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Lysis of human red blood cells 2: effect of contact time on cosolvent induced hemolysis

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Abstract

The degree of hemolysis induced by several cosolvent formulations is evaluated at various contact times using the dynamic in vitro method developed by Krzyzaniak (Krzyzanik, J.F., Raymond, D.M. and Yalkowsky, S.H., Lysis of Human Red Blood Cells 1: Effect of Contact Time on Water Induced Hemolysis. PDA J. Pharm. Sci. & Tech., 50 (1996) 223–226). Hemolysis is shown to increase with cosolvent concentration and to be sigmoidally related to the logarithm of the formulation:blood contact time. With this information, a physiologically realistic in vitro method with a formulation:blood ratio of 0.1 and a contact time of 1 s has been developed and used to estimate the amount of hemolysis occurring after an intravenous injection of some commonly used cosolvent formulations. © 1997 Elsevier Science B.V.

Keywords: Cosolvent; Dynamic; Hemolysis; In vitro

1. Introduction

Cosolvents are commonly used in intravenous formulations to increase the solubility of poorly water soluble drugs. When using cosolvents, there is a risk of lysis of red blood cells with the subsequent release of hemoglobin and other cellular components into the blood stream. The increase of free hemoglobin in the plasma is associated with many undesirable medical conditions including: renal dysfunction (Lucké, 1946; Lalich, 1955; Jaenike, 1966; Myers et al., 1966; Birndorf and Lopas, 1970; Saitoh et al., 1993), splenomegaly (Berkow, 1992), jaundice and kernicterus (Berkow, 1992). Therefore, the potential of a cosolvent vehicle to induce intravascular hemolysis should be determined prior to its clinical administration.

When developing an in vitro method to evaluate intravascular hemolysis, the dynamics of the intravenous injection must be considered. As a

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cosolvent formulation is injected into a vein, the formulation is rapidly mixed with blood resulting in a decrease in the cosolvent concentration. The concentration of the cosolvent in the blood after the injection is dependent on its concentration in the formulation and the ratio of injection rate to the blood flow rate at the injection site. The formulation and blood remain in contact at this ratio until the vein in which the injection occurred is joined by other veins, after which, the formulation:blood ratio progressively decreases until the formulation and its cosolvent components are completely diluted by the total volume of blood in the body.

The amount of hemolysis occurring after an injection is dependent on the hemolytic nature of the cosolvent, its concentration in the blood after the initial dilution, and the time it takes for further dilution to occur. For a formulation of a fixed composition, hemolysis is dependent only upon the formulation:blood ratio and upon the time it takes for the formulation to be diluted enough so that it becomes non-hemolytic.

The two analogous in vitro parameters that are typically used to describe the hemolysis produced by a particular formulation are the formulation:blood ratio and the formulation:blood contact time. Several in vitro methods have been developed to evaluate hemolysis in which these parameters are specified (Husa and Adams, 1944; Grosicki and Husa, 1954; Fort et al., 1984; Al-Assadi et al., 1989; Obeng and Cadwallader, 1989; Lowe et al., 1995). Neither the large formulation:blood ratios nor the long contact times commonly used in these methods are able to simulate the brief contact time of the diluted formulation components with blood that is associated with an intravenous injection.

In an attempt to develop a more physiologically realistic model, Reed and Yalkowsky (1985) first determined that in vitro hemolysis is dependent on the formulation:blood ratio. They developed an in vitro method using a formulation:blood ratio of 0.1 and a relatively short 2 min contact time to evaluate the hemolytic potential of pharmaceutical cosolvents. While this formulation:blood ratio is reasonable, the 2 min contact time is still too long to accurately model hemolysis occurring after an intravenous injection. The aim of this study is to determine the dependence of contact time upon cosolvent induced hemolysis and to develop a realistic in vitro method for its evaluation.

2. Materials and methods

The percent hemolysis produced by various formulations was determined using the apparatus and procedure developed by Krzyzaniak et al. (1996). A brief description of this method is given below.

2.1. Reagents

Cosolvents commonly used in parenteral formulations were used to prepare each test solution. Ethanol was purchased from Quantum Chemical Co. (Anaheim, CA). Propylene glycol, glycerin, and polyethylene glycol (PEG 300, PEG 400, PEG 3350), were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium Phosphate Dibasic (Na_2HPO_4), Sodium Phosphate Monobasic (NaH_2PO_4), and Sodium Chloride were also purchased from Sigma Chemical Co. (St. Louis, MO) and used to prepare the Sorensen's phosphate buffer. All chemicals were used as received.

2.2. Reconstituted blood

Expired human red blood cells were obtained from the American Red Cross, washed with normal saline, and reconstituted to a 40% hematocrit with isotonic Sorensen's phosphate buffer (pH 7.4).

2.3. Test solutions

Each test solution was prepared by adding distilled water to the appropriate volume of cosolvent to yield either an iso-osmotic or a hypertonic solution. In order to avoid hemolysis due to osmotic effects which are independent of the hemolytic potential of the cosolvent, hypotonic test solutions were used. The role of osmotic pressure in producing hemolysis has previously been described (Krzyzaniak et al., 1996).

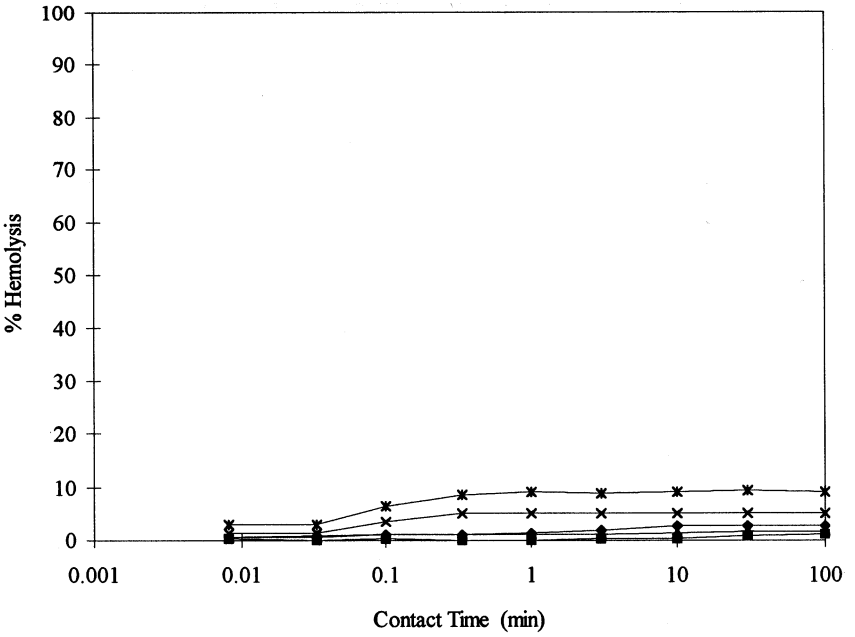

Fig. 1. The effect of contact time on hemolysis induced by five concentrations of ethanol at a ratio of 0.1 ml of test solution to 1 ml of blood. Percentage ethanol (v/v): (◆) 1.6, (■) 10, (▲) 50, (×) 70 and (*) 90.

2.4. In vitro hemolysis testing

In general, a test solution was injected at a predetermined rate into a constant flow of reconstituted blood. The formulation and blood remained in contact for a given amount of time until the hemolytic reaction was quenched by dilution with normal saline. An aliquot of the quenched sample was transferred to a test tube and centrifuged at 3000 rpm for 10 min. The absorbance of hemoglobin in the supernatant was measured at 540 nm using a spectrophotometer. Percent hemolysis was calculated from the absorbances of the sample, normal saline (a non-hemolytic blank), and water (a positive control) as described by Krzyzaniak et al. (1996).

The procedure described above was used to determine the effect of contact time on cosolvent induced hemolysis at a formulation:blood ratio of 0.1 and contact times ranging from 0.5 s to 6000 s (100 min). A formulation:blood ratio of 0.1, i.e. one part formulation to ten parts blood, was used in this study since it has previously been suggested to be a reasonable ratio for describing the initial dilution of a formulation by blood that accompanies an intravenous injection (Reed and Yalkowsky, 1985; Ward and Yalkowsky, 1992). With the use of this ratio, the injection of a 40% cosolvent solution will produce a 3.6%-cosolvent solution in blood. At the end of the designated contact time, this mixture was diluted approximately one hundred fold with normal saline to quench further hemolysis. Note that the final volume of both the formulation and blood in the quenching solution was kept constant for each contact time.

The contact time was varied by adjusting the formulation injection rate, the blood flow rate, and the volume of the mixing tube. For example, the following experimental conditions were used to achieve a formulation:blood ratio of 0.1 and a 0.5-s contact time: test solution flow rate of 0.6 ml/min, blood flow rate of 6 ml/min, tube diameter of 1/32 inch I.D. and a tube length of 5 cm. Note that many combinations of the injection rate, flow rate, and volume of the mixing tube can produce the same contact time and formulation:blood ratio.

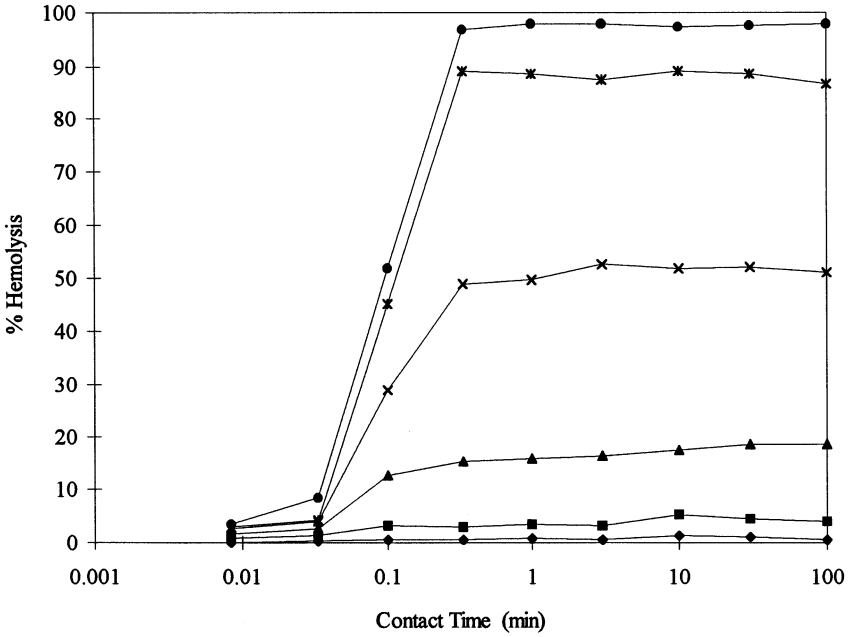

Fig. 2. The effect of contact time on hemolysis induced by six concentrations of propylene glycol at a ratio of 0.1 ml of test solution to 1 ml of blood. Percentage propylene glycol (v/v): (◆) 2.1, (■) 20, (▲) 30, (×) 40, (∗) 50, and (●) 60.

3. Results and discussion

3.1. Effect of contact time on hemolysis

The hemolysis induced by solutions containing ethanol, propylene glycol, glycerin, and polyethylene glycol 300 at contact times ranging from 0.5 s to 6000 s are given in Figs. 1–4, respectively. It is clear from these figures that hemolysis is sigmoidally dependent on the logarithm of contact time. Each sigmoidal curve can be characterized by a minimum hemolysis occurring at short contact times and a maximum hemolysis occurring at long contact times. The minimum and maximum hemolytic potentials are shown to be dependent on the cosolvent used as well as its concentration in the formulation.

In vitro methods which use a long contact time will generally determine hemolysis at the plateau corresponding to maximum hemolysis. For example, hemolysis is at a maximum value at all contact times above 1 min for all of the ethanol, propylene glycol, and glycerin solutions as shown in Figs. 1 and 2, and Fig. 3, respectively. The percent hemolysis determined at this plateau is not representative of the amount induced at the injection site since the contact time of the initially diluted cosolvent sample with blood is brief during injection, and the cosolvent is rapidly diluted with the total volume of blood in the body.

From the above discussion, it is clear that in vitro methods which use a large formulation:blood ratio and a long contact time overestimate the hemolytic potential of the cosolvent system. Since cosolvent induced hemolysis is dependent on the cosolvent concentration in the formulation, formulation:blood ratio, and the formulation:blood contact time before complete dilution, such methods are therefore unable to accurately evaluate hemolysis occurring after an injection. The inability of these methods to accurately evaluate intravenous hemolysis is confirmed by the data of Fort et al. (1984). Their study shows the comparison of in vivo hemolysis data to in vitro hemolysis data obtained from a method which uses a formulation:blood ratio of 100, i.e. 5 of test solution to 0.05 ml heparinized dog blood, and a contact time of 20 min.

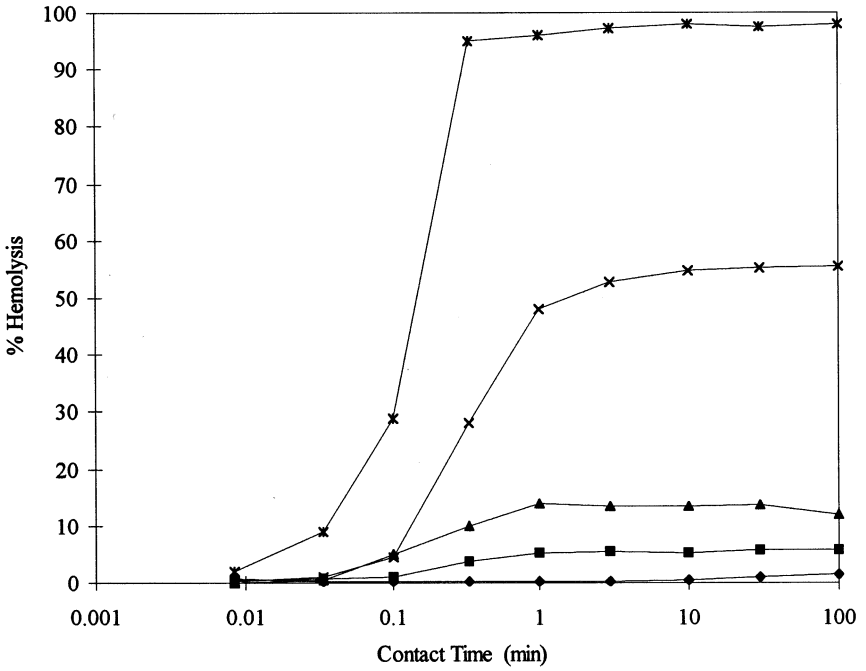

Fig. 3. The effect of contact time on hemolysis induced by five concentrations of glycerin at a ratio of 0.1 ml of test solution to 1 ml of blood. Percentage glycerin (v/v): (◆) 2.0, (■) 10, (▲) 15, (×) 20 and (∗) 50.

Note that test solutions were considered non-hemolytic when the percent hemolysis determined by their in vitro method was less than 20%. Since their comparison results in a false-positive result for all non-hemolytic cosolvent formulations tested, cosolvent induced hemolysis must be evaluated using a more physiologically realistic formulation:blood ratio and contact time.

Although in vitro methods which use large formulation:blood ratios and long contact times are unable to accurately model an intravenous injection, they are potentially useful as an indication of cell damage resulting from intramuscular injections. This is because the muscle cells remain in contact with the undiluted formulation for a prolonged period of time. The muscle damaging potential and physico-chemical properties of many injectables are studied by Oshida et al. (1979). In their study, an in vitro method with a formulation:blood ratio of 10 and a contact time of 30 min is used to determine the presence of hemolysis. A strong correlation between hemolysis and muscle lesions is indicated after evaluating the toxic effects of several parenteral formulations.

3.2. Hemolytic potentials of cosolvents

A dramatic increase in hemolysis is observed with increasing contact time for each of the cosolvents studied as indicated in Table 1. The data show that at all contact times glycerin is the most hemolytic followed by propylene glycol, PEG 300, and ethanol. However, no difference in the hemolytic potential is observed for ethanol and polyethylene glycol 300 at a 1-min contact time. The data in this table also shows that 50% solutions of ethanol and PEG 300 are almost non-hemolytic at a contact time of 1 min or less while 50% propylene glycol is only minimally hemolytic at a 1-s contact time. This is consistent with the fact that 40% propylene glycol is commonly used in marketed intravenous products. Note that the results of testing a propylene glycol solution at long contact times suggest that propylene glycol is unacceptable for intravenous injection.

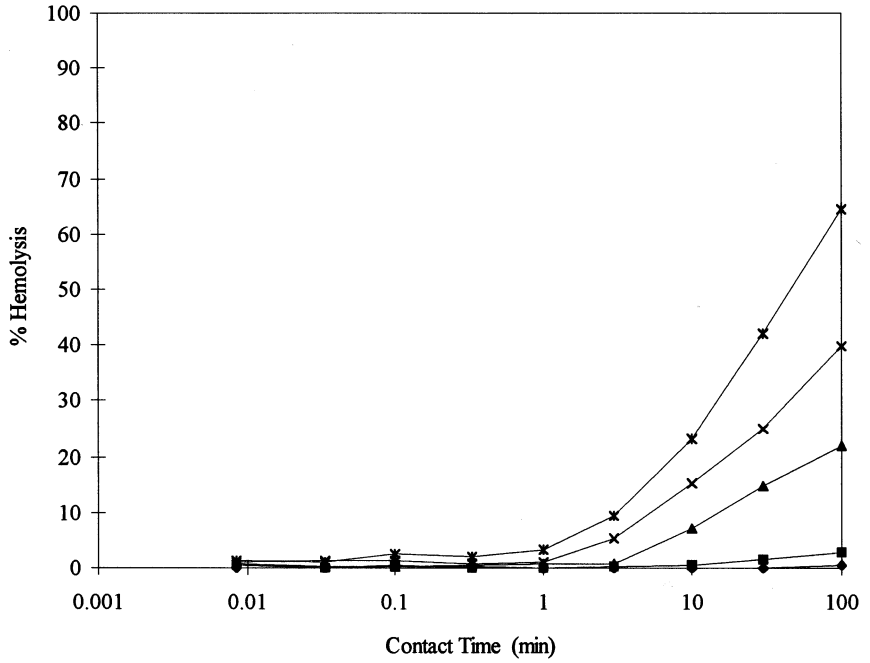

Fig. 4. The effect of contact time on hemolysis induced by five concentrations of polyethylene glycol 300 at a ratio of 0.1 ml of test solution to 1 ml of blood. Percentage polyethylene glycol 300 (v/v): (◆) 7.5, (■) 20, (▲) 40, (×) 60 and (∗) 80.

The degree of hemolysis induced by 40% solutions of PEG 300, PEG 400, and PEG 3350 at various contact times is given in Fig. 5. From Fig. 5, PEG 300 can be seen to be the most hemolytic while PEG 3350 is the least hemolytic. Interestingly, the PEG 300 solution is more hemolytic than the PEG 400 solution on an equimolar basis. The hemolytic potentials could not be distinguished for equimolar PEG 400 and PEG 3350 because both test solutions induce very little hemolysis at all contact times. It is important to note that all three of the polyethylene glycols are non-hemolytic at short contact times.

4. Conclusion

As previously discussed, a physiologically realistic formulation:blood ratio and contact time should be used to evaluate intravascular hemolysis. When using this method, hemolysis can be evaluated during the short period of time in which the initially diluted formulation components mix with blood, and before they are further diluted by the general circulation. It should be noted that the formulation:blood ratio and contact time will depend on many factors including the site of injection and physical condition of the subject.

Table 1
Percent hemolysis induced by 50% (v/v) cosolvent in water

Contact time	Ethanol	PEG 300	Propylene glycol	Glycerin
1 s	<1	<1	3	5
1 min	1	1	88	96
100 min	2	28	87	98

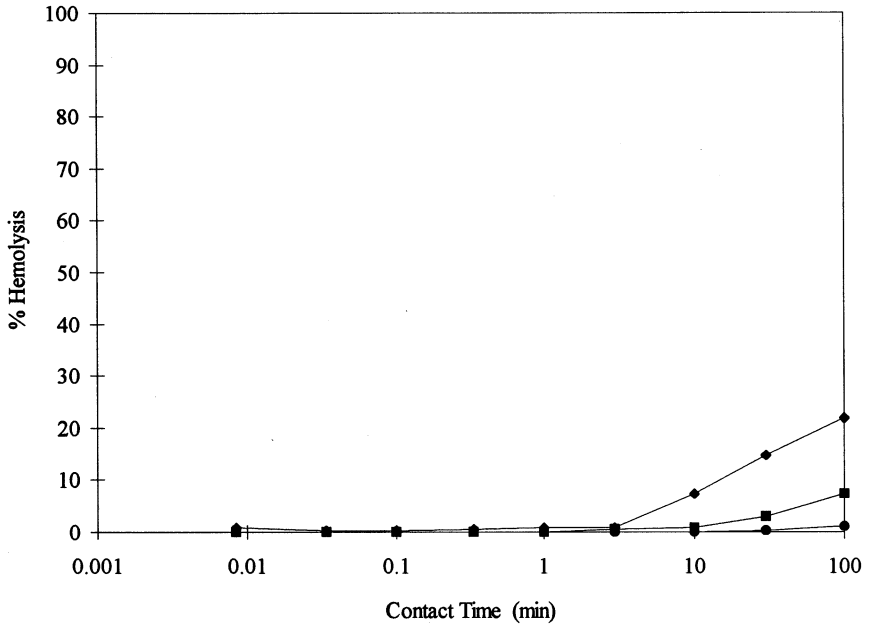

Fig. 5. The effect of contact time on hemolysis induced by polyethylene glycols 300, 400, and 3350 at a ratio of 0.1 mL of test solution to 1 mL of blood. 40% Polyethylene glycol (v/v): (◆) PEG 300, (■) PEG 400 and (●) PEG 3350.

Although no single formulation:blood ratio and contact time is appropriate for all in vivo situations, the degree of hemolysis occurring in vitro with a ratio of 0.1 and a contact time of about 1 s is believed to a reasonable approximation for hemolysis occurring in the human after an injection. This ratio would represent 1 mL of a formulation injected over 15 s, i.e. injection rate of 4 ml/min, into a vein with a blood flow of 40 ml/min. A 1-s contact time corresponds to the time in which the formulation becomes non-hemolytic as it is rapidly diluted in the blood stream.

The hemolytic potential of several pharmaceutical vehicles intended for intravenous administration has been determined using the ratio and contact time described above (Table 1 and Figs. 1–5). This data indicates that the cosolvent formulations used in this study are generally non-hemolytic which is consistent with the fact that they are commonly used in marketed intravenous formulations.

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