A Spin Filter Method for Continuous Evaluation of Hemolysis

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Abstract □ A novel method for quantifying hemolysis is described. This method uses a spin filter to separate the free hemoglobin from the red blood cells suspended in the test solution. This procedure enables the use of a closed loop system that continuously measures hemolysis spectrophotometrically. It is shown that hemolysis does not always stop after the solution has been quenched with normal saline. In fact, the process of hemolysis induced by chemicals such as potassium oleate is relatively slow.

1. Introduction

Hemolysis is defined as the destruction of red blood cells in such a manner that hemoglobin is liberated into the medium in which the cells are suspended.[1] Increases in free hemoglobin in the plasma resulting from hemolysis are associated with serious medical conditions such as renal dysfunction, splenomegaly, jaundice and kernicterus.[2]

In 1936, Wokes[3] defined the hemolyzing concentration of a solution as the concentration that will produce a supernatant liquid that is faintly pink after half of an hour of contact time with freshly shed normal human blood. This method for detecting hemolysis does not quantify the amount of hemolysis produced and is not sensitive enough to detect hemolysis that could not be seen by the naked eye. Husa and Adams[4] developed an in vitro method for evaluating hemolysis that became the standard parenteral screening method for many years. This method involves the incubation of red blood cells with a large excess of test solution. In this method the supernatant is measured for absorbance at 540 nm (the absorbance maximum for hemoglobin). The use of a spectrophotometer provides a sensitive method for quantifying the extent of hemolysis. Although the Husa and Adams method is generally quite useful, three main problems have been noted.[5] The first problem with this procedure is that the presence of nonaqueous solvents and metal ions produce changes in the absorbance spectrum of hemoglobin and therefore can give inaccurate results. The second problem with this method is that the formulation:blood ratio of 50 is unrealistically high for modeling intravenous injections. The third problem is that this method used a very long contact time (30 min) of formulation with blood before separation and analysis of hemoglobin. This contact time does not mimic the dilution that occurs naturally after injection of the formulation into the human vein.

In 1985, Reed and Yalkowsky[6] developed a more physiologically realistic model using a formulation:blood ratio of 0.1 and a 2-min contact time of blood with the test solution. However, this contact time is still too long to accurately model hemolysis that occurs after an intravenous injection.[2] Reed and Yalkowsky also addressed the issue of alterations in the absorbance spectrum of hemoglobin caused by the presence of nonaqueous solvents and metal ions that are sometimes present in parenteral injections by washing the red blood cells and ghosts that remained after mixing the test solution with blood. The intact red blood cells were then lysed with water, and the fraction of healthy cells was determined. This procedure, which is analogous to a back-titration, allowed all spectral analysis of hemoglobin to be performed in a purely aqueous medium.

Obeng and Cadwallader[7] were the first to develop a dynamic method that mimics hemolysis occurring in the body following an intravascular injection. Their method utilizes an in vitro flow system that provided a reasonable simulation of the mixing that occurs at the intravenous injection site, taking into consideration tubing diameter, blood flow rate, injection volume, and injection time. In this method, Obeng and Cadwallader injected the formulation into a flowing stream of red blood cells. Then the formulation and blood mixture entered a large volume of saline, which was assumed to quench the hemolytic reaction. The saline mixture was then centrifuged and the supernatant was analyzed with spectrophotometer. Although this method is capable of measuring the effect of contact time, this was not done to any significant extent. Krzyzaniak and coworkers[8] developed a dynamic in vitro method that is able to quantify hemolysis at short contact times (1 s) with a formulation:blood ratio of 0.1. This contact time of 1 s provides a more physiologically realistic model of the injection site.

Both the Reed and Yalkowsky and the Obeng and Cadwallader methods assume that quenching the test solution with saline stops the hemolytic reaction. However, some red blood cells that are slightly damaged could still break some time after the solution has been quenched. This reaction could not be detected by the previous methods because all other methods quantify hemolysis at one point of time after the reaction has been quenched. Another problem with all previous methods is that they determine the degree of hemolysis at a relatively long time after quenching because of a lengthy centrifugation step. They cannot be used to evaluate the very early reaction between red blood cells and the test solution.

The aim of this investigation is to develop a method for quantifying hemolysis that is physiologically realistic and that is able to evaluate the very early reaction between red blood cells and the test solution.

2. Materials and Methods

2.1 Materials—The blood used in this investigation was obtained from the American Red Cross (Tucson, AZ). Sodium lauryl sulfate, (SLS), poly(ethylene glycol) 400 (PEG 400), propylene glycol (PG), and sodium chloride were purchased from Sigma Chemical Company. Tween 80, glycerine, potassium oleate, and

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Table 1—Tested Compounds and Their Concentrations

compound	concentration (% w/v in normal saline
potassium oleate	1
sodium lauryl sulfate (SLS)	1
polysorbate 80 (Tween 80)	12
polyethylene glycol 400 (PEG 400)	50
propylene glycol (PG)	50
glycerine	50

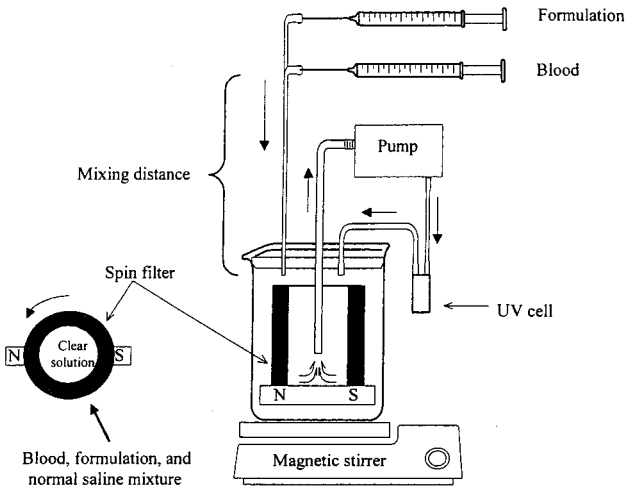

Figure 1—The spin filter apparatus for evaluating hemolysis.

red food color (red dye) were purchased from Biotech, Mallinckrodt, TCI America, and McCormick & Company Inc., respectively. All chemicals were used as received. These compounds were chosen because they are commonly used in pharmaceutical formulations. Each of these were admixed with normal saline to the concentration listed in Table 1.

2.2 Methods—*2.2.1. Spin Filter Method*—Figure 1 illustrates the experimental apparatus. Whole blood was washed three times with normal saline to remove the damaged red blood cells, buffy coat, and free hemoglobin. The washed red blood cells were reconstituted to a 40% hematocrit with Sorensen phosphate buffer (pH 7.4). The intact red blood cells were then pumped through Tygon tubing (1/32 in. i. d.) with a syringe pump. The vehicles were injected into the blood flow with the aid of syringe pump. The formulation-blood mixture at the ratio of 0.1 remained in contact for one second. These formulation:blood ratio and contact time were obtained by a blood flow rate of 6 mL/min, a formulation flow rate of 0.6 mL/min, and a mixing distance of 5 cm in the approximately 1 mm i. d. tubing. The mixture then was diluted with a large amount of normal saline. The normal saline containing the formulation:blood mixture was continuously sampled with a centrifugal filtering system (0.3 μm Whatman, Gamma-12 high efficiency in-line units) as described by Shah.[9] The spin filter allows only the contents of lysed red blood cells mixed with normal saline to enter the spectrophotometer. The spin filter, which rotates at a speed of ~142 rpm, throws off by centrifugal force erythrocytes, ghosts, and other matter that would clog a stationary filter. Using this spin filter apparatus, the supernatant and the cells are rapidly separated without centrifugation. The filtered solution was then allowed to circulate through the spin filter until the hemolytic reaction has terminated. Red dye was used to determine the lag-time in this method. A pump was used to circulate the solution through the spin filter and flow cell in the spectrophotometer. Hemolysis was then calculated with the following formula:

$$\%\text{Hemolysis} = \frac{A_F - A_0}{A_{100} - A_0} \times 100 \qquad (1)$$

where A_F = absorbance of hemoglobin in the filtered solution after injecting test solution, A_0 = absorbance of hemoglobin in the filtered solution after injecting normal saline, and A_{100} = absorbance of hemoglobin in the filtered solution after total cell lysis with water. Because the filtered solution is continuously sampled, a percent hemolysis versus time profile is generated. This profile describes both the extent of hemolysis and the time frame of the hemolytic reaction.

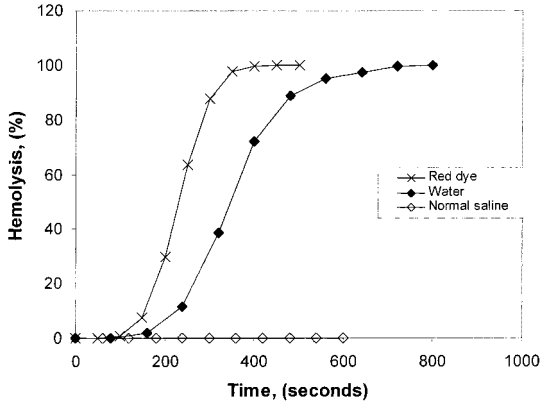

Figure 2—Percent hemolysis versus time profile for the blanks used on the spin filter method; that is, normal saline and water. The Y axis for red dye was obtained considering its absorbance at the plateau as 100% hemolysis and eq 1.

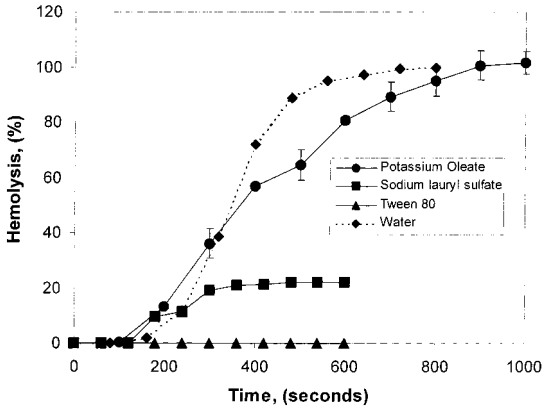

Figure 3—Hemolysis induced by surfactants.

3. Results

Figure 2 shows the percent hemolysis versus time profile for normal saline and water. The Y axis data for the red dye were obtained by the normalization of the absorbance data at 540 nm with the absorbance at t_{100} (A_{100} or absorbance at the plateau) and eq 1. All samples were run in triplicate at room temperature.

The six pharmaceutical vehicles were divided into two categories, surfactants and cosolvents. The first category includes SLS, potassium oleate, and Tween 80. The second category includes PG, PEG 400, and glycerine. The percent hemolysis produced by each vehicle at each time point was measured three times and their mean and standard deviation was determined. A cumulative graph of the percent hemolysis versus time for the surfactants and cosolvents are presented in Figures 3 and 4, respectively.

4. Discussion

Red dye data (Figure 2) indicate that the spin filter method has a lag-time of ~100 s. This figure also shows 0% hemolysis at 600 s for normal saline, indicating that red blood cells are not destroyed during the experiment. Interestingly, this figure shows that the hemolysis produced by water is a time-dependent process. The time to reach 100% hemolysis is ~700 s (almost 2 min longer that

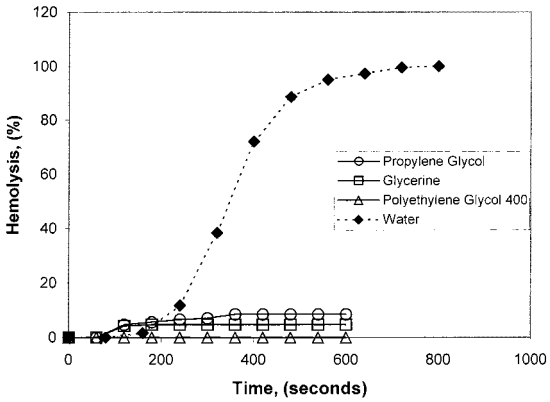

Figure 4—Hemolysis induced by cosolvents.

the red dye). These data indicate that hemolysis is not stopped instantaneously by quenching, and that the spin filter method is able to measure both the extent of hemolysis and the time required for the hemolysis process to occur.

Figures 3 and 4 show that the hemolytic reactions for the cosolvents PG and glycerine are less complete and more rapid than the reactions produced by the surfactants potassium oleate and SLS, at the tested concentrations. Also, these figures indicate that the cosolvent PEG 400 and the surfactant Tween 80 are not hemolytic, at the tested concentrations, which is consistent with the results of previous investigations.[2,10]

Figure 3 shows that the hemolytic reaction produced by potassium oleate is slower than the reaction produced by water. Potassium oleate takes longer to produce complete hemolysis than does water. However, Figure 3 shows that potassium oleate is as hemolytic as water at longer times. In addition, Figure 3 shows that although SLS is less hemolytic than water, it reaches its maximum value in less time than does water.

5. Conclusions

Unlike other methods, the spin filter method for evaluating hemolysis provides a means of assessing hemolysis that occurs after quenching. If it is assumed that the mixture of the blood from an injected vein with blood from other parts of the body is equivalent to quenching of hemolysis, then the spin filter method provides a more realistic simulation of the process of hemolysis, as it would occur in the body following an intravascular injection, than other methods. This method is able to determine not only the extent of hemolysis but also the estimated time frame of the reaction.

References and Notes

1. *Stedman's Medical Dictionary*, 26th ed.; Spraycar M., Ed.; Williams & Wilkins: Baltimore, MD; 1995; p 780.
2. Krzyzaniak, J. F.; Raymond D. M.; Yalkowsky, S. H. Lysis of human red blood cells 2: Effect of contact time on cosolvent induced hemolysis. *Int. J. Pharm*. **1997**, *152*, 193−200.
3. Wokes, F. Isotonic solutions for injection. *Quart. J. Pharm. Pharmacol.* **1986**, *9*, 455−459.
4. Husa, W.; Adams, J. R. Isotonic solutions. II. The permeability of red corpuscles to various substances. *J. Am. Pharm. Assoc.* **1944**, *33*, 329−332.
5. Ward, G. H.; Yalkowsky, S. H. The role of the effective concentration in interpreting hemolysis data. *J. Parenteral Sci. Technol*. **1992**, *46*, 161−162.
6. Reed, K. W.; Yalkowsky, S. H. Lysis of human red blood cells in the presence of various cosolvents. *J. Parenteral Sci. Technol*. **1985**, *39*, 64−68.
7. Obeng, E. K.; Cadwallader, D. E. In vitro dynamic method for evaluating the hemolytic potential of intavenous solutions. *J. Parenteral Sci. Technol*. **1989**, *43*, 167−172.
8. Krzyzaniak, J. F.; Raymond, D. M.; Yalkowsky, S. H. Lysis of human red blood cells 1: Effect of contact time on water induced hemolysis. *PDA J. Pharm. Sci. Technol*. **1996**, *50*, 223−226.
9. Shah, A. C.; Peot, C. B.; Ochs, J. F. Design and evaluation of a rotating filter-stationary basket in vitro dissolution test apparatus I: Fixed fluid volume system. *J. Pharm. Sci.* **1973**, *62*, 671−676.
10. Krzyzaniak, J. F.; Yalkowsky, S. H. Lysis of Human Red Blood Cells 3: Effect of Contact Time on Surfactant-Induced Hemolysis. *PDA J. Pharm. Sci. Technol.* **1998**, *52*, 66−69.

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