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Myotoxicity studies of injectable biodegradable in-situ forming drug delivery systems

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Abstract

The objective of the study was to investigate the potential in-vitro and in-vivo myotoxicity of different in-situ forming biodegradable drug delivery systems, namely in-situ Microparticle (ISM) systems and polymer solutions (in-situ implant systems). The acute myotoxicity was evaluated in-vitro using the isolated rodent skeletal muscle model by measuring the cumulative creatine kinase (CK) efflux. For the in-vivo study, following intramuscular injection (i.m.) into male Sprague Dawley rats, the area under the plasma CK-curve was used to evaluate muscle damage. The formulations included ISM-systems [a poly (lactide)-solvent phase dispersed into an external oil phase] and poly (lactide) solutions (in-situ implant systems). Phenytoin and normal saline served as positive and negative controls, respectively. Poly (lactide) in different solvents (in-situ implant systems) resulted in 14.4–24.3 times higher CK-values compared to normal saline, indicating a high myotoxic potential. With the ISM-system, the CK-release was significantly lower, decreased with a lower polymer phase: oil phase ratio, and approached the values of normal saline at a ratio of 1:4. Bupivacaine HCl- and Buserelin acetate- containing ISM-systems resulted in significantly lower CK-levels when compared to the corresponding drug formulation in normal saline. The in-vivo studies confirmed the in-vitro data and showed good muscle compatibility of the ISM-systems.

Keywords: Biodegradable polymers; Creatine kinase; Intramuscular injection; Microparticles; Muscle damage; Myotoxicity

1. Introduction

Various intramuscular or subcutaneous controlled drug delivery systems in the form of implants or microparticles have been developed based on biodegradable polymers such as polylactides (PLA) or poly-lactide-co-glycolides (PLGA; Ogawa et al., 1988; Lewis, 1990). The preparation of biodegradable implants and especially of microparticles is complicated and involves multiple step processes and formulation parameters to be controlled (Jalil and Nixon, 1990). Additional issues are scale-up and costs.

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As an alternative to solid implant or microparticle formulations, liquid drug-polymer formulations have been developed, which form implants in-situ upon injection and contact with body fluids (Dunn et al., 1990; Tipton and Fujita, 1991; Shah et al., 1993). PLA- or PLGA-polymers are dissolved in water-miscible solvents, such as *N*-methyl-2-pyrrolidone (NMP) or dimethylsulfoxide (DMSO). Upon injection of the drug-containing polymer solution, the polymer solidifies at the site of injection and forms an implant. This technology has been utilized for the delivery of model proteins, LHRH-antagonists, narcotic antagonists, growth factors, anti-inflammatory agents and antibiotics (Tipton and Fujita, 1991; Coonts et al., 1993; Duysen et al., 1993; Lambert and Peck, 1993; Radomsky et al., 1993). The drug release follows the Higuchi square root of time relationship (Higuchi, 1963) with a high burst release. Some disadvantages of the in-situ implant system are the high burst release, the potential solvent toxicity and the high viscosity of the polymer solution possibly causing injection problems.

As an alternative to microparticles or in-situ implant systems, a novel in-situ forming microparticle system (ISM) was developed (Bodmeier, 1997; Kranz and Bodmeier, 1998). These ISM-systems consist of an internal, drug-containing polymer-solvent phase (polymer phase) dispersed into an external phase (for example an oil phase). Upon injection of this dispersion, the internal polymer phase releases the drug in a controlled release fashion. Solvents for the polymers are for example NMP, DMSO, 2-pyrrolidone or PEG 400, which are able to form highly concentrated polymer solutions. Peanut oil, an oil for injection, can be chosen as a biocompatible external oil phase. The ISM systems have a significantly reduced initial burst release and a lower viscosity (the viscosity is primarily controlled by the external and not by the internal polymer phase) and therefore easier injectability when compared to the polymer solutions (Bodmeier, 1997; Kranz and Bodmeier, 1998). In addition, the preparation process for ISM is simple, when compared with the classical techniques for the preparation of microparticles (for example solvent evaporation or organic phase separation techniques).

The objective of this study was to investigate the potential in-vitro and in-vivo myotoxicity of in-situ microparticle (ISM) systems and polymer solutions (in-situ implants). The myotoxicity was assessed by measuring the cumulative release of creatine kinase (CK) from an isolated rat extensor digitorum longus (EDL) muscle after injection of the test formulation. This in-vitro system has been used for its potential to discriminate between the myotoxicity of various parenteral formulations (Brazeau and Fung, 1989a,b). For the in-vivo myotoxicity studies, the area under the plasma CK-curve was used to evaluate the muscle damage caused by these formulations (Brazeau and Fung, 1989b).

2. Materials and methods

2.1. Materials

The following chemicals were obtained from commercial suppliers and used as received: poly(d,L-lactide) (PLA, R 203, M_W 25 700) and poly(D,L-lactide-co-glycolide) (PLGA, RG 502, M_W 16 000, Boehringer Ingelheim, Ingelheim, Germany), dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany), 2-pyrrolidone (Soluphor®), pluronic F 68 (BASF AG, Ludwigshafen, Germany), peanut oil (Henry Lamotte GmbH, Bremen, Germany), aluminum-monostearate (Fluka Chemie AG, Buchs, Swiss), *N*-methyl-2-pyrrolidone (NMP), bupivacaine hydrochloride (Sigma Aldrich Company, St. Louis, USA), buserelin acetate (Hoechst-Marion-Roussel, Frankfurt, Germany), phenytoin (Dilantin®, Parke Davis, Berlin, Germany), 0.9% NaCl solution (normal saline, Abbott Laboratories, Chicago, USA). All chemicals were at least reagent grade.

2.2. Preparation of the in-situ forming drug delivery system

For the preparation of the in-situ forming drug delivery systems, solutions of 40% (w/w) PLA or PLGA in 2-pyrrolidone, NMP or DMSO were prepared. Polymer solutions (15 μl) were injected

into the EDL muscle and served as in-situ implant system. The ISM-systems were prepared as previously described (Bodmeier, 1997; Kranz and Bodmeier, 1998). Briefly, the polymer phase was emulsified into the peanut oil phase in a polymer to oil phase ratio of 1:2, 1:4 and 1:10 using an Ultra-Turrax Homogenizer (M 133/1281-0, Biospec Products INC, Bartlesville, USA). The polymer concentration was varied between 10 and 40% (w/w) PLA for 2-pyrrolidone. For the preparation of the bupivacaine hydrochloride- or buserelin acetate-containing ISM systems, 2 mg drug/ml (based on the total formulation) were dissolved in the polymer phase. This was compared to bupivacaine hydrochloride- or buserelin acetate-containing normal saline solutions (2 mg/ml), respectively.

2.3. In-vitro myotoxicity studies

Extensor digitorum longus (EDL) muscles (approximately 200 mg) were isolated from male Sprague Dawley rats as previously described (Brazeau and Fung, 1989a,b, 1990). Male Sprague Dawley rats (250–300 g) were sacrificed via cervical dislocation following the administration of an anesthetic dose of sodium pentobarbital. The EDL muscles were injected with the formulation (15 μl) using a 100-μl Hamilton syringe 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 carbonated (95% O_2/5% CO_2) balanced salt solution (BSS). The solutions were drained and fresh BSS added at 30-min intervals. The drained solutions at 30, 60, 90 and 120 min were analyzed for CK using a commercially available spectrophotometric kit (Sigma, No. 47, St. Louis, MO) and a Beckmann DU 7400 spectrophotometer (Beckmann Instruments, Fullerton, CA, USA) at 340 nm. Myotoxicity was calculated from the sum of the CK (U/L) over the 120-min period, as described in earlier studies (Brazeau and Fung, 1989a,b, 1990). Possible spectrophotometric and kinetic interferences were tested in preliminary experiments and ruled out for all solvents and formulations (Brazeau and Fung, 1989a). Phenytoin (50 mg/ml in normal saline) and normal saline served as positive and negative controls, respectively (Brazeau and Fung, 1989b).

To test the acute myotoxicity of the different solvents or polymer solutions (2-pyrrolidone, NMP, DMSO), the formulations (15 μl) were injected into the EDL rodent muscles as described above.

2.4. In-vivo myotoxicity

Sterile silastic cannulas were implanted into the right atria of male Sprague–Dawley rats (250–300 g) following the injection of an anesthetic cocktail as previously reported (Millard et al., 1982; Bryan et al., 1983). The cannulas were filled with heparinized (100 U/ml) normal saline solution to prevent blood clotting and maintain cannula access. The rats were given 7 days to recover prior to the study to allow CK-levels to stabilize at baseline. Following i.m. injection of 0.3 ml of the polymer solution (40% PLA in 2-pyrrolidone) and the ISM systems (polymer:oil phase ratios of 1:2 and 1:4, respectively) into the right musculus rectus, blood samples (0.5 ml) were collected via the right atria at 0, 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 h. The blood samples were centrifuged immediately after collection and plasma was stored at −20°C for analysis of CK. Red blood cells were reconstituted in heparinized (40 U/ml) normal saline solution (0.20 ml) and reinjected into the rat to maintain blood volume. Heparinized (100 U/ml) normal saline solution was used to fill the catheter between periods of sample collection. Myotoxicity was assessed by the area under the plasma CK curve calculated by the linear trapezoidal rule. Phenytoin (50 mg/ml in normal saline) and normal saline served as positive and negative controls, respectively (Brazeau and Fung, 1989b).

Protocols for the in-vitro and in-vivo study were approved by the Animal Care and Use Committee at the University in Florida in accordance with National Institute of Health Guidelines.

2.5. Data analysis

Data are presented as the mean and standard error of the mean with $n = 4–6$ muscles per treat-

ment for in-vitro studies and $n = 4$ for the in-vivo studies. Statistical analysis of cumulative CK-release between the different treatments was conducted using analysis of variance (ANOVA) and the Fisher's Post-Hoc test with $P < 0.05$ considered statistically significant.

3. Results and discussion

The isolated rodent skeletal muscle model is a well-studied model for addressing acute toxicity or tissue damage following intramuscular injection (Brazeau and Fung, 1989a,b). Its advantage is the possibility to rapidly screen formulations for their potential to cause acute tissue damage, as measured by the extent of CK-release following i.m. injections, and to compare the extent of this toxicity to previously established non-toxic or myotoxic formulations. It is therefore a suitable model for the screening of the myotoxicity of solvents suggested for in-situ forming drug delivery systems (Shively et al., 1995) and to evaluate the new ISM-system (Bodmeier, 1997; Kranz and Bodmeier, 1998). The limitation of the model is that it does not allow the determination of toxic effects following repeated injections or the chronic myotoxicity after administration of long-acting dosage forms. The isolated muscle system has been shown to retain muscle viability for approximately 120 min (Brazeau and Fung, 1989a). In order to eliminate the possibility that decreased muscle viability contributed to an increased release of creatine kinase, the myotoxicity of the formulations was determined at 120 min post-injection.

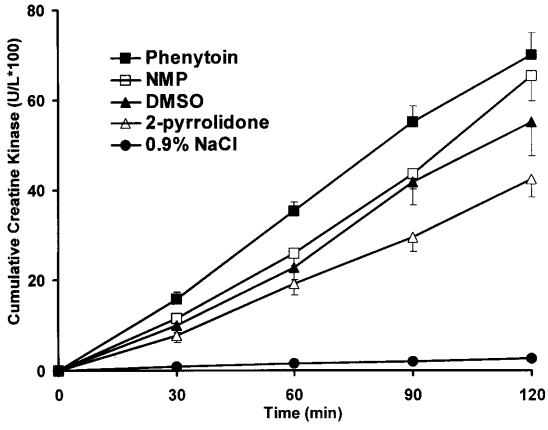

Fig. 1. Cumulative CK-release following the injection of *N*-methyl-2-pyrrolidone (NMP), dimethylsulfoxide (DMSO), 2-pyrrolidone, phenytoin (positive control) and 0.9% NaCl (negative control).

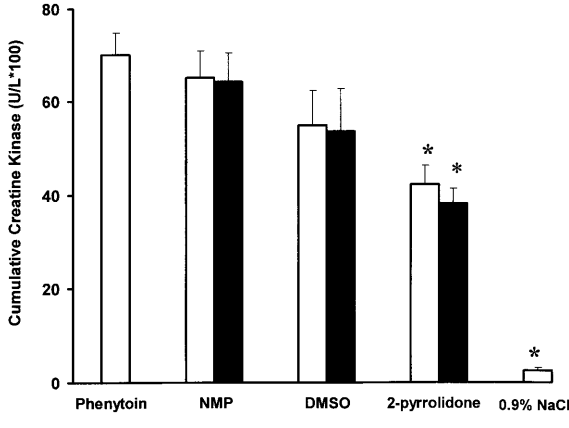

Fig. 2. Cumulative CK-release after 120 min following the injection of (□) pure solvents and (■) 40% PLA-solutions. (*) denotes significantly different values compared to phenytoin.

The development of an in-situ forming drug delivery system requires solvents that are able to form concentrated polymer solutions (> 100 mg/ml) in order to achieve a high drug entrapment and suitable drug release profiles (Shively et al., 1995). In addition, the solvents have to be biocompatible. NMP, DMSO and 2-pyrrolidone were chosen, because they are known to have an intravenous LD_{50} of greater than 2 ml/kg (Lambert and Peck, 1995; BASF Technical Information, 1999). For the i.m. administered in-situ forming drug delivery systems, it is necessary to choose solvents, which cause low muscle damage and pain following administration. The cumulative CK-release profile over 120 min following the injection of NMP, DMSO and 2-pyrrolidone compared to the positive and the negative control is shown in Fig. 1. To compare the myotoxicity of different formulations, the cumulative CK-release after 120 min is plotted in Figs. 2–5. The injection of phenytoin was approximately 24-times more myotoxic than the negative control, normal saline, which is non-myotoxic as measured by enzyme release and histological examinations (Fig. 2; Stei-

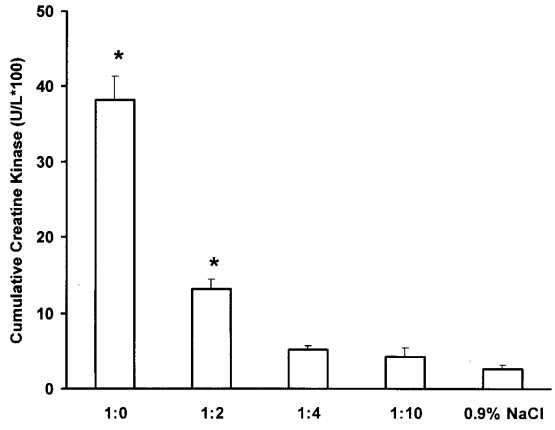

Fig. 3. Effect of the polymer phase: oil phase ratio on the cumulative CK-release after 120 min following injection of a 40% PLA-solution in 2-pyrrolidone (1:0, in-situ implant) and of various ISM-systems containing 40% PLA-solutions in 2-pyrrolidone (1:2, 1:4, 1:10). (*) denotes significantly different values compared to 0.9% NaCl.

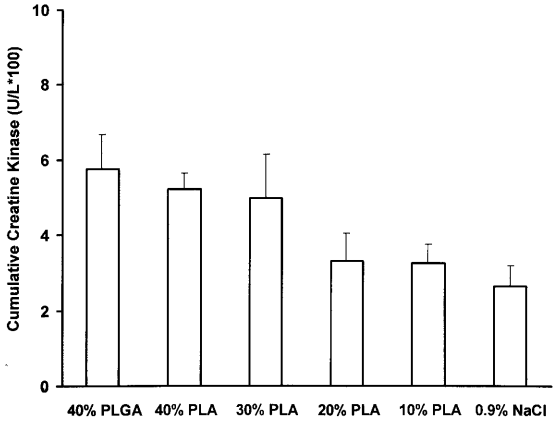

Fig. 4. Effect of the polymer type and polymer concentration on the cumulative CK- release after 120 min following injection of ISM-systems (40% polymer in 2-pyrrolidone, polymer:oil phase ratio of 1:4).

ness et al., 1978; Surber and Sucker, 1987). These findings were in good agreement with the findings reported in a previous study (Brazeau and Fung, 1989a) and showed the potency of the EDL muscle model to distinguish between the myotoxicity of different formulations by measuring the CK-efflux. The rank order of myotoxicity of the solvents was 2-pyrrolidone < DMSO < NMP. There was no statistical difference between the myotoxicity of NMP and DMSO versus the positive control, whereas the myotoxicity of 2-pyrrolidone was statistically lower than the positive control (1.7 times). Therefore, 2-pyrrolidone is chosen when formulating ISM-systems. Nevertheless, all pure solvents show a relatively high myotoxic potential, which is statistically higher than the negative control (16.0–24.4 times higher myotoxicity than normal saline). The injection of 40% PLA-solutions using these solvents showed no significant reduction of the CK-efflux when compared to the pure solvents (14.4–24.3 times higher myotoxicity than normal saline). The injection of polymer solutions (in-situ implants; Dunn et al., 1990; Tipton and Fujita, 1991; Shah et al., 1993) has a high acute myotoxic potential and therefore the potential to cause damage at the injection-site.

A novel biodegradable drug delivery system, the in-situ microparticle (ISM) system, has been developed as an alternative to classical microencapsulation techniques (Bodmeier, 1997; Kranz and Bodmeier, 1998). The ISM-system consists of an internal polymer phase (drug, biodegradable polymer and solvent) dispersed in an external phase, for example an oil for injection. After injection, the internal polymer phase comes in contact with body fluids and releases the drug in a

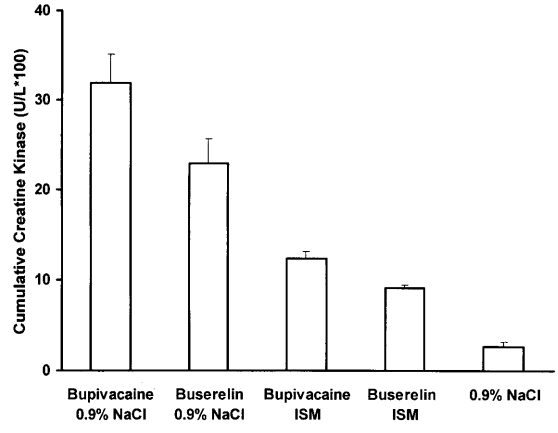

Fig. 5. Cumulative CK-release after 120 min following the injection of bupivacaine HCl or buserelin acetate in normal saline solution and ISM-systems (2 mg/ml) (40% PLA in 2-pyrrolidone, polymer: oil phase ratio of 1:4). (*) denotes significantly different values compared to a drug solution in 0.9% NaCl.

controlled release fashion. In this study, ISM-systems with various polymer solvents and peanut oil, commonly used oil for injection, as external phase were evaluated. The injection of peanut oil alone leads to CK-values, which were statistically lower than the negative control (0.347 U/l*100 for the peanut oil vs. 2.647 U/l*100 for the negative control). These findings were in good agreement with a study showing that oily/drug formulations cause less local muscle damage than aqueous/drug formulations (Svendsen, 1983). Therefore, peanut oil is well tolerated and becomes an attractive candidate for reducing the myotoxicity of the polymer solutions (in-situ implants) through the formation of a dispersion with an internal polymer phase (which is therefore not immediately in contact with muscle tissue) and an external peanut oil phase.

The CK-release after injection of a 40% PLA-solution in 2-pyrrolidone as the solvent (denoted 1:0 in the figure; in-situ implant system) was compared with ISM-systems prepared with a varying ratio of internal polymer phase to external oil phase (Fig. 3). The pure polymer solution showed the highest CK-values. With the ISM-systems, the CK-levels were significantly lower and decreased with increasing amount of external oil phase. At a polymer to oil phase ratio of 1:2 the new ISM drug delivery system shows 5.4 times lower CK-efflux compared to phenytoin, but the CK values are 4.9 times higher than following injection of normal saline. By increasing the content of the oil phase to a polymer to oil phase ratio of 1:4 or 1:10, the CK-release was not statistically different from normal saline. Similar results were also obtained with NMP and DMSO as the solvents. The novel ISM-system therefore showed a lower acute myotoxicity when compared to the pure polymer solutions and was highly compatible at higher amounts of external oil phase in the formulation. With the polymer solutions, the muscle tissue comes into immediate contact with the solvent after injection, while with the ISM-system, the external oil phase initially forms a partial barrier between the muscle tissue and the internal polymer phase, thus resulting in a better compatibility.

The polymer concentration (10–40%) and the type of polymer (PLA or PLGA) did not affect the CK-efflux for ISM systems prepared at a polymer to oil phase ratio of 1:4 (Fig. 4). No statistically significant differences were observed between the formulations. The addition of polymers to the solvents, even at high polymer levels, did not lead to an increased acute myotoxicity, thus indicating the good biocompatibility of PLA and PLGA. PLA and PLGA are commonly used in drug delivery systems, surgical sutures, grafts and various prosthetic devices for parenteral use for more than 20 years (Athanasiou et al., 1996). In addition, there is no difference between the CK-levels of the new ISM-system and the CK-levels of commercially available microparticle formulations, which have been tested previously (Brazeau et al., 1996).

The ability of the ISM-systems to protect against drug-induced muscle damage was investigated using bupivacaine hydrochloride (local anesthetic) and buserelin acetate (gonadorelin analogue) incorporated into ISM-systems containing 40% PLA in 2-pyrrolidone and a polymer to oil phase ratio of 1 to 4 (Fig. 5). Both drugs were selected because of an interest in developing controlled drug delivery systems for local (bupivacaine hydrochloride) and systemic (buserelin acetate) administration. Buserelin acetate has been studied in controlled drug delivery systems for systemic administration. Bupivacaine hydrochloride is a useful candidate for the development of a long-acting formulation for local administration of an anesthetic, since it would be useful in the treatment of chronic orofacial pain. The drug-containing ISM-systems were compared to drug-containing normal saline solutions (2 mg drug/ml formulation). Bupivacaine hydrochloride and buserelin acetate normal saline solutions had 12.0 and 8.7 higher CK-levels than normal saline, respectively. Both drugs therefore significantly increased the acute myotoxicity. The incorporation of bupivacaine hydrochloride and buserelin acetate into the ISM system resulted in a significantly lower muscle damage when compared to the corresponding formulation of these drugs in normal saline (61.3 and 60.0%, respectively). The bupivacaine hydrochloride- and buserelin acetate-con-

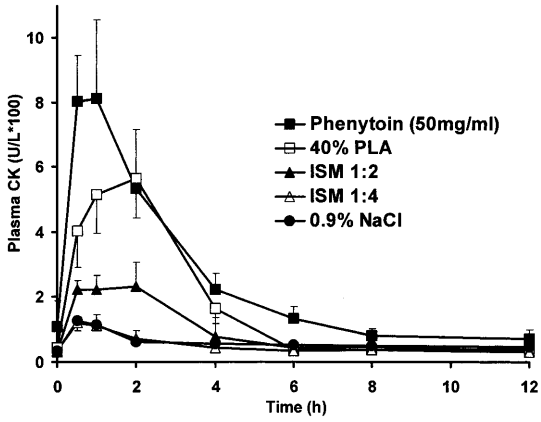

Fig. 6. Mean plasma CK-levels versus time following the intramuscular injection of 40% PLA in 2-pyrrolidone (in-situ implant), ISM-systems (40% PLA in 2-pyrrolidone, polymer to oil phase ratios of 1:2 and 1:4), phenytoin (positive control) and of 0.9% NaCl (negative control).

taining ISM-systems showed 2.4 and 1.8 times higher CK-levels than comparable drug-free ISM-systems, which was caused by the myotoxic effect of the drug. In contrast to the comparable drug-free ISM-system, the CK-levels following the injection of the drug-containing ISM systems were statistically higher than the negative control. However, these results indicated that drug-containing ISM formulations reduced the severity of muscle damage following i.m. injection when compared to the aqueous drug solutions. ISM systems deliver drugs in a controlled release manner with a reduced initial burst (Bodmeier, 1997; Kranz and Bodmeier, 1998). Buserelin acetate and bupivacaine hydrochloride will be released from the ISM-systems over an extended period of time, which decrease the initial drug-muscle-tissue interaction and consequently reduce muscle damage. A pharmacokinetic study, demonstrating the potential of the ISM to deliver drugs at biologically active levels, will be published soon.

Based on the in-vitro myotoxicity data, in-vivo studies were performed with selected formulations (Fig. 6). The area under the plasma CK-curve for 12 h (U-h/L, mean and SEM) was used to assess muscle damage. Peak plasma CK-level occurred at 0.5–2 h following injection, which was in close agreement with a previously published report (Brazeau and Fung, 1989b). The CK-plasma levels then fell off to normal values after 6 h. No further increase in plasma CK was observed up to 72 h (data not shown). The injection of phenytoin was approximately four times more myotoxic than the injection of normal saline ($29.4 \times 10^2 \pm 7.5 \times 10^2$ vs. $7.1 \times 10^2 \pm 1.2 \times 10^2$). Besides the positive control, the polymer solution (40% PLA in 2-pyrrolidone, in-situ implant) resulted in the highest plasma CK-levels. ISM-systems with a polymer to oil phase ratio of 1:2 were 1.9 times and the ISM systems with a ratio of 1:4 were 3.4 times less myotoxic than animals injected with the polymer solution. The plasma curve of 1:4 ISM-system was very similar to the one of normal saline ($5.9 \times 10^2 \pm 1.4 \times 10^2$ for the ISM vs. $7.1 \times 10^2 \pm 1.2 \times 10^2$ for the normal saline solution). The in-vivo results correlated very well with the results obtained with the in-vitro model, thus proving its value for rapidly screening formulations.

In conclusion, ISM-systems showed good muscle compatibility in in-vitro and in-vivo myotoxicity studies when compared to in-situ implant forming polymer solutions. This simple drug carrier formulation in liquid form has the potential to become an attractive alternative for parenteral controlled drug delivery systems.

Acknowledgements

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