The Biocompatibility of Parenteral Vehicles—*In Vitro/In Vivo* Screening Comparison and the Effect of Excipients on Hemolysis

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ABSTRACT: The hemolytic potential for a series of intravenous solutions was determined by an in vitro *testing procedure; the solutions were subsequently administered intravenously to rats and evaluated for* in vivo *biocompatability. Each test solution contained an excipient from one or more of the following categories: nonaqueous cosolvents; complexing agents; surfactants. The test results indicate that the* in vitro *hemolysis values closely predict the* in vivo *test results. Further, a commonly used parenteral cosolvent, propylene glycol, was found to produce a large hemolytic response which can be alleviated by the addition of either a tonicifying agent or polyethylene glycol 400. These findings present useful information when formulating a parenteral vehicle utilizing an organic cosolvent.*

Introduction

Drug compounds which display minimal solubility in an aqueous solution present a challenge when formulating a biocompatible intravenous injectable preparation. In this investigation, the compound of interest, a dihydropyridine compound, possesses very low intrinsic aqueous solubility of 12 μg/mL (1). For clinical and toxicological testing, intravenous solutions containing up to 10 mg/mL are required. A series of formulations were prepared which solubilize the dihydropyridine compound in the desired concentration range. These solutions contain excipients from one or more of the following categories: (*i*) nonaqueous cosolvents such as ethanol, propylene glycol (PG), polyethylene glycol 400 (PEG 400), dimethylisosorbide (DMI), and dimethylacetamide (DMA); (*ii*) complexing agents such as nicotinamide; and (*iii*) surfactants such as poloxamer (pluronic L64) and polyoxyethylated vegetable oil (Emulphor® EL-719).

Incorporation of the various cosolvents and excipients can adversely impact the biological compatibility of the parenteral vehicle. Thus, *in vivo* biocompatibility of each solution was evaluated in rats after administration. Urine and blood samples were collected for examination, and the physical appearance at the site of injection as well as the general condition of the rat were monitored. In conjunction with the *in vivo* testing, *in vitro* tests were performed to determine the hemolytic potential of each solution.

During the course of testing, propylene glycol proved to have a high degree of hemolytic potential as well as nonbiocompatible properties; these results coincide with previously reported findings (2–4). Because propylene glycol is a widely used cosolvent for parenteral administration (5, 6), various excipients were evaluated in combination with PG to determine their tandem effect on hemolytic potential. These additional solutions were not designed to solubilize the dihydropyridine compound. Instead, the solutions were selected to examine whether a parenteral vehicle containing PG can exhibit biocompatibility.

Experimental

In vivo *Biocompatibility Testing*

Spraque-Dawley derived CD male rats were used. The rats were between 8–12 weeks of age at the time of treatment. Twelve groups, each composed of five rats, were given a single intravenous bolus dose (2.5 mL/kg) of either test or control vehicle once daily for two weeks. Each dose was delivered over approximately 10 sec via the tail vein. No attempt was made to inject the test vehicle at the same site each day. Clinical observations such as swelling and/or bruising at the site of injection, pallor and activity level, were recorded at least once weekly. Body weights were recorded weekly and terminally. Urine samples were collected on study day 1 or 2 (for observation of an acute toxicity reaction) and on study day 13 (for observation of a more chronic reaction). Blood samples for hematology and clinical chemistry evaluations were collected on study day 15, the day after the final dose.

Vehicles 2–12 listed below were chosen for evaluation based on their ability to solubilize the subject drug under development. As such, a systematic examination of all possible combinations of the solvents used, was not undertaken. All vehicles prepared for testing had an apparent pH in the range of 5.0–7.4.

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Formulation No.	*Composition*
1	Isotonic saline solution-control
2	12% Ethanol, 15% PG, 20% PEG 400 (in ABS)
3	12% Ethanol, 15% PG, 20% PEG 400 (in water)
4	10% Ethanol, 40% PEG 400 (in ABS)
5	10% Ethanol, 40% PEG 400 (in water)
6	10% Ethanol, 40% PG (in ABS)
7	40% Dimethylisosorbide (in acetate buffer)
8	10% Ethanol, 20% nicotinamide (in water)
9	10% Ethanol, 30% PG, 10% nicotinamide (in water)
10	15% DMA, 15% nicotinamide (in water)
11	15% Ethanol, 6% pluronic L64 (in SAB)
12	7% Emulphor EL 719 (in SAB)

ABS = acetate buffered saline (0.9% sodium chloride, 0.012% glacial acetic acid, adjusted to a pH of 5.2 ± 0.2).

SAB = sorbitol acetate buffer (5.0% sorbitol, 0.012% glacial acetic acid, adjusted to a pH of 5.2 ± 0.2).

In vitro *Hemolysis Testing*

Formulations were tested for hemolytic potential according to the method developed by Reed and Yalkowsky (3). The method calls for mixing equal volumes of test vehicle with whole human citrated blood; the remaining intact red blood cells are washed several times with saline, then lysed with water. The hemoglobin concentration is determined by spectrophotometer and compared to a control sample (Formulation 1) treated in the same manner.

Excipient Effect on Propylene Glycol Hemolytic Activity—In vitro/In vivo *Evaluation*

A 15% propylene glycol water solution was selected as the control. Various concentrations of sodium chloride, sorbitol, and polyethylene glycol 400 were added to the PG control solution. These solutions were tested for hemolytic potential by the *in vitro* testing method described above. Based on the hemolysis data obtained, further testing of certain solutions was performed *in vivo* for their biocompatibility, following the procedure outlined above. The solutions selected for this additional *in vivo* work are listed below.

Formulation No.	*Composition*
13	15% PG in water
14	15% PG, 20% PEG 400 in water
15	15% PG, 1.8% NaCl in water
16	15% PG, 9% sorbitol in water

Results and Discussion

Formulations 2 through 12 were selected for testing based on their ability to solubilize more than 10 mg/mL of a sparingly water soluble dihydropyridine compound. A few formulations contain only subtle modifications of excipients such as water vs. buffered saline (Formulations 2 and 3, and 4 and 5); these were compared for the buffer and salt effects on biocompatibility.

A summary of *in vivo* test results are presented in Tables I and II. With the exception of animals treated with vehicles 8 and 10, all other vehicle-treated animals showed a normal increase in body weight comparable to the control group (Table I).

Animals in all groups, except the saline control and Formulation 12, exhibited changes at the tail vein injection area such as: swelling, encrustation, and/or discoloration. These changes were slight, or slight to moderate for vehicles 2, 3, 4, 5, 7, 9, and 10, and were slight to marked for vehicles 6, 8, and 11 (Table I).

Hematological changes attributed to the experimental regimen were manifested as a decrease in erythrocyte, hemoglobin, and hematocrit values; these changes were most apparent for animals treated with vehicles 8 and 9 and were seen to a lesser extent with vehicles 6 and 12. Treatment-related clinical chemistry changes showed slightly increased A/G:G (albumin to globulin) ratios following treatment with vehicles 3 and 5 and a slightly decreased A/G:G ratio following treatment with vehicle 8. Other clinical manifestations such as inactivity (vehicles 6, 7, and 11) and pallor (vehicles 8, 9, and 11) were observed (Table II).

The *in vivo* hemolysis testing results along with visual observations and dipstick testing results of urine samples from *in vivo* studies, are presented in Table III. Treatment-related urinary changes consisted of discolored urine and occult blood present in urine. Discolored, red-

TABLE I. Body Weight Change and Vein Irritation Results After i.v. Administration of Test Vehicles

Formulation No.	Body Weight Change (gm) ± SD	Perivascular Irritation[a]
1	31.4 ± 4.2	0
2	29.6 ± 2.3	1
3	32.6 ± 7.4	2
4	29.4 ± 10.5	2
5	30.8 ± 11.3	1
6	26.0 ± 9.7	3
7	27.4 ± 9.9	1
8	−4.8 ± 18.3	3
9	13.6 ± 20.7	1
10	−10.6 ± 12.3	2
11	26.2 ± 11.6	3
12	44.4 ± 10.0	0
13	41.0 ± 5.0	1
14	42.6 ± 8.1	0
15	40.0 ± 5.0	0
16	45.8 ± 3.3	0

[a] Visual inspection of tail vein.
1 = slight.
2 = moderate.
3 = marked.

TABLE II. Hematological Results Observed After i.v. Administration of Test Vehicles

Formulation No.	Red Blood Cell Count mil/cumm ± SD	Hemoglobin gm/100 mL ±SD	Hematocrit % ± SD	A/G: G ±SD
1	8.16 ± 0.34	14.7 ± 0.9	39.8 ± 2.6	1.5 ± 0.3
2	8.24 ± 0.27	15.0 ± 0.5	40.0 ± 1.2	1.5 ± 0.4
3	8.16 ± 0.29	14.8 ± 0.7	39.4 ± 2.1	2.1 ± 0.2
4	7.67 ± 0.47	14.3 ± 0.5	38.0 ± 2.3	1.9 ± 0.3
5	7.75 ± 0.43	14.5 ± 1.0	38.6 ± 2.3	2.1 ± 0.2
6	7.38 ± 0.49	13.6 ± 0.8	35.8 ± 1.8	1.3 ± 0.1
7	7.77 ± 0.31	14.5 ± 0.5	38.6 ± 0.9	1.5 ± 0.2
8	6.82 ± 0.44	12.0 ± 0.6	32.4 ± 1.9	0.7 ± 0.1
9	6.71 ± 0.36	12.1 ± 1.2	33.2 ± 2.8	1.2 ± 0.5
10	8.14 ± 0.27	14.9 ± 0.2	39.5 ± 0.6	1.3 ± 0.3
11	7.83 ± 0.23	14.1 ± 0.8	37.0 ± 2.1	1.4 ± 0.4
12	7.46 ± 0.38	12.9 ± 0.5	34.8 ± 1.5	1.3 ± 0.2
13	7.97 ± 0.35	14.7 ± 0.2	39.5 ± 1.0	1.5 ± 0.3
14	7.73 ± 0.46	14.4 ± 0.8	37.4 ± 2.2	1.5 ± 0.3
15	7.96 ± 0.33	14.7 ± 0.2	39.8 ± 0.7	1.2 ± 0.1
16	7.78 ± 0.24	14.5 ± 0.5	39.8 ± 1.5	1.3 ± 0.2

brown (bloody) urine was noted visually at each sampling interval and was primarily seen in animals given vehicle 6 or 9. Red-brown urine was also noted at study day 1 or 2 for several animals given vehicle 7 or 8. Occult blood was detected at each sampling interval and primarily observed for vehicles 6, 7, 8, 9, and 10.

The *in vitro* hemolytic data corresponds quite well with the results observed during *in vivo* testing. Generally, those solutions demonstrating a high hemolytic value by *in vitro* testing also produce negative physical changes with *in vivo* testing. The data indicate that the solutions most prone to elicit an *in vivo* hemolytic response contain excipients such as nicotinamide, propylene glycol, and dimethylisosorbide.

Because propylene glycol is a commonly used cosolvent for parenteral formulations, a diminishing of its toxicological effects is desired. Using the same *in vitro* and *in vivo* techniques described above, two tonicifiers (sodium chloride and sorbitol) and polyethylene glycol 400 were evaluated individually in 15% propylene glycol solution. The *in vitro* results for sodium chloride and sorbitol are shown in Figures 1 and 2, respectively. As expected with these compounds, hemolysis caused by the 15% propylene glycol solution was decreased with the addition of each of the tonicifying agents (2, 4).

According to the osmotic calculation, a 2.0% propylene glycol solution in water is iso-osmotic to human red blood cells. The fact that a 15% propylene glycol aqueous solution causes a high degree of hemolysis suggests the solution is hypotonic to red blood cells. The tonicifying agents,

TABLE III. Comparison of *In Vitro* and *In Vivo* Testing Data: % Hemolysis vs. Urinary Observations

		Day 1		Day 13	
Formulation No.	In Vitro % Hemolysis[a]	Urine Color[b]	Occult Blood[c]	Urine Color	Occult Blood
1	0	straw	none-trace	straw	none-trace
2	2	straw	none-trace	straw	none-slight
3	7	straw	none	straw	none
4	0	straw	none	straw	none
5	3	straw	none	straw	none-moderate
6	94	red brown	marked	straw-red brown	marked
7	23	straw-red brown	marked	straw	none-trace
8	63	straw-red brown	slight-marked	straw	none-trace
9	78	straw-red brown	marked	straw-red brown	marked
10	53	straw	none-marked	straw	none-marked
11	1	straw	none-trace	straw	none-trace
12	0	straw	none-trace	straw	none
13	80	yellow	marked	straw-pink	none-marked
14	22	straw	none	straw	none
15	27	straw	none	straw	none
16	27	straw	none-trace	straw	none

[a] For the *in vitro* % hemolysis data, the standard deviation for all samples is approximately ±5%.
[b] Visual inspection.
[c] Dipstick test.

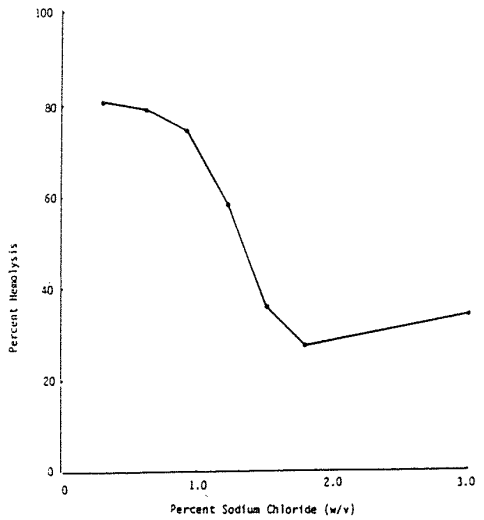

Figure 1—Effect of sodium chloride on the hemolytic potential of a propylene glycol solution (15% w/v).

sodium chloride and sorbitol, achieve substantial or maximal protection in the propylene glycol vehicle at concentrations of 1.8% for sodium chloride and 20% for sorbitol. These concentrations are also greater than the concentration needed to make an isotonic aqueous solution (that is, 0.9% for sodium chloride and 5.0% for sorbitol). These tonicifying agents are effective in preventing hemolysis of the propylene glycol-water vehicle through their colligative properties. In general, colligative properties can be assessed by freezing point depression (using an osmometer); but because 15% propylene glycol itself does not freeze by this method, the effect of the tonicifying agent on this property cannot be evaluated.

Figure 3 shows hemolysis as a function of the addition of polyethylene glycol 400 (PEG 400) to a 15% propylene glycol solution. The graph again shows a decline in hemolysis with the addition of PEG 400. For reference, PEG 400 in water, is iso-osmotic with 0.9% sodium chloride at a concentration of 11.6% (7). PEG 400 exhibits the greatest substantial protective effect at a concentration of about 20%. As observed with the tonicifying agents, a larger concentration than that needed for an iso-osmotic solution is required for red blood cell (RBC) protection in propylene glycol.

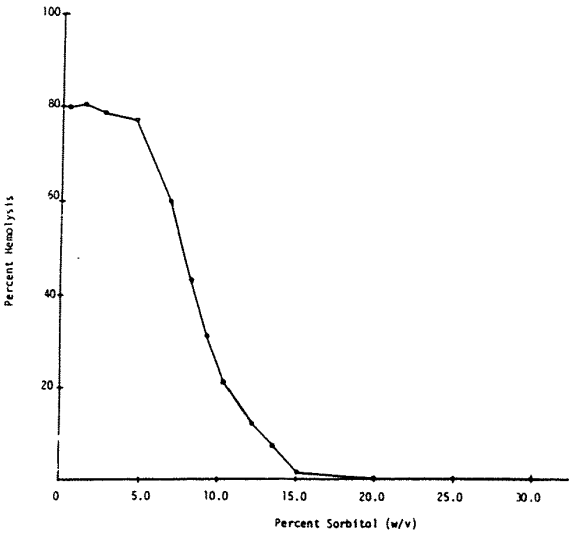

Figure 2—Effect of sorbitol on the hemolytic potential of a propylene glycol solution (15% w/v).

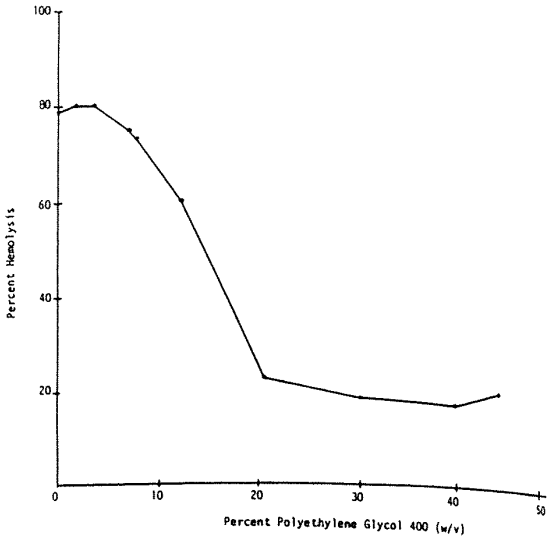

Figure 3—Effect of polyethylene glycol 400 on the hemolytic potential of a propylene glycol solution (15% w/v).

The reduction of hemolysis by PEG 400 in the propylene glycol–water system may be attributed to: (*1*) an osmotic effect of PEG 400; and (*2*) a potential complex formation, through hydrogen bonding, between the propylene glycol and PEG 400. This complex formation thereby modifies the hemolytic property of propylene glycol.

The finding that PEG 400 demonstrates protective effects against PG is useful in formulating a cosolvent system for lipophilic drugs because it: (*i*) provides a more biocompatible solution through its protective effect on RBCs against PG; and (*ii*) increases the solubility power of the cosolvent system.

To evaluate the correlation of the *in vitro* hemolytic values to an *in vivo* response, solutions 13–16 were prepared and tested *in vivo* following the procedure outlined above. All the samples chosen gave approximately 60% decrease for *in vitro* hemolysis. For sodium chloride and PEG 400 this represented the greatest protective effect achievable. For sorbitol, a 9% concentration level was chosen to compare with the two solutions prepared above; maximum *in vitro* protection, however, was found to occur at 20%. Animals given vehicle 13 (15% PG, 85% water) exhibited occult blood in their urine on study days 1 and 13. One of the samples collected on study day 13 was pink in color (presumably due to blood in the urine). Animals given vehicles 14–16 did not exhibit any form of urinary changes. For all vehicles, no other treatment-related changes were seen for any other parameter measured: clinical condition, body weights, hematology or clinical chemistry (Tables I, II and III). The results confirm that

the *in vitro* test results are predictive of *in vivo* findings, and the addition of either 9% sorbitol, 1.8% sodium chloride or 20% PEG 400 improves the *in vivo* acceptability of the 15% PG solution.

The general finding that *in vivo* toxicology data (hemolytic potential) can be gauge by *in vitro* testing is useful when formulating a parenteral preparation. An ideal formulation would contain excipients which are nontoxic, nonirritating, and nonsensitizing. But for physical or chemical reasons, these criteria cannot always be met; the addition of solvents and excipients may be necessary to achieve a desired drug solubility. This study has demonstrated that various solvents and excipients such as PG, DMI, and nicotinamide are detrimental to red blood cells and exhibit negative side effects *in vivo*; the adverse effects produced by propylene glycol can be diminished by the incorporation of either a tonicifying agent or PEG 400. This latter finding suggests that nonaqueous cosolvents, in general, might be made more biocompatible by combining with certain additives. Further formulation development with a nonaqueous cosolvent should entail an *in vitro* hemolysis screening using various additives (for example, tonicifying agents, or alternate excipients, such as PEG 400) to determine suitability. Then a concentration vs. hemolysis profile should be generated for the excipient-cosolvent combination to determine optimal *in vitro* biocompatibility.

It should be noted that although *in vitro* hemolysis data successfully predicts *in vivo* hemolysis, additional unrelated adverse effects may become apparent during *in vivo* testing (as was seen with vehicle 3, 5, and 11). Therefore, after initial *in vitro* screening for hemolysis, the proposed vehicles should ultimately be screened *in vivo* prior to being judged acceptable for parenteral use.

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