Arch Pharm Res Vol 27, No 2, 259-264, 2004

Archives of Pharmacal Research
http://apr.psk.or.kr

Pharmacokinetics of PEG-Hemoglobin SB1, a Hemoglobin-Based Oxygen Carrier, after Its Intravenous Administration in Beagle Dogs

Oh-Seung Kwon[1], Uoo Tae Chung, and Youn Bok Chung

National Research Laboratory (NRL) of PK/PD, College of Pharmacy, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea and [1]Bioanalysis and Biotransformation Research Center, Korea Institute of Science and Technology, Seoul 136-790, Korea

(Received December 4, 2003)

The purpose of the present study was to investigate the pharmacokinetics of PEG-hemoglobin SB1, a modified bovine hemoglobin with polyethylene glycol, after its single and multiple administration in beagle dogs. For this purpose, the analytical method of free hemoglobin in the plasma was developed and validated. Excellent linearity (r^2=0.999) was observed in the calibration curve data, with the limit of quantification of 0.005 g/dL. The precision and the deviation of the theoretical values for accuracy were always within ±15% in both the between- and the within-day results. The method was tested by measuring the plasma concentrations following intravenous administration to beagle dogs and was shown to be suitable for pharmacokinetic studies. In a single dose study, the plasma half-life ($t_{1/2}$) increased and the total body clearance (CL_t) decreased with the dose (*i.e.*, 0.017 to 0.75 gHb/kg as PEG-hemoglobin SB1) in both sexes. The volume of distribution at steady-state (Vd_{ss}) showed no difference with the dose. In contrast, the values of $t_{1/2}$, CL_t and the area under the plasma concentration-time curve (AUC) after the multiple dose were significantly different from those of the single dose administration. The values of $t_{1/2}$ in the multiple administration were about two times higher than that of the single dose. As a result, $t_{1/2}$ of hemoglobin after the administration of PEG-hemoglobin SB1 was about 15-30 h, indicating the PEG modification of the hemoglobin lead to a prolongation of plasma concentration of the protein. Therefore, these observations suggested that the PEG modification of hemoglobin is potentially applicable in the hemoglobin-based therapeutics.

Key words: Hemoglobin, Polyethylene glycol (PEG), Pharmacokinetics, Plasma half-life

INTRODUCTION

Hemoglobin is responsible for the transport of oxygen from the lungs to other tissues in the body (Winslow *et al.*, 1995). Thus, hemoglobins and its variants from humans have been studied extensively (Suzuki *et al.*, 1989; Fago *et al.*, 1997; Hardison *et al.*, 1998). The crystal structures of a number of different hemoglobins have been determined and their function in transport of oxygen, carbon dioxide and nitric oxide has been elucidated (Sharma *et al.*, 1987; Brunori *et al.*, 1966).

A number of hemoglobin-based therapeutics are currently under development by a number of pharmaceutical companies (Reah *et al.*, 1997; Gould *et al.*, 1998; Przybelski *et al.*, 1996; Kasper *et al.*, 1996). Hemoglobin therapeutics have been designed as oxygen-carrying fluids useful as a blood replacement during surgery and in trauma, enhancers of radiation therapy, and scavengers of nitric oxide (Hess 1996a; Scott *et al.*, 1997). Once removed from the red blood cell, hemoglobin cannot be used for these therapeutic indications without modification due to renal toxicity (Savitsky *et al.*, 1978). In addition, the plasma half-life of unmodified hemoglobin is short and the affinity with the oxygen in plasma is apparently poor for an efficient delivery of oxygen (Hess, 1996b; Sakai *et al.*, 2000; Phillips *et al.*, 1999). A number of modifications have been proposed for hemoglobin to circumvent the limitation of the native human oxygen carrier [e.g., crosslinking α, β dimers; poly-

Correspondence to: Youn Bok Chung, National Research Laboratory (NRL) of PK/PD, College of Pharmacy, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea
E-mail: chungyb@chungbuk.ac.kr

merizing hemoglobin; or binding polymers to the surface of hemoglobin (Iwashita *et al.*, 1988; Kluger *et al.*, 1992; Chatterjee *et al.*, 1986; Ritchie *et al.*, 2000)].

One such product, PEG-hemoglobin SB1 is currently in clinical trials for treatment as a blood replacement agent. PEG-hemoglobin SB1 is a modified bovine hemoglobin with polyethylene glycol (PEG). PEG is a nontoxic, amphiphilic polyether that is heavily hydrated in aqueous solution. Polymer modified hemoglobin with PEG is apparently free of renal toxicity or vasoconstriction activity (Gould *et al.*, 1998), indicating that the modified protein may be practically applicable. Moreover, the use of PEG-modification is an attractive means of overcoming problems with short half-life for the protein. In the present study, therefore, the pharmacokinetics of PEG-hemoglobin SB1 were investigated after its single and multiple administration in beagle dogs. Our findings indicate that the modification leads to a prolongation of plasma level of the hemoglobin and that the PEG modification of the protein is potentially applicable in the hemoglobin-based therapeutics.

MATERIALS AND METHODS

Materials

PEG-hemoglobin SB1 was obtained from SunBio Co. Ltd. (Korea). Dipotassium hydrogenphosphate and potassium dihydrogenphosphate was purchased from Showa Chemical Industries (Tokyo, Japan). All other reagents were commercial products and of analytical grade. Male and female beagle dogs (Sam Tac, Kyunggi-Do, Korea) aged 5 months on a normal laboratory diet were used throughout the study.

Analysis of free hemoglobin in plasma

The analytical method of plasma free hemoglobin was developed and validated. The concentration of free hemoglobin was calculated by the following procedures, using UV/Vis spectrophotometer (Jasco Co., Ltd., V-530, Japan).

Eight working standards covering the concentration range of PEG-hemoglobin SB1 (0.005-3.0 g/dL), by appropriate dilution of the stock solution in pH 7.5 phosphate buffer. The optical absorbances of the working standards were first measured at 560, 576 and 592 nm, and corrected absorbance, $2y - (x + z)$ (viz, x, y and z were absorbances at 560, 576 and 592 nm, respectively), calculated. Then, the corrected absorbance was plotted against the hemoglobin concentration and calibration curve constructed. For the determination of hemoglobin concentration in plasma samples, the corrected absorbance [i.e, $2y - (x + z)$] was calculated and the concentration of free hemoglobin estimated by the use of the calibration curve.

Validation criteria

Linearity

The linearity of the assay was assessed by preparing concentration of PEG-hemoglobin SB1 (0.005-3.0 g/dL) and by plotting the corrected absorbance versus measured concentration.

Precision and accuracy

The precision and accuracy of the method were determined by the analysis of three quality control samples for each concentration (0.005-3.0 g/dL) during the same day (repeatability) and on three consecutive days (reproducibility). The accuracy of the assay was determined by preparing known concentrations of PEG-hemoglobin SB1 and then the determination of the concentration for the samples.

Stability

Stability of the assay was assessed in samples of PEG-hemoglobin SB1 (0.005-3.0 g/dL) in dog plasma. The concentration of the sample was evaluated at 1, 2, 15, 18, 24, 48, and 72 h of the preparation.

Pharmacokinetic study

PEG-hemoglobin SB1 was administered intravenously at the dose of 2.5 mL/kg, 5.0 mL/kg, and 10 mL/kg of dosing solution (0.7 gHb/10 mL) in both sexes, male and female beagle dogs.

In a single dose study, blood samples were collected immediately before the administration and up to 48 h after the dose (2.5-10 mL/kg of 0.7 gHb/10 mL solution). In a multiple dosing study, the animal were given intravenously at the dose of 2.5-10 mL/kg every 2 days for 2 weeks. In both experimental designs, plasma was obtained by centrifugation (10,000 g for 2 min) of blood samples and immediately frozen at -70°C until analysis.

Pharmacokinetic analysis

Non-compartmental methods were used to determine pharmacokinetic parameters. Computer-based calculations were carried out using WinNonLin. The elimination rate constant (k) was calculated from the semi-log regression on the terminal phase of the plasma concentration-time curve. Plasma half-life ($t_{1/2}$) was calculated using the euqation of $t_{1/2} = 0.693/k$. Area under the plasma concentration-time curve from time zero to infinity (AUC) was calculated from the equation $AUC = AUC_t + C_t/k$, where C_t is the last quantifiable concentration. Area under the plasma concentration-time curve from zero to time of last quantifiable concentration (AUC_t) was calculated using linear trapezoidal approximation. In addition, the parameters such as the steady-state volume of distribution (Vd_{ss}), the total plasma clearance (CL_t), the mean residence time

(MRT) were calculated by standard methods.

Statistical analysis

Comparison between two means was performed using the unpaired Student's *t*-test. One-way analysis of variance was used to test for significant difference between groups. Statistical significance was defined as $p<0.05$.

RESULTS AND DISCUSSION

Validation of analysis method

Fig. 1 illustrates the calibration curve of PEG-hemoglobin SB1 in plasma samples. Excellent linearity ((function) = 0.064 × (concentration) + 0.0001, $r^2 = 0.999$) was observed between the corrected absorbance [i.e., 2y – (x + z); x, y and z represent absorbances at 560, 576 and 592 nm, respectively] and the concentrations. The calibration curve data obtained throughout the validation study are summarized in Table I. According to these results, the limit of quantification was 0.005 g/dL.

The between-day results (reproducibility) of quality control are given in Table I. For each level, the imprecision did not exceed 15% (min: 0.01%, max: 2.21%) and the mean values were always within ±15% deviation of the theoretical values for accuracy (min: 1.06%, max: 11.1%). The acceptance criteria were also fulfilled for the within-day results (repeatability).

PEG-hemoglobin SB1 samples, prepared in plasma of beagle dogs, was proved to be stable within 3-day period at 4°C. No significant degradation was observed, indicating the storage condition provided a reasonable stability within 3-day period. The storage condition was used throughout the study and the sample always assayed in 3-day of the collection.

Table I. Validation results of precision and accuracy of the analysis of free hemoglobin in plasma samples

Concentration (ng/mL)	Precision (CV%)			Inaccuracy (%)		
	Intra-day[a]	Inter-day[a]	Mean[b]	Intra-day[a]	Inter-day[a]	Mean[b]
0.005	0.01	0.01	0.01	6.25	4.67	6.54
0.01	8.66	0.01	5.97	4.17	11.10	6.44
0.05	3.86	0.01	2.58	2.08	1.59	1.35
0.1	2.53	0.91	3.38	5.73	1.06	3.43
0.5	1.09	2.07	2.06	3.12	7.51	4.15
1.0	2.47	2.21	2.27	2.40	5.71	2.91
3.0	1.00	2.07	1.51	0.24	1.06	0.45

[a]Each value was calculated from the different three experiments.
[b]The values were calculated from the data of both intra- and inter-day (n=6).

Pharmacokinetics after the single dose

Fig. 2 shows the plasma concentration of hemoglobin-time profiles after the intravenous administration of PEG-hemoglobin SB1 at the dose of 0.175 gHb/kg, 0.35 gHb/kg and 0.7 gHb/kg, respectively, in male beagle dogs. Non-compartmental methods were used to evaluate pharma-

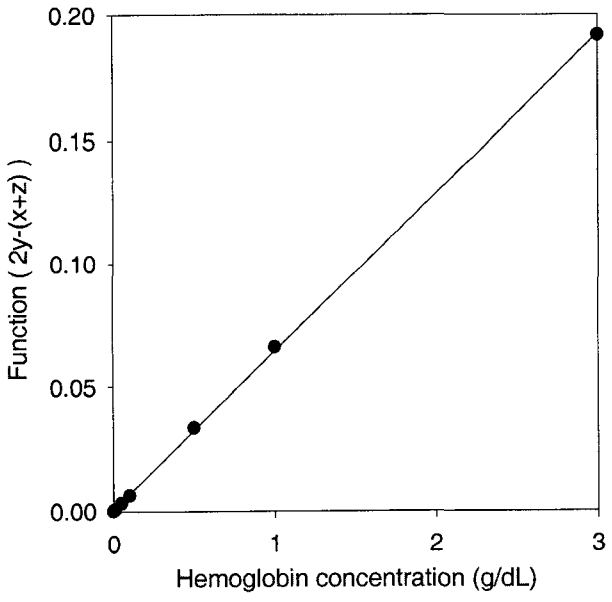

Fig. 1. Calibration curve of hemoglobin in plasma. Linearity (y = 0.064x + 0.0001, $r^2 = 0.999$) was observed between the corrected absorbance [i.e., 2y – (x + z); x, y and z represent absorbances at 560, 576 and 592 nm, respectively] and the hemoglobin concentrations.

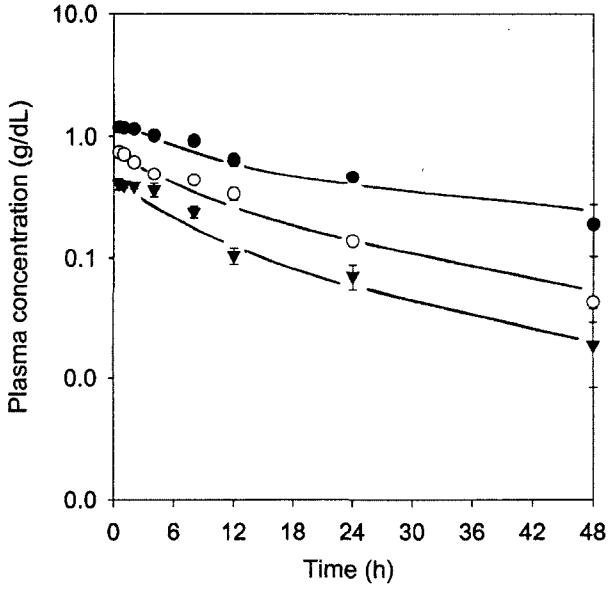

Fig. 2. Plasma concentration of hemoglobin-time profiles after the single intravenous administration of PEG-hemoglobin SB1 at the dose of 0.175 gHb/kg (▼), 0.35 gHb/kg (○) and 0.7 gHb/kg (●), respectively, in male beagle dogs. Each point represents the Mean±S.E. of three male dogs.

Table II. Pharmacokinetic parameters of hemoglobin after the intravenous administration of PEG-hemoglobin SB1 after the single intravenous dose in male beagle dogs[a,b]

Parameter	Dose (gHb/kg)		
	0.175	0.35	0.70
K (h^{-1})	0.093 ± 0.010	0.072 ± 0.0012	0.043 ± 0.0073
$t_{1/2}$ (h)	7.69 ± 0.92	9.68 ± 0.17	17.04 ± 2.97
AUC ($g \cdot h \cdot dL^{-1}$)	5.56 ± 0.88	11.70 ± 1.47	35.43 ± 5.97
MRT (h)	16.77 ± 3.28	15.81 ± 1.74	33.49 ± 5.78
V_{dss} (dL/kg)	0.671 ± 0.04	0.611 ± 0.012	0.852 ± 0.063
CL_t (dL/h/kg)	0.042 ± 0.0058	0.040 ± 0.005	0.027 ± 0.004

[a] Mean ± S.E. (n=3). [b] g/dL

Table III. Pharmacokinetic parameters of hemoglobin after the intravenous administration of PEG-hemoglobin SB1 at the single intravenous dose in female beagle dogs[a,b]

Parameter	Dose (gHb/kg)		
	0.175	0.35	0.70
K (h^{-1})	0.12 ± 0.016	0.080 ± 0.0064	0.048 ± 0.0027
$t_{1/2}$ (h)	6.19 ± 1.01	8.83 ± 0.73	14.43 ± 0.82
AUC ($g \cdot h \cdot dL^{-1}$)	5.09 ± 1.12	14.01 ± 0.64	32.58 ± 1.27
MRT (h)	16.93 ± 7.58	22.07 ± 0.20	26.60 ± 2.14
V_{dss} (dL/kg)	0.682 ± 0.160	0.711 ± 0.029*	0.622 ± 0.146
CL_t (dL/h/kg)	0.049 ± 0.010	0.032 ± 0.002	0.023 ± 0.004

[a] Mean ± S.E. (n=3). [b] g/dL.
* Significantly different from the male dogs (Table II) ($p < 0.05$).

cokinetic parameters (Table II) such as the elimination rate constant (k), the plasma half-life ($t_{1/2}$), the steady-state volume of distribution (V_{dss}), the total plasma clearance (CL_t), the mean residence time (MRT) and the area under the curve (AUC). The mean terminal plasma half-life ($t_{1/2}$) of the hemoglobin at the dose of 0.175 and 0.35 gHb/kg were 7.69 and 9.68 h, respectively, indicating no significant difference between two doses. However, the half-life (i.e., 17.0 h) at the highest dose (i.e., 0.75 gHb/kg) was approximately twice the half-lives found in the lower doses. Accordingly, the values of CL_t decreased by about 60% with the dose from 0.017 to 0.75 gHb/kg. The values of V_{dss} showed no difference with the dose.

In the case of female beagle dogs (Fig. 3, Table III), the pharmacokinetic parameters after the intravenous administration of PEG-hemoglobin SB1 were comparable with those of male beagle dogs (Table II). That is, the value of $t_{1/2}$ increased by two times whereas CL_t decreased by about 50% with an increase in the dose from 0.017 to 0.75 gHb/kg. Similarly, the V_{dss} did not show any statistical difference with respect to the dose.

Pharmacokinetics after the multiple dose

Fig. 4 shows the plasma concentration of hemoglobin-time profiles after the multiple administration every 2 days for 2 weeks at the dose of 0.175 gHb/kg, 0.35 gHb/kg and 0.7 gHb/kg of PEG-hemoglobin SB1, respectively, in male beagle dogs. The plasma concentrations were declined multi-exponentially in a similar manner of the single ad-

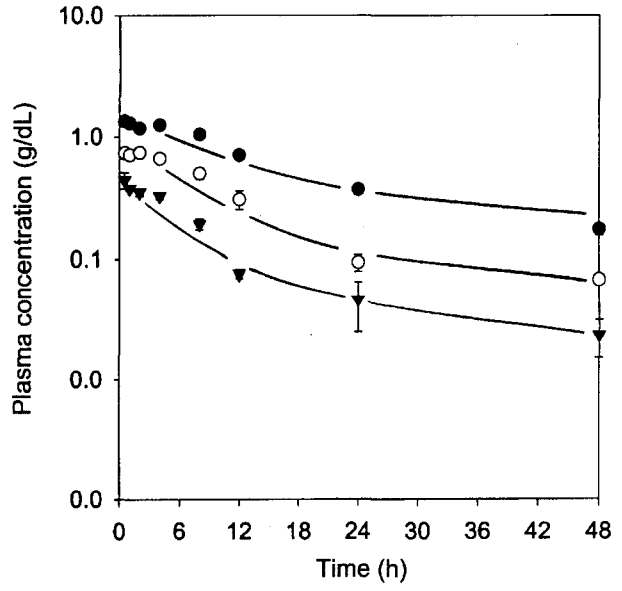

Fig. 3. Plasma concentration of hemoglobin-time profiles after the single intravenous administration of PEG-hemoglobin SB1 at the dose of 0.175 gHb/kg (▼), 0.35 gHb/kg (○) and 0.7 gHb/kg (●), respectively, in female beagle dogs. Each point represents the Mean ±S.E. of three female dogs.

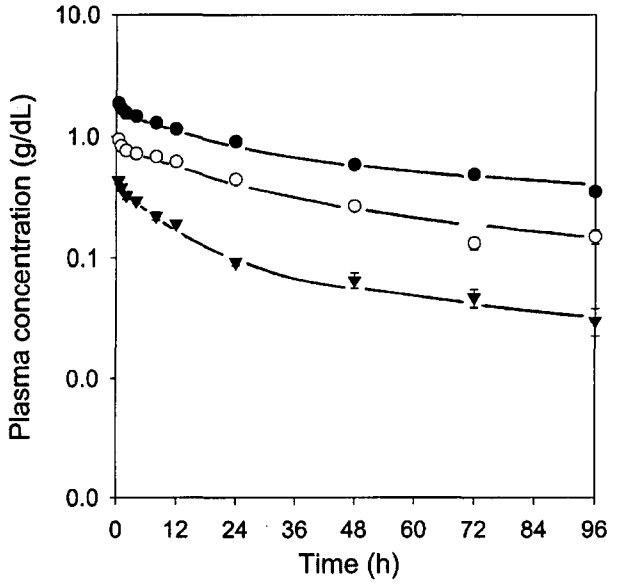

Fig. 4. Plasma concentration of hemoglobin-time profiles after the multiple intravenous administration every 2 days for 2 weeks at the dose of 0.175 gHb/kg (▼), 0.35 gHb/kg (○) and 0.7 gHb/kg (●) of PEG-hemoglobin SB1, respectively, in male beagle dogs. Each point represents the Mean±S.E. of three male dogs.

ministration (Fig. 2). The pharmacokinetic parameters after the multiple administration of PEG-hemoglobin SB1 in male beagle dogs were summarized in Table IV. The plasma half-life ($t_{1/2}$) of hemoglobin was elevated by about two times whereas the values of CL_t decreased by about 50% with the dose. In addition, the values of $t_{1/2}$, CL_t and AUC after the multiple administration were significantly different from those of the single dose in male beagle dogs (Table II). The values of $t_{1/2}$ in the multiple administration were about two times higher than that of the single dose.

In the case of the multiple administration of PEG-hemoglobin SB1 in female beagle dogs (Fig. 5, Table V), the

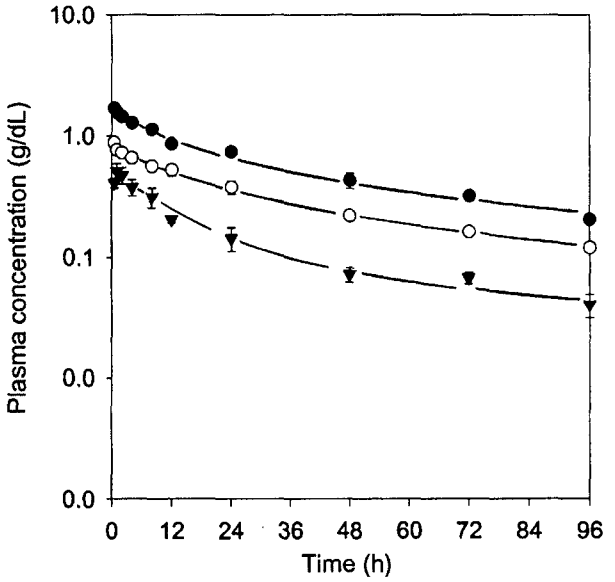

Fig. 5. Plasma concentration of hemoglobin-time profiles after the multiple intravenous administration every 2 days for 2 weeks at the dose of 0.175 gHb/kg (▼), 0.35 gHb/kg (○) and 0.7 gHb/kg (●) of PEG-hemoglobin SB1, respectively, in female beagle dogs. Each point represents the Mean±S.E. of three female dogs.

Table IV. Pharmacokinetic parameters of hemoglobin after the intravenous administration of PEG-hemoglobin SB1 at the multiple intravenous dose every 2 days for 2 weeks in male beagle dogs[a,b]

Parameter	Dose (gHb/kg)		
	0.175	0.35	0.70
$K(h^{-1})$	0.043 ± 0.0059#	0.024 ± 0.0018##	0.018 ± 0.0007#
$t_{1/2}$ (h)	16.72 ± 2.65#	29.12 ± 2.08##	37.98 ± 1.45##
AUC ($g \cdot h \cdot dL^{-1}$)	11.37 ± 1.32#	32.75 ± 2.43##	99.14 ± 3.05##
MRT (h)	59.60 ± 17.20	38.75 ± 1.88##	79.11 ± 5.68##
V_{dss} (dL/kg)	1.137 ± 0.207	0.535 ± 0.016#	0.717 ± 0.037
CL_t (dL/h/kg)	0.020 ± 0.003#	0.014 ± 0.001##	0.009 ± 0.0003##

[a]Mean ± S.E. (n=3). [b]g/dL

#Significantly different from the single dose in male dogs (Table II) ($p < 0.05$). ##Significantly different from the single dose in male dogs (Table II) ($p < 0.01$).

Table V. Pharmacokinetic parameters of hemoglobin after the intravenous administration of PEG-hemoglobin SB1 at the multiple intravenous dose every 2 days for 2 weeks in female beagle dogs[a,b]

Parameter	Dose (gHb/kg)		
	0.175	0.35	0.70
$K(h^{-1})$	0.039 ± 0.0014##	0.023 ± 0.0009##	0.025 ± 0.0019*##
$t_{1/2}$ (h)	17.69 ± 0.61##	30.31 ± 1.18##	28.55 ± 2.18*##
AUC ($g \cdot h \cdot dL^{-1}$)	14.71 ± 1.90#	35.92 ± 1.15##	65.10 ± 3.72**##
MRT (h)	51.07 ± 9.57#	58.15 ± 5.56*##	55.41 ± 3.76*##
V_{dss} (dL/kg)	0.789 ± 0.120	0.734 ± 0.094	0.765 ± 0.013
CL_t (dL/h/kg)	0.016 ± 0.002#	0.013 ± 0.0004##	0.014 ± 0.0008**

[a]Mean ± S.E. (n=3). [b]g/dL

*Significantly different from the multiple dose in male dogs (Table VI) ($p<0.05$). **Significantly different from the multiple dose in male dogs (Table VI) ($p<0.01$).

#Significantly different from the single dose in female dogs (Table III) ($p<0.05$). ##Significantly different from the single dose in female dogs (Table III) ($p<0.01$).

pharmacokinetic parameters were comparable with those of male beagle dogs (Table IV). The value of $t_{1/2}$ increased by two times whereas CL_t decreased by about 50% with the dose. Moreover, the values of $t_{1/2}$, CL_t and AUC after the multiple administration were significantly different from those of the single dose in female beagle dogs (Table III). The plasma concentrations after the multiple dose were maintained for the long periods compared with the single dose. The values of $t_{1/2}$ in the multiple administration were about two times higher than that of the single dose.

In summary, the plasma half-life ($t_{1/2}$) of hemoglobin were about 15-30 h after the single and multiple administration of PEG-hemoglobin SB1. The prolongation of plasma half life for PEG modified hemoglobin after the multiple-dosing situation was particularly noted in this study. Since the short half life was one of the limitation in the hemoglobin-based therapeutics, the prolongation of half life of the protein by PEG modification suggests that the modification may have a practical application.

CONCLUSION

The simple and convenient analytical method of free hemoglobin in plasma was developed and validated. The coefficient of variation for all the criteria of validation were less than 15%, indicating that the assay is valid for pharmacokinetic study with the protein. The sample was found to be very stable upon storage in 4°C.

The value of $t_{1/2}$ increased by two times whereas CL_t decreased by about 50% with the dose after both the single and the multiple administration of PEG-hemoglobin SB1. The plasma half-life ($t_{1/2}$) of hemoglobin after the administration of PEG-hemoglobin SB1 was about 15-30

h. These findings indicated that the plasma concentration of hemoglobin could be maintained for the long periods with PEG modifications and the prolongation by PEG modification may be clinically relevant.

ACKNOWLEDGEMENT

This study was supported by a National Research Lab. (NRL) program (#M1-0302-00-0069) of the Ministry of Science and Technology, Republic of Korea.

REFERENCES

Brunori, M., Noble, R. W., Antonini, E., and Wyman, J., The reaction of the isolated alpha and beta chains of human hemoglobin with oxygen and carbon monoxide. *J. Biol. Chem.,* 225, 5238-5243 (1966).

Chatterjee, R., Welty, E. V., Walder, R. Y., Pruitt, S. L., Rogers, P. H., Arnone, A. and Walder, J. A., Isolation and characterization of a new hemoglobin deritive cross-linked between the alpha chains (lysine 99 alpha 1-lys 99 alpha 2). *J. Biol. Chem.,* 261, 9929-9937 (1986).

Fago, A., Bendixen, E., Malte, H., and Webcr, R. E., The anodic hemoglobin of anguilla molecular basis for allosteric effects in a root-effect hemoglobin. *J. Biol. Chem.,* 272, 15628-15635 (1997).

Gould, S. A., Moore, E. E., Hoyt, D. B., Burch, J. M., Hae-nd, J. B., Garcia, J., DeWoskin, R., and Moss, G. S., The first randomized trial of human polymerized hemoglobin as blood substitute in acute trauma and emergent surgery. *J. Am. Coll. Surg.,* 18, 113-122 (1998).

Hardison, R. C., Chui, D. H. K., Riemer, C. R., Miller, W., Carver, M. F. H., Molchanova, T. P., Efremov, G. D., and Huisman, T. H. J., Access to a syllabus fo human hemoglobin variants (1996) *via* world wide web. *Hemoglobin,* 22, 113-127 (1998).

Hess, J. R., Alternative oxygen carriers. *Curr. Opin. Hematol.,* 3, 492-497 (1996a).

Hess, J. R., Blood substitutes. *Sem. Hematol.,* 33, 369-378 (1996b).

Iwashita, Y., Yabuki, A., Yamaji, K., Iwasaki, K., Okami, T., Hirata, C., and Kosaka, K., A new resuscitation fluid "stabilized hemoglobin" preparation a characteristics. *Biomat. Artif. Cells Artif. Organs,* 16, 271-280 (1988).

Kasper, S., Walter, M., Grune, F., Bischoff, A., Erasmi, H., and Buzello, W., Effects of a hemoglobin-based oxygen carrier (HBOC-201) on hemodynamics and oxygen transport in patients undergoing preoperative hemodilution for elective abdominal aortic surgery. *Anesth. Analg.,* 83, 921-927 (1996).

Kluger, R., Wodzinska, J., Jones, R. T., Head, C., Fujita, T. S., and Shih, D. T., Three-point cross-linking: potential red cell substitutes from the reaction of trimesoyl tris-(methyl phosphate) with hemoglobin. *Biochemistry,* 31, 7551-7559 (1992).

Phillips, W. T., Klipper, R. W., Awasthi, V. D., Rudolph, A. S., Cliff, R., Kwasiborski, V., and Goins, B. A., Polyethylene glycol-modified liposome-encapsulated hemoglobin: a long circulating red cell substitute. *J. Pharmacol. Exp. Ther.,* 288, 665-670 (1999).

Przybelski, R. J., Daily, E. K., Kisicki, J. C., Mattia Gold-berg, C., Bounds, M. J., and Colburn, W. A., Phase I study of the safety and pharmacologic effects of diaspirin cross-linked hemoglobin solution. *Crit. Care Med.,* 24, 1993-2000 (1996).

Reah, G., Bodenham, A. R., Maliick, A., Daily, E. K., and Przybelski, R. J., Initial evaluation of diaspirin cross-linked hemoglobin (DCLHb) vasopressor in critically ill patients. *Crit. Care Med.,* 25, 1480-1488 (1997).

Ritchie, A. J., Hartshom, S., Crosbie, A. E., Callingham, B. A., Latimer, R. D., and Vuylsteke, A., The action of diaspirin cross-linked haemoglobin blood substitute on human arterial bypass conduits. *Eur. J. Cardiothorac. Surg.,* 18, 241-245 (2000).

Sakai, H., Tomiyama, K. I., Sou, K., Takeoka, S., and Tsuchida, E., Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. *Bioconjug. Chem.*,11, 425-432 (2000).

Savitsky, J. P., Doczi, J., Black, J., and Arnold, J. D., A clinical safety trial of stroma-free hemoglobin. *Chin. Pharmacol. Ther.,* 23, 73-80 (1978).

Scott, M. G., Kucik, D. F., Goodnough, L. T., and Monk, T. G., Blood substitutes: evolution and future applications. *Clin. Chem.,* 43, 1724-1731 (1997).

Sharma, V. S., Traylor, T. G., and Gardiner, R., Reaction of nitric oxide with heme proteins and model compound of hemoglobin. *Biochemistry,* 26, 3837-3843 (1987).

Suzuki, T., Takagi, T., and Ohta, S., Primary structure of a dimeric haemoglobin from the deep-sea seep clam calyptogena soyoae. *Biochem. J.*, 260, 177-182 (1989).

Szebeni, J, Wassef, N. M., Hartman, K. R., Rudolph, A. S., and Alving, C. R., Complement activation *in vitro* by the red cell substitute, liposome-encapsulated hemoglobin: mechanism of activation and inhibition by soluble complement receptor type 1. *Transfusion,* 37, 150-159 (1997).

Winslow, R., Hemoglobin-based red cell substitutes: unsolved issues and further directions. In: Tuchida E, editor. *Artificial Red Cell.* Chichester: John & Sons, 17-30 (1995).