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D-Phenylglycine Improves L-Dopa Binding to Serum Albumin

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ABSTRACT

D-phenylglycine-L-dopa, a dipeptide synthesized in this laboratory for improving the oral absorption of L-dopa, showed better absorption and distribution in rats. We assumed that the extensive distribution might explain its sustained release of brain dopamine. Since protein binding is a main factor of drug distribution, we investigated the effect of D-phenylglycine on the binding of L-dopa to human serum albumin. The degree of the binding of D-phenylglycine, L-dopa and D-phenylglycine-L-dopa to serum albumin was determined. Free and bound portion of test compounds were separated with ultrafiltration method and the assay of free drug portion was conducted with reversed phase HPLC. The LOQ for D-phenylglycine, L-dopa and D-phenylglycine-L-dopa was 0.5 μg/mL, 0.1 μg/mL and 0.5 μg/mL, respectively. Assay methods were validated by determining the precision and accuracy of interday and intraday variations. The coefficient of variation (CV) was within 12% and the relative error (RE) was within 10% (n = 3). The recovery rate was 95.4% - 98.1% for D-phenylglycine, 91.9 % - 98.8% for L-dopa and 96.8% - 97.9% for D-phenylglycine-L-dopa, respectively (n = 3). D-Phenylglycine showed higher serum albumin binding than L-dopa did at various concentrations. At a concentration of 600 μM, the degree of albumin binding of D-phenylglycine, L-dopa, D-phenylglycine-L-dopa was 27.98%, 8.20% and 19.18%, respectively. The albumin binding of L-dopa at this concentration increased by 2.4 folds when chemically bound to D-phenylglycine. The number of the binding sites of L-dopa increased by 5.8 folds and the binding constant Ka1 increased by 3.8 folds when L-dopa was chemically bound to D-phenylglycine. With the affinity to serum albumin, D-phenylglycine showed its possibility as a delivery moiety for drugs with limited distribution to use the body protein as a reservoir.

Key words: D-phenylglycine, L-dopa, D-phenylglycine-L-dopa, albumin, protein binding

INTRODUCTION

Studies indicated that orally absorbable amino-β-lactams could enter the circulatory system through the intestinal oligopeptide transporter, PepT1[1-7]. As D-Phenylglycine is a common moiety in molecules of amino-β-lactams, we synthesized D-phenylglycine-L-dopa[8] to improve the low and inconsistent bioavailability associated with L-dopa[9-14]. This dipeptide showed 50 times higher plasma concentration than that of L-dopa after intestinal perfusion in rats[8]. Another study also indicated that this dipeptide exhibited larger volume of distribution and longer biological half-life than that of L-dopa after oral administration in rats and in rabbits[8]. We assumed that the large volume of distribution might explain its sustained release of brain dopamine. As protein binding is one of the factors that affect drug volume of distribution[15-17], it is interesting to investigate if the protein binding of D-phenylglycine contributes to the high volume of distribution associated with D-phenylglycine-L-dopa. It would also be interesting to understand if D-phenylglycine is feasible as a moiety for designing sustained release prodrugs for drugs with high clearance or poor pharmacokinetic profile. We therefore designed a comparative protein binding assay on D-phenylglycine, L-dopa and D-phenylglycine-L-dopa.

Studies were conducted in human serum albumin[18]. Free and protein-bound drug portions were separated with ultrafiltration method[19-20]. The analysis of free drugs was conducted with reversed phase HPLC. Assay methods were validated by determining the precision and accuracy of interday and intraday variations. The recovery of drugs was determined. The degree of serum albumin binding of D-phenylglycine, L-dopa and D-phenylglycine-L-dopa were compared. The binding kinetics was also investigated[21-23].

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MATERIALS AND METHODS

I. *Materials*

D-Phenylglycine-L-dopa was synthesized in this laboratory[8]. D-(-)-α-Phenylglycine and L-dopa were purchased from Acros Organics (New Jersey, USA). Human albumin was purchased from Sigma Chemical (Steinheim, Germeny). Sodium chloride was from Riedel-de Haen (Seelze, Germany). Ortho-phosphoric acid 85%, potassium dihydrogen phosphate, di-Sodium hydrogen phosphate and acetonitrile were from Merck (Darmstadt, Germany). Sodium hydroxide was from Riedel-de Haen (Seelze, Germany). Branson Sonifier 450 sonicator, Kubota 2010, Eppendorf 5415C centrifuge, Model 905 incubator (Cherng Huei Instrument Co.) and Ystral Laboratory series x10/20 Homogenizer were used for the preparation of test solutions. Osmolarity of

test solutions was determined with Wescor 5500 vapor pressure osmometer (Wescor Company, Logan, UT, USA). Samples after incubation were vortexed in a Scientific Industries Vortex Genie2 (Bohemia, USA), centrifuged with Hettich Mikro 22R (Tuttlingen, Germany), and filtered with Millipore Amicon Microcon YM-30 Centrifugal Filter Units (Bedford, USA).

II. *Test Solutions*

Isotonic Sorensen phosphate buffer solution was prepared by adding potassium dihydrophosphoric acid (1.78 g), disodium hydrophosphoric acid (7.61 g) and sodium chloride (9.00 g) in deionized water (1000 mL) and adjusted to pH 7.4 by 3N sodium hydroxide solution. Isotonic human serum albumin solution was prepared by adding 10 mg of human serum albumin in 125 μL of Sorensen phosphate buffer solution. Stock solutions of test drugs was prepared by adding 10 mg of test compounds in 10 mL of Sorensen phosphate buffer and adjusted to 1000 μg/mL. Stock test solutions were prepared by serial dilution of the stock solutions (1000 μg/mL) with Sorensen phosphate buffer.

III. *Chromatography*

Samples were assayed in a HPLC system consisting of a Jasco PU-1580 pump (Tokyo, Japan), a Jasco UV-1575 detector (Tokyo, Japan), a Jasco AS-1555-10 autosampler (Tokyo, Japan), and a Jasco Borwin integretor (Tokyo, Japan). Samples were analyzed with a Merck LiChrospher 100 RP-18e reversed-phase column (5 μm, 250×4 mm, Darmstadt, Germany). D-Phenylglycine and L-dopa were eluted at a flow rate of 1.0 mL/min with 0.1 M phosphate buffer solution adjusted to pH 2.0 with ammonium hydroxide solution, and detected by UV at 236 nm of wavelength. D-Phenylglycine and L-dopa (50 μg/mL) were used as each other's internal standard. D-Phenylglycine-L-dopa, by adding acetanilide as the internal standard, was eluted at a flow rate of 1.2 mL/min with a solvent system comprising acetonitrile and water at a ratio of 85:15 and was detected by UV at 197 nm of wavelength.

IV. *Validation of Assay Methods*

HPLC assay methods were validated by determining the precision and accuracy of intraday and interday analysis of serum standards over a period of 6 days. The limit of detection (LOD) and the limit of quantitation (LOQ) were determined to establish the standard curve for HPLC assay. Recovery of test subject was also determined.

V. *Protein Binding Assay*

Ultrafiltration method was adopted for protein binding assay. In order to measure the drug binding on the filtering membrane, drug solutions (500 μL of 0.1 μg/mL, 1 μg/mL, 10 μg/mL and 100 μg/mL) were first placed in a Microcon YM-30 filter, of which particles with molecular weight lower than 30,000 dalton were filtered, and centrifuged at 5312 g for 10 minutes. The filtrates were then subjected to HPLC assay.

Drug solutions (250 μL of 0.1 μg/mL, 1 μg/mL, 10 μg/mL and 100 μg/mL) were incubation with 250 μL of human serum albumin (1.23×10^{-3} M, 8%) at 37°C for 5 minutes and then diluted to a final solution with human serum albumin at a concentration of 6.15×10^{-4} M (4%). The solutions were then vortexed and incubated at 37° C for 10 minutes. Afterwards, the test solutions (230 μL) were then placed in a Microcon YM-30 filter and centrifuged at 531 g for 10 minutes. The filtrates were diluted and subjected to HPLC assay.

VI. *Data Analysis*

Data analysis was performed on Visual dBase and SPSS/PC$^+$ and was presented as mean±S.D. of n experiments. Differences were evaluated by one-way ANOVA ($p < 0.05$). Parameters of albumin binding kinetics were derived by establishing the r/[D] vs r Scatchard plot. One binding-site model (equation 1), two binding-site model (equation 2), and one nonspecific binding model (equation 3) were adopted for the calculation of the parameters, where r represents moles of bound drug per mole of protein, Ka_1 and Ka_2 represent binding constants (L/mol), [D] represents free drug concentration, and n represents the number of drug binding sites (sites/L)[24].

$$r = \frac{nKa[D]}{1 + Ka[D]} \quad (1)$$

$$r = \frac{n_1Ka_1[D]}{1 + Ka_1[D]} + \frac{n_2Ka_2[D]}{1 + Ka_2[D]} \quad (2)$$

$$r = \frac{n_1Ka_1[D]}{1 + Ka_1[D]} + Ka_2[D] \quad (3)$$

RESULTS AND DISCUSSION

D-phenylglycine, L-dopa and D-phenylglycine-L-dopa were assayed with HPLC. The retention time of D-phenylglycine, L-dopa and D-phenylglycine-L-dopa was 6.1, 10.6 and 4.9 min, respectively.

The limit of detection (LOD) and the limit of quantitation (LOQ) for D-phenylglycine was 0.05 μg/mL and 0.5 g/mL, respectively. It demonstrated good linearity at concentration range between 0.1 ~ 100 μg/mL (0.66 ~ 661.55 μM) in intraday assays (regression coefficient (r) > 0.9998, n = 3, relative error (R. E.) +0.49 ~ +4.97%, coefficient of variation (C. V.) < 12%) and interday assays (r > 0.9997, R. E. +0.61 ~ +9.82 %, C. V. < 10%) assays.

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Table 1. The degree of protein binding of D-Phenylglycine, L-Dopa and D-Phenylglycine-L-dopa.

Concentration of test drug (μg/mL)	D-Phenylglycine		L-Dopa		D-Phenylglycine-L-dopa	
	Concentration of bound drugs (μg/mL)	% binding	Concentration of bound drugs (μg/mL)	% binding	Concentration of bound drugs (μg/mL)	% binding
1	0.31 ± 0.05	30.67	0.10 ± 0.03	10.00	0.22 ± 0.03	22.33
5	1.56 ± 0.06	31.27	0.56 ± 0.06	11.13	1.20 ± 0.10	23.93
10	2.98 ± 0.12	29.80	0.90 ± 0.16	9.03	2.32 ± 0.07	23.20
30	9.55 ± 1.48	31.84	2.41 ± 0.74	8.03	5.45 ± 0.64	18.17
50	14.05 ± 1.47	28.09	4.98 ± 1.91	9.95	9.60 ± 0.64	19.19
100	25.97 ± 4.21	25.90	7.62 ± 1.44	7.62	18.22 ± 1.34	18.22
300	71.10 ± 6.27	23.70	20.35 ± 5.25	6.78	52.18 ± 9.24	17.39
500	121.83 ± 7.46	24.37	26.49 ± 6.91	5.30	77.98 ± 13.79	15.60
1000	198.54 ± 15.06	19.85	36.35 ± 8.38	3.64	106.54 ± 17.76	10.65
2000	332.72 ± 65.29	16.64	79.86 ± 25.85	3.99	216.29 ± 52.51	10.81

Table 2. D-Phenylglycine, when chemically bound to L-dopa, increased the degree of protein-binding of L-dopa.

Compound	Molecular weight	concentration equivalent to 600 μM (μg/mL)	Degree of albumin binding* (%)	ratio
D-Phenylglycine	151.2	90.72	27.98	3.4**
L-Dopa	197.2	118.32	8.20	1.0
D-Phenylglycine-L-dopa	330.3	198.18	19.18	2.4**

*The degree of albumin binding was derived via regression analysis from the data of Table 1.
**One Way ANOVA, $p < 0.05$.

The recovery rate for D-phenylglycine was between 95.4% - 98.1% (n = 3).

The LOD and LOQ for L-dopa was 0.01 μg/mL and 0.1 μg/mL, respectively. Good linearity was demonstrated at concentration range of 0.1 ~ 100 μg/mL (0.51 ~ 507.13 μM) in intraday assays (r > 0.9998, n = 3, R. E. +0.46 ~ +9.27%, C. V. <10%) and interday assays (r> 0.9995 R. E. +0.76 ~ +8.17%, C. V. < 7.2%) assays. The recovery rate was between 91.9 % - 98.8% (n = 3).

The LOD and LOQ for D-phenylglycine-L-dopa was 0.05 μg/mL and 0.5 μg/mL respectively. Good linearity at concentration range between 0.1 ~ 100 μg/mL (0.30 ~ 303.63 μM) in intraday assays (r > 0.9996, n = 3, R. E. +0.76 ~ +8.37%, C. V. < 5%) and interday assays (r > 0.9992 R. E. +0.91 ~ +9.06%, C. V. < 6.1%) were demonstrated. The recovery rate of D-phenylglycine-L-dopa was between 96.8% - 97.9% (n = 3).

The concentration of the bound portion to the concentration of total test compound was analyzed. Dose dependent binding at concentration range of 0-2000 μg/mL was observed for D-phenylglycine, L-dopa and D-phenylglycine-L-dopa (Figure 1). The degrees of albumin binding between D-phenylglycine, L-dopa and D-phenylglycine-L-dopa were compared (Table 1). Both D-phenylglycine-L-dopa and D-phenylglycine showed higher binding to human serum albumin than L-dopa at various concentrations. At concentration of 600 μM, for example, the bound portion of D-phenylglycine, L-dopa and D-phenylglycine-L-dopa to serum albumin was 27.98%, 8.20% and 19.18%, respectively. The albumin binding of D-phenylglycine was 3.4 times stronger than that of L-dopa. The albumin binding of L-dopa, when chemically bound to D-phenylglycine, increased by 2.4 folds at this concentration (Table 2).

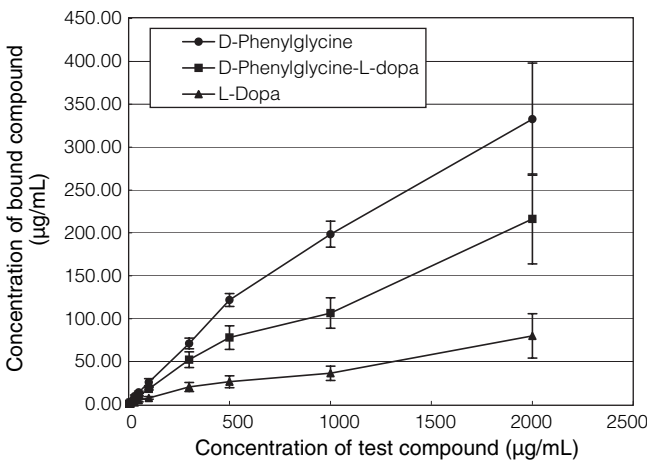

Figure 1. Comparison of serum albumin binding of D-phenylglycine, L-dopa and D-phenylglycine-L-dopa.

The kinetics of serum albumin binding of the three compounds was also investigated, and the Scatchard's plot of r/[D] vs r was established (Figure. 2). The parameters

were calculated according to the one binding-site model, two binding-site model and non-specific binding model[24]. Akaike's information criterion (AIC) was used for evaluation and for the selection of the best fit model[25]. As judged by AIC, D-phenylglycine exhibited higher possibility of two binding-site interaction with serum albumin than L-dopa and the dipeptide D-phenylglycine-L-dopa. In the D-phenylglycine case, the Ka1 calculated from two binding-site model was larger than the Ka1 calculated from one binding-site model (0.56 vs 0.11), indicating a possibility of allosteric induction when D-phenylglycine is bound to serum albumin. Based on the data calculated from one binding-site model, the binding constant (Ka1) of D-phenylglycine was lower than that of L-dopa (0.11 vs 0.30) (Table 3). However, its binding site (n, sites/L) was 10 times more to that of L-dopa (6.00 vs 0.59). When D-phenylglycine was chemically bound to L-dopa, the binding sites (n, sites/L) of the dipeptide to

Table 3. The kinetic parameters of the binding of D-Phenylglycine, L-Dopa and D-Phenylglycine-L-dopa to human serum albumin calculated based on Scatchard plot.

	D-Phenylglycine	L-Dopa	D-Phenylglycine-L-dopa
One binding-site model			
n (sites/L)	6.00	0.59	3.41
Ka_1 (L/mol)	0.11	0.30	1.13
AIC	32.92	0.17	23.06
Two binding-site model			
n_1 (sites/L)	1.32	1.09	0.39
Ka_1 (L/mol)	0.56	0.15	1.28
n_2 (sites/L)	8.01	1.91	4.38
Ka_2 (L/mol)	0.07	0.06	0.08
AIC	20.16	43.19	34.58
non-specific binding			
n (sites/L)	2.73	0.91	2.01
Ka_1 (L/mol)	0.39	0.20	0.82
Ka_2 (L/mol)	0.34	0.07	0.20
AIC	28.51	46.06	38.92

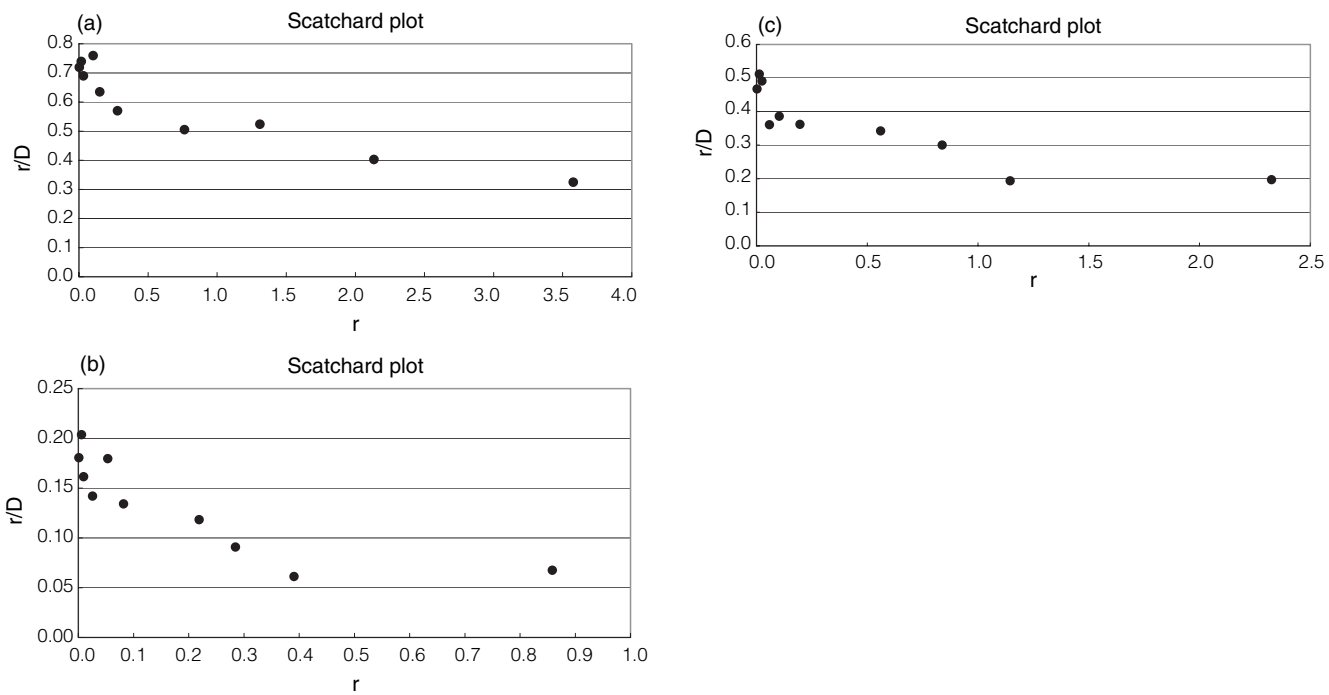

Figure 2. Scatchard plot of the binding of (a) D-phenylglycine (n = 3), (b) L-dopa (n = 3) and (c) D-phenylglycine- L-dopa (n = 3) to human serum albumin. r denotes moles of test compound bound to albumin per mole of protein. D denotes free drug concentration.

serum albumin increased by 5.8 folds (3.41 vs 0.59) and the Ka1 increased by 3.8 folds (1.13 vs 0.30).

CONCLUSIONS

This study demonstrated that D-phenylglycine increased the binding of L-dopa to human serum albumin. In comparison to L-dopa, the higher affinity of D-phenylglycine-L-dopa to albumin came from both an increased binding constant and the number of binding sites. With the affinity of D-phenylglycine to serum albumin, this moiety showed its possibility as a delivery tool for drugs with high clearance and short biological half life to use the body protein as drug reservoir.

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