Biodegradation of Poly(L-aspartic acid-*co*-valine) and Its Derivatives

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ABSTRACT: Biodegradable poly(L-aspartic-acid/valine) (PAV) was synthesized by thermal polycondensation. The molar ratios of aspartic acid/valine were 9 : 1, 8 : 2, 7 : 3, 6 : 4, and 5 : 5, respectively. The chemical structure of PAV was confirmed by ^{13}C-NMR, DSC, FTIR, X-ray, and GPC measurements. The degradation of PAV was studied in PBS buffer solution (pH 7.4 and 8.2) and in aqueous solution with enzymatic hydrolysis in which papain and trypsine acted as catalyst. It showed that the biodegradation rate of PAV was increased when the content of valine in copolymer increased. 3-Hydroproplamine was used in a succinimide ring-open reaction to obtain hydrophilic products (PHPAV). Stability tests showed that humidity, light, and temperature had no remarkable effect on PHPAV, however, it could be degraded in base solution. Biocompatibility and hematological tests were investigated. No deaths were found in the mice treated with PHPAV solution up to a 7.5 g/kg dose. No differences in number of red cells and glutamic pyruvic and creatinuria transaminase activities were found between the experiment and the control groups. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 46–51, 2006

Key words: L-aspartic acid; valine; polycondensation; degradation

INTRODUCTION

Poly(amino acid)s and their copolymers have received great interest from the biodegradable medical fields for many years.[1–4] Owing to their low tissue toxicity and high enzymatic degradability at desired sites, poly(amino acid)s have been frequently considered as a potential matrix system for controlled release of bioactive agents.[5–8] Thermal polycondensation using acid catalyst with reduced pressure was a useful method for the production of poly(amino acid)s and their derivatives. Poly(succinimde- *co*-6-aminocaprioic acid),[9] poly(succinimde-*co*-ω-amino acid),[10] and poly-[(aspartic acid)-*co*-lysine][11] were reported by thermal polycondensation technique. Furthermore, the relationship between structure and properties of poly(aspartic acid)s[12] and one- and two- dimensional ^{1}H- and ^{13}C-NMR characterization of poly(succinimde) were also performed.[13,14]

In our previous article, the use of poly(3-hydroxypropyl)-DL-aspartamide (PHPA) as a drug carrier for norethindrone, 5-Fu, was described.[15,16] We think that it was necessary to optimize the rate of release and degradation of the matrix by copolymer synthesis. One of the useful and effective ways to realize the aim was to form co-poly- amino-acids. In the present study, we reported the acid catalyst polycondensation of aspartic acid with valine product poly-(aspartamide/valine) (PAV) at different molar ratios. Their chemical structures were conformed by ^{13}C-NMR, DSC, FT-IR, X-ray, and GPC measurements. The effects of light, temperature, humidity, and pH on PHPAV were investigated. Enzymatic (using trysin and papain) and hydrolytic (PBS buffer) degradation behavior *in vitro* was carried out to simulate *in vivo* polymer degradation from the perspective of their possible use as biomedical materials. 3-Hydroxypropylamine was used in a succinimide ring-open reaction to get poly-(3-hydroxypropyl)- aspartamide/valine (PHPAV) (Scheme 1). The copolymers were of interest in terms of expanding their potential for biodegradable material.

EXPERIMENTAL PROCEDURE

Materials

L-Aspartic acid and valine were from Shanghai Chemical Co. (China). 3-Hydroxypropyl- amino, *N*,*N*′-dimethylformamide, trypsin, and papain were from Sigma Chemical Co. Shanghai, China and were used without further purification. All reagents were analytical grade.

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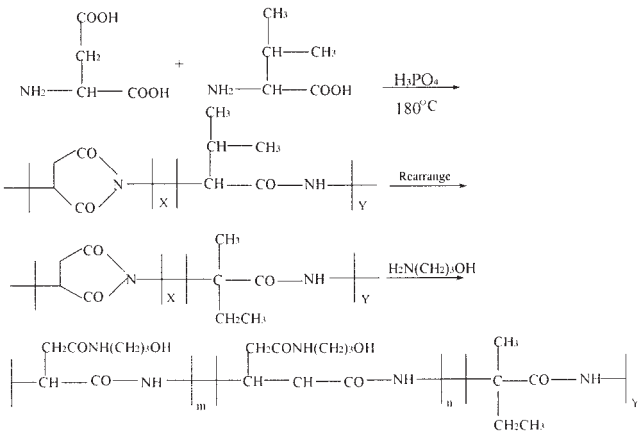

Scheme 1 Route of synthesis.

Laboratory animal test

ICR mice (18–25 g) were used for testing acute toxicity and hematological study. All animals were housed in standard conditions and were allowed free access to food and water. Mice were fasted 12 h before the experiments.

Instrumentation

The ^{13}C-NMR spectra were obtained using a Bruker AM-400 NMR spectrometer in $CDCl_3$ at 25°C using 5-mm o.d. sample tubes. The X-ray scanning experiment was carried out using D/MAX-IIIB. Thermal analysis was performed on a thermal analysis system (Perkin–Elmer, DSC-7) at a heating rate of 5°C/min. Molecular weight of PHPAV was determined by GPC using a Shimadzu LC-6A with a refractive index detector. The FTIR spectra were recorded on a Mattson Instrument Int. Alpha Centanri. The sample was pressed into a KBr disc.

Synthesis of PAV and PHPAV

Synthesis of PAV

A mixture of L-aspartic acid (10.6 g, 0.08 mol), valine (2.3 g, 0.02 mol) and 85% phosphoric acid (5 ml) was heated at 160°C for 4 h in a 500-ml eggplant-shaped flask under reduced pressure (about 10 mmHg) using a rotary evaporator. The reaction system changed from a syrupy heterogeneous liquid to a glassy solid. After completion of the reaction, the pale brown glassy product was homogeneously dissolved by adding DMF (50 ml), precipitated in distilled water, dried at room temperature under reduced pressure. The yield of PAV-8 was 66.2%. The synthesis procedure was repeated for aspartic acid and valine with molar ratios of 9 : 1, 7 : 3, 6 : 4, and 5 : 5 (mol/mol), respectively.

PBS and enzymatic degradation of PAVs

Enzymatic degradation study *in vitro* was performed using both trysin and papain. The concentration of the enzymatic solution was 1.0 mg/10 ml: PAV (100 mg) was exposed to the solution of hydrolases at 25°C. The PAV was removed from the enzyme solution for 72 h, filtered, weighed, and vacuum dried at 60°C until constant weight was achieved. The results were calculated by following formula: $\% = [(W_o - W_i) / W_o] \times 100$, where W_o was the primary weight of sample and W_i was the point time weight of sample.

The hydrolytic degradation study was performed in pH 7.4 and 8.2 in PBS (0.1 mol/L) at 37°C. PAV (100 mg) was exposed to the PBS buffer and was removed from the solution for 72 h, filtered, weighed, and vacuum dried at 60°C until a constant weight was achieved.

Preparation of hydrophilic PHPAV

3-Hydroxypropylamine (9.0 ml, 0.12 mol) was added dropwise to a continuously stirred solution of PAV-8 (6.8 g) in 50 ml DMF. The reaction temperature was maintained in the range of 0–10°C while 3-hydroxypropylamine was being dropped and was kept at room temperature for 4 h. It was then added dropwise to 200 ml *n*-propanol. A white product was obtained, filtered, and washed with acetone until the product was neutralized. Finally, it was dried under vacuum at room temperature; 8.6 g of product was obtained.

Evaluation of PHPAV property

Stability of PHPAV

Effect of humidity on PHPAV. Three aliquots of 100 mg PHPAV-8 were weighed in a small open flask and placed in a constant temperature (37.0°C ± 0.1) oven. The humidity was kept at 70% (KCl solution) for 10 days.

Effect of light on PHPAV. Three aliquots of 100 mg PHPAV-8 were weighed and placed in a box kept at 2,000 LX of light to irradiate for 10 days.

Effect of pH on PHPAV. Twenty-one aliquots of 20 mg PHPAV-8 were dispersed in 50 ml of buffer solution, with pH varied at 2, 4, 6, 7, 8, 10, and 12, kept at 37.0°C ± 0.1 for 4 h. Each sample was neutralized with either 1 mol/L NaOH or 1 mol/L HCl.

Effect of temperature on PHPAV. Nine aliquots of 100 mg PHPAV-8 were placed with a thermostat and maintained at temperatures of 40.0 ± 0.1, 60.0 ± 0.1, and 80.0 ± 0.1°C for 10 days. Each sample was dissolved in distilled water and analyzed by GPC. Other ratios of PHPAV were also used.

TABLE I
The Properties of PAV

Sample code	Copolymer (mol/mol)	Yield (%)	T_g(°C)	T_d(°C)	ASP/valine[a]	Product color
PAV-9	9:1	69.5	86.1	398.0	9.2:0.8	White
PAV-8	8:2	66.2	83.8	396.1	8.3:1.7	White
PAV-7	7:3	64.0	75.1	392.0	6.9:3.1	White
PAV-6	6:4	57.2	69.9	390.1	5.7:4.3	Pale red
PAV-5	5:5	46.8	67.8	370.3	5.0:5.0	Pale red

[a] Calculate from ^{1}H-NMR in DMF.

Biocompatibility test

Acute toxicity study

Acute toxicity was evaluated in the five groups of mice (each group consisted of 5 males and 5 females). Exact solution of PHPAV-8 was dissolved in distilled water and injected intraperitoneum once only (injected volume: 20 ml/kg body wt). Five different dosages of PHPAV-8 were used: 3.5, 4.5, 5.5, 6.5, and 7.5 g/kg. The same dosages were used for other ratios of PHPAV.

Hematological study

One hundred twenty mice (60 males and 60 females) were used for the study. The first group of 60 mice was treated with PHPAV-8 solution (i.m.); concentration of PHPAV-8 was 0.12 g/ml in distilled water. The second group of 60 mice, as the control group, was injected with pure saline (1 ml/100 g). The mice were treated for 8, 15, 22, 28, 35, and 42 days, respectively. The same dosages were used for other ratios of PHPAV.

RESULTS AND DISCUSSION

Recently, many studies have been performed to synthesize new polymeric materials with better biodegradability properties as drug carriers. Since almost all poly(amino acid)s are based on amino and formed amide, it was difficult to create a degradable poly(amino acid) with cleavable ester bonds. As a consequence of this demand, copolymerization has been used and specific degradation tests for their evaluation have been developed. Here we synthesized a new copolymer, poly(aspartic acid/valine) with a functional side-chain group, by adequate selection of constituent amino acids.

Synthesis and properties of PAVs

The PAVs were prepared by melting aspartic acid and valine with different molar ratios at a high temperature (160°C) for 4.5 h. Phosphoric acid was used as catalyst under a reduced pressure. Azeotropic removal of water from the reaction mixture was carried out throughout the reaction. After reaction, distilled water was used to precipitate PAVs. The properties of PAVs are summarized in Table I. The results indicate that the yields of PAVs decreased with increasing valine content in copolymer. It indicated that the solubility of PAVs was increased in water. The colors of the powder product changed from white to pale red when the content of the valine was increased.

The solubility behavior of PAVs was compatible with the proposed reaction scheme. It was insoluble in common organic solvents, but was soluble in *N*,*N*′-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO).

PAVs were characterized using DSC, X-ray, ^{13}C-NMR, and FTIR techniques to confirm their chemical structure. It showed that the glass transition temperature (T_g) varied from 67.8°C for PAV-5 to 86.1°C for PAV-9, and the decomposed temperature (T_d, 5% weight loss) changed from 370.3°C for PAV-5 to 398.0°C for PAV-9. It was noted that T_g and T_d were increased with the increased content of valine in the copolymer. In addition, poly(succinimide) and poly(succinimde-amide) were easily hydrolyzed for conversion in to poly(aspartic acid) and its derivatives and exhibited biodegradability.

As showed in Figure 1, the ^{13}C-NMR DEPT spectrum of PAV-8 agreed well with the chemical structure showed in Scheme 1. It showed that a structure rearrangement process happened during the thermal polycondensation. The peaks of PAV at 173.3, 173.7 ppm (– CO–), δ 47.46 ppm (–CH–), and δ 32.84 (–CH_2) were assigned to the aspartamide unit, while shifts at 169.7, 169.1 ppm (–CO–), δ 51.81 ppm (–C–), δ 37.81 ppm (–CH_2), and δ 21.20 ppm, 23.50 ppm (–CH_3) were assigned to the valine unit.

The X-ray patterns of PAVs are shown in Figure 2. It showed that PAVs were amorphous. Two peaks occurred: one at $2\theta = 18°$, which could be assigned to the aspartamide unit, and the other at $2\theta = 31.5°$, which could be assigned to the valine unit. When the valine content increased in the copolymer (from PAV-9 to PAV-5), the peak at $2\theta = 31.5°$ became wider. This indicated that the extent of the amorphous phase was increased in PAVs.

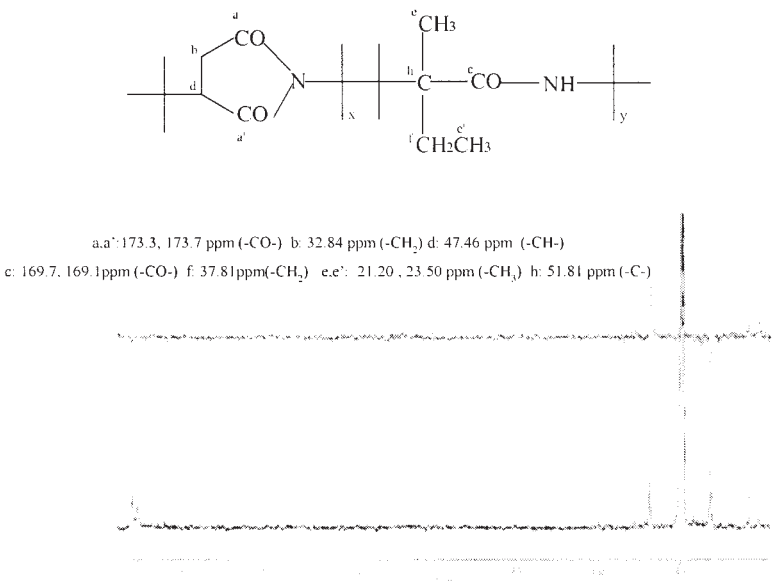

Figure 1 ^{13}C-NMR DEPT spectrum and assignment for PAV-8 measured in $CDCl_3$.

FTIR spectra of PAVs are shown in Figure 3. This provides evidence of the PAVs formation. The O=C–N stretch vibration characterizes peaks of succinimde end groups, which appeared from 1780 to 1720 cm^{-1}. When the ratio of valine in copolymer was increased, the stretch vibration characteristic peaks of protons assigned to methyl and methylene appeared at 3000–2800 cm^{-1} and the in-plane bending vibration characteristic peaks of protons assigned to methyl and methylene appeared at 1380 and 1480 cm^{-1}, respectively. The structure of PAV was confirmed from X-ray patterns, DSC, ^{13}C-NMR, and FTIR spectra analysis.

PBS and enzymatic degradation of PAVs

To evaluate the degradation of PAVs in the presence of PBS buffer solution (pH 7.4 and 8.2), we calculated the weight of the starting polymer and the weight loss of the polymer after 72 h. The results are show in Figure 4. It is noted that PAV-5, PAV-6, PAV-7, and PAV-8, were degraded more strongly than PAV-9. PAV-9 showed almost no biodegradation in both pH 7.4 and 8.2 buffer solutions. We could say that with an increase in the molar ratio of valine in the copolymer, the rate of biodegradability increased. The weight loss of PAV-5 was calculated in pH 7.4 and 8.2 buffer solutions at 120 h. Only 9.8% of PAV-5 remained in the pH 8.2 solution, and 50.2% of PAV-5 was present in the pH 7.4 buffer solution, indicating that the rate of degradation for PAV-5 was faster in the weak base solution because of the high concentration of valine presence in the copolymer.

Trypsin and papin were used as catalysts for the biodegradation of PAVs; they were typical hydrophobic amino acids, especially in the second amino acid

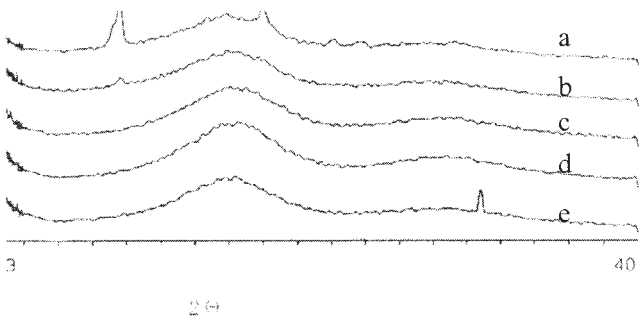

Figure 2 X-ray spectra of PAV: (a) PAV-5, (b) PAV-6, (c) PAV-7, (d) PAV-8, and (e) PAV-9, respectively.

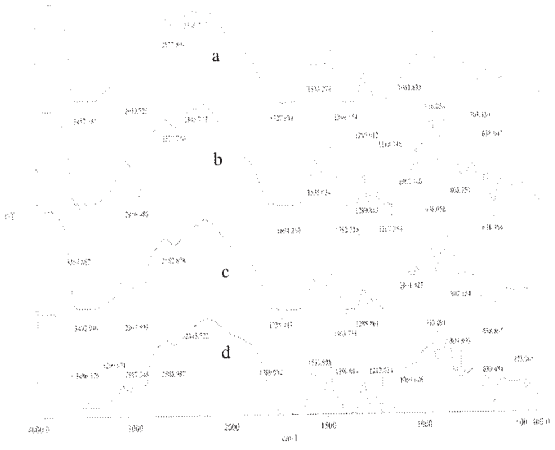

Figure 3 FTIR spectra of (a) PAV-8, (b) PAV-7, (c) PAV-6, (d) PAV-5.

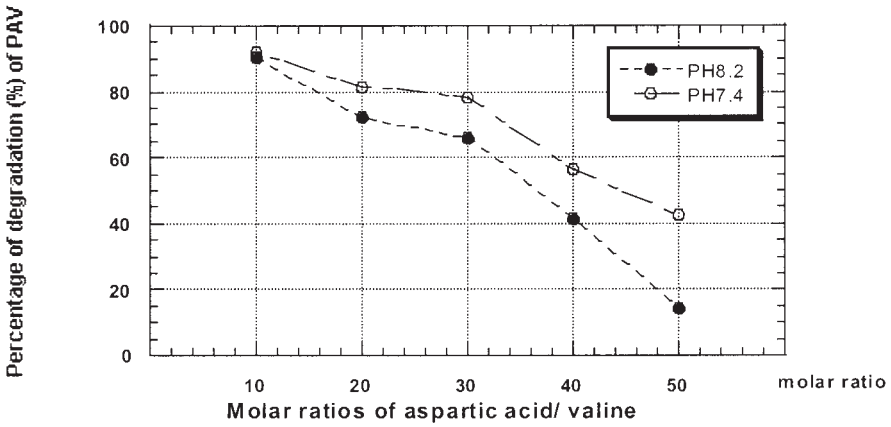

Figure 4 Percentage of degradation (%) of PAV at pH 7.4 and pH 8.2 PBS buffer solutions (0.1*M* 37°C) for 72 h.

on the amino group side from the cleaved bond. As expected, the biodegradability of PAVs increased with an increase in the content of valine. Figure 5 showed that the enzymes strictly recognized the structure of valine in the copolymer. For example, when the content of valine increase from 20%(PAV-8) to 50%(PAV-5), the weight loss of the polymer, by the trypsin-catalyzed reaction, increased from about 32 to 60%; while using papain as a catalyst, the weight loss increased from 42 to 91.2%.

Enzymatic degradation of PAV-5 was carried out using papain and trypsin acting as catalysts in aqueous solution at 120 h. More than 95% of PAV-5 dissolved and degraded in papain, implling that the degradation study could be significantly influenced by the composition of the copolymer. In the present study, the biodegradability of polymer was estimated by of the weight loss of the solid polymer, thus, the results would depend on the solubility of the polymer fragments from both hydrolysis and enzyme hydrolysis. It was suggested that a synergistic manner appeared with hydrolysis and enzyme hydrolysis for the copolymer. The explanation of this behavior might be related to the fact that the enzyme selectively attacked the amorphous phase of the polymer.

An attempt to identify the degradation products was unsuccessful, as the separation of the products from the buffer solution containing enzymes was difficult. Furthermore, the PHLC analysis did not provide useful information on the structure of the products.

Synthesis and properties of PHPAV

As a potential material for a drug carrier, 3-hydroxypropylamine, which was an active spacer, was used for the ring opening of poly(aspartic acid/valine). The results and properties are summarized in Table II. GPC traces showed that the molecular weights of PHPAVs were in the range of 3.86 to 7.76×10^4. *n*-Propanol was used to precipitate PHPAV-9 and PHPAV-8. An equimolar solution of *n*-propanol and ethyl acetate (1 : 1) was used for PHPAV-7, PHPAV-6, and PHPAV-5. The colors changed from white to pale red with the increased content of valine in the copolymer. All of the products should be kept in a closed container.

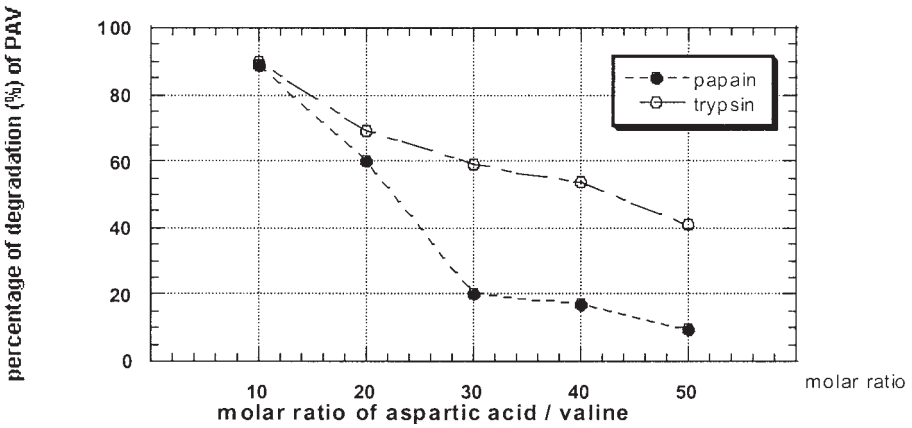

Figure 5 Percentage of degradation (%) of PAV using trypsin and papain as catalyst (1 mg/10 ml 37°C) for 72 h.

Stability of PHPAV

The stability of PHPAV was evaluated *in vitro* (PHPAV-8 for instance). No change was observed for PHPAV kept in an opened flask for 1 week. From this observation, it could be kept either 1 year or more in a closed container or 2 to 3 years in a vacuum container or in inert gas. The pH experiments showed that it was most stable in the range of pH 2–7. But, at a high pH range, especially when pH was greater than pH 12, the stability of PHPAV was slightly degraded due to the easier cloven of the ester linkage. This was confirmed by the presence of a small-molecular-weight PHPAV fragment in the GPC measurement. Effects of humidity, light, and temperature (from 25° to 80°C) on the PHPAV were also investigated. There was no remarkable effect on the molecular weight for PHPAV.

Acute toxicity and hematological studies of PHPAV

No death was found in the treated mice, i.e., with PHPAV solution up to a dosage of 7.5 g/kg. Therefore, under the experimental conditions, it was impossible to determine the LD_{50} in mice.

Results of hematological studies are shown in Table III. (PHPAV-8, for this example). It could be seen that treatment of the mice with PHPAV-8 solutions did not cause any change in hemoglobin level. No difference was found in the number of red cell (Hg), glutamic pyruvic (ALT), and creatinuria (Cr) transaminase activities between the treated groups and those of the control groups.

Further investigations of a model drug conjugating to PHPAV copolymer and *in vitro* and *in vivo* release details are in progress.

CONCLUSION

Poly(aspartic acid/valine) with different molar ratios could be synthesized by acid- catalyzed thermal polycondensation of aspartic acid with valine. 3-Hydroxypropylamine was used to succinimide ring-opening reaction to get poly(3-hydroxypropyl)- aspartamide/valine. The structures and properties analyses indicated that the content of valine in the copolymer could control the degradation rate of PHPAV.

TABLE II
Properties of PHPAV[a]

Sample cord	Yield(%)	M_w	M_n	M_w/M_n
PHPAV-9	70.6	46,300	37,900	1.22
PHPAV-8	71.5	38,600	36,100	1.07
PHPAV-7	70.9	51,700	47,300	1.09
PHPAV-6	69.2	77,600	70,100	1.11
PHPAV-5	68.3	62,500	57,900	1.08

[a] Chromatography condition, the column Diol-150ϕ7.5 250 mm; water as mobile phase; flow rate of 1 ml/min; column temperature 40°C, and glucosan standards.

TABLE III
Hematological Parameters in Mice Treated for 8–42 Days with PHPAV-8 and Physiological Saline 0.12 g/ml[a]

Treatment (day)	Hg (g/1000 ml)	ALT (μmol/L)$\times 10^3$	Gr (μmol/ml)$\times 10^3$
8			
PHPAV-8	13.1 ± 0.1	32.1 ± 0.8	—
Saline	13.8 ± 1.0	30.8 ± 1.2	20.0 ± 1.5
15			
PHPAV-8	12.4 ± 0.7	45.0 ± 10.0	33.4 ± 5.7
Saline	12.8 ± 1.1	45.6 ± 7.1	22.9 ± 1.2
22			
PHPAV-8	12.5 ± 0.8	50.6 ± 15.9	24.3 ± 7.5
Saline	13.2 ± 0.6	40.8 ± 8.5	23.2 ± 6.1
28			
PHPAV-8	12.5 ± 0.7	51.2 ± 4.4	30.5 ± 4.2
Saline	13.0 ± 0.4	35.6 ± 7.8	38.7 ± 2.9
35			
PHPAV-8	12.8 ± 0.2	58.8 ± 8.6	25.7 ± 4.3
Saline	12.8 ± 0.3	57.0 ± 8.2	18.3 ± 5.7
42			
PHPAV-8	12.4 ± 0.8	51.0 ± 4.8	37.9 ± 2.8
Saline	14.1 ± 0.3	47.5 ± 7.2	32.7 ± 3.2

[a] Values are means ± SD.

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