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Report

Tissue Tolerance of Intramuscular Injectables and Plasma Enzyme Activities in Rats

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The local tissue damage after intramuscular injection caused by various commercially available injection solutions was determined in the albino rat, by measuring plasma activities of creatine phosphokinase, aspartate aminotransferase, and lactic dehydrogenase, the tissue activity of creatine phosphokinase, and macroscopic changes in the muscle at the injection site (gastrocnemius muscle). The plasma enzyme activities were determined 2, 6, 18, and 28 hr after the injection. After 28 hr the animals were sacrificed for macroscopic inspection of the injection site and for the determination of tissue enzyme activity. The tissue injury caused by the test substances correlated well with the elevated creatine phosphokinase activity (2 hr). The elevations of aspartate aminotransferase (18 hr) and lactate dehydrogenase (2 hr) activity as well as the loss of tissue creatine phosphokinase activity were less indicative of differences between test preparations. The i.p. administration of some of the test preparations caused increased enzyme activity without muscle damage, which could interfere with the test results. The creatine phosphokinase determination indicates the damage occurring immediately after the administration of the test solution, and the macroscopic inspection offers the possibility to obtain some information on the evolution of the muscular lesion.

KEY WORDS: tissue tolerance; intramuscular; injection; rat; plasma enzyme activity.

INTRODUCTION

Numerous morphological methods have been proposed for determining local tissue damage due to intramuscular injection of drugs and their vehicles (1–5). However, few of the suggested methods are suitable for routine testing of a wide range of formulations—as required in particular by the pharmaceutical industry.

In the past decade, determination of plasma creatine phosphokinase activity in the blood of animals and humans has become increasingly important as a means of quantifying local tissue damage following i.m. injection (6–8). However a series of problems has been described in the literature. Pronounced fluctuations in creatine phosphokinase activity have been determined in rabbits because of escape reactions shown by the animals before and during the treatment (6,9) or inconsistent administration of the injection solution. To avoid these problems it was suggested to familiarize the animals with the experimental handling without actual intervention (9). It was also proposed to determine the decrease in the creatine phosphokinase activity in the tissue at the site of injection (10). The topography of the muscle into which the injection is to be made can be critical depending on the injection site and the species used (2,5,11). As a result it is difficult to produce homogeneous muscle damage in all animals or volunteers of a trial group. The rabbit is the only animal species widely used for routine testing of the local toxicity following a single injection, while biochemical methods of determining local tissue damage in the rat (12) are scarce in the literature. A reason for this may be the small muscle mass available (9) and the fact that the small test volumes of injection solution used in small laboratory animals are less toxic than the larger volumes used in humans (3,5). Creatine phosphokinase as well as aspartate aminotransferase and lactate dehydrogenase are enzymes found in skeletal muscle, myocardium, and brain tissue (13,14). The determination of the plasma activities of aspartate aminotransferase has also been proposed as a means of assessing local toxicity in dogs (9,15). However, aspartate aminotransferase elevation was found to be a less powerful indicator than creatine phosphokinase elevation. The relative organ concentration of these enzymes is significantly greater in the skeletal muscle of rats than in the rabbit muscle (13).

The object of the present study was to determine local tissue damage caused by various injection solutions after intramuscular injection in the albino rat, using biochemical and morphological methods.

To this end, the increase in plasma activities of the enzymes creatine phosphokinase, aspartate aminotransferase, and lactate dehydrogenase, the decrease in the activity of creatine phosphokinase in the tissue, and macroscopic changes in the muscle at the injection site were determined. The results provide a trial design for screening local tissue damage.

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MATERIALS AND METHODS

Injection Solutions. Commercially available formulations were used in the study (Table I). All the injection solutions, except diclofenac, are aqueous solutions. Diclofenac contains propylene glycol and benzyl alcohol. Two formulations of novaminsulfon-Na (Siegfried, Zofingen, Switzerland) were prepared according to the Ph.Helv.VI[4] by ourselves (100 mg, 250 mg/ml water).

Determination of Enzyme Activity. Enzyme activity was determined by means of a Centrifi-Chem 400 centrifugal analyzer and the ⟨ROCHE⟩ Diagnostica test combinations. In the text, the following abbreviations are used: CPK, creatine phosphokinase; ASAT, aspartate aminotransferase; and LDH, lactate dehydrogenase. The normal enzyme values in the plasma and the tissue determined by the applied method are shown in Tables II and III. Plasma enzyme activity was determined 2, 6, 18, and 28 hr after the injection. After 28 hr the animals were sacrificed (CO_2) and the gastrocnemius muscle (pars medial) was dissected for the macroscopic inspection and the determination of tissue enzyme activity.

Various authors have described increased enzyme activity as a result of the test preparations itself, without essential muscle damage (8,16). A preliminary trial ($N = 6$ animals) showed an increase in plasma enzyme activity determined 2, 4, and 24 hr after i.p. administration of 0.2 ml of test solution for some of the applied preparations (Table II). The activity of all three enzymes rose sharply after the administration of diclofenac and promethazine. For hyoscine butylbromide a marked rise was observed for the LDH activity only. The i.p. administration of cefotaxime interfered with the enzyme assay, the cause of which could not be determined. Prior to the experiment, the animals were acclimatized for 4 days and familiarized with the manipulations (without actual intervention), thus eliminating the risk of incidental fluctuations in enzyme activities, particularly that of CPK (6), because of escape reactions. Preliminary experiments showed that following intramuscular injection in rats ($N = 10$), CPK and LDH activities peak after 2 ± 0.5 hr, and ASAT activity after 18 ± 4 hr. CPK and LDH activity returned to normal levels after 6–10 hr, and ASAT activity after about 72 hr (17).

Sample Preparation. For plasma samples, blood was transferred to heparinized tubes, then centrifuged at 12,000 rpm and 20°C for 4 min. Blood was taken from the eye (retroorbital vein plexus) by glass pipette, with the animals under dosed ether narcosis. It has been shown that neither repeated blood sampling on its own nor ether narcosis affected relevant enzyme activities (17). For tissue samples, the gastrocnemius muscle (pars medial) was dissected, weighted, cut up very finely, added to 4 ml Tris–acetate buffer, pH 7.4 (18), homogenized by ice cooling (Polytron PTA Z OSM), and subsequently centrifuged at 6000 rpm and 4°C for 20 min.

Table I. Commercially Available Drugs and Special Formulations

Active substance	Dosage (mg/ml)	Brand name	Manufacturer
Imipramine (INN rec.)	12.5	Tofranil	Geigy
Hyoscine butylbromide	20	Buscopan	Boehringer In CH
Lidocaine (INN rec.)	20	Xylocaine, 2%	Vifor
Promethazine (INN rec.)	25	Phenergan	Specia
Diclofenac (INN rec.)	25	Voltaren	Geigy
Cefotaxime (INN rec.)	250	Claforan	Hoechst
Sodium noramidopyrine methanesulfonate (INN prop.) or novaminsulfon-Na	500	Novalgin	Hoechst
	100	—	Siegfried
	250	—	Siegfried

Animals and Housing. Ten male rats (220 ± 10 g) per group of the Füllinsdorf Albino SPF breed (Breeding Station, Füllinsdorf, Switzerland) were housed in five double cages and were allocated cages 4 days before the start of the experiment on a randomized basis. During the experiment they had free access to water and standardized feed NAFAG 850 (NAFAG, Gossau, Switzerland).

Site of Injection and Dosage. For i.m. injections, 0.1 ml test solution in the left and 0.1 ml in the right gastrocnemius muscle (pars medial) were administered via a 0.5 × 4-mm cannula. For i.p. injections, 0.2 ml test solution was administered i.p. via a 0.44 × 25-mm cannula. Consistent and precise intramuscular injection, without loss of solution or penetration of the injection solution between the muscle faecies, as well as prompt and clean dissection was achieved by using the gastrocnemius muscle (pars medial) (17,19).

Macroscopic Evaluation of Tissue Damage. After complete dissection, the gastrocnemius muscle (pars medial) was halved longitudinally through the site of injection and evaluated as to the following visible tissue and structural lesions (5).

Degree of irritation	Score	Finding
No finding	0	No visible changes
Mild	1	Slight hyperemia and discoloration
Moderate	2	Marked discoloration compared with surrounding tissue
Severe	3	Brown discoloration with small necrotic lesions
Very severe	4	Marked necrotic changes, abscess, involving the entire muscle

[4] Abbreviations used: CPK, creatine phosphokinase (EC 4.1.2.13); ASAT, asparate aminotransferase (EC 2.6.1.1); LDH, lactate dehydrogenase (EC 1.1.1.27); AUC, area under the curve; Ph.Helv.VI, *Pharmacopoea Helvetica Editio Sexta;* NAFAG, Naehr- und Futtermittel AG; SAS, Statistical Analysis System; GLM, general linear models; REGWF, option of the GLM procedure that performs the Ryan–Einot–Gabriel–Welsch multiple *F* test on all main-effect means in the means statement.

Table II. Plasma Enzyme Activities After i.p. Administration of 0.2 ml Test Solution per Animal ($N = 6$)

Enzyme	Time (hr)	Normal range	Enzyme activity (IU/liter ± SD) NaCl, 0.9%	Hyoscine butylbromide, 20 mg/ml	Lidocaine, 20 mg/ml	Imipramine, 12.5 mg/ml	Novaminsulfon-Na, 500 mg/ml	Promethazine, 25 mg/ml	Diclofenac, 25 mg/ml
CPK	2	50–200	74.2 ± 25.9	135.0 ± 47.8	127.0 ± 26.5	53.4 ± 65.5	69.0 ± 25.6	386.0 ± 136.3	852.0 ± 166.6
	4		64.5 ± 37.0	59.8 ± 17.2	149.4 ± 34.4	43.0 ± 21.4	57.4 ± 14.4	252.0 ± 61.9	308.0 ± 156.3
	24		54.3 ± 27.6	107.1 ± 25.4	102.5 ± 30.2	65.3 ± 20.3	84.2 ± 32.2	295.2 ± 70.3	129.8 ± 71.6
ASAT	2	25–45	34.2 ± 4.8	53.5 ± 4.0	35.4 ± 8.0	44.4 ± 13.6	46.0 ± 10.7	81.8 ± 5.6	270.6 ± 85.4
	4		39.0 ± 8.2	47.0 ± 5.1	47.8 ± 12.1	39.6 ± 13.9	46.4 ± 11.6	95.6 ± 15.1	322.0 ± 86.1
	24		36.2 ± 6.7	35.3 ± 3.4	35.9 ± 3.8	35.4 ± 3.6	43.8 ± 15.2	99.8 ± 11.5	100.6 ± 9.0
LDH	2	100–200	181.9 ± 62.7	728.0 ± 189.4	131.4 ± 13.0	149.0 ± 68.1	127.4 ± 105.9	375.0 ± 85.0	1270.5 ± 330.1
	4		159.9 ± 85.2	354.4 ± 95.4	156.6 ± 66.0	131.6 ± 43.4	171.6 ± 40.6	375.0 ± 99.5	846.6 ± 151.7
	24		215.8 ± 62.9	822.6 ± 218.3	123.2 ± 47.6	153.9 ± 70.9	148.3 ± 52.7	1010.0 ± 234.5	1019.2 ± 254.5

A mean rating was calculated by adding the individual scores (two per animal, left and right leg, i.e., 20 per group) and dividing by 20.

Statistical Evaluation. Differences in substance activity were investigated for the parameters 2-hr CPK, 2-hr LDH, 18-hr ASAT, 28-hr tissue CPK, AUC-CPK, AUC-LDH, and AUC-ASAT (trapezoidal rule) using a one-way analysis of variance in accordance with the GLM procedure of the SAS (20). A log-normal distribution of the values was assumed. The Ryan–Einot–Gabriel–Welsch F test (option REGWF of the GLM procedure) was used for paired comparison of the formulations. In this test, several F tests are calculated sequentially so that the overall test contains the multiple-level $\alpha = 5\%$; i.e., the probability of erroneously obtaining a significant difference with one or more test substances is less than 5%. The Kruskall–Wallis test was used to obtain the scores (NPAR1Wdy procedure of the SAS) (20). In addition, mean values were calculated for the scores of the left and right muscle. The Kruskall–Wallis test is a nonparametric analogue to one-way analysis of variance. Wilcoxon tests (nonparametric analogue to two-sample t test) were used for paired comparison. To maintain the multiple level of $\alpha = 5\%$, the adjusted P values of the individual tests were compared by means of the Bonferoni–Holm procedure (21).

RESULTS

The plasma enzyme activity versus time profiles for the various test solutions are shown in Fig. 1. Data pertinent to individual experiments are listed in Tables II and III. The tissue injury caused by all the test substances was differentiated on the basis of the elevated CPK activity. The elevation of ASAT and LDH activity was not indicative of significant differences between the test preparations (see Fig. 2).

Table III. Plasma and Tissue CPK Activity After 2 hr and 28 hr; Score Rating After 28 hr and AUC of Plasma CPK Activity (28 hr) Following Intramuscular Injection of Various Test Solutions[a]

No.	Test solution	Plasma CPK (2 hr) ± SD IU/liter	R1	R2	AUC plasma CPK (0–28 hr) IU/liter	R1	R2	Tissue CPK (28 hr) ± SD IU/g moist weight	R1	R2	Score (28 hr)	R1	R2
0	No treatment	111.7 ± 38.4			200.7 ± 45.0			2924.4 ± 195.4			0		
1	NaCl, 0.9%	114.7 ± 38.1	1	1	219.8 ± 58.0	1	1	2812.8 ± 168.3	1	1	0.1	1	1
2	Hyoscine butylbromide, 20 mg/ml	219.4 ± 57.5	2		273.5 ± 51.7	2		2632.9 ± 235.6	2		1.0	2	
3	Lidocaine, 20 mg/ml	335.6 ± 63.4	3		363.7 ± 54.5	3		2525.0 ± 321.6	3		1.4	3	
4	Imipramine, 12.5 mg/ml	974.5 ± 274.1	4		875.6 ± 207.3	4		1578.7 ± 318.7	4		3.9	5	
5	Novaminsulfon-Na, 500 mg/ml	1451.5 ± 292.4	5	4	1254.3 ± 260.0	5	4	1213.9 ± 182.0	5	4	3.8	4	4
6	Novaminsulfon-Na, 250 mg/ml	655.7 ± 105.3		3	631.5 ± 83.3		3	1449.1 ± 245.0		3	3.5		3
7	Novaminsulfon-Na, 100 mg/ml	344.0 ± 49.2		2	400.4 ± 56.3		2	1890.0 ± 549.2		2	2.7		2
	Promethazine, 25 mg/ml	—[b]			—[b]			1272.6 ± 114.5			3.9		
	Diclofenac, 25 mg/ml	—[b]			—[b]			1186.2 ± 152.5			4.0		
	Cefotaxime, 250 mg/ml	—[b]			—[b]			—[b]			2.5		

[a] The investigation was performed in two test groups: test group 1, solutions 1, 2, 3, 4, and 5, and test group 2; solutions 0, 1, 5, 6, and 7. R1, ranking of original values within the first test group; R2, ranking of original values within second test group.

[b] No measurement made (see Table II).

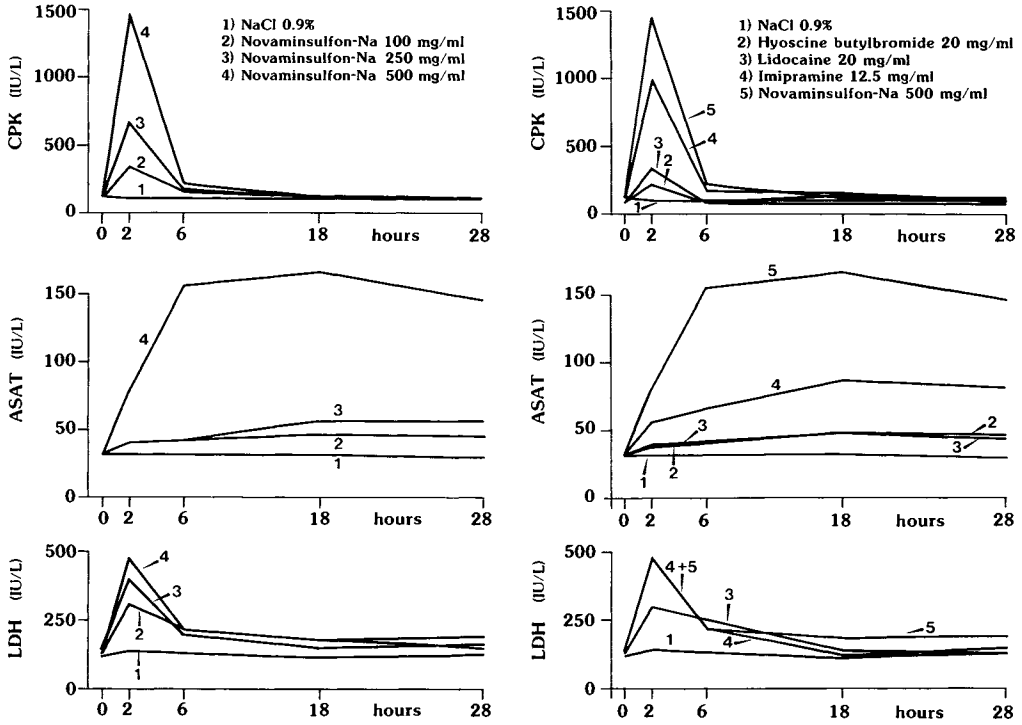

Fig. 1. Plasma enzyme activity after i.m. injection of various test solutions in rats.

Determination of the areas under the curve (28 hr) for the activity of the three enzymes produced the same result as the spot measurements 2-hr CPK, 18-hr ASAT, and 2-hr LDH (Fig. 2).

Macroscopic evaluation of muscle lesions by the rating system as well as the determination of the decrease in tissue CPK activity did not reveal more significant differences between the test solutions (see Fig. 2). The pronounced decrease in tissue CPK activity after i.m. administration of promethazine and diclofenac was not included in the statistical evaluation, since a rise in CPK activity was observed after i.p. administration (see Tables II and III). Ranking of 2-hr CPK, AUC-CPK, tissue CPK values, and score rating in the summary (see Table III) shows a close correlation, except the score rating with imipramine and novaminsulfon-Na, 500 mg/ml, where the ranks are exchanged.

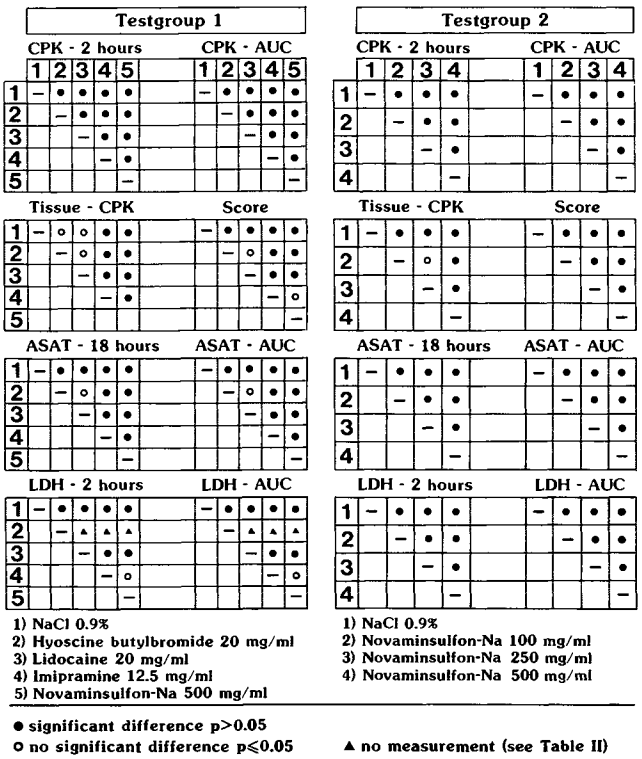

Fig. 2. Differentiation between the test solutions.

DISCUSSION

A number of morphological procedures have been proposed for the determination of local tissue damage resulting from intramuscular injections. Only a small number of these methods, however, is suitable for screening tests. Biochemical methods, such as that used to quantify plasma creatine phosphokinase activity in rabbits after i.m. injection, have simplified the determination of local tissue damage that results from injection of various solutions and are now widely used.

The present investigation has shown that biochemical methods of determining CPK, ASAT, and LDH activity for the purpose of ascertaining muscle damage can also be performed in rats. The high degree of standardized of the experimental conditions and the preparatory procedures, as proposed by Steiness *et al.* (6), led to relatively small variations (CPK, ≤22%; ASAT, ≤12%; LDH, ≤21%).

As various authors report, the peak elevation of CPK activity in rabbits is subject to considerable fluctuations. Therefore spot measurements are valid only when the differences in the elevation of activity are very pronounced; small

differences are difficult to interpret (8,17). In view of the short interval to peak elevation in the rat, this model is particularly suited for determination of CPK and LDH (2 ± 0.5 hr). The time interval to the peak elevation of ASAT activity was not as manifest as it was for the CPK and LDH activity (see Fig. 1). The elevation was pronounced only following the i.m. administration of novaminsulfon-Na, 500 mg/ml. The other test solutions produced less conspicuous rises. Therefore this enzyme may be less suitable as a parameter in routine investigations of local tissue damage.

The increase in activity of 2-hr CPK allowed significant differences in the tissue-damaging effects of all the injection solutions to be ascertained. This was not possible in all cases on the basis of ASAT and LDH activities. A decrease in tissue CPK activity, as proposed by Svendsen *et al.* (10), was similarly unsuitable in all cases for differentiation of tissue damage. Furthermore, the determination of this parameter is a very costly procedure and its use in routine testing cannot by justified.

Determination of AUC over a 28-hr period tallied with the findings of spot measurements of 2-hr CPK, 18-hr ASAT, and 2-hr LDH. However, the determination of a time course of enzyme activity has no advantages in evaluating local toxicity, while it involves greater stress for the test animals. Spot measurement of peak activity is a means of obtaining a rapid and reliable evaluation.

Ranking of the original values for 2-hr CPK, AUC-CPK, tissue CPK, and score rating shows a measure of agreement in nearly all cases, except the score rating with imipramine and novaminsulfon-Na, 500 mg/ml (Table III). However, statistically significant differentiation in test groups 1 and 2 was possible only in the case of 2-hr CPK and AUC-CPK (Fig. 2).

Macroscopic evaluation according to the method proposed by Shintani *et al.* (5) allowed a ranking of the test solutions referring to their tissue-damaging effect. However, a statistically significant differentiation was not possible in all cases.

The following potential limitation of the test procedures should be noted. The i.p. administration of the dose scheduled for i.m. tolerability tests can, on its own, lead to release of the enzymes under investigation (promethazine, diclofenac, hyoscine butylbromide). Instances of increased CPK activity without muscle injury have been reported by Meltzer (16). The evaluations of CPK activity were explained by a decrease in body temperature caused by the compounds. The reasons for the increased activity following i.p. administration of diclofenac and of LDH activity after hyoscine butylbromide are unknown. Greatly reduced enzyme activity, such as that following cefotaxime injection, is also possible. This phenomenon was observed after i.p. and i.m. administration. Interference with the enzyme assay in the presence of cefotaxime or inhibition of enzymatic activity at the site of injection may be possible. Therefore, results may be false positive in the case of cefotaxime or false negative for hyoscine butylbromide (LDH).

The experiments reported here show with regard to muscle damage that determination of 2-hr CPK activity and macroscopic inspection of the injection site are superior to the other parameters determined. The determination of 2-hr CPK activity is suitable not only for rapid evaluation of tissue toxicity but also for screening purposes. However, distorting factors by such tests may be possible. The inspection of the injection site provides important evidence but is a more exacting procedure. Both tests have to be considered as complementary. The CPK determination indicates events occurring immediately after the administration of the test solution, while morphologic injection provides information on the muscular lesion over a longer time period.

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