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RESEARCH ARTICLE

Assessment of the Myotoxicity of Pharmaceutical Buffers Using an In Vitro Muscle Model: Effect of pH, Capacity, Tonicity, and Buffer Type

Jintana Napaporn,[1] Maike Thomas,[1] Kari A. Svetic,[1] Zahra Shahrokh,[2] Gayle A. Brazeau[1]

[1] *Department of Pharmaceutics, College of Pharmacy, University of Florida, Box 100494 JHMHC, Gainesville, Florida 32610*
[2] *Genentech Inc., Department of Pharmaceutical R&D, 1 DNA Way, South San Francisco, California 94080*

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ABSTRACT

The purpose of the present study was to investigate the myotoxicity of three buffers containing carboxylic acid groups (i.e., acetate, succinate, and citrate) as a function of their pH, capacity, and tonicity. The myotoxicity of these buffers in the range of pH 2–6 and 0.001–0.1 M buffer capacity was assessed using cumulative creatine kinase (CK) release from an isolated rodent muscle model following injection. Phenytoin and 0.9% NaCl injection were used as positive and negative controls, respectively. Buffer solutions were prepared. A lower pH and higher buffer capacity was linked to increased myotoxicity for the acetate buffers. However, for succinate and citrate buffers, pH appeared to influence the extent of myotoxicity, whereas buffer capacity did not seem to have an effect. When either NaCl or trehalose was used as a tonicity-adjusting agent at pH 6, isotonic 0.01 M buffer solutions dramatically lowered the cumulative CK release compared to those that were not isotonic. Isotonic succinate buffers displayed the lowest myotoxicity, whereas citrate buffers displayed the highest values. Citrate buffers containing three carboxylic acid groups showed higher myotoxicity than succinate buffers and acetate buffers at 0.001 and 0.01 M buffer capacities, whereas acetate buffer produced higher cumulative CK release than citrate and succinate buffers at 0.1 M buffer capacity. The myotoxicity of pharmaceutical buffers containing carboxylic acid groups appears to be directly affected by lowering the pH of the solution.

KEY WORDS: *Buffer; Capacity; Creatine kinase; Intramuscular injection; Myotoxicity; pH; Tonicity.*

Address correspondence to Jintana Napaporn. Fax: (352) 392-4447. E-mail: jintana@grove.ufl.edu

INTRODUCTION

The intramuscular injection of drugs is a well-established and frequently used procedure of drug administration because it can provide relatively fast drug systemic availability when oral or intravenous administration is or cannot be achieved because of the biopharmaceutical characteristics of the drug (e.g., gastrointestinal instability or low aqueous solubility). Furthermore, the intramuscular route provides a means for sustained drug delivery and is conceivably an important route for the delivery of new therapeutic peptides and/or gene products (1). Unfortunately, the incidence of injection site complications following single or multiple intramuscular injections in humans can be as high as 20% depending upon the formulation and the site of injection (2). These complications range from minor discomfort and pain to skeletal muscle damage (defined as myotoxicity), which will potentially limit clinical acceptance. Myotoxicity following the intramuscular injection of a parenteral formulation could be a function of the therapeutic agent (e.g., phenytoin, cephalosporins, local anesthetics) or the presence of other excipients (e.g., solvents, buffer) in the formulation (1,2).

Many commercial formulations use pharmaceutical buffer systems (primarily acetate, succinate, citrate, phosphate) for one or all of the following reasons: (*a*) to reduce discomfort to the patients, (*b*) to ensure requisite drug stability and solubility, and (*c*) to control the therapeutic activity of the drug substance (3). Parenteral formulations using these buffers and other excipients have the potential to cause damage and/or pain following injection. Citrate buffer has been reported to cause significantly more pain than 0.9% NaCl injection after subcutaneous administration in patients (4). Furthermore, the degree of pain was related to the injected concentration of citrate buffer. Also, buffers containing carboxylic acids (acetate, succinate, citrate) have been shown to cause red blood cell agglutination and/or hemolysis in patients (5). Although these isolated studies and anecdotal clinical reports indicate that buffers used in parenteral formulations may be responsible for the pain and/or damage following injection, a systematic investigation of buffers and buffer properties with respect to their potential to cause tissue damage has not been conducted to date.

On the basis of limited data on parenteral formulations and their excipients, solutions to be applied are likely to cause toxicity if their pH is extremely high or low compared to the pH of the relevant body fluid or tissue. Of possible greater significance than the actual pH of the solution is the buffer capacity and the volume to be used relative to the volume of body fluid in which the solution will come into contact following injection (6). It can, therefore, be hypothesized that myotoxicity of a formulation with a large pH difference between the solution being administered and the physiological environment in which it is applied will be minimized by lowering the buffer capacity of the solution. For parenteral products using buffers, the desired pH range, capacity, tonicity, buffer type, and tissue damage are critical factors that formulation scientists must consider in the development and optimization of these formulations.

Creatine kinase (CK) release is often used as a marker of damage to skeletal muscle both in vitro and in vivo (7). In the present studies, an in vitro technique that measures the release of cytosolic enzyme CK from an isolated rat muscle model was employed to screen a series of buffers for their potential to cause acute skeletal muscle damage following an intramuscular injection (8). The specific purpose of the present study was to investigate the myotoxicity of buffers containing a carboxylic acid group (i.e., acetate, succinate, and citrate) as a function of their pH, capacity, tonicity, and buffer type. These investigations provide the first myotoxicity data for three buffer systems commonly used in intramuscular formulations. These studies also illustrate a rational approach for testing of intramuscular injection buffer systems, accounting for buffer-induced myotoxicity.

MATERIALS AND METHODS

Materials

The muscle incubation medium, balanced salt solution (BSS), contains 116 mM NaCl, 5.4 mM KCl, 5.6 mM dextrose, 26.2 mM $NaHCO_3$, and 0.001 mM sodium phenol red in Sterile Water for Irrigation (Baxter, Deerfield, IL). The pH was adjusted to 7.4 with 1 M HCl. Acetic acid, succinic acid, citric acid, and their salts were obtained from Fisher Scientific Co. (Fair Lawn, NJ) and Sigma Chemical Co. (St. Louis, MO), respectively. Sterile Water for Irrigation was used in preparation of all buffer solutions. Phenytoin (Dilantin®) and 0.9% NaCl injection, the positive and negative controls, were manufactured by Parke-Davis (Morris Plains, NJ), and Abbott Laboratories (North Chicago, IL), respectively. All other chemicals were at least reagent grade, the highest grade commercially available, and were obtained from Sigma Chemical Co.

Preparation of Test Formulation

The buffer solutions to be tested at 0.001, 0.01, and 0.1 M buffer capacity were prepared by using the Henderson–Hasselbalch equation and the Van Slyke equation according to their pK_a values. To evaluate the effect of tonicity, 0.01 M buffer solutions at muscle physiological pH (pH 6) were prepared isotonically using either NaCl or trehalose as tonicity-adjusting agents. The second tonicity study was conducted using NaCl in the range of 0–2.7% w/v. To compare buffer types and to test the possible relationship between buffer capacity, number of carboxylic acids, and myotoxicity, buffer solutions were prepared by varying buffer capacity from 0.001 to 0.1 M at pH 6.

CK Activity in In Vitro Interference Assay

All of the test formulations were evaluated to determine whether they stimulated or inhibited CK activity. Briefly, as described previously (9), rabbit muscle CK Type I (lyophilized form, Sigma Chemical Co.) was prepared by dissolving approximately 1 mg of the enzyme in 10 ml of BSS at pH 7.4. A given aliquot of this solution was spiked into incubation vessels containing the BSS at 37°C and bubbled with 95% O_2/5% CO_2. The test solution was added to the test incubation vessel, while the same volume of 0.9% NaCl injection served as control. The amount of CK, approximately 500 U/L, was the same in both the test and control incubation vessels. All studies were conducted for 30 min. CK was determined spectrophotometrically at 340 nm using a commercially available kit (no. 47-UV, Sigma Chemical Co.). This assay is based upon the change in the absorbance caused by a reduction of nicotinamide adenine dinucleotide (NAD^+) to reduced nicotinamide adenine dinucleotide (NADH). The degree of interference with CK activity was evaluated by the ratio of CK activity in the presence of the test solution to the activity measured in the absence of the test solution.

In Vitro Myotoxicity Protocol

Rodent extensor digitorum longus (EDL) muscles (approximately 100–200 mg) were isolated from male Sprague Dawley rats as previously described (10). Briefly, rodents were administered an anesthetic dose of sodium pentobarbital and sacrificed via cervical dislocation as approved by the Animal Care and Use Committee at the University of Florida in accordance with the National Institutes of Health Guidelines. The EDL muscles were injected with the test solution (15 µl) using a 100 µl Hamilton syringe (Reno, NV) equipped with a needle guard to control the depth and angle of injection. The injected muscles were placed into a Teflon®-coated plastic basket and immersed in 9 ml of carbogenated (95% O_2/5% CO_2) BSS. The viability of this isolated muscle in this system was previously shown to be 2 hr (8). The solutions were drained and fresh BSS added at 30-min intervals. These drained solutions were analyzed for CK activity at 30, 60, 90, and 120 min. The assay was run at 30°C and validated using a standard (Accutrol Normal). Myotoxicity was assessed by the cumulative release of CK into the incubation medium over a 2-hr period (11).

Data Analysis

Data were presented as the mean and standard error of mean with $n = 12$ samples per test solution for in vitro interference assay and $n = 4$ muscles per treatment for in vitro myotoxicity studies. Statistical analysis of cumulative CK activity in the different treatments was performed using the SAS program for completely randomized factorial design to screen for the main effect and the interaction effect of pH and buffer capacity for each buffer type. A Duncan's post-hoc test with $p < 0.05$ was considered to be statistically significant in the different treatments. A subsequent breakdown analysis was applied in order to analyze for the simple effects of pH and buffer capacity at the particular level of each factor (12). The differences between the positive control (Dilantin) and negative control (0.9% NaCl injection) were analyzed using analysis of variance (ANOVA) with $p < 0.05$ considered to be statistically significant.

RESULTS

Buffer Studies

In all treatment groups, the muscles were similar in length and weight, ranging from 3.5 to 4.5 cm in length and 90 to 125 mg in weight. The cumulative CK release of the positive (Dilantin) and negative (0.9% NaCl injection) controls is shown in Fig. 1. The myotoxicity of Dilantin was 14 times higher ($p < 0.05$) than that of the 0.9% NaCl injection.

The second series of studies was conducted to determine if the buffer solutions altered the activity of CK (Table 1). The interference assay showed that only the

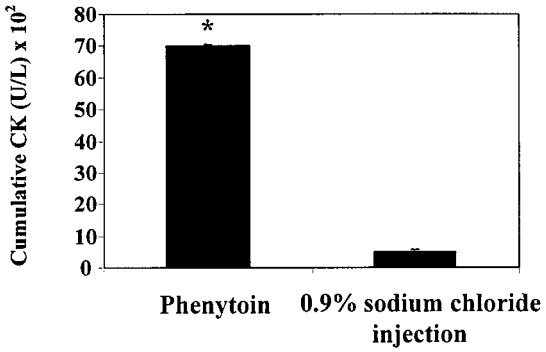

Figure 1. Myotoxicity of positive control (Dilantin) compared to negative control (0.9% NaCl injection). *Statistically significant compared to 0.9% NaCl injection ($p < 0.05$, $n = 4$, mean ± SEM).

0.1 M pH 2 citrate and 0.001 M pH 2 succinate solutions significantly increased the activity of CK enzyme by 23 and 83%, respectively ($p < 0.05$).

The cumulative CK release of acetate buffers ranged from 350 to 1100 U/l [Fig. 2(a)]. Only cumulative CK release at 0.1 M solutions for pH 4 and 6 was significantly higher than that for 0.9% NaCl injection ($p < 0.05$). There was a trend of increasing CK release with increasing buffer capacity and decreasing pH, although values were not statistically different from 0.9% NaCl injection or from each other. This suggests that the acetate buffers with lower pH and higher buffer capacity are more myotoxic ($p < 0.05$).

For succinate buffers, the maximum cumulative CK release was similar to that of the acetate buffers [Fig. 2(b)]. A comparison of pH 2 and 5 buffer solutions at a constant buffer capacity suggests that pH influences the myotoxicity more than does buffer capacity. At all three buffer capacities, the myotoxicities of pH 2 buffers were statistically higher from 0.9% NaCl injection, while pH 5 buffers were not different ($p < 0.05$).

The myotoxicity of citrate buffers, in the range of 500–1750 U/l, was greater than those of acetate and succinate buffers [Fig. 2(c)]. Most citrate buffers tested (8/9) exhibited significantly higher cumulative CK release than did 0.9% NaCl injection. Only 0.1 M pH 6 buffer was not different from 0.9% NaCl injection ($p < 0.05$). Statistical analysis revealed that pH is the important consideration with respect to the myotoxicity of this buffer species. In addition, a higher buffer capacity (0.1 M) is associated with less myotoxicity of this buffer at muscle pH 6 (e.g., pH 6).

Table 1

In Vitro Interference Assay of CK Activity of Tested Buffer Solutions at 30 min (37°C) (n = 12)

Treatment	CK Activity Ratio, Mean (SD)
Acetate buffer solutions	
0.001 M pH 4	0.86 (0.01)
0.01 M pH 4	1.07 (0.11)
0.1 M pH 4	0.93 (0.04)
0.001 M pH 6	1.07 (0.11)
0.01 M pH 6	1.06 (0.09)
0.1 M pH 6	1.06 (0.08)
0.01 M pH 6 isotonic with NaCl	0.90 (0.05)
0.01 M pH 6 isotonic with trehalose	0.96 (0.05)
Succinate buffer solutions	
0.001 M pH 2	1.83 (0.34)[a]
0.01 M pH 2	0.99 (0.03)
0.1 M pH 2	1.16 (0.11)
0.001 M pH 5	1.15 (0.15)
0.01 M pH 5	1.08 (0.09)
0.01 M pH 5	1.01 (0.06)
0.001 M pH 6	0.97 (0.07)
0.01 M pH 6	1.01 (0.05)
0.1 M pH 6	0.96 (0.04)
0.01 M pH 6 isotonic with NaCl	0.97 (0.06)
0.01 M pH 6 isotonic with trehalose	0.99 (0.07)
Citrate buffer solutions	
0.001 M pH 2	0.99 (0.11)
0.01 M pH 2	0.96 (0.07)
0.1 M pH 2	1.23 (0.21)[a]
0.001 M pH 4	0.95 (0.13)
0.01 M pH 4	0.90 (0.07)
0.1 M pH 4	1.07 (0.06)
0.001 M pH 6	0.93 (0.07)
0.01 M pH 6	1.05 (0.10)
0.1 M pH 6	1.18 (0.12)
0.01 M pH 6 isotonic with NaCl	0.98 (0.05)
0.01 M pH 6 isotonic with trehalose	0.90 (0.06)

[a] Statistically higher than enzyme activity ratio = 1.0 (0.9% NaCl injection), $p < 0.05$.

Tonicity Studies

Previous reports from the literature indicate that there is no hemolysis when red blood cells (RBCs) are exposed to NaCl between 0.7 and 1.4% (w/v) (13). To investigate if this observation is consistent in muscle, NaCl solutions of various concentrations ranging from hypotonic to hypertonic, including 0, 0.2, 0.45, 0.7, 0.9, 1.4, 1.8, and 2.7%, were tested for their myotoxic potential (Fig. 3).

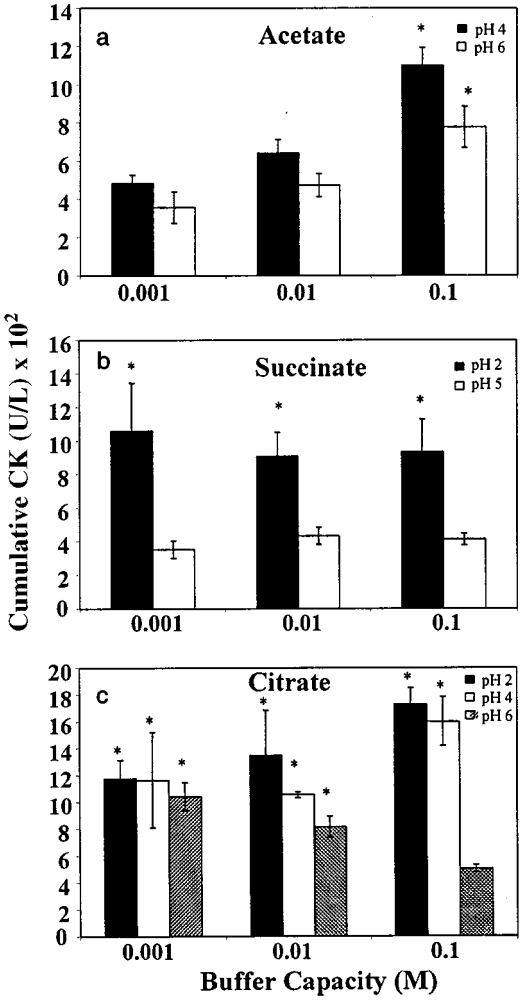

Figure 2. Myotoxicity of buffers at 0.001, 0.01, 0.1 M buffer capacities. (a) Acetate buffer; (b) succinate buffer; (c) citrate buffer. *Statistically significant compared to 0.9% NaCl injection ($p < 0.05$, $n = 4$, mean ± SEM).

CK release following the injection of each solution was linear over the 2-hr period. In the absence of NaCl, myotoxicity was seven times higher than that with 0.9% NaCl. Myotoxicity was dramatically reduced with 0.2% NaCl, but was significantly higher by threefold compared to that with 0.9% NaCl injection. There was no significant difference in myotoxicity observed in the range of 0.45–2.7% NaCl.

Buffer Tonicity Studies

Previous tonicity studies suggested that higher buffer capacities (and most often hypertonic solutions) cause higher toxicity compared to lower buffer capacities. However, it was unclear whether myotoxicity could be reduced when hypotonic buffers were made isotonic with appropriate tonicity-adjusting agents. Because 0.1 M buffer capacity is hypertonic, 0.01 M buffers were made isotonic using either NaCl or trehalose at pH 6. Isotonic buffer solutions showed dramatically lower cumulative CK than the corresponding hypotonic, pH 6 solutions ($p < 0.05$), as shown in Fig. 4. Furthermore, isotonic

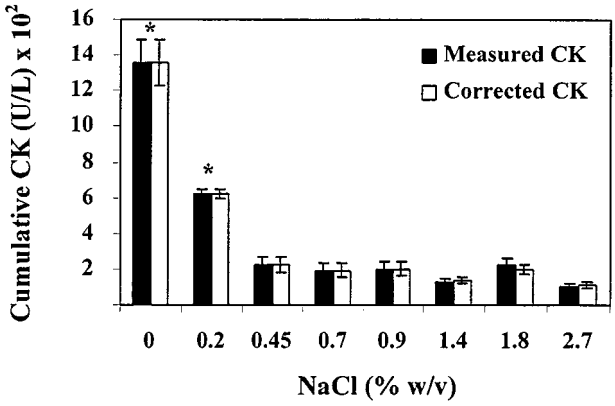

Figure 3. Myotoxicity of saline solutions in concentrations varying from 0 to 2.7% w/v. The corrected cumulative CK was calculated from the degree of interference of test solution compared to 0.9% NaCl injection obtained during interference assay. *Statistically significant from 0.9% NaCl injection ($p < 0.05$, $n = 4$, mean ± SEM).

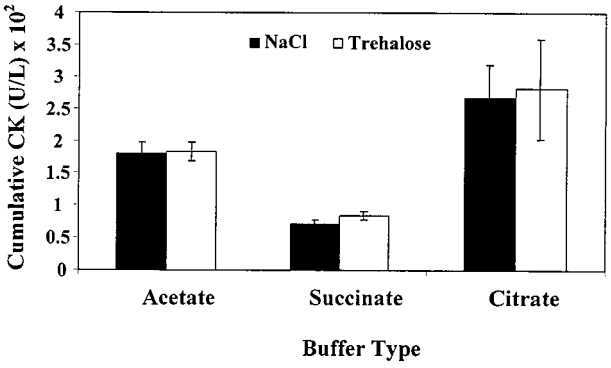

Figure 4. Myotoxicity of 0.01 M pH 6 buffer solutions to screen the effect of buffer tonicity. Solutions were prepared isotonically using either NaCl or trehalose as tonicity agent ($n = 4$, mean ± SEM).

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