Novel Techniques for *In Vivo* Hemolysis Studies in Guinea Pigs

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ABSTRACT. The *in vivo* toxic-hemolytic studies using small experimental animals are complicated by difficulties in preventing hemolysis during repeated collection of blood specimens and in measuring hemoglobin concentration in small amounts of plasma sample. To solve these problems we tried to develop the new techniques for the *in vivo* hemolysis studies using guinea pigs. The hemolysis accident was minimized to 2.75 mg/dl by collecting the blood directly into heparinized microhematocrit tubes by small longitudinal incision in the auricular artery. The hemoglobin in a small amount of sample (10 μ l) was determined by the new analytical system using a microflow spectrophotometer with a modified cyanmethemoglobin method. The standard curve of the hemoglobin concentration in the system revealed a line of $Y=1.8X + 0.79$ ($r=0.999$), $CV<1\%$ with a minimum detectable concentration of 1.25 mg/dl. By using the new techniques, it was found that the plasma hemoglobin concentration in normal animals were 7.27 ± 0.44 mg/dl (mean $\pm$ S.E.). The *in vivo* hemolytic activity of saponin was observed dose-dependently at doses of 30–50 mg/kg, i.v. in the guinea pigs. It is concluded that the present techniques are useful for *in vivo* hemolytic studies in small experimental animals such as guinea pigs.—**KEY WORDS:** cyanmethemoglobin, guinea pig, hemoglobin, hemolytic effect, saponin.

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The hemolytic tests are commonly conducted in *in vitro* toxicological screening and in bioassay guided the isolation of the toxic-hemolytic substances. On the other hand, the *in vivo* hemolytic studies are conducted for the substances or their metabolites which are active only *in vivo* [1, 3, 10]. For the toxicological screening it is necessary to test hemolytic activity of a compound both *in vitro* and *in vivo*, which provides comprehensive information of the compound on the toxic and regenerative changes of the red blood cells (RBC) system. The *in vivo* hemolysis studies using small experimental animals are complicated by difficulties in preventing hemolysis during collecting of blood specimens and in measuring plasma hemoglobin, which may lead to incorrect evaluation in plasma hemoglobin determination. Several methods have been developed to minimize the collection artifact and to measure the low concentration of plasma hemoglobin in humans [2, 4, 6, 8, 9, 11]. However, these methods are still not sufficient for the *in vivo* hemolysis studies in small experimental animals such as guinea pigs, rats and mice, which require a repeated sampling of blood specimens and the determination of the hemoglobin concentration in small amounts of plasma sample. To solve these problems, we tried to develop the new techniques for the *in vivo* hemolysis studies in small experimental animals using guinea pigs.

MATERIALS AND METHODS

Animals: Male Hartley guinea pigs weighing 200–250 g were used. The animals were given chow pellets (CG-3

Nihon Clea, Japan) and tap water *ad libitum*, but fasted for 24 hr before the experiment, except for drinking water.

Blood collection: To increase the blood flow, the animals were warmed at 38°C with the heating plate and were applied with xylene topically on the ear before the sampling. In the preliminary experiments, the blood sampling by a syringe with a needle increased the hemolysis as technical errors. Therefore, the blood samples (50–70 μ l) were collected directly into a heparinized microhematocrit tubes (75 mm, Drummond Sci.Co., Broonall, Pennsylvania, U.S.A.), by small longitudinal incision of the auricular artery. The blood collected was centrifuged at 12,000 rpm for 5 min with the hematocrit centrifuger (Himac CT12D, Hitachi, Tokyo, Japan). The plasma (20–30 μ l) obtained was used for determination of the hemoglobin concentration.

Determination of plasma hemoglobin concentration: The hemoglobin concentration was assayed with a kit of Hemoglobin test Wako (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) by the cyanmethemoglobin method. As the kit is designed for determination of blood hemoglobin, the method was modified for the determination of the plasma hemoglobin as follows; plasma sample (10 μ l) was mixed with the reaction solution (50 μ l) and incubated for 10 min at room temperature, though in the original method the blood sample was diluted 251 times with the reaction solution. The mixture was passed through a membrane filter (0.2 μ m, LCR4-LG, Japan Millipore Ltd, Tokyo, Japan), and the filtrate was used as a sample solution. Ten microlitres of the sample solution was loaded into the following analytical system for measuring the hemoglobin concentration.

Analytical system for measuring hemoglobin concentration: Figure 1 shows a schematic diagram of the analytical system. The system is made up of a pump (655A-11, Hitachi, Tokyo, Japan), an auto injector (Sil-9A,

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Fig. 1. Schematic diagram of the apparatus for determination of plasma hemoglobin.

Shimadzu, Kyoto, Japan), a detector (SPD-10AV, Shimadzu, Kyoto, Japan) and an integrator (D-2500, Hitachi, Tokyo, Japan). Two columns of Zorbax ODS (4.6×250 mm, DuPont, Wilmington, U.S.A.) and Asahipak ODP-50 (4.6×250 mm, Asahi Chemical Industries Ltd., Tokyo, Japan) were connected between the pump and the injector, which work only as a damper for the flow pulse. Additionally, two capillary tube coils (0.3 mm ID, 100 cm length) were connected in front of and behind of the detector. Deionized water was used as a carrier solvent and the flow rate was set up at 0.5 ml/min. The hemoglobin concentration of the sample solution was determined from the peak area ($\text{mV} \times \text{sec}$) on the absorbance at 540 nm.

Preparation of hemoglobin standard: For the comparison of the present method and the modified cyanmethemoglobin method developed by Moore *et al.* [8], the hemoglobin standard in high concentration (300 mg/dl) was prepared from the hemolysate as follows; blood was collected from the saphena vein in guinea pigs using a heparinized disposable syringe, and the RBC was hemolyzed by deionized water and the ultrasonic treatments.

In vivo experimental hemolysis: Saponin (Wako Pure Chemicals Industries Ltd., Osaka, Japan) was dissolved in physiological saline and the solution was filtered through a sterile membrane filter ($0.45 \mu\text{m}$, Minisart NML, Sartorius, Goettingen, Germany). The saponin solution was injected into the saphena vein of guinea pigs, and blood samples were collected immediately before, and at 0.5, 1, 2, 3, 4 and 5 hr after the injection. Control animals were treated with physiological saline.

RESULTS

The linearity of the present method was tested with a series of dilutions of the cyanmethemoglobin standard (17.9, 35.8 and 71.6 mg/dl). Figure 2 shows the relationship between the hemoglobin concentration and the peak area ($\text{mV} \times \text{sec}$) of the present analytical system. The regression analysis revealed a line of $Y = 1.8X + 0.79$ ($r=0.999$) and the coefficient variation (CV) was less than 1% ($n=5$) throughout the entire hemoglobin concentration range: Y:

Fig. 2. Standard curve of the hemoglobin in the analytical system. Hemoglobin standard solutions: 17.9, 35.8 and 71.6 mg/dl, injection volume: $10 \mu\text{l}$, solvent: deionized water, flow rate: 0.5 ml/min, detector: 540 nm, regression line: $Y=1.8X + 0.79$ ($r=0.999$), $\text{CV}<1\%$ ($n=5$). An insert chart shows the peak area with the absorbance of each standard solution; 1: blank, 2: 17.9 mg/dl, 3: 35.8 mg/dl and 4: 71.6 mg/dl.

peak area ($\text{mV} \times \text{sec}$), X: hemoglobin concentration (mg/dl). The minimum detectable hemoglobin concentration in the system was 1.25 mg/dl.

The values of peak area depend on both the hemoglobin and the plasma background. Therefore, to correct the hemoglobin concentration in the plasma, background value should be subtracted from the measured values. Since the hemoglobin-free plasma is not available, the peak areas were investigated in 24 plasma samples which were judged visually as the hemolysis did not occur. The mean value and S.E. of the peak area in these samples was $9.61 \pm 0.06 \text{ mV} \times \text{sec}$, and the range was between 9.03 and $9.98 \text{ mV} \times \text{sec}$. The lowest value ($9.03 \text{ mV} \times \text{sec}$) was regarded as a background value.

The present method was compared with the modified cyanmethemoglobin method developed by Moore *et al.* [8], using the hemoglobin standard and the fresh pooled plasma. As shown in Fig. 3, comparable results were obtained by both methods over the range of 4.47–286.4 mg/dl. A linear regression analysis showed a good correlation ($r=0.999$) between both methods, though the concentrations in the present method were slightly higher.

The control levels of the plasma hemoglobin concentration are important in the evaluation of the hemolytic effect with agents. The plasma hemoglobin concentrations in the normal animals were determined, which were then corrected with the plasma background. Plasma hemoglobin concentrations in 52 untreated control animals are shown in Fig. 4. The mean value and S.E. of the plasma hemoglobin concentration was $7.27 \pm 0.44 \text{ mg/dl}$ and the range was between 2.75 mg/dl and 15.57 mg/dl.

Fig. 3. Plasma hemoglobin concentrations measured with the present analytical system (○) and the modified cyanmethemoglobin method (●). Hemoglobin standard: 17.9, 35.8, 71.6, 143.2 and 286. mg/dl.

Fig. 4. The plasma hemoglobin concentrations in untreated guinea pigs. Each point represents individual values of 52 animals. Mean value and S.E. are shown by the column and the bar, respectively.

In order to examine whether the present techniques were applicable or not in the *in vivo* study, the changes of the plasma hemoglobin concentrations after administration of saponin (10–50 mg/kg, i.v.) were observed in guinea pigs. As shown in Fig. 5, the plasma hemoglobin concentration before the injection was 6.53 ± 0.60 mg/dl ($n=6$), and that

Fig. 5. The changes of plasma hemoglobin concentrations after single intravenous injection of saponin or saline in guinea pigs. (○: 20, □: 30, ●: 40 and ▲: 50 mg/kg saponin, ○: saline).

in the saline control changed slightly in the range of 6.19 to 8.68 mg/dl. With saponin (30, 40 and 50 mg/kg), the plasma hemoglobin concentrations increased dose-dependently; they increased mildly during the first 30 min, thereafter increased rapidly, reached the highest concentrations at 1 hr, and gradually decreased. The concentrations were still higher than that of the saline treatment even 5 hr after the injection. With saponin of less than 20 mg/kg, the hemoglobin concentrations were in the control level.

DISCUSSION

In the present study, the novel techniques were developed for the determination of the plasma hemoglobin in guinea pigs from two points of view, i.e. how to minimize the hemolysis accident and how to measure the plasma hemoglobin concentration in small amounts of sample.

In the analytical system for measuring hemoglobin concentration, the high stability of the flow rate was essential and it was maintained by using two columns as pulse dampers. Additionally, the pressure balance in the system was obtained with the 2 capillary tube coils. These devices made it possible to get a linear relationship between the measured area and the hemoglobin concentration, and to measure the hemoglobin concentration in small amounts of sample (10 μ l).

Since the hemoglobin concentration in plasma is very low compared to that in blood, the cyanmethemoglobin method for the blood hemoglobin was modified for determination of plasma hemoglobin. The sample (10 μ l) was diluted 6 times with the reaction solution, and measured the area (mVx sec) at 540 nm with 10 μ l of the sample. Moore *et al.* [8] have proposed the modified cyanmethemoglobin method for the plasma hemoglobin, in which the plasma samples (25–300 μ l) were diluted 3.3–40

times with the reaction solution to get 1 ml of the sample for measurement with a spectrophotometer at 540 nm. The results by the two methods show that the plasma hemoglobin concentrations measured by the present method were compatible with those by Moore *et al.* In the physiological condition, the hemoglobin concentration in plasma was presumed to be almost zero. However, the normal level of the plasma hemoglobin concentration was reported to be 5–10 mg/dl, owing to the hemolysis accident during and after the blood collection [5–7, 11]. To minimize the hemolysis accident the heparin mist-sprayed tube has been recommended for blood collection in plasma hemoglobin assays [5]. Langdon and Villafranca [7] used the heparin fine mist nonsiliconed glass tubes, resulting in the normal level of 0.5–2.5 mg/dl in beagle dogs. Chalmers and Snell [2] also used the heparin Vacuette tube, resulting in the normal level of 6–35 mg/dl in humans. In the present study, the blood sample was collected directly into the heparinized microhematocrit tube by the longitudinal incision of the auricular artery, resulting in the normal level of plasma hemoglobin of 7.27 ± 0.44 mg/dl (mean $\pm$ S.E. $n=52$) in guinea pigs. This suggests that with the present techniques the hemolysis accidents were minimized as well as those of the previous methods.

In the *in vivo* hemolytic study, Copeland, *et al.* [4] reported that the hemoglobin concentration due to the collection artifact is less than 10 mg/dl in humans, and the concentration over 20 mg/dl was considered due to abnormal physiologic causes. In the present study the normal level in the guinea pigs was less than 10 mg/dl, and the plasma hemoglobin concentration increased in the range of 30 to 600 mg/dl with saponin treatment. The dose dependent hemolytic effect with saponin shows reasonable changes of the plasma hemoglobin concentrations. It is concluded that the present techniques are useful for the *in vivo* hemolytic studies in small experimental animals such as guinea pigs.

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