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Research Article

Use of an *in Vitro* Model for the Assessment of Muscle Damage from Intramuscular Injections: *in Vitro–in Vivo* Correlation and Predictability with Mixed Solvent Systems

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The potential of binary mixtures of propylene glycol–water, ethanol–water, and polyethylene glycol 400–water to cause skeletal muscle damage (myotoxicity) following intramuscular injection was examined with an *in vitro* model using the isolated rat muscle. At moderate concentrations (20–40%, v/v) of the organic cosolvent, the order of myotoxicity was propylene glycol > ethanol ≫ polyethylene glycol 400. The *in vitro* results were then compared with *in vivo* toxicity in rabbits after injection of normal saline, 40% (v/v) polyethylene glycol 400, 40% (v/v) propylene glycol, indocyanine green in normal saline, and indocyanine green in 40% (v/v) propylene glycol. Employing the area under the creatine kinase activity curve from 0 to 72 hr as the index of skeletal muscle damage, an excellent *in vitro–in vivo* correlation was observed. The basic myotoxicity relationships obtained from the binary cosolvent systems were then used to examine the myotoxicity of ternary organic cosolvent mixtures. Several mixed solvent systems with the same theoretical molar solubilization power for a model compound, diazepam, were selected to determine (1) if myotoxicity can be reduced by changing the composition of the ternary mixtures and (2) if myotoxicity of the individual components is additive. For the solvent systems containing propylene glycol, ethanol, and water, the total myotoxicity equaled the sum of the individual myotoxicity of each component. In contrast, for the solvent systems containing polyethylene glycol 400, the total myotoxicity was only half of the sum of individual toxicities. These results suggest that polyethylene glycol 400 in mixed cosolvent systems might have a protective effect on the myotoxicity generated by intramuscular injections.

KEY WORDS: myotoxicity; creatine kinase; propylene glycol; ethanol; polyethylene glycol 400; myotoxicity, *in vitro* and *in vivo*.

INTRODUCTION

Intramuscular administration is frequently employed in drug therapy for prompt action, when intravenous or oral administration is unsuitable (1–3). Many intramuscular formulations for lipophilic drugs utilize aqueous organic cosolvents, viz., ethanol, propylene glycol, polyethylene glycol, glycerol, and dimethylacetamide, to provide adequate solubility (4). Propylene glycol, ethanol, and polyethylene glycol 400 are among the most commonly used organic cosolvents in injectable formulations, e.g., of hydralazine, lorazepam, phenytoin, digoxin, phenobarbital, pentobarbital, and diazepam (4). However, parenteral administration of the organic cosolvents can cause tissue damage and hemolysis (5–13). The potential of these solvent mixtures to cause skeletal muscle damage (myotoxicity) have not been systematically characterized.

In previous work, we had developed an *in vitro* technique that measures the release of creatine kinase from an isolated rat muscle model to screen agents for their potential to cause skeletal muscle damage (14). A good rank order correlation was obtained between this *in vitro* technique and the *in vivo* myotoxicity of a number of pharmaceutical formulations, as indicated by circulating creatine kinase levels and histological evaluation (which are the commonly utilized indices of skeletal muscle damage both clinically and experimentally) (14). We determine here the myotoxicity of binary mixtures of propylene glycol–water, ethanol–water, and polyethylene glycol 400–water. These *in vitro* results are then validated with *in vivo* studies on creatine kinase activity in male New Zealand white rabbits.

The *in vitro* myotoxicity model was applied to the rational design of intramuscular injection systems. The composition of the solvent system of an intramuscular formulation should cause minimal skeletal muscle damage and patient discomfort, with optimal pharmaceutical (e.g., solubility, stability, and injectability) and biopharmaceutical (e.g., rate and extent of absorption) properties. A series of mixed solvent systems, each possessing equivalent theoretical molar solubility for a model compound (diazepam) was compared to the commercially utilized and quite myotoxic vehicle by evaluating their myotoxicity with the isolated *in*

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vitro rat muscle model. We intended to determine whether skeletal muscle damage can be reduced by a change in the solvent composition, while maintaining equal solubilization power for the drug, and whether the myotoxicities of each component are additive.

These investigations, to our knowledge, provide the first myotoxicity data for three individual organic cosolvents commonly utilized in intramusclar formulations. These studies also illustrate a rational approach for the design and testing of intramuscular injection solvent systems, accounting for both vehicle myotoxicity and drug solubility.

MATERIALS AND METHODS

Materials

Propylene glycol, ethanol, and polyethylene glycol 400 were obtained from Fisher Scientific Company, Aaper Alcohol and Chemical Company, and Sigma Chemical Company, respectively. Double-distilled water was used in the preparation of the binary and ternary organic cosolvent–water mixtures. All other chemicals were at least reagent grade and obtained from J. T. Baker Chemical Company or Fisher Scientific Company.

Myotoxicity Screening

Skeletal muscle damage was determined using a previously described *in vitro* isolated rate muscle model (14). Briefly, male Sprague Dawley rats were sacrificed, and the extensor digitorum longus (EDL) muscle isolated and removed. These muscles were injected with 15 μl of the test solution and placed into a 37°C balanced salt solution bubbled with 95% O_2–5% CO_2. The myotoxicity of the injected solutions was evaluated by the cumulative release of creatine kinase (an intracellualr cytosolic enzyme) into the incubation medium over a 2-hr period. Creatine kinase activity was measured using the CK reagent (Sigma Chemical Company, St. Louis, Mo.) as described previously. Possible spectrophotometric and kinetic interferences by the test solutions were ruled out in preliminary experiments.

In Vitro Organic Cosolvent Myotoxicity

Binary mixtures of propylene glycol–water, ethanol–water, and polyethylene glycol 400–water (0–100%, v/v, organic cosolvent) were prepared and tested for their *in vitro* myotoxicity. Four to nine muscle preparations were used at each concentration of the organic cosolvent. In order to use these myotoxicity data in subsequent studies, the data for each individual organic cosolvent (myotoxicity vs molar concentration) were empirically fitted to a sigmoidal curve (propylene glycol–water mixtures and ethanol–water mixtures) or to a straight line (polyethylene glycol 400–water mixtures between 20 and 80%).

In Vivo Organic Cosolvent Myotoxicity

In order to validate the *in vitro* myotoxicity results, two *in vivo* studies were carried out. First, the myotoxicity of normal saline, 40% (v/v) propylene glycol, and 40% (v/v) polyethylene glycol 400 was investigated. The skeletal muscle damage caused by the above solvent systems was evaluated using the suggested drug safety guidelines proposed by the Pharmaceutical Manufacturers Association for musculoirritant effects of drugs (15). Male New Zealand white rabbits (2.0–4.2 kg) were familiarized with the investigators and the experimental surroundings prior to the start of the experiment. The animals were then injected with 1 ml of each test solution in a three-way crossover randomized design, with a minimum of 2 weeks between each phase of the experiment. The injection was made using a 23-gauge 1-in. needle into the midlumbar muscles. The injection sites were randomly rotated to assure that no single muscle area received more than one injection. Blood samples (1 ml) were obtained from the central artery or the marginal vein of the ear at −1.0, −0.75, −0.25, 0.5, 1, 2, 4, 6, 12, 24, 48, and 72 hr after injection. The animals were given access to water from 4 hr on. The samples were stored at −20°C until analyzed (not longer than 4–5 days) and serum creatine kinase activity levels were analyzed using a commercial CK Reagent (Sigma Chemical Company, St. Louis, Mo.). Serum creatine kinase activity was corrected in each animal by subtracting the mean baseline creatine kinase activity (determined from the −1.0-, −0.75-, and −0.25-hr samples). The area under the corrected serum creatine kinase activity versus time curve between 0 and 72 hr was calculated using the linear trapezoidal rule. The reported values are the mean and standard deviation of five animals.

A second *in vivo* experiment was later carried out to include the presence of a myotoxic compound in an intramuscular injection solution. Indocyanine green (5 mg/kg) in normal saline or in 40% (v/v) propylene glycol (total injection volume of 0.5 ml/kg) was injected into the midlumbar muscles of male New Zealand white rabbits (two rabbits per treatment). Heparinized blood samples (1.5 ml) were collected at 0, 0.33, 0.66, 1.0, 1.5, 2, 4, 6, 8, 12, 24, 72, 120, 168, and 240 hr after injection. The data were treated as previously described. Plasma concentrations of indocyanine green were examined using high-performance liquid chromatography (16). The *in vitro* myotoxic potentials of these indocyanine green solutions were determined using the isolated rat muscle model.

Ternary Cosolvent–Water Mixtures

A series of ternary mixtures (viz., propylene glycol–ethanol–water, polyethylene glycol 400–ethanol–water, and polyethylene glycol 400–propylene glycol–water) was selected based on their equivalent ability to solubilize diazepam. The mixtures were chosen by using the linear solubilization relationships of Yalkowsky and associates (17,18). In this theoretical relationship, Eq. (1),

$$\log(S_m/S_w) = f_1\delta_1 + f_2\delta_2 \quad (1)$$

the log ratio of drug solubility in the ternary solvent mixture (S_m) to that in water alone (S_w) is estimated as a linear combination of the product of the volume fraction of each cosolvent (f_1, f_2) and the solubilization slope for the drug in the respective cosolvent (δ_1, δ_2). Assuming that this ideal linear relationship exists for diazepam solubility in these ternary mixtures, a series of solutions having theoretically equivalent diazepam molar solubility to a reference solution was prepared (Table I). The reference solution was 40% (v/v)

Table I. Ternary Cosolvent Mixtures with Theoretically Equivalent Isomolar Solubility for Diazepam[a]

Number	% (v/v) PG	% (v/v) EtOH	% (v/v) PEG 400	% (v/v) DDW
1[b]	40	10	—	50
2	30	16.5	—	53.5
3	20	23.2	—	56.8
4	10	29.4	—	60.6
5[c]	10	10	—	80
6	34.2	—	20	45.8
7	—	16.5	28	55.5
8	20	—	33.3	46.7
9	—	10	37.6	52.4

[a] PG, propylene glycol; EtOH, ethanol; PEG 400, polyethylene glycol 400; DDW, double-distilled water.
[b] Reference solution.
[c] Exception: solvent mixture does not solubilize equal amounts of diazepam compared to other mixtures.

propylene glycol–10% (v/v) ethanol–water, the cosolvent system used currently in the commercial injection. The individual slope value of the diazepam solubilization curve (δ_1) in propylene glycol–water, ethanol–water, and polyethylene glycol 400–water mixtures was obtained from the literature (18). These mixtures were tested for their *in vitro* myotoxicity as described previously.

Organic Cosolvent Additivity Studies

For the above ternary mixtures, the predicted myotoxicity values were calculated assuming that the total creatine kinase release was an additive function of the damage caused by each cosolvent and water [Eq. (2)].

$$CK_{Total} = CK_A + CK_B + CK_{DDW} \quad (2)$$

In this relationship, CK_{Total} is the predicted total cumulative creatine kinase release. CK_A and CK_B are the predicted values of the cumulative creatine kinase release caused by the individual cosolvents in the mixture. These values are obtained from the empirically fitted myotoxicity curves for the individual organic cosolvents (Fig. 2). The value for CK_{DDW} (the myotoxicity caused by double-distilled water) was the mean value obtained from the two separate series of experiments involving each of the two cosolvents.

Statistical Analysis

Values are presented as the mean ± SD. Data analysis was performed using one-way analysis of variance followed by a Tukey's test for differences between the mean values.

RESULTS AND DISCUSSION

In Vitro Organic Cosolvent Myotoxicity

The cumulative creatine kinase release following the injection of different binary cosolvent water mixtures was linear over 2 hr (r^2 values between 0.94 and 0.99). Representative profiles for the cumulative release of creatine kinase versus time following the injection of propylene glycol–water, ethanol–water, and polyethylene glycol 400–water (at an intermediate percentage of organic cosolvent) are shown in Fig. 1. The myotoxicity as a function of the percentage (v/v) of organic cosolvent in the injection mixture is shown for all three cosolvents in Fig. 2. In the propylene glycol–water and ethanol–water mixtures, a higher myotoxicity was associated with a higher organic cosolvent concentration. For the polyethylene glycol 400–water mixtures, the solutions containing 20% (v/v) and 30% (v/v) polyethylene glycol 400 exhibited a smaller creatine kinase release compared to the injection of double-distilled water. Beyond these concentrations, higher polyethylene glycol 400 levels brought about higher observed myotoxicities, similar to that observed for the other two organic cosolvents.

Similar results demonstrating increased tissue damage following exposure to solutions with higher concentrations of propylene glycol and ethanol have been reported in the literature. In rabbits, the levels of serum creatine kinase progressively increase following the intramuscular injection of glass-distilled water, 10% propylene glycol in glass-distilled water, and 50% propylene glycol in glass-distilled water, respectively (5). Likewise, subcutaneous administration of increasing concentrations of propylene glycol in water to dogs caused a corresponding increase in the degree of local tissue irritation (6). Erythrocyte hemolysis studies also showed that formulations with lower concentrations of ethanol caused less *in vivo* hemolysis in rats and *in vitro* hemolysis in dogs (7).

At moderate cosolvent concentrations (20–40%, v/v), propylene glycol was found to be more myotoxic than ethanol and polyethylene glycol 400 ($P < 0.05$) (Fig. 2). Propylene glycol was found to be approximately 3 times more myotoxic than polyethylene glycol 400 at 40% (v/v) organic cosolvent and 10 times more myotoxic than polyethylene glycol 400 at 20% (v/v) organic cosolvent. Ethanol was found to exhibit myotoxicity values intermediate to those of propylene glycol and polyethylene glycol 400. Studies investigating the hemolysis of red blood cells by the organic cosolvents have also demonstrated the same rank order (19).

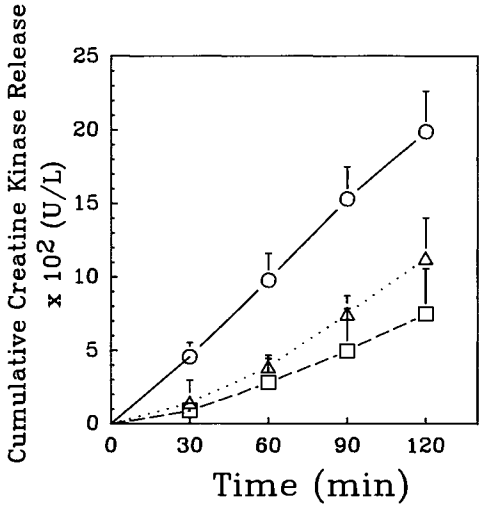

Fig. 1. Cumulative creatine kinase release over 2 hr for 40% (v/v) propylene glycol (○), 39% (v/v) ethanol (△), and 40% (v/v) polyethylene glycol 400 (□). Each curve is the mean + SD of six to eight muscles.

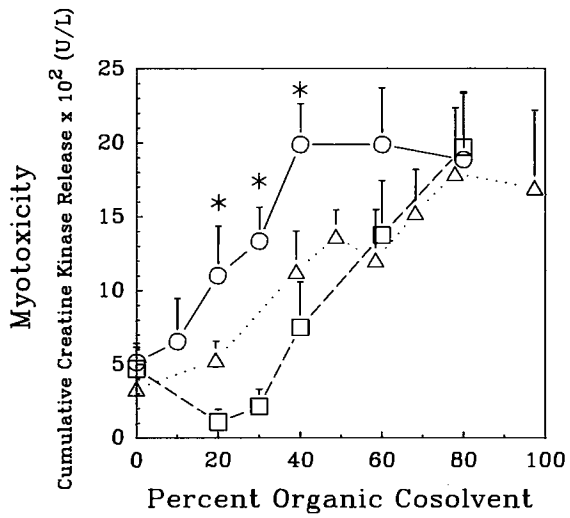

Fig. 2. Cumulative creatine kinase release over 2 hr versus percentage (v/v) of propylene glycol (○), ethanol (△), and polyethylene glycol 400 (□). Each value is the mean + SD of between four and nine muscles. Statistical significance, $P < 0.05$ (*).

In Vivo Organic Cosolvent Myotoxicity

Validation of these *in vitro* myotoxicity results for propylene glycol and polyethylene glycol 400 was carried out by investigating the changes in serum creatine kinase activity following the intramuscular injection of 40% (v/v) propylene glycol, 40% (v/v) polyethylene glycol 400, and normal saline in rabbits (Fig. 3). Serum creatine kinase activity increased following the injection of all three solvent systems. Serum creatine kinase activity returned to baseline within 72 hr of injection. Serum creatine kinase activity was higher following the intramuscular injection of 40% (v/v) propylene glycol compared to 40% (v/v) polyethylene glycol 400. Normal saline injection caused only a small increase in serum creatine kinase activity over baseline levels. The corrected area under the serum creatine kinase activity versus time curve following the propylene glycol injection ($1.71 \pm 0.68 \times 10^5$ U-hr/liter) was statistically larger ($P < 0.05$) compared to those after the injection of polyethylene glycol 400 ($0.62 \pm 0.16 \times 10^5$ U-hr/liter) and normal saline ($0.21 \pm 0.18 \times 10^5$ U-hr/liter). Similar to the *in vitro* myotoxicity results, the injection of 40% (v/v) propylene glycol caused serum creatine kinase activity levels that were approximately three times higher than the injection of 40% (v/v) polyethylene glycol 400. This finding is consistent with a previous literature observation that the intravenous administration of polyethylene glycol solutions resulted in fewer adverse hematologic changes than propylene glycol solutions (7).

To validate further the *in vitro* isolated rat muscle model and to examine the presence of a myotoxic agent in an intramuscular solvent system, indocyanine green in normal saline and in 40% (v/v) propylene glycol was injected into rabbits in a subsequent study. Indocyanine green was used as the model compound since it is not known to have any systemic or local pharmacologic effect(s) that could alter the release of compounds (either endogenous or exogenous) from the intramuscular site. The presence of indocyanine green in normal saline and in 40% (v/v) propylene glycol caused a substantial increase in creatine kinase activity (Fig. 4) compared to the enzyme activity in the solvent system alone (Fig. 3). As before, creatine kinase activity returned to baseline levels within 72 hr. There was no detectable plasma levels of indocyanine green up to 240 hr in the four animals.

In Vitro–in Vivo Correlation

The results of the correlation between the *in vivo* and the *in vitro* results are shown in Fig. 5. There is an excellent linear correlation in the degree of skeletal muscle damage caused by the various injection systems as measured by the two experimental methods ($r^2 = 0.985$). This finding validates the usefulness of the isolated *in vitro* rat muscle model

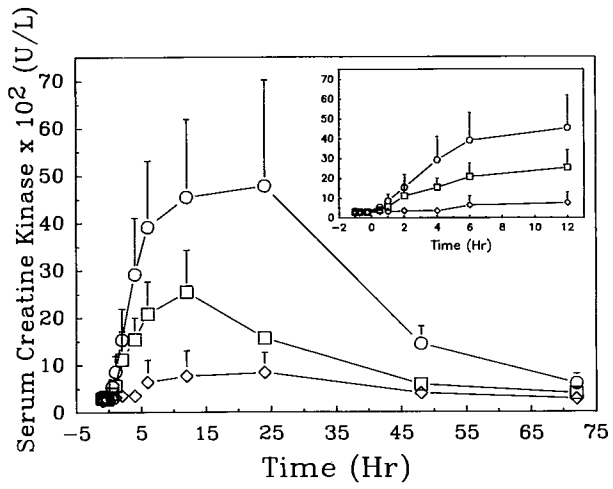

Fig. 3. Mean serum creatine kinase activity versus time following the intramuscular injection of 40% (v/v) propylene glycol (○), 40% (v/v) polyethylene glycol 400 (□), and normal saline (◇) into rabbits. The inset is the serum creatine kinase activity between 0 and 12 hr. Each curve is the mean + SD of five animals.

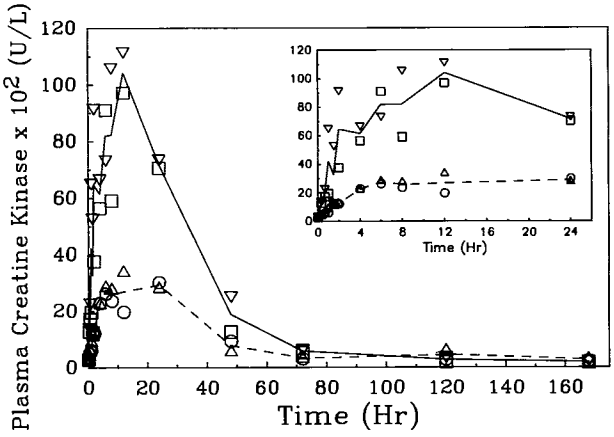

Fig. 4. Individual plasma creatine kinase activity versus time curves following the injection of indocyanine green in normal saline (Rabbit G, ○; Rabbit J, △) or indocyanine green in 40% (v/v) propylene glycol (Rabbit H, □; Rabbit I, ▽). The solid line and dashed line are the mean values of the two rabbits for indocyanine green in 40% (v/v) propylene glycol and indocyanine green in normal saline, respectively. The inset is the plasma creatine kinase activity between 0 and 24 hr.

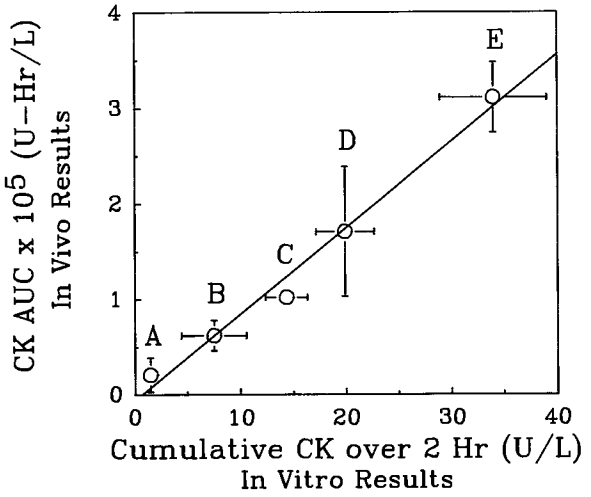

Fig. 5. Correlation between the *in vivo* and the *in vitro* myotoxicity methods. The solid line is the result of the linear regression. The various investigated treatments are (A) normal saline, (B) 40% (v/v) polyethylene glycol 400, (C) indocyanine green in normal saline, (D) 40% (v/v) propylene glycol, and (E) indocyanine green in 40% (v/v) propylene glycol. For the *in vitro* data, the values are the mean ± SD (N = 6–9). For the *in vivo* data, the values are the mean ± SD (N = 5) for solutions A, B, and D and are the mean ± range (N = 2) for solutions C and E.

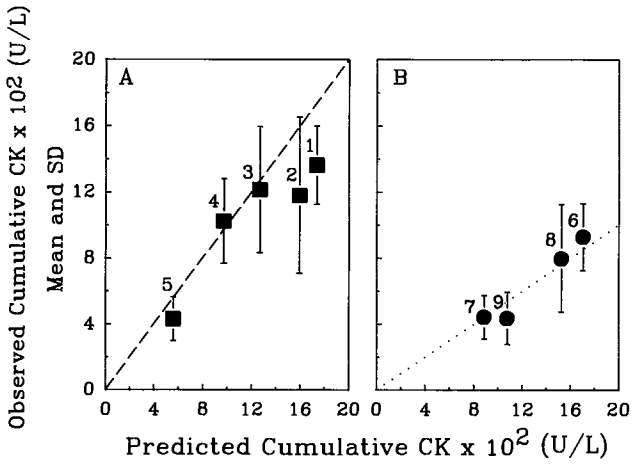

Fig. 6. Observed cumulative creatine kinase release over 2 hr versus predicted values for solvent systems containing polyethylene glycol–ethanol–water (A) and for systems containing polyethylene glycol 400–ethanol–water and polyethylene glycol 400–propylene glycol–water (B). The numbers represent the solvent mixtures listed in Table I. The dashed line is the identity line, slope = 1 (A), and the dotted line is the half-identity line, slope = 0.5 (B). Each value is the mean ± SD of results from five to seven muscles.

system for the screening of injection systems with respect to their potential to cause *in vivo* skeletal muscle damage.

Optimization of Mixed Organic Cosolvent Systems

The individual myotoxicity curves for each individual organic cosolvent was then utilized to examine whether the myotoxicity of mixed solvent systems (containing two organic cosolvents and water) can be predicted from simple additivity. To choose a series of mixed solvent systems, we elected to assign a common characteristic for them, viz., they should possess the same solubilization power for diazepam as the 40% (v/v) propylene glycol–10% (v/v) ethanol–water system. This approach is similar to one frequently faced by pharmaceutical formulators who must choose the least toxic solvent system among a number of possible candidates possessing a similar acceptable physicochemical characteristic.

Initially, three ternary mixtures of propylene glycol–ethanol–water with theoretically equivalent molar solubilizing power for diazepam (mixtures 2–4 in Table I and Fig. 6A) to the reference mixture (mixture 1 in Table I and Fig. 6A) were examined. An additional mixture, which did not have this solubilization property (mixture 5 in Table I and Fig. 6A), was also included in the examination. The total myotoxicity of these propylene glycol–ethanol–water mixtures appeared to be an additive function of the components in the mixture since there was reasonable agreement between the predicted creatine kinase values and the experimentally observed values (Fig. 6A). The agreement was better in those mixtures where the concentration of propylene glycol in the mixture was lower (mixtures 3–5). Further examination reveals that the three mixed solvent systems with theoretically equivalent diazepam solubilization (mixtures 2–4) showed no notable reduction in the magnitude of the myotoxicity compared to the reference solution (mixture 1).

Our previous *in vitro* and *in vivo* data on organic cosolvent-induced skeletal muscle damage suggested that polyethylene glycol 400 is less myotoxic than propylene glycol. Furthermore, polyethylene glycol 400 has been shown to have equivalent solubilizing power for diazepam when compared to propylene glycol (18). The combination of these two pieces of experimental information suggested the possibility that a complete or partial substitution of polyethylene glycol 400 in place of propylene glycol in the formulation of diazepam could maintain adequate drug solubility, while reducing the degree of skeletal muscle damage. Consequently, we examined four additional ternary solvent systems containing polyethylene glycol 400 (mixtures 6–9 in Table I and Fig. 6B). The myotoxicities of these mixtures were generally lower than those of the mixtures containing propylene glycol–ethanol–water. The observed myotoxicities of these mixtures were, on the average, 50% lower than the predicted values (Fig. 6B). This result suggested the possibility that the presence of polyethylene glycol 400 in these ternary injection mixtures may have a protective effect on organic cosolvent-induced skeletal muscle damage. This finding is consistent with those of Korttila and co-workers, who have demonstrated that the intramuscular injection of a diazepam formulation which utilizes half the normal amount of propylene glycol and included polyethylene glycol (Macrogel 300) to solubilize diazepam caused less patient discomfort and pain and exhibited equivalent or higher serum levels of diazepam (20).

Another possible explanation is that the total myotoxicity caused by ternary solvent mixtures containing polyethylene glycol 400 was not an additive function of the myotoxicity of the individual components in a mixture. The nature of the composite interaction of these injection mixtures with skeletal muscle fibers is unknown. It may be related to the

mechanism by which individual organic cosolvents cause skeletal muscle damage following intramuscular injection. These mechanisms have yet to be elucidated.

These experiments represent, to our knowledge, the most systematic investigation of the degree of skeletal muscle damage following the intramuscular administration of the three selected organic cosolvents. Polyethylene glycol 400 has been shown to be less myotoxic than propylene glycol and ethanol, although the reason for this difference is presently unknown. From these studies, we have illustrated an experimental approach which may allow the pharmaceutical formulator to screen rapidly and systematically a series of intramuscular injection solvent systems for their potential to cause skeletal muscle damage. These myotoxicity data, in conjunction with the required physicochemical properties and biopharmaceutical characteristics of the drug, can define the intramuscular solvent systems that should be used.

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