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Intestinal Absorption of Stable Cyclic Glycylphenylalanine: Comparison with the Linear Form*

TAKASHI MIZUMA, SATOSHI MASUBUCHI AND SHOJI AWAZU

Department of Biopharmaceutics and Drug Rational Research Center, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192–03, Japan

Abstract

The absorption, especially the stability and transportability, of the cyclic peptide cyclic glycylphenylalanine (cyclo(Gly-Phe)) and the linear peptides glycylphenylalanine, glycyl-D-phenylalanine and phenylalanylglycine have been studied in rat small intestine.

Linear peptides were degraded on the mucosal side and only glycyl-D-phenylalanine appeared on the serosal side. However, cyclo(Gly-Phe) was stable on the mucosal side and appeared on the serosal side. Furthermore, the absorption clearance of cyclo(Gly-Phe) was higher than that of glycyl-D-phenylalanine. In the presence of the peptidase inhibitor bestatin, the degradation of linear peptides was reduced and linear peptides appeared on the serosal side, but only phenylalanylglycine, which is transported by the oligopeptide transporter, was absorbed faster than cyclo(Gly-Phe). The absorption clearance of cyclo(Gly-Phe) was reduced as its concentration was increased from 125 μM to 500 μM. Furthermore, the absorption clearance of cyclo(Gly-Phe) at 125 μM was reduced at 4°C or in the presence of glycylsarcosine and cephalexin, which are transported by the oligopeptide transporter.

These results indicated that cyclo(Gly-Phe) was stable enough to be absorbed and was transported in part by the oligopeptide transporter rather than completely by passive diffusion.

Peptide and protein drugs must be transported without metabolic degradation to the systemic circulation to perform their pharmacological action. Although active transport of oligopeptide by the intestinal H^+/oligopeptide cotransporter have been reported (Ganapathy & Leibach 1985; Fei et al 1994; Tamai et al 1994), intestinal absorption of intact peptide is generally poor because of metabolic degradation by peptidase. Our previous kinetic study on the intestinal absorption of peptides such as kyotorphin (Mizuma et al 1997) and sugar-coupled leucine enkephalin (Mizuma et al 1996) indicated that metabolic degradation in intestinal tissue during the absorption process was the rate-limiting factor in peptide absorption. In this study we have investigated the intestinal absorption of cyclic glycylphenylalanine (cyclo(Gly-Phe)) and of the linear peptides glycylphenylalanine, glycyl-D-phenylalanine and phenylalanylglycine in terms of stability and transport in the rat intestine.

Materials and Methods

Materials

Glycylphenylalanine (Gly-Phe), glycyl-D-phenylalanine (Gly-D-Phe), phenylalanylglycine (Phe-Gly), glycylsarcosine, cephalexin and bestatin were purchased from Sigma (St Louis, MO). Cyclic glycylphenylalanine was purchased from Bachem Feinchemikalien AG (Switzerland). Other chemicals of analytical grade were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Intestinal absorption

Study of the intestinal absorption of peptides was performed with the everted small intestine (Mizuma et al 1992). Briefly, male Wistar rats (180–230 g, Japan SLC, Shizuoka, Japan) were fasted overnight, anaesthetized with ether, and the intestinal blood was removed by saline perfusion. The upper, middle or lower parts of the small intestine were removed and everted. The upper part of small intestine (10 cm) was the region between 2 cm and 12 cm below the Treitz ligament. The middle part (10 cm) was the region between points 5 cm above and below the half-way point between the Treitz ligament and the ileocaecal junction. The lower part of small intestine (10 cm) was the region between 2 and 12 cm above the ileocaecal junction. The everted small intestine was connected to a disposable 10-mL plastic syringe in a manner similar to that reported by Doluisio et al (1969) and placed, in a beaker, in 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; 30 mL) containing the peptide under investigation and through which gas (95% O_2, 5% CO_2) was bubbled at 37°C. The serosal side was filled with incubation medium (5 mL) containing no peptide. The serosal solutions were mixed and sampled by the method of Doluisio et al (1969). When necessary, peptidase inhibitor was added to the medium on the mucosal and serosal sides. Incubation media (100 μL) were sampled from both the serosal and the mucosal sides for periods up to 60 min. The samples were mixed with internal standard solution (250 μM *p*-aminosalicylic acid in 10% perchloric acid; 100 μL) for sub-

Correspondence: T. Mizuma, Department of Biopharmaceutics, Tokyo University of Pharmacy and Life Science, 1432–1 Horinouchi, Hachioji, Tokyo 192-03, Japan.
E-mail: mizuma@ps.toyaku.ac.jp

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sequent HPLC assay. The mixture was centrifuged at 11 000 *g* for 5 min by means of a KM-15200 (Kubota Ltd, Tokyo, Japan) bench-top centrifuge. The resultant supernatant was analysed by HPLC.

HPLC assay

Peptides and their metabolites were determined by reversed-phase HPLC. The HPLC system was consisted of a 655A-11 pump, a 655A UV detector (operated at 210 nm) and a D-2500 integrator (all from Hitachi, Tokyo, Japan). Compounds were separated on a 15 cm × 6 mm i.d. Tosoh (Japan) ODS 80TM column; the flow rate was set at 1·5 mL min^{-1}. For assay of Gly-Phe and Gly-D-Phe, the mobile phase was 1:9 methanol–0·05% aqueous phosphoric acid; the retention times of Gly-Phe (or Gly-D-Phe) and internal standard under these conditions were 10·4 and 12·2 min, respectively. For assay of Phe-Gly, the mobile phase was 1:19 methanol–0·05% aqueous phosphoric acid; the retention times of Phe-Gly and internal standard under these conditions were 10·6 and 16·7 min, respectively. For assay of cyclo(Gly-Phe), the mobile phase was 3:17 methanol–0·05% aqueous phosphoric acid; the retention times of cyclo(Gly-Phe) and internal standard under these conditions were 11·2 and 8·4 min, respectively.

Data analysis

Elimination clearance, CL_{eli}, from the mucosal side was calculated by use of equation 1 (Mizuma et al 1996).

$$CL_{eli} = X_{eli}/AUC_{muc,0-60} \quad (1)$$

where X_{eli} is the amount eliminated from the mucosal side, and is calculated by use of the equation:

$$X_{eli} = (C_{60} - C_0) \times V \quad (2)$$

and $AUC_{muc,0-60}$, which is the area under the concentration curve of peptide on the mucosal side from time 0 to 60 min, is calculated by the trapezoidal rule (Gibaldi & Perrier 1982). C_{60} and C_0 are the concentrations of peptide on the mucosal side at 60 and 0 min, respectively and V is the volume of medium on the mucosal side.

For comparison of cyclic and linear peptides the absorption clearance, CL_{abs}, was calculated by use of equation 3 (Mizuma et al 1996), because the mucosal concentrations of linear peptides changed drastically with time.

$$CL_{abs} = X_{abs}/AUC_{muc,0-60} \quad (3)$$

where X_{abs} is the amount of peptide absorbed from the mucosal to the serosal sides in 60 min.

To study the effect of transport inhibitors on the absorption of cyclo(Gly-Phe), CL_{abs} was calculated from the initial rate of absorption by use of equation 4 (Mizuma et al 1992).

$$CL_{abs} = V_{10-30}/C_{muc,0} \quad (4)$$

where V_{10-30} is initial absorption rate calculated from the amount absorbed during the period from 10 to 30 min after the start of incubation, and $C_{muc,0}$ is the mucosal concentration at time 0. Statistical treatment was performed by Dunnett's multiple-comparison post-test after analysis of variance or by Student's unpaired *t*-test (two-tailed).

Results

The time-course of mucosal elimination and serosal appearance of the peptides

Fig. 1 shows the time-courses of mucosal elimination and serosal appearance of Phe-Gly, Gly-Phe, Gly-D-Phe and cyclo(Gly-Phe) in the upper part of small intestine. Phe-Gly was eliminated from the mucosal side slower than Gly-Phe but much faster than Gly-D-Phe. Cyclo(Gly-Phe) was eliminated from the mucosal side more slowly than any linear dipeptide. Cyclo(Gly-Phe) and Gly-D-Phe appeared on the serosal side, whereas Gly-Phe and Phe-Gly did not. Similar profiles were observed for the absorption of these peptides in the middle and lower parts of small intestine.

Comparison of the stability of cyclic and linear peptides in the upper, middle and lower parts of small intestine

The elimination clearances of peptides in the upper, middle and lower parts of small intestine are listed in Table 1. The order of elimination clearance was Gly-Phe > Phe-Gly > Gly-

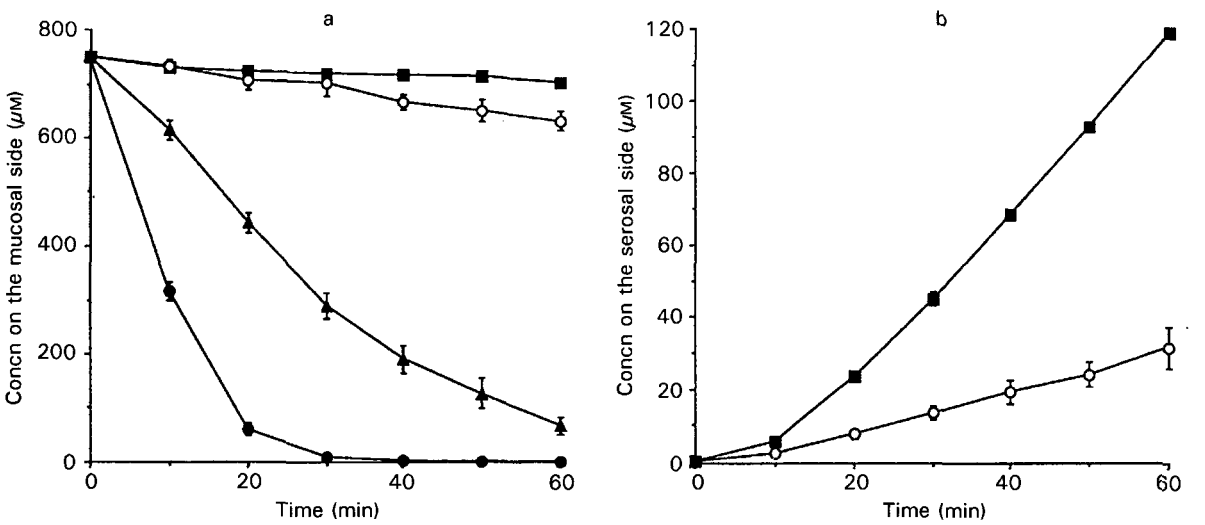

FIG. 1. Time-courses of the concentrations of cyclo(Gly-Phe) (■), Gly-Phe (●), Gly-D-Phe (○) and Phe-Gly (▲) on the mucosal (a) and serosal (b) sides of the small intestine. Data are means ± s.e. (n = 3–7). Gly-Phe and Phe-Gly were not detected on the serosal side.

Table 1. Elimination clearance of cyclo(glycylphenylalanine), glycylphenylalanine, glycyl-D-phenylalanine and phenylalanylglycine in the upper, middle and lower regions of small intestine.

Peptide	Upper	Middle	Lower
Cyclo(glycylphenylalanine)	3·36 ± 0·19	1·91 ± 0·55	0·673 ± 0·356
Glycylphenylalanine	277·6 ± 9·4*	335·6 ± 41·3*	274·9 ± 25·6*
Glycyl-D-phenylalanine	8·67 ± 1·44*	14·6 ± 1·74*	19·7 ± 2·0*
Phenylalanylglycine	99·1 ± 7·1*	152·6 ± 29·6*	104·0 ± 5·9*

Data are means ± s.e. (μL min^{-1} cm^{-1}; n = 3–7). The initial concentration of peptide on the mucosal side was 750 μM. *$P < 0.05$, significantly different from result for cyclo(glycylphenylalanine).

Table 2. Absorption clearance of cyclo(glycylphenylalanine), glycylphenylalanine, glycyl-D-phenyl alanine and phenylalanylglycine in the upper, middle and lower regions of small intestine.

Peptide	Upper	Middle	Lower
Cyclo(glycylphenylalanine)	1·26 ± 0·01	1·34 ± 0·10	1·12 ± 0·04
Glycylphenylalanine	ND	ND	ND
Glycyl-D-phenylalanine	0·350 ± 0·068*	0·273 ± 0·024*	0·133 ± 0·017*
Phenylalanylglycine	ND	ND	ND

Data are means ± s.e. (μL min^{-1} cm^{-1}; n = 3–7); ND, not detected. The initial concentration of peptide on the mucosal side was 750 μM. *$P < 0.05$, significantly different from result for cyclo(glycylphenylalanine).

D-Phe > cyclo(Gly-Phe) in all parts of the small intestine. The elimination clearance of Phe-Gly or Gly-Phe in the middle region of the small intestine was higher than in other regions whereas the lower the region of the small intestine, the greater was the elimination clearance of Gly-D-Phe. Cyclo(Gly-Phe) was stable in all parts of small intestine.

Comparison of absorption clearance of cyclic and linear peptides in the upper, middle and lower parts of small intestine

The absorption clearance of the peptides in the upper, middle and lower parts of the small intestine are listed in Table 2. Absorption clearance of cyclo(Gly-Phe) was much higher than that of any linear peptides in any parts of small intestine. Although only Gly-D-Phe among three linear peptides appeared on the serosal side, its absorption clearance was much lower than that of cyclo(Gly-Phe). Absorption clearance of cyclo(Gly-Phe) was higher in the middle region of the small intestine than in the other regions.

Effect of peptidase inhibitor on the mucosal elimination and serosal appearance of Phe-Gly, Gly-Phe, Gly-D-Phe and cyclo(Gly-Phe)

Fig. 2 shows the time-courses of the concentrations of the dipeptides on the mucosal and serosal sides of the small intestine in the presence of bestatin. Bestatin reduced the rate of elimination of Gly-Phe and Phe-Gly from the mucosal side, whereas the elimination rate of cyclo(Gly-Phe) and Gly-D-Phe was unchanged. Phe-Gly appeared fastest among the four peptides. However, the other linear peptides appeared more slowly than did cyclo(Gly-Phe).

Effect of peptidase inhibitor on elimination and absorption clearance

Elimination clearance of the peptides from the mucosal side and absorption clearance in the presence of bestatin are listed in Table 3. The elimination clearances of Gly-Phe and Phe-Gly were significantly reduced in the presence of bestatin, and the absorption clearance of Gly-Phe was similar to that of Gly-D-Phe. The absorption clearance of Phe-Gly in the presence of bestatin was highest among these dipeptides.

Effect of transport inhibitors on the absorption clearance of cyclo(Gly-Phe)

Absorption clearance of cyclo(Gly-Phe) at 125 μM was reduced in the presence of a 10 mM concentration of the dipeptide glycylsarcosine (Table 4). The absorption clearance was also reduced in the presence of cephalexin, and at 4°C. Cephradine (10 mM) also inhibited absorption clearance to an extent similar to cephalexin, though not significantly (data not shown). The absorption clearance of cyclo(Gly-Phe) was reduced as the concentration of cyclo(Gly-Phe) was increased from 125 to 500 μM.

Discussion

The linear dipeptides, Gly-Phe and Phe-Gly, did not appear on the serosal side of the small intestine even though they were eliminated from the mucosal side faster than cyclo(Gly-Phe) (Fig. 1). However, in the presence of bestatin the rate of elimination of Gly-Phe and Phe-Gly were remarkably reduced, and the serosal appearance of Gly-Phe and Phe-Gly was observed (Fig. 2). These results indicate that elimination of Gly-Phe and Phe-Gly from the mucosal side occurred primarily as a result of degradation by peptidase. Furthermore, not only the rate of absorption but also the absorption clearance of all of linear peptides in this study were increased in the presence of bestatin (Tables 2 and 3), indicating that these dipeptides were unstable in intestinal tissue during the absorption process. The instability of peptides in intestinal tissue during the absorption process has also been observed for

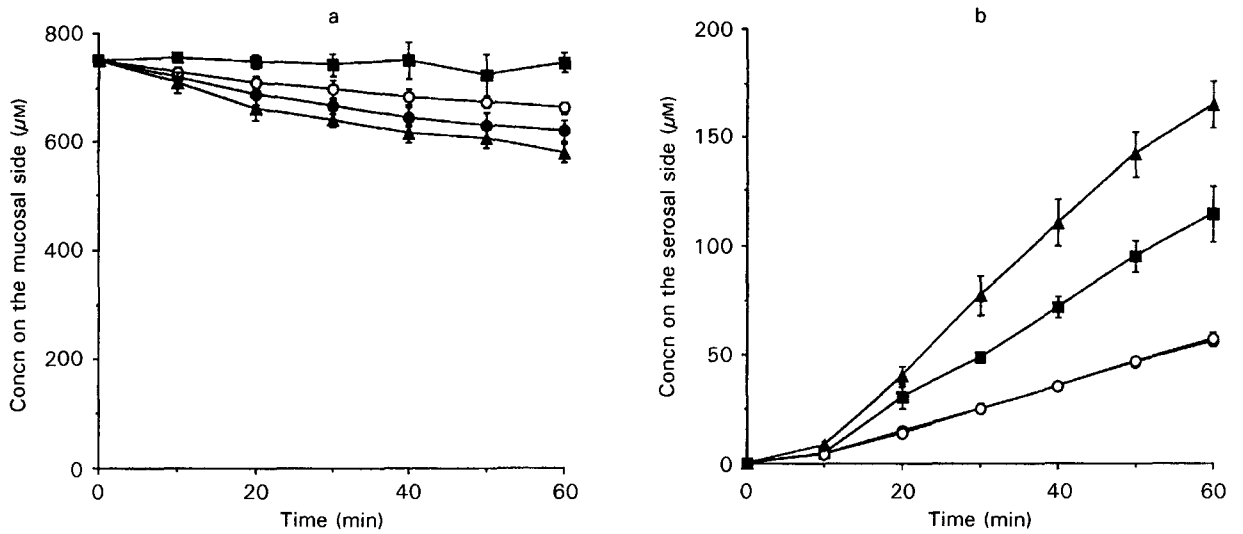

FIG. 2. Time-courses of the concentrations of cyclo (Gly-Phe) (■), Gly-Phe (●), Gly-D-Phe (○) and Phe-Gly (▲) on the mucosal (a) and serosal (b) sides of the small intestine in the presence of 500 μM bestatin. Data are means ± s.e. (n = 3). Closed circles (b) are in close proximity to open circles.

Table 3. Effect of bestatin (500 μM) on elimination and absorption clearances of glycylphenylalanine, glycyl-D-phenylalanine and phenylalanylglycine in the upper region of small intestine.

Peptide	Elimination clearance	Absorption clearance
Glycylphenylalanine	9·67 ± 1·74*	0·645 ± 0·034*
Glycyl-D-phenylalanine	6·22 ± 0·85	0·629 ± 0·024*
Phenylalanylglycine	13·1 ± 1·5*	1·96 ± 0·18*

Data are means ± s.e. (μL min^{-1} cm^{-1}; n = 4). The initial concentration of peptide on the mucosal side was 750 μM. *$P < 0·05$, significantly different from the results obtained for the upper region of the small intestine in the absence of bestatin (Tables 1 and 2).

the intestinal absorption of enkephalin and kyotorphin (Mizuma et al 1996, 1997). The increase of the absorption clearance of Phe-Gly in the presence of bestatin indicated that even though Phe-Gly was transported by the oligopeptide transporter in the intestine (Ganapathy & Leibach 1985; Fei et al 1994), the instability of Phe-Gly through intestinal tissue resulted in poor absorption. Furthermore, although bestatin was reported to be transported by the oligopeptide transporter (Inui et al 1992), the absorption clearances of the three linear peptides were increased in the presence of bestatin. This result indicates that bestatin was more effective at peptidase inhibition than at transport inhibition, and that metabolic degradation of Phe-Gly by peptidase in intestinal tissue was the rate-limiting factor in the absorption; similar behaviour has been observed for kyotorphin (Mizuma et al 1997) and for sugar-coupled leucine enkephalin (Mizuma et al 1996).

In the absence of bestatin, cyclo(Gly-Phe) appeared on the serosal side faster than any of the linear dipeptides. In the experiment on the absorption of cyclo(Gly-Phe), assumed metabolites of cyclo(Gly-Phe) such as Gly-Phe or Phe-Gly were not detected, indicating that cyclo(Gly-Phe) was stable during transport in the intestine. The absorption clearance of cyclo(Gly-Phe) was higher than that of Gly-D-Phe, and was even higher than those of three linear peptides, except for Phe-Gly in the presence of bestatin.

Table 4. Inhibition of the absorption clearance of cyclo(glycylphenylalanine) by glycylsarcosine and cephalexin.

Conditions	Absorption clearance
Control (125 μM)	1.39 ± 0.07
10 mM Glycylsarcosine	1.09 ± 0.04*
10 mM Cephalexin	1.12 ± 0.07*
At 4°C	0.249 ± 0.027*
Control (500 μM)	1.16 ± 0.04

Data are means ± s.e. (μL min^{-1} cm^{-1}; n = 3 or 4). *$P < 0·05$ significantly different from result obtained with 125 μM control.

The stability of cyclo(Gly-Phe) was observed not only in the upper part of the small intestine but also in the middle and lower parts, whereas linear peptides were degraded in all parts of the small intestine (Table 1). Furthermore, in all parts of small intestine the absorption clearance of cyclo(Gly-Phe) was much higher than those of the linear dipeptides (Table 2). These results indicate that the cyclic dipeptide was more stable and more absorbable in all parts of the small intestine than were the linear forms of the dipeptides.

The absorption clearance of cyclo(Gly-Phe) at 125 μM was reduced at 4°C and in the presence of glycylsarcosine,

cephalexin (Table 4) and cephradine (data not shown). Because glycylsarcosine, cephalexin and cephradine were reported to be transported by the oligopeptide transporter (Nakashima et al 1984; Ganapathy & Leibach 1985; Fei et al 1994), it was assumed that cyclo(Gly-Phe) would also be transported in the intestine both by the oligopeptide transporter and by passive diffusion. It was also considered that the small, but definite contribution of oligopeptide transporter-mediated transport to the intestinal absorption of cyclo(Gly-Phe) caused a slight decrease of the absorption clearance of cyclo(Gly-Phe) as the concentration of cyclo(Gly-Phe) was increased (Table 4).

Because cyclic arginyltyrosine, cyclokyotorphin, was reported to be a more potent analgesic than linear kyotorphin (Sakurada et al 1982), the cyclic oligopeptide must have some potential in terms not only of intestinal absorption but also pharmacological effect. Furthermore, it has been suggested that cyclic dipeptides other than cyclo(Gly-Phe) are transported by the oligopeptide transporter (unpublished results). Study of these is now in progress.

In conclusion, this study showed that the cyclic form of Gly-Phe was stable and absorbable in all parts of the small intestine, and implied that it was transported in part by the oligopeptide transporter. This result might also enable the design of a peptidomimetic drug which is sufficiently stable to be transported by the oligopeptide transporter.

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