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RESEARCH ARTICLE

Comparative Hemolytic Activity of Undiluted Organic Water-Miscible Solvents for Intravenous and Intra-Arterial Injection

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ABSTRACT: In humans, nonaqueous solvents are administered intravascularly in two kinds of situations. They have been used in subcutaneous or intramuscular pharmaceutical formulations to dissolve water-insoluble drugs. The need for these vehicles had increased in recent years, since the drug development process has yielded many poorly water-soluble drugs. The use of water-miscible nonaqueous solvents is therefore one of the approaches for administering these products as reference solutions useful in formulation bioequivalence studies.

The intravascular use of organic solvents has also gained importance owing to a new approach for the treatment of cerebral malformations using precipitating polymers dissolved in water-miscible organic solvents. At present, the solvent most commonly used for the liquid embolics to solubilize the polymers is dimethyl sulfoxide, which exhibits some local and hemodynamic toxicities. In order to find new, less toxic vehicles for pharmaceutical formulations for the intravenous and intra-arterial routes and for embolic materials, 13 water-miscible organic solvents currently used (diluted with water) for pharmaceutical applications, were evaluated in this study. Their hemolytic activity and the morphological changes induced when mixed with blood (1:99, 5:95, 10:90 solvent:blood) were estimated *in vitro*. From these data, the selected organic solvents could be subdivided into four groups depending on their hemolytic activity: very highly hemolytic solvents (ethyl lactate, dimethyl sulfoxide), highly hemolytic solvents (polyethylene glycol 200, acetone), moderately hemolytic solvents (tetrahydrofurfuryl alcohol, N-methyl-2-pyrrolidone, glycerol formal, ethanol, Solketal, glycofurol) and solvents with low hemolytic activity (propylene glycol, dimethyl isosorbide, diglyme).

KEYWORDS: hemolysis, hemocompatibility, blood, intravenous, intra-arterial, intravascular injection, water-miscible organic solvents, parenterals

Introduction

Nonaqueous solvents are useful vehicles in intravascular formulations, because they permit the formulator to develop parenteral solutions of poorly soluble drugs.

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Their usefulness has been reinforced with the present development of combinatorial chemistry and high throughput biological screening leading to new more lipophilic and less water-soluble chemicals. In addition, efforts at the early discovery stage have concentrated on optimizing *in vitro* activity of the chemicals instead of the physicochemical properties of the compounds. As a result, many discovery compounds are poorly water-soluble (1,2). Therefore, the use of water-miscible nonaqueous solvents is one of the approaches for the administration of poorly water-soluble drugs, and

for the development of intravenous formulations useful as reference solutions for determining bioavailability of orally administered chemicals.

The selected organic solvents must be nonsensitizing, nonirritating, nontoxic, miscible with water and body fluids, must not exert any pharmacological activity, and the viscosity should allow for easy injection. Moreover, pharmaceutical formulations injected intravenously or, more rarely, intra-arterially, e.g., for contrast media, must be hemocompatible. No such solvent presently exists, and the choice of a non-aqueous solvent for parenteral use is then a compromise among these many factors.

The use of organic solvents, up to concentrations of 100%, has been described in intramuscular or subcutaneous pharmaceutical injectable formulations for dissolving water-insoluble drugs like digoxin, diazepam, or steroids (3,4). Since the early '90s, preparations containing organic solvents have gained importance for the treatment of vascular disorders such as aneurysms or arteriovenous malformations. This new approach is based on the endovascular embolization of these malformations with precipitating polymers dissolved in water-miscible organic solvents (5-8). Dimethyl sulfoxide is commonly used in these preparations as a solvent, but has been reported as toxic for intravascular application due to its local dose-related side effects on animal vessel and pig brain tissue (9-11), as well as its systemic effects such as a cardiovascular toxicity (12-14). A preliminary trial conducted in our laboratory has highlighted its strong hemolytic activity.

In a previous study (15), the hemodynamic toxicity of 13 water-miscible organic solvents diluted with water for use in pharmaceutical injectable formulations was evaluated in order to find new organic solvents useful for dissolving embolic materials and less toxic than dimethyl sulfoxide. However, no local hemotoxicity of the solvents was conducted during this study and no comparative results are available in the literature.

It is the purpose of this paper to investigate the hemotoxicity of selected water-miscible solvents as possible vehicles for pharmaceutical formulations for the intravenous and the intra-arterial routes, by determining their hemolytic activity and their ability to induce morphological changes in the erythrocytes. The hemolysis estimation was conducted using a method adapted from Husa et al. (16,17) measuring the release of oxyhemoglobin from damaged erythrocytes (18,19), and assuming that the amount of oxyhemoglobin in serum is directly proportional to the degree of lysis of the red blood cells. Morphological changes were also investigated on blood-smears after mixing various amounts of solvents with blood. Note that, in this study, all the solvents were tested undiluted with saline or water.

Materials and Methods

Organic Solvents

The solvents were selected based on their suitable viscosity for injection through needles and microcatheters, their ability to solubilize polymeric materials, as well as their previous use, at least at low concentration, in injectable formulations (20-23).

Ethyl lactate (EtL), dimethyl sulfoxide (DMSO), tetrahydrofurfuryl alcohol (THFA), N-methyl-2-pyrrolidone (NMP), glycerol formal (GF), ethanol (EtOH), Solketal™, acetone and diglyme were purchased from Fluka (Buchs, Switzerland). Glycofurol 75 was a gift from Hoffman-la-Roche (Basle, Switzerland) and dimethyl isosorbide (DMI) was supplied by ICI Chemicals (Essen, Germany).

Propylene glycol (PG) and polyethylene glycol 200 (PEG 200) (Fluka, Buchs, Switzerland) were added to this study due to their current use in pharmaceutical parenteral formulations (3,24,25), even if they have been shown to be unable to dissolve polymers for embolization.

Saline solution (sodium chloride 0.9%) and distilled water were used as reference values.

Determination of the Hemolytic Activity

Blood samples were taken in the morning from a fasted human volunteer (blood group O, Rh +) using 4 ml lithium-heparin tubes and a nonvacuum mode.

The solvents were mixed with blood in 1:99, 5:95, and 10:90 ratios into Eppendorf cups to a final volume of 400 ml using a Vortex. The mixtures were then incubated for 90 min in a water bath at 37° C. Cell corpuscular debris were pelleted by centrifugation of 10 min at 1000 rpm (18). The release of oxyhemoglobin from damaged RBC was

assayed after diluting 100 ml in NaCl 0.9% to be in the absorbance range of 0.1 to 1.0 at 415 nm using a Perkin Elmer spectrophotometer lambda bio 10 (Überlingen, Germany). The results are expressed as mean SEM of six determinations.

As 100% reference value, the total hemolysis after mixing blood with distilled water in the 1:9 ratio was determined using the same methodology. Pure blood (control) and sodium chloride 0.9% mixed with blood in a 9:1 ratio were added to the study in order to eliminate any error due to intrinsic erythrocyte fragility.

Data were statistically analyzed using a Student's t-test (unpaired samples) for comparison between the results obtained with the control and between solvent concentrations.

Morphological Evaluation

Solvents were mixed in 1:99, 5:95 and 10:90 ratios with fresh healthy donor blood samples using sterile potassium-EDTA tubes. Immediately after mixing with blood by inverting the tubes, blood-smears were made by spreading one drop on a slide and spread, then stained with the Giemsa solution.

Blood cells morphology, especially the red blood cells (RBC), was determined using a Nikon Optiphot-2 microscope (Tokyo, Japan) with an oil immersion 100/1.25 objective.

Results and Discussion

Determination of the Hemolytic Activity

To determine the RBC lysis, investigators have commonly used the method developed by Husa et al. (16,17,26-34) using large volumes of solutions containing organic water-miscible solvent diluted with saline mixed with small volumes of blood (about 99:1 ratio), which do not reflect the conditions found in in vivo intravascular administration. To compensate for the rapid dilution of solvents in blood when injected intravascularly, Reed et al. (19,35) as well as Stenz et al. (18), modified Husa's method. They utilized the same hemoglobin analysis procedure, which determines the amount of oxyhemoglobin released in the plasma as a direct function of the number of cells hemolyzed, but used solvent-blood ratios of 1:1 to 1:9.

To our knowledge, hemolytic activity of undiluted organic solvents has been rarely reported in the literature. Investigations commonly deal with binary solvent systems with water and many of them have highlighted the difficulty in preventing hemolysis even when iso-osmotic concentrations of undiluted solvents were used. These studies have also confirmed that the addition of sodium chloride in the binary solutions frequently prevents RBC hemolysis. As an example, DMSO, PEG 200, PG and EtOH give 100% hemolysis even when used at iso-osmotic concentrations in aqueous solutions (4,16,28,30-34). Addition of sodium chloride in these aqueous solutions prevented hemolysis for concentrations up to 40% DMSO, 25% PEG 200, 32% PG and 10% EtOH. All these studies were conducted according to Husa's method which used solvent-blood ratios from 95:5 to 99:1, not adapted to the conditions of in vivo intravascular administration.

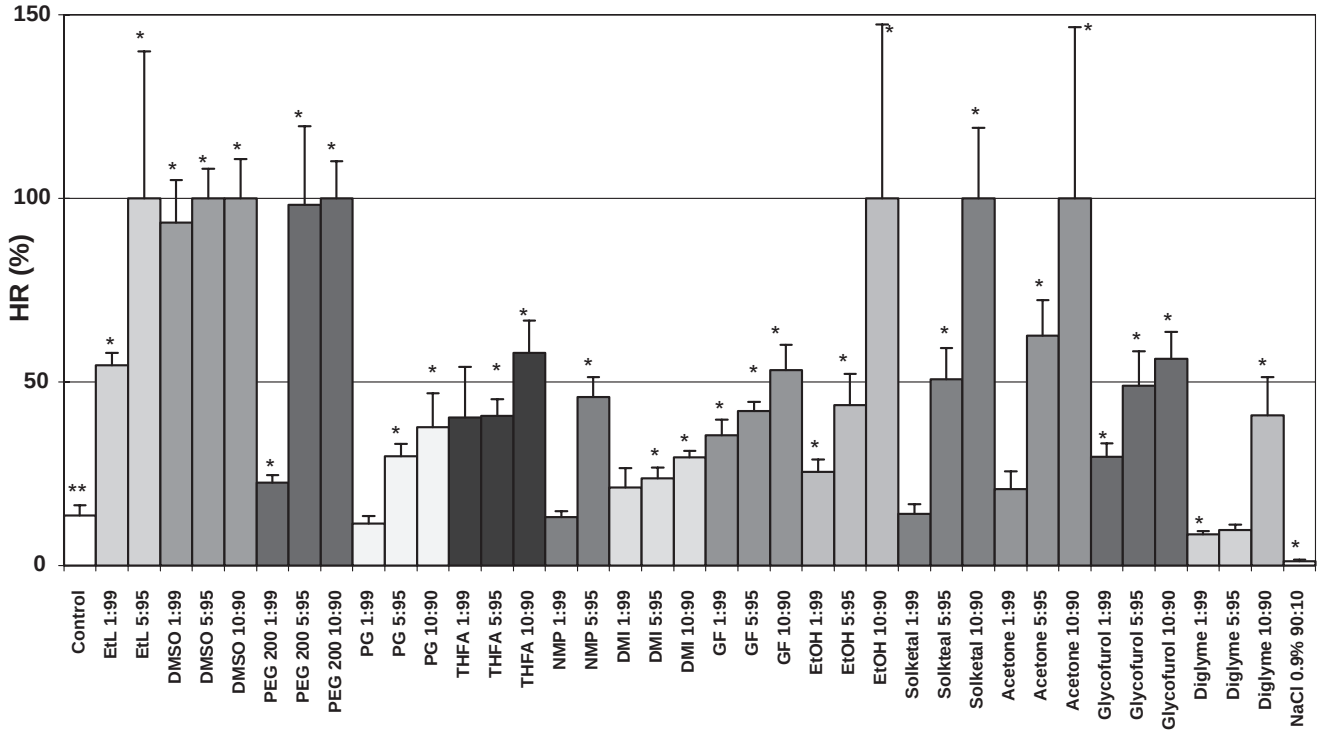
Reed and coworkers (19,35) have reported the hemolytic activity of solvents diluted in saline solution using their method developed for intravascular applications. They have determined that solutions of 5.1% DMSO, 9.7% PEG 200, 5.7% PG, 39.5% DMI and 21.2% EtOH in saline are responsible for 50% of human RBC hemolysis.

In our study, the methodology proposed by Stenz et al. (18) was applied to various amounts of organic solvents not diluted in aqueous solutions but directly mixed with human blood. The degree of human RBC hemolysis after 90 min at 37° C is shown in Figure 1. No results could be obtained for EtL 10:90 and NMP 10:90 ratios because they induced blood coagulation.

The 13 organic solvents could be subdivided into four groups regarding their 50% hemolytic activity:

- very highly hemolytic solvents (at concentration of less than 1:99 ratio): EtL, DMSO
- highly hemolytic solvents (at concentration of less than 5:95 ratio): PEG 200, acetone
- moderately hemolytic solvents (at concentration of less than 10:90 ratio): THFA, NMP, GF, EtOH, Solketal, glycofurol
- slightly hemolytic solvents (at concentration at least 10:90 ratio): PG, DMI, diglyme.

Figure 1: Percent of free hemoglobin released in plasma (SEM) for solvents mixed with blood in given ratios.



* $p < 0.05$, Student's t-test, unpaired samples, comparison with control

** Blood alone

Except for THFA and DMI, RBC hemolysis is, as expected, strongly dependent on the concentration. Statistical comparison between solvents and the control showed significant different hemolytic activity ($p < 0.05$). Only low concentrations of PG, NMP, Solketal, acetone, and diglyme gave results comparable with the control. Surprisingly, diglyme in 1:99 ratio with blood shows a significant protective activity towards RBC preventing RBC hemolysis.

Hemolysis results from this study were consistent with those reported in the literature except for PEG 200 and PG. For PEG 200, Reed et al. (19) found a 50% hemolysis from concentration of 9.7%, whereas our results demonstrated a 50% hemolysis from ratios with blood of less than 5:95. It is worth noting that, in their study, PEG 200 was mixed prior to blood with NaCl 0.9%, which is well known as a RBC protective agent when used in concentrations ranging from 0.45 to 0.9% (16). Data found for PG are controversial: 50% hemolysis is reported for a concentration of 5.7% PG (19), whereas this study shows a 50% hemolysis for ratios with blood higher that 10:90. This difference cannot be attributed to abnormal erythrocyte fragility, which was absent as shown using NaCl 0.9%, but could be due to a difference in the procedure used, as Reed et al. (19) used an incubation time of 2 min and a temperature of 25° C instead of 90 min and a temperature of 37° C as in our study.

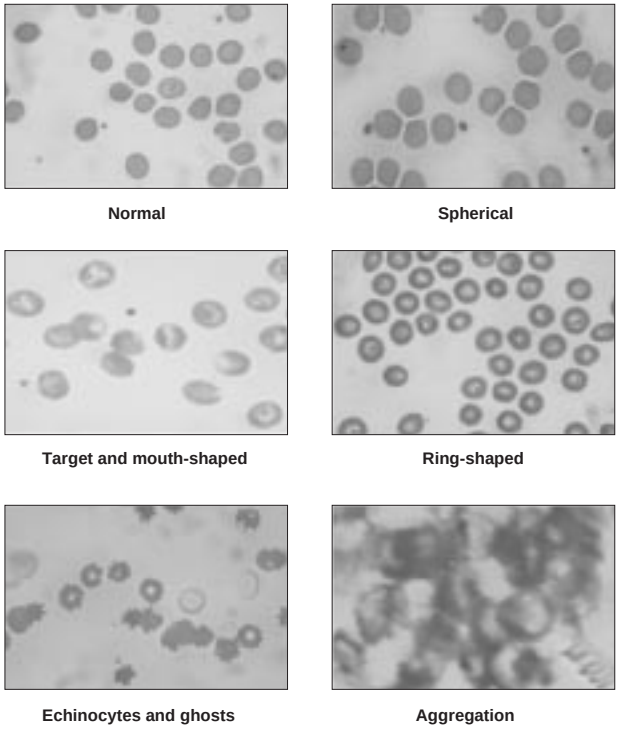
Morphological Evaluation

Observations of the blood-smears showed that the majority of the solvents tested modify RBC morphology (Table 1, Figure 2). It has to be stressed that this morphological effect is probably only present at the injection site, then decreases due to the blood flow diluting the organic solvent. Two criteria were investigated to classify the solvents according to their hemocompatibility. Use of EtL, PEG 200, NMP, Solketal, acetone and glycofurol for intravascular applications could give some problems at the injection site due to their aggregating properties at any ratio with blood. Moreover, these solvents induced strong morphological modifications, like ghosts, ring-shaped RBC and echinocytes.

Table 1: Morphology of the RBC on blood-smears (Giemsa coloration) after their mixture with solvents in given ratios.

Solvent / blood ratio		RBC morphology	RBC aggregation
NaCl 0.9%	1:99	Normal RBC	-
	5:95	Normal RBC	-
	10:90	Normal RBC	-
EtL	1:99	Ring-shaped cells	+
	5:95	Ghosts	+
	10:90	Lysis	+
DMSO	1:99	Ring-shaped cells	-
	5:95	Ring-shaped cells	+
	10:90	Ghosts	+
PEG 200	1:99	Stomatocytes, spherocytes	+
	5:95	Ring-shaped cells, lysis	+
	10:90	Lysis	+
PG	1:99	Ring-shaped cells	-
	5:95	Spherocytes	-
	10:90	Various shapes	-
THFA	1:99	Spherocytes	-
	5:95	Spherocytes, ring-shaped cells	+
	10:90	Spherocytes, ring-shaped cells	+
NMP	1:99	Normal RBC	+
	5:95	Ghosts	+
	10:90	Lysis	+
DMI	1:99	Spherocytes	-
	5:95	Ring-shaped cells	+
	10:90	Ring-shaped cells	+
GF	1:99	Spherocytes	-
	5:95	Spherocytes, target-cells	-
	10:90	Spherocytes	-
EtOH	1:99	Normal RBC	-
	5:95	Stomacytes, target-cells	-
	10:90	Spherocytes	+
Solketal	1:99	Ring-shaped cells	+
	5:95	Ghosts	+
	10:90	lysis	+
Acetone	1:99	Ring-shaped cells	+
	5:95	Ring-shaped cells	+
	10:90	Ring-shaped cells, coagulation	+
Glycofurol	1:99	Echinocytes, ring-shaped cells	+
	5:95	Lysis	+
	10:90	Lysis	+
Diglyme	1:99	Spherocytes, target cells	-
	5:95	Spherocytes	+
	10:90	Spherocytes	+

Figure 2: Morphology of the RBC.



The other seven water-miscible organic solvents generated no aggregation or significant modification of the RBC when administered undiluted with water. DMSO, THFA, DMI and diglyme in a 1:99 ratio with blood did not agglutinate RBC, even if DMSO strongly modified the RBC morphology (ring-shaped erythrocytes). EtOH did not aggregate RBC until a ratio with blood of 5:95, whereas no aggregation of the RBC was induced when solutions of PG and GF with blood in 10:90 ratio were used. It is worth remembering that this effect on the RBC morphology is only an indication of the solvent toxicity at the injection site and that rapid dilution by the blood flow occurred when injected *in vivo*. The measurement of the hemolysis is therefore a more useful method for estimating solvent toxicity.

Conclusion

The present study shows that, of the 13 vehicles tested, 9 organic solvents (PG, THFA, NMP, DMI, GF, ETOH, Solketal, glycofurol, diglyme) are potential candidates, even when used without dilution with water, as vehicles for the intravascular administration based on their low or moderate hemolytic activity. A clear preference is given to DMI and diglyme because mixtures with blood in the 10:90 ratio, and perhaps higher, do not exhibit hemolysis and because previous results have highlighted their low hemodynamic toxicity. THFA, GF, EtOH, Solketal and glycofurol could probably be utilized because of their slight hemolytic activity at a blood ratio of less than 10:90, even if local RBC aggregation could happened. It is worth remembering that the low viscosity of EtOH and its thrombotic properties are useful for embolization. PG and NMP cannot be retained as possible vehicles due to their high hemodynamic toxicities, as well as EtL, DMSO, PEG 200 and acetone. RBC morphological studies only give indicative data with regards to the possible injection of these solvents due to the rapid dilution *in vivo* and show that all selected solvents modify the morphology of the RBC, even at low concentration.

This study showed that undiluted organic water-miscible solvents could be proposed as vehicles for the solubilization of drugs or embolic materials using intravenous or intra-arterial routes. The lack of comparative results means that studies considering mutagenicity, genotoxicity and local toxicity will need to be conducted.

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