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Report

Use of the Peptide Carrier System to Improve the Intestinal Absorption of L-α-Methyldopa: Carrier Kinetics, Intestinal Permeabilities, and *In Vitro* Hydrolysis of Dipeptidyl Derivatives of L-α-Methyldopa

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Intestinal permeabilities of five dipeptidyl derivatives of L-α-methyldopa (I) were studied by an *in situ* intestinal perfusion method. The dipeptides displayed a significant increase in their permeabilities compared to L-α-methyldopa. The increases ranged from 4 to 20 times. These results suggest that the peptide transport system is less structurally specific than the amino acid transport systems and can be used as an absorption pathway for peptide analogues. The kinetic advantage demonstrated by the dipeptide, L-α-methyldopa-L-phenylalanine, over the amino acid analogue, L-α-methyldopa, suggests that the peptide carrier would be a possible route for improving the intestinal absorption of pharmacologically active amino acid analogues. Furthermore, the preliminary results of *in vitro* hydrolysis studies of selected dipeptidyl derivatives indicate that the peptide carrier system could be used as a base for a prodrug strategy.

KEY WORDS: dipeptides; intestinal permeability; L-α-methyldopa; L-α-methyldopa-L-phenylalanine; uptake kinetics; peptide carrier system; *in vitro* hydrolysis.

INTRODUCTION

L-α-Methyldopa (I) is a poorly absorbed antihypertensive agent and an amino acid analogue. Its oral bioavailability has been studied in both humans and rats (1–3). Its mechanism of absorption is carrier-mediated via an amino acid carrier (2,4,5). Amino acid carriers are structurally restrictive, and there are at least four distinctive carriers. Each carrier is primarily responsible for the transport of a specific type of amino acid (6). This structural restriction is thought to be the main reason for I to be poorly absorbed since the α-methyl group severely hinders the binding of the substrate to the carrier as in the case of α-aminoisobutyric acid (7). Attempts have been made to improve the absorption of I by changing its amino acid characteristics so that the compound can be absorbed passively, and the results obtained have been favorable (1,8–10). In the present study, the possibility of using another approach, namely, the peptide carrier, was explored to determine if it can be used as a means of improving the absorption of I by making peptide analogues of I. Three basic reasons underlie the synthesis of these dipeptides: first, the peptide carrier is likely to have a more relaxed structural requirement and be generally more efficient than amino acid carrier(s) (11); second, most peptides are hydrolyzed to their constituent amino acids before entering the systemic circulation (11); and third, the derivatizing groups, amino acids, are natural substances with a low toxicity potential. In this paper, the synthesis and stability of the dipeptidyl derivatives of I under experimental conditions and intestinal permeabilities and preliminary *in vitro* intestinal hydrolysis of selected dipeptides are presented.

EXPERIMENTAL

Materials

L-α-Methyldopa was obtained from Sigma Chemical Co. (St. Louis, Mo.). Di-*t*-butyldicarbonate, *N,N'*-dicyclohexylcarbodiimide (DCC), and trifluoroacetic acid (TFA) were purchased from Pierce Chemical Co. (Rockford, Ill.). 1-Hydroxybenzotriazole monohydrate and thionyl chloride were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). All materials including buffer components were used as obtained.

Perfusate Solution

The perfusate solution consisted of a pH 5.5 phosphate buffer, a substrate or peptide, and PEG 4000 as a water transport marker (12), with a trace amount of ^{14}C-PEG 4000. Sodium chloride was added to adjust the perfusate solution to a final osmolarity of 300 ± 10.

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Substrate Solution

The substrate solution used in the *in vitro* hydrolytic experiments consisted of a pH 6.5 Bis–Tris buffer and 1 mmol of substrate per liter.

Animal Surgery

The procedure has been published previously (12) and was used without modification.

Assay

Samples were taken at predetermined time intervals depending on the flow rate, and a portion of the samples was stabilized by quenching it to pH 1.0 for high-performance liquid chromatographic (HPLC) assay. The assay for L-α-methyldopa has been published elsewhere (13) and was used without modification. The mobile phases for the dipeptides were similar, consisting of acetonitrile and a 0.05 *M*, pH 2.5, ammonium phosphate buffer. The fractions of the organic phase varied between 10 and 20% depending on the peptide.

Stabilities of Dipeptides in the Blank Perfusate

The samples were shown to be stable in both the perfusate buffer (pH 5.5) and the pH 1.0 quenched blank perfusate solution. The blank perfusate solution was obtained by passing the perfusate buffer through a segment of intestine at the same flow rate as in the permeability experiment. The results showed that more than 95% of the dipeptides remained after 220 min at 37°C. In addition, no degradation was observed in acidified perfusate (pH 1) over a 300-min test interval. Since the average residence time of a dipeptide in the intestinal lumen is less than 15 min, the amount of the peptide lost because of degradation is negligible. The hypothesis that these dipeptides have a good stability toward the intestinal luminal enzymes was further confirmed by failure to detect any L-α-methyldopa in the perfusate (<0.001 m*M*) when up to 10 m*M* L-α-methyldopa-L-phenylalanine was used in the perfusion experiment. This contrasts strongly with other L-phe-containing dipeptides, which generally undergo 5–15% hydrolysis (by detection of L-phe) at a 1 m*M* concentration under the same conditions (unpublished results).

In Vitro Hydrolysis Studies of the Derivatives

The technique was adapted according to the studies by Boullin *et al.* (14) and Gardner and Plumb (15) with slight modification. A new tissue preparation was made at the beginning of each experiment.

After the anesthesia was induced by i.m. injection of urethane (2.5 g/kg), an abdominal incision 4–5 cm in length was made to expose the intestine. After locating the intestinal segment between the beginning of the jejunum and the end of the ileum, a small opening was made at each end. To the small opening at the upper jejunum, 40–50 ml of a 250 m*M* sucrose solution at 4°C was pushed slowly through the intestinal segment to clean the intestine. When the intestine was clean, the rat was sacrificed, after which the intestinal segment was taken out and its length measured. Using surgical scissors, the intestinal segment was cut open and its interior exposed. The mucosal layer was scraped, collected, and diluted 1:1 (v/v) using an ice-cold sucrose solution. The collected suspension of the mucosal cells was then subjected to ultrasonication for a total of 180 sec, with 15 sec intervals between each 60 sec of sonication. The suspension was then centrifuged at 2500 rpm for 4 min. The supernatant was stored in an ice bath and used in the subsequent hydrolysis experiments.

Cell homogenates were prepared as follows. After removal of the mucosal layer, 15 cm of jejunum was immersed into 5 ml of sucrose solution at 0°C. The segment was then cut into small pieces and homogenized in a tissue homogenizer. The homogenate was kept in an ice-cold bath until used.

The kinetic studies were performed as follows. Five hundred microliters each of the following solutions was placed in a test tube: for control experiments, pH 6.5 Bis–Tris buffer, sucrose solution, and substrate solution; for blank experiments, mucosal cell or wall homogenate suspension and buffer solution (twice); and for hydrolysis experiments, buffer solution, substrate solution, and mucosal cell or wall homogenate suspension. The mixture was vortexed for 20 sec and five to seven 200-μl samples were taken after the onset of an experiment. As soon as the sample was taken, it was immediately mixed with two parts of acetonitrile and centrifuged at 11,500 rpm for 90 sec. The supernatant was then analyzed by HPLC.

Synthesis of Prodrugs

(S)-N-*[(1,1-Dimethylethyl)oxy-carbonyl]-2-methyl-3-(3,4-dihydroxyphenyl)alanine (Boc-L-α-methyldopa) (II).* The synthesis of II was accomplished according to the method of Kaiser *et al.* (16). The yield was 56.2%. NMR [$(CH_3)_2$-SO-d_6]: d 1.18 (s, 3H, CH_3), 1.39 [s, 9H, $(CH_3)_3$], 2.75, 3.00 (AB, d, J = 13.51 Hz, 2H, CH_2), 6.32–6.6 (m, 3H, Ar-H), 6.53 (s, 1H, NH).

(S)-*2-Methyl-3-(3,4-dihydroxyphenyl)alanine Methyl Ester Hydrochloride (L-α-Methyldopa Methyl Ester · HCl) (III).* L-α-Methyldopa (2.11 g, 10.0 mmol) was esterified with $SOCl_2$-CH_3OH following the procedure published by Bodor *et al.* (17) for L-dopa esterification. The yield was 92%. NMR (D_2O): d 1.44 (s, 3H, CH_3), 2.89, 3.07 (AB, d, J = 14.44 Hz, 2H, CH_2), 3.65 (s, 3H, OCH_3), 6.42–6.71 (m, 3H, Ar-H).

L-2-Methyl-3-(3,4-dihydroxyphenyl)alanyl-L-phenylalanine (L-α-Methyldopa-L-phe) (IV). A solution of Boc-L-α-methyldopa (0.5 g, 1.6 mmol) and *N,N'*-dicyclohexylcarbodiimide (0.31 g, 1.5 mmol) in anhydrous dioxane (20 ml) was added to the solution of phenylalanine methyl ester hydrochloride (0.32 g, 1.5 mmol) and triethyl amine in anhydrous dioxane (30 ml) at 0°C under a N_2 atmosphere. Then 1-hydroxybenzotriazole monohydrate (0.23 g, 1.5 mmol) in DMF (2 ml) was added and the reaction mixture was stirred at 0°C for 4 hr. After stirring for 16 hr at ambient temperature, the dicyclohexyl urea formed during the reaction was filtered off and the filtrate was concentrated. The residue was taken in ethyl acetate and washed successively with 5% aqueous sodium bicarbonate, 1 *N* HCl and H_2O. The organic layer was then dried ($MgSO_4$) and

Table I. Wall Permeabilities (P_w* ± SE) of L-α-Methyldopa and Its Dipeptidyl Derivatives

Compound	Concentration (m*M*)		
	1.0	0.1	0.01
L-α-Methyldopa (I)	0.41 (0.11)	0.4 (0.22)	0.43 (0.14)
Gly-I		4.34 (0.27)	
Pro-I		1.68 (0.23)	
I-pro		5.41 (0.55)	
Phe-I		5.29 (1.57)	
I-phe	4.30 (0.30)	10.22 (0.45)	10.9 (1.8)

concentrated to yield the desired Boc-dipeptidyl methyl ester (90% yield), which was purified by column chromatography (19:1 chloroform/methanol as eluate).

The methyl ester group was hydrolyzed by treating with 1 equiv of 0.5 *N* ethanolic sodium hydroxide at 0°C for 30 min (monitored by HPLC). Then the reaction mixture was acidified, concentrated, and extracted with ethyl acetate. The organic layer was concentrated to give Boc-L-α-methyldopa-phe, which was taken to the next step without further purification.

The Boc group was removed by treating with TFA/dichloromethane (1:1) for 30 min at room temperature and the crude product after removing the solvent was purified by semipreparative HPLC (reversed-phase, C-18 column, 12 × 25 cm, Vydac), isocratic condition, 87% H_2O and 13% CH_3CN (both containing 0.1% TFA, 280 nm), to yield 0.3 g (53%) of pure L-α-methyldopa-phe. NMR (D_2O): d 1.27 (s, 3H, CH_3), 2.71–3.23 (m, 5H, $2CH_2$, CH), 6.5–6.72 (m, 3H, dopa AR-H), 7.13–7.26 (m, 5H, phe AR-H).

The L-α-methyldopa-pro was synthesized from Boc-L-α-methyldopa and proline methyl ester hydrochloride following the procedure described above. The gly-L-α-methyldopa, phe-L-α-methyldopa, and pro-L-α-methyldopa were prepared from the appropriate Boc amino acids and L-α-methyldopa methyl ester hydrochloride. Final purification of the peptides was carried out by HPLC with isocratic conditions ranging from 5 to 15% acetonitrile. The purity of the dipeptides was further checked by analytical HPLC. The dipeptides were subjected to final analysis by high-field NMR (IBM WP 270-MHz spectrometer) and FAB-MS (NIH Facility, Michigan State University, East Lansing), and the result confirmed their structures.

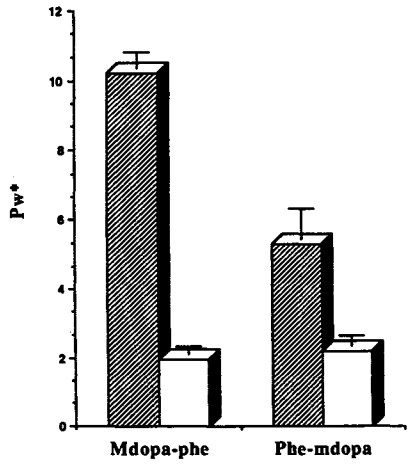

Fig. 1. Inhibition of dipeptidyl derivatives of L-α- methyldopa by 50 m*M* L-gly-L-gly. Hatched bars represented the permeability before inhibition; open bars, that after inhibition.

RESULTS AND DISCUSSION

Selection of the Perfusate pH

The effect of the pH of the perfusate on the permeability was performed for L-pro-L-phe. The permeability results did not show a significant difference among pH 4, 5.5, 7, and 8 (unpublished results). Since L-α-methyldopa is highly susceptible to oxidation, it was decided that the perfusion experiment would be performed at pH 5.5. In addition, pH values in this range have also been used by other investigators to study peptide absorption (18–22). Since our experiment is a steady-state experiment at pH 5.5, a substantial change in the perfusate pH is unlikely. When the outlet perfusate was checked for pH changes in the L-α-methyldopa perfusion experiments, the pH changes were very minimal (less than 0.1 pH unit).

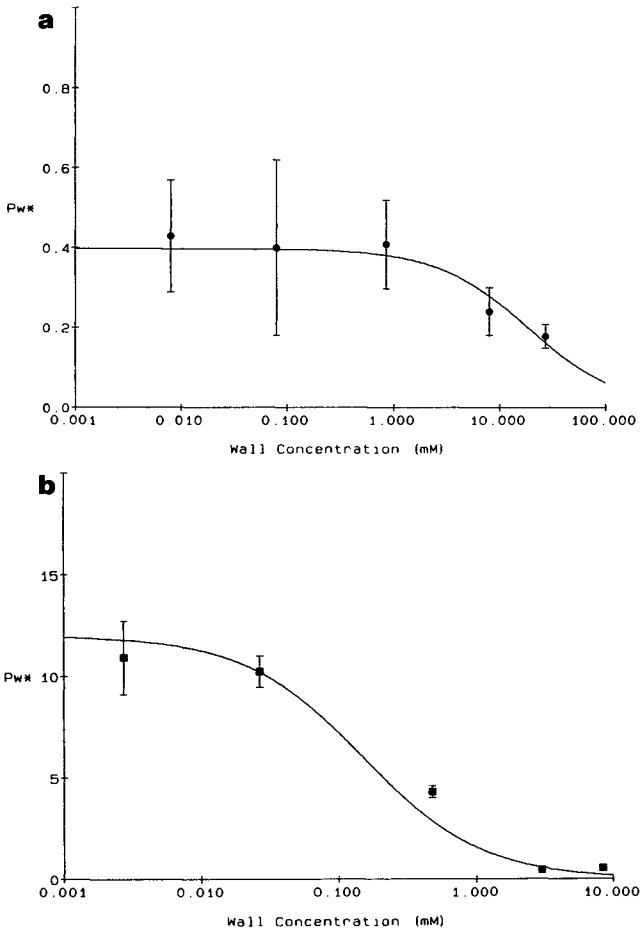

Fig. 2. Comparison of carrier kinetics in the transport of L-α-methyldopa and L-α-methyldopa-L-phenylalanine. (a) L-α-methyldopa; (b) L-α-methyldopa-L-phenylalanine. Note that the two vertical axes have a 20-fold scale difference.

Table II. Kinetic Parameters of L-α-Methyldopa, L-Phenylalanine, and L-α-Methyldopa-L-Phenylalanine

Compound	K_m (mM)	J_{max}* (mM)	p_c*	Ref. No.
L-α-Methyldopa	18.6 (4)[a]	7.4 (1.1)	0.40 (0.04)	13
L-Phenylalanine	9.5 (3.1)	48 (20)	5.3 (1.4)	12
L-α-Methyldopa-L-phe	0.15 (0.03)	1.8 (0.27)	12.2 (0.31)	

[a] Estimated standard deviation of the parameter.

Intestinal Membrane Permeabilities

The permeabilities were calculated from the experimental data according to the method published (12,23). The results of this analysis are summarized in Table I. Comparison with the parent compound, L-α-methyldopa, indicates that the dipeptides showed significant increases in permeabilities, ranging from 4 to 20 times ($P < 0.01$). Using a concentration of 50 mM L-gly-L-gly to coperfuse with I-phe or phe-I, it was possible to decrease the permeabilities of these two compounds substantially (Fig. 1). The results indicated that the absorption of these dipeptides is by the same carrier system responsible for the absorption of other peptides. Furthermore, the substantial increases in the permeabilities of the dipeptides over that of L-α-methyldopa agree with the current understanding of the intestinal transport of amino acids and peptides (6,11) and indicate that the peptide carrier is less structurally specific than the amino acid carriers.

Carrier Uptake Kinetics

In order to gain a better understanding of the transport characteristics of the dipeptides, a full permeability vs concentration study was performed for L-α-methyldopa-L-phenylalanine (IV). The choice was based on the following: first, the derivative has the highest permeability; and second, the derivative has the best chance to survive the luminal enzymatic attack (e.g., the pancreatic enzymes) before it is absorbed since the α-methyl group is known to reduce significantly the enzymatic hydrolysis rates. The uptake kinetics of IV are presented in Fig. 2b. The uptake kinetics of I are also presented for comparison (Fig. 2a). Using weighted nonlinear regression to Eq. (1), the K_m and J_{max}* were obtained (Table II).

$$P_w^* = J_{max}^*/(K_m + C_w^*) \quad (1)$$

where $C_w^* = C_o(1 - P_{eff}^*/P_{aq}^*)$

Table III. Summary of the *in Vitro* Hydrolysis Results (pH 6.5)

Compound	Intestinal portion	% hydrolyzed (after 60 min)
Gly-phe	Mucosal layer	99.5 (6)[a]
	Cell homogenate	99.5 (6)
Gly-mdopa	Mucosal layer	18 ± 7 (2)
	Cell homogenate	16.5 ± 3.5 (2)
Mdopa-phe	Mucosal layer	16 ± 6 (3)
	Cell homogenate	22 ± 10 (4)
Phe-mdopa	Mucosal layer	25 ± 10 (2)
	Cell homogenate	28 ± 12 (2)

[a] The results generally represent the average of two to six experiments as shown in the parentheses.

An important parameter for a compound that is transported via a carrier-mediated process is the carrier permeability (P_c*) (12), which is defined as

$$P_c^* = J_{max}^*/K_m \quad (2)$$

This is particularly true under physiological conditions, when the concentration of the substrate is generally in the range of a tenth of a millimolar, while the K_m of the carrier is in the range of several millimolar or higher (12,24). Although P_c* is defined as a ratio of J_{max}* over K_m, it is determined independently by applying the weighed nonlinear regression to Eq. (3) (Table II).

$$P_w^* = P_c^*/(1 + C_w^*/K_m) \quad (3)$$

From the results in Table II it is clear that the dipeptide has a much lower K_m and higher P_c* than L-α-methyldopa. The permeability at a low concentration, P_c*, is 20-fold higher than that of L-α-methyldopa, which would give the dipeptide form of L-α-methyldopa a significantly larger absorption rate. Based on the correlation between the fraction of the dose absorbed and the membrane permeability (25), the dipeptide would be well absorbed (100%).

The value of K_m for the dipeptide (L-α-methyldopa-L-phenylalanine) is the lowest that has been measured with this technique for intestinal mucosal tissue. It is difficult to compare these results with other studies of small peptides since those peptides may undergo significant hydrolysis. Furthermore, different methodologies may also contribute to the difference in reported K_m values. Given the complex kinetic processes that are occurring during absorption, the molecular significance of these K_m values is at present uncertain. However, the overall absorption rate would be significantly larger for these dipeptides than for L-α-methyldopa when dosed below the apparent K_m.

In Vitro Intestinal Hydrolysis Tests

In testing the hydrolysis of the L-α-methyldopa dipeptidyl derivatives, it was found that these dipeptide analogues were more slowly hydrolyzed in the test system than gly-phe. However, as shown in Table III, significant hydrolysis does occur, suggesting that these dipeptidyl derivatives of L-α-methyldopa may be prodrugs and that this strategy may be a viable prodrug strategy. Further studies are under way to investigate both the permeability and the hydrolysis of these dipeptides.

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