratio) dissolved in purified water were incubated at 37ºC for 3 to 4 weeks. The reaction mixture was concentrated by evaporation at 40ºC under reduced pressure. The resultant solution was applied to Sephadex G-25 superfine column (55 mm I.D., 75 cm length) and eluted with water at a flow rate of 3.5 ml/min. Each fraction eluted was checked by the HPLC under the conditions described below. The fractionated solution containing CcpLE was concentrated by evaporation described above, and was freeze-dried. The crude CcpLE dissolved in the mobile phase (25% acetonitrile, 2% methanol, 1.5% acetic acid in water) was applied to a preparative ODS column (30 mm I.D., 30 cm length), and eluted with the same mobile phase at a flow rate of 2.5 ml/min. Finally, the purified CcpLE was obtained by freeze-drying after evaporation of methanol. CcpLE was confirmed by molecular ion peaks $[M+H]^+$ at 882.8 (m/z) and $[M+Na]^+$ at 904.7 by electrospray ionization (ESI) method. Furthermore, the ninhydrin reaction of CcpLE was negative, indicating that the amino group of LE was reacted with sugar. Sugar moiety was confirmed by the phenol sulfuric reaction.

Synthesis of N-t-butyloxycarbonyl-L-kyotorphin (Boc-KTP). Boc-KTP was synthesized from Boc-Tyr and Arg(NO_2)-OBzyl by the standard DCC method of peptide synthesis as follows. Boc-Tyr (1.055g) and Arg(NO2)-OBzl (1.974g) were dissolved in the mixture of DMF (8 ml) and triethanolamine (580 µl) by stirring for 30 min at room temperature. DCC (890 mg) dissolved in ethylacetate was added to the solution kept at 0ºC on ice by dropwise, and was stirred for 2 hr, and then further stirring of reaction mixture was performed at room temperature under light-protection for 46 hr. The formation of Boc-Tyr-Arg(NO_2)-OBzyl was confirmed by TLC and HPLC according to the method described below. The reaction mixture was filtrated through paper filter. The resultant filtrate was concentrated by evaporation at 40ºC under reduced pressure. The residue was dissolved in 10 ml of ethylacetate, and byproduct was removed by extraction with water. The extraction was performed 3 times. Ethylacetate fraction was evaporated, and crude Boc-Tyr-Arg(NO_2)-OBzyl was obtained. Boc-Tyr-Arg(NO_2)-OBzyl was dissolved in the mobile phase (45% acetonitrile, 1.5% acetic acid) for the following liquid chromatography, and was applied to a preparative liquid chromatography column (ODS, 30-50 µm particle size, 30 mm id., 30 cm length, GL Science, Tokyo, Japan). Each fraction (10 ml) eluted at a flow rate of 2.5 ml/min was collected to a tube. Boc-Tyr-Arg(NO_2)-OBzyl contained in fractions which was confirmed by HPLC assay, was evaporated. Boc-Tyr-Arg(NO_2)-OBzyl was dissolved in methanol (5 ml) in a flask, which was filled with nitrogen gas. Into the solution, Pd-C was added, and the flask was filled with hydrogen gas. The solution was stirred for 1 day under light-protection at room temperature. After confirming deprotection of NO_2 group by HPLC or TLC assay, the solution was filtrated through celite on glass fiber sheet. The filtrate was evaporated. The crude Boc-KTP was dissolved in the mobile phase, and was applied to the semipreparative column (TSKgel ODS-80TM, 7.8 mm i.d., 30 cm length, Tosoh, Tokyo, Japan). The flow rate of mobile phase (20% acetonitrile, 1.5% acetic acid in water) was 3.0 ml/min. The fractions containing Boc-KTP were collected, and evaporated. Purified Boc-KTP was recrystalized with a mixture of ethylacetate and hexane. Identification of Boc-KTP was performed by ESI mass spectrum showing molecular ion peaks[M+H]+ at 438.3 (m/Z) and [M+Na]+ at 460.3. The ninhydrin reaction was negative, but after hydrochloric acid treatment it was positive, indicating the presence of Boc moiety at amino group of Tyr in the synthesized Boc-KTP.

TLC. Sample was spotted on the TLC plate (6 cm x 5 cm), and developed with the solvent mixture of n-butanol, methanol, water and acetic acid (10:8:2:1). After UV-detectable (λ=253.7nm) spot was checked, the ninhydrin reagent was sprayed upon the TLC plate. After the TLC plate was heated, the spot was checked. As another procedure, before the ninhydrin reagent was sprayed, diluted perchloric acid was sprayed upon the plate, and the plate was heated.

HPLC. ODS-80TM column (6 mm i.d., 15 cm length) and mobile phase consisted of 30% acetonitrile, 0.05% phosphoric acid were used. The UV detector was set at 274 nm. The assay condition other than these were described below.

Intestinal absorption. Intestinal absorption of peptides were performed with the everted small intestine (15). Male Wistar rats (180-230g, JAPAN SLC. Inc., Japan) fasted overnight were anesthetized with ether, and the intestinal blood was removed by saline perfusion. The jejunum was removed and everted. The everted small intestine of 10 cm was placed in 30 ml of incubation medium (113.3 mM NaCl, 4.83 mM KCl, 1.214 mM KH_2PO_4, 1.205 mM $MgSO_4$, 16.96 mM $NaHCO_3$, 10.18 mM Na_2HPO_4, 0.645 mM $CaCl_2$, pH 7.4) containing peptide at 250 µM in a beaker at 37ºC. The serosal side was filled with 5 ml of the

incubation medium without peptides. When necessary, peptidase inhibitors were added to the medium. Incubation media (100μl) were sampled from both the serosal and the mucosal sides at every 10 min until 60 min. The samples of CcpLE absorption experiment were mixed with 100 μl of 10% perchloric acid containing 12.5 mM L-phenylalanine as an internal standard of CcpTGG and 25 mM sodium 1-naphthalenesulfonate as an internal standard of CcpLE for HPLC assay. The samples of the absorption experiments of KTP, D-KTP and Boc-KTP were mixed with 100μl of internal standard solution (200μM tryptophan in 10% perchloric acid for KTP, D-KTP and Ret-KTP, or 100 μM Sodium 1-naphthalensulfonate in acetonitrile for Boc-KTP) for the following HPLC assay. The mixture was centrifuged at 11,000 x g for 5 min by a benchtop centrifuge KM-15200 (KUBOTA, Japan). Twenty-five microliter of the resultant supernatant was applied to HPLC. The absorption experiment of phenol red (PR, 1 mM) was performed by the same method.

Metabolism in intestinal homogenates. Ten cm of intestine was isolated according to the method described in the intestinal absorption experiment, and was homogenized by Physcotron homogenizer (Nichion Irika, Tokyo, Japan) in the same buffer as in the absorption experiment to prepare 10% intestinal homogenate (W/V). Metabolic reaction was started by mixing 500 μM peptide solution (0.6 ml) with homogenate (0.5-2.5%) (0.6 ml) after preincubation for 5 min at 37 ºC. The reaction mixture was periodically sampled. The procedure for the peptide assay was the same as in the absorption experiment.

Assay of LE, cellobiose-coupled LE (CcpLE) and its metabolites. LE, CcpLE and its metabolite (cellobiose-coupled tyrosylglycylglycine, CcpTGG) were determined by reversed-phase HPLC. For the assay of LE and CcpLE, an octyl column (TSKgel OCTYL-80Ts, 4.6 mm I.D., 15 cm length, Tosoh Corp., Japan) and the mobile phase composed of 18% acetonitrile, 3% methanol and 0.05% phosphoric acid were used. UV detector (274 nm) and fluorescence detector (Ex 278nm, Em 305nm) were used for the purification and the transport experiments, respectively. For the assay of CcpTGG, HPLC condition followed the previous method (13).

Assay of phenol red (PR). Incubation media (100 ml) sampled from the serosal side and the mucosal side, were mixed with 2.6 ml of 1 N sodium hydroxide. PR in the solution was determined by dual wavelength spectrophotometry (λ1: 610 nm, λ2: 560 nm) using Spectrophotometer 557 (Hitachi, Japan).

Assay of KTP, Boc-KTP and their metabolites. KTP, Boc-KTP and their metabolites were determined by reversed-phase HPLC. The HPLC system was consisted of a pump (Twincle, Jusco Co, Tokyo, Japan), a UV detector (Shimadzu, Kyoto, Japan), a fluorescence detector (821-FP, Jasco) and an integrator (D-2500, Hitachi Ltd, Tokyo, Japan). For the assay of KTP, D-KTP and Ret-KTP, the mobile phase composed of 7% methanol and 0.05% phosphoric acid in pure water and an ODS column (80TM, 6 mm i.d., 15 cm length, Tosoh Corp., Japan) were used. For the assay of Boc-KTP, the mobile phase composed of 20% acetonitrile and 0.05% phosphoric acid in water and an ODS column (A-312, 6 mm i.d., 15 cm length, YMC, Japan) were used. UV detector (274 nm) and fluorescence detector (Ex 278nm, Em 305nm) was used for the purification, absorption experiments, respectively, and a flow rate was 1.5 ml/min.

RESULTS AND DISCUSSION

The absorption experiment of LE or cellobiose-coupled LE showed that LE did not appear on the serosal side, but cellobiose-coupled LE appeared (Fig. 1 in our report (1)). Although the elimination clearance of cellobiose-coupled LE was lower than that of LE (Fig. 2 in our report (1)), cellobiose-coupled LE was shown to be metabolized. This indicates that cellobiose-coupling stabilized LE against aminopeptidase, but the stabilization was not complete. In the presence of peptidase inhibitors, the elimination clearances of LE and cellobiose-coupled LE from the mucosal side were decreased. However, LE did not appear on the serosal side. Furthermore, the absorption clearance of cellobiose-coupled LE, which is calculated by normalization with mucosal concentration according to Eq. 8, was increased in the presence of peptidase inhibitors. On the contrary, these peptidase inhibitors did not increase the absorption clearance of unmetabolizable compound, phenol red, indicating that the passive transport was not increased by these inhibitors. Therefore, it was indicated that cellobiose-coupled LE was metabolized not only on the mucosal (luminal) side but also in the intestinal tissue during absorption process. Thereby, the effect of peptidase inhibitors on the stability and intestinal absorption was evaluated by the metabolic inhibition model, which incorporated the metabolic activity in intestinal tissues into the calculation of the absorption clearance (Eq. 1 and Fig. 2).

The relationship of the intestinal absorption and metabolic clearances, which was shown in Fig. 3 of our report (1), indicated that the higher the metabolic clearance was, the lower the absorption clearance was, and that the relationship was hyperbolic. The overall transport clearance was 0.654 μl/min/cm. This value was comparable to that (0.653 μl/min/cm) of cellobiose-coupled leucine enkephalinamide (16), which is resistant to the endopeptidases. On the other hand, when the remaining activity was estimated from the elimination clearance from the mucosal side, the overall transport of CcpLE was much higher (6.95 μl/min/cm) than that of CcpLEamide. These values indicate that the metabolic inhibition model can predict the absorbability. It was also shown that the intestinal absorption of CcpLE can be improved 8 times by complete inhibition of metabolism according to the Eq. 4.

The metabolic inhibition model was also applicable to the intestinal absorption of KTP (2). KTP did not appear on the serosal side. However, in the presence of peptidase inhibitors such as o-phenanthrolin, tryptophan hydroxamate and bestatin, KTP appeared on the serosal side. Therefore, the metabolic inhibition model was also applied to the evaluation of the effect of peptidase inhibitors on the improved absorption. The overall transport clearance of KTP was 0.247 μl/min/cm. Meanwhile, the absorption clearance (the overall transport clearance) of D-KTP, peptidase-resistant isomer of KTP, was 0.516 μl/min/cm, and was approximately similar to that of KTP. This indicates that the metabolic inhibition model approximately predicts the absorbability of KTP as well, and that the absorption clearance of KTP can be increased by metabolic inhibition.

On the other hand, no correlation of the absorption clearance with the elimination clearance was observed in the absorption of KTP and Boc-KTP (Fig. 6a and b in our report (2)). The results with KTP and Boc-KTP as well as CcpLE indicate that the elimination activity from the mucosal side does not affect the absorption clearance.

Drug metabolism affect the bioavailability of drug following oral administration, but the contribution of intestinal metabolism to the bioavailability has not been evaluated. The estimation of two hybrid parameters is enough for the prediction of the absorbability by the metabolic inhibition model, although

a number of parameters are incorporated into Eq. 1. Therefore, this model, which predicts the extent of the contribution of metabolizability to the absorbability, is useful.

REFERENCES

1. Mizuma, T., Ohta, K., Koyanagi, A., Awazu, S. 1996. Improvement of intestinal absorption of leucine enkephalin by sugar coupling and peptidase inhibitors. *J. Pharm. Sci.* **85**, 854-857.
2. Mizuma, K., Koyanagi, A., Awazu, S. 1997. Intestinal transport and metabolism of analgesic dipeptide, kyotorphin: rate-limiting factor in intestinal absorption of peptide as drug. *Biochim. Biophys. Acta* **1335**, 111-119.
3. Takagi, H., Shiomi, H., Ueda, H., Amano, H. 1979. A novel analgesic dipeptide from bovine brain is a possible Met-enkephalin releaser. *Nature* **282**, 410-412.
4. Janicki, P. K., Lipkowski, A. W. 1983. Kyotorphin and D-kyotorphin stimulate Met-enkephalin release from rat striatum in vitro. *Neurosi. Lett*. **43**, 73-77.
5. Matsubayashi, K., Kojima, C., Kawajiri, S., Ono, K., Takegoshi, T., Ueda, H., Takagi, H. 1984. Hydrolytic deactivation of kyotorphin by the rodent brain homogenates and serum. *J. Pharmacobio-Dyn.* **7**, 479-484.
6. Ueda, H., Ming, G., Hazato, T., Katayama, T., Takagi, H. 1985. Degradation of kyotorphin by a purified membrane-bound-aminopeptidase from monkey brain: potentiation of kyotorphin-induced analgesia by a highly effective inhibitor, bestatin. *Life Sci.* **36**, 1865-1871.
7. Akasaki, K., Tsuji, H. 1991. An enkephalin-degrading aminopeptidase from rat brain catalyzed the hydrolysis of a neuropeptide, kyotorphin (L-Tyr-L-Arg). *Chem. Pharm. Bull.* **39**, 1883-1885.
8. Orawski, A. T., Simmons, W. H. 1992. Dipeptidase activities in rat brain synaptosomes can be distinguished on the basis of inhibition by bestatin and amastatin: identification of a kyotorphin (Tyr-Arg)-degrading enzyme. *Neurochem. Res.* **17**, 817-820.
9. Geary, L. E., Wiley, K. S., Scott, W. L., Cohen, M. L. 1982. Degradation of exogenous enkephalin in the guinea-pig ileum: relative importance of aminopeptidase, enkephalinase and angiotensin converting enzyme activity. *J. Pharmacol. Exp. Ther.* **221**, 104-111.
10. Cohen, M. L., Geary, L. E., Wiley, K. S. 1983. Enkephalin degradation in the guinea-pig ileum: effect of aminopeptidase inhibitors, puromycin and bestatin. *J. Pharmacol. Exp. Ther.* **224**, 379-385.
11. Yamaoka, K., Tanigawara, Y., Nakagawa, T., Uno, T. 1981. A pharmacokinetic analysis program (MULTI) for microcomputer. *J. Pharmacobio-Dyn.* **4**, 879-885.
12. Gibaldi, M., Perrier, D. 1982. Pharmacokinetics. Marcel Dekker, Inc. New York. 1-43.
13. Mizuma, T., Sakai, N., Awazu, S. 1994. Na^+-Dependent transport of aminopeptidase-resistant sugar-coupled tripeptides in rat intestine. *Biochem. Biophys. Res. Commun.* **203**, 1412-1416.
14. Gray, G. R. 1974. The direct coupling of oligosaccharides to protein and derivatized gels. *Arch. Biochem. Biophys.* **163**, 426-428.
15. Mizuma, T., Ohta, K., Hayashi, M., Awazu, S. 1992. Intestinal active absorption of sugar-conjugated compounds by glucose transport system: implication of improvement of poorly absorbable drugs. *Biochem. Pharmacol.* **43**, 2037-2039.
16. Mizuma, T, Ohta, K. and Awazu, S., unpublished data.