



Muscle Relaxant Property of Company X. Compound X (Study No. YYY)

DATE

This study was conducted in compliance with Institutional Animal Care and Use Committee regulations at one of NeuroDetective International's network laboratories.

This study was conducted under the terms of Statement of Work #QQQ, Laboratory Services Agreement between Company and NeuroDetective International, dated

Sponsor

Company

Sponsor Representative: Dr. XXX

Study Monitor: Dr. Forrest Haun
NeuroDetective International

Study Director: Dr. YYY

Testing Facility: NeuroDetective network laboratory

COMPLIANCE STATEMENT
NEURODETECTIVE INTERNATIONAL
Muscle Relaxant Property of Company Compound X

The study was initiated on DATE and the in-life phase ended on DATE, . The study was completed on DATE (date of first draft of final report).

The study was conducted in compliance with Institutional Animal Care and Use Committee regulations at a specific NeuroDetective network laboratory. As a non-GLP study it does not fall within the scope of the "Good Laboratory Practice" regulations of the United States Food and Drug Administration (21 CFR Part 58 and subsequent amendments).

I, the undersigned, hereby declare that the study described in this report was planned, performed and reported under my control as Study Director and that the report provides a true and accurate record of the data generated. I declare further that the original data sheets are archived in my laboratory at the testing facility described above.

Date _____
Dr. YYY
Study Director

This report has been peer reviewed and approved by:

Date _____
Forrest Haun, Ph.D.
Study Monitor

Date: _____
Dr. XXX
Sponsor Representative

OBJECTIVE

The objective of this study was to assess the muscle relaxant properties of a test compound, designated X in three rat behavior models.

This study was conducted in the laboratory of Dr. YYY at a NeuroDetective network laboratory, under the supervision of NeuroDetective International, Inc. The experimental procedures used for this study and protocol described below were approved by the Institutional Animal Care and Use Committee at the NeuroDetective network laboratory (protocol #####).

METHOD AND MATERIALSAnimals

Fifty-one male Sprague-Dawley rats, weighing 230 – 350g at the beginning of the study, were used. These animals were obtained from a commercial supplier (Harlan Laboratories, Houston, Texas, USA). The animals were housed in pairs and maintained on a 12:12 light:dark cycle with free access to water and food. The animals were maintained and cared for in accordance to the guidelines outlined by the International Association for the Study of Pain.

Experimental Design

The test and control items were administered once each by oral gavage as outlined below.

Treatment Group	Dose Level (mg/kg)	Dose Conc. (mg/mL)	Dose Volume (mL/kg)	Number of animals
1. Vehicle ^(a)	0	0	1 ml/kg	11
2. Carisoprodol	560	(b)	(b)	10
3. Low Dose X	2.5	0.25	1 ml/kg	10
4. Mid Dose X	10	1.0	1 ml/kg	10
5. High Dose X	20	2.0	1 ml/kg	10

- (a) Group 1 animals received the control/vehicle test item (2% Carboxymethylcellulose) alone.
- (b) Carisoprodol was initially prepared at 560 mg/ml and dosed at 1 ml/kg (560 mg/ml), per instructions from the Sponsor. This solution was used to dose 3 animals in Group 2. However the excessive viscosity of the resulting suspension raised concern about control of injection volume, and so the remaining 7 animals in Group 2 received the same dose but in a volume of 3 ml/kg (186.7 mg/ml). The animals from these two groups showed similar results in all behavior tests, and so their data was combined.

Reason for Choice of Route of Administration

Previous work has demonstrated that X is orally available and results in significant X systemic exposure.

Reason for Choice of Test System

The models were chosen because they are well-validated tools to assess the impacts of test items on skeletal muscle relaxation, locomotor activity and grip strength.

Materials:Test Items:

Identity:	X
Chemical Name	
Description	
Batch/Lot No.:	
Retest Date:	DATE
Purity:	>95%
Storage	Store at room temperature, protected from light
Handling	Reference MSDS
Manufacturer:	
Supplier:	Company X

Identity	Carisoprodol
Chemical Name	2-[[[(aminocarbonyl)oxy]methyl]-2-methylpentyl isopropylcarbamate
Description	White, crystalline powder
Batch	20433
Retest Date	November 2015
Purity:	99.8%
Storage	Store at room temperature
Handling	Reference MSDS
Manufacturer	Laboratorio CHimico Internazionale Milan, Italy
Supplier:	Mutual Pharmaceutical Company, Inc.

Vehicle Item

Component 1: 2% methylcellulose

Identity	Carboxymethylcellulose, 400 cps
Description	White powder
Batch	B74375-2
Storage	Room temperature
Handling	Standard laboratory precautions and as per MSDS
Supplier:	Calbiochem

Component 2: Water

Supplier	Distilled tap water via Millipore Direct Q-3
----------	--

Preparation of Dosing Formulations

The test and control items were prepared on the day of dosing (for each replicate) according to the instructions provided by the Sponsor. On the day of testing, animals were randomly assigned to drug condition, and then each received an oral administration of either X (one dose), vehicle only, or positive control, using a standard B-D 1ml syringe attached to a standard gavage needle.

Behavioral testing commenced on all animals beginning 1 hour after drug administration and was performed sequentially as follows locomotor activity, then grip strength and finally

morphine-induced Straub-tail (this test was performed last in order to avoid morphine-induced alteration in locomotor activity and grip strength). The total time to complete all three tests was approximately 100 - 110 minutes from the time of dosing. This time frame consists of direct testing time and the period of time between behavioral assays. For example, following the post-drug administration period, grip strength was tested (10 min), followed by open field (5 min of testing plus a few minutes between tests – 7 min total), followed by morphine induced Straub tail test (20 min post-morphine, a few minutes for the injection and between test period, a minute for assessment – 23 min total).

All behavioral testing was performed “blind” to the specific drug compound administered to each animal. Additional measures to ensure “blindness” included: (1) having one person in charge of creating and coding the drug solutions, randomization of animals to drug treatment, and breaking of drug code; (2) having another person in charge of behavioral testing, and other daily project related duties (i.e. daily monitoring of animal health, etc); and finally (3) having a third person in charge of data entry and management.

Behavior Testing

Assessment of the properties of the test compound utilized the following tests:

Locomotor activity

Each animal was placed within a circular open field chamber (50 cm diameter) made of galvanized steel covered with black contact paper. The black contact paper enhances the image of the white animal and ensures 100% tracking accuracy. The horizontal locomotor activity of the animals was recorded from an overhead camera, linked to image analysis software (Ethovision XT ver. 7.0, Noldus Information Technology, Leesburg, Virginia, USA). Total distance traveled in a 5 minute time period was recorded.

Grip strength

Each animal's forepaw grip strength was assessed using a standard strain gauge (Mark-10 series 5, M5-200). The animal was held gently, then lowered toward the strain gauge device and allowed to grasp the device rod with its forepaws. The animal was slowly pulled away from this rod until its grip was released. The force exerted on the gauge at the time of grip release was recorded. Each animal was given three trials, and the maximum force readings of the three trials were averaged to determine the grip strength for each animal. The testing procedure took approximately 10 minutes to complete.

Morphine-induced Straub tail

Each animal received a subcutaneous injection of 15 mg/kg morphine at a volume of 1 ml/kg. 20 minutes after the administration of morphine, the animal was scored for the presence of Straub tail, defined as a horizontal elevation of the tail when the tail is positioned over the edge of a table. A three-category scoring rubric was used, where 0 = absent, 1 = mild, and 2 = moderate to severe. The behavioral assessment was performed within a one minute period of time.

Statistical Analysis

Non-parametric statistics were used to analyze the Straub tail data since both variables (Treatment Group and Score) are categorical, and the results are reported as the Likelihood Ratio for the probability of the given frequencies occurring randomly. For the locomotor activity (open field) data, a one-way ANOVA for the 5 groups was used to analyze the total distance traveled (in cm) during the

five minute test period in the open field. For the grip strength data, the three trials were averaged for a single grip strength value in grams of force (gF) for each animal, and the group results analyzed using a one-way ANOVA. In addition, weight of the animals at the end of testing was analyzed, using a one-way ANOVA.

RESULTS

Straub Tail

An overall 5 x 3 Chi-Square test evaluating the impact of drug treatment (vehicle, positive control, 2.5 mg/kg X, 10 mg/kg X, and 20 mg/kg X) on Straub score (0, 1 or 2) was not significant, $LR(8) = 10.37$. Moreover a separate 3 x 3 Chi-Square test comparing only the three X drug groups was also not significant, $LR(4) = 4.15$. However, individual 2 x 3 Chi-Square tests comparing Vehicle and the different treatment groups showed that carisoprodol and both higher doses of X (10 mg/kg and 20 mg/kg) had significantly lower frequencies of the most severe Straub tail response (category 2), compared to Vehicle, as well as to the lowest dose of X (2.5 mg/kg), $LR(2) = 6.20$, $p < 0.05$ (Figure 1a).

Comparisons of the mean scores for each group showed that animals dosed with 2.5 mg/kg X also demonstrated a significantly more severe mean Straub tail response compared to those dosed with carisoprodol, as did the vehicle group, $(2) = 6.58$, $p < 0.05$ (Figure 1b). The two higher doses of X were not significantly different from carisoprodol in their mean Straub tail score.

Locomotor Activity

There was no significant overall effect of group on total locomotor activity (distance traveled), $F(4,46) = .49$, n.s. Fisher's post-hoc tests showed no individual differences among the groups in open field activity level (Figure 2).

Grip Strength

There was no significant effect of X on grip strength relative to vehicle (Figure 3). Overall there was a significant treatment effect, $F(4,46) = 2.53$, $p = 0.05$. Additional analysis to explore the significant overall main effect of treatment utilizing post-hoc tests (Fisher's LSD) showed that animals receiving carisoprodol had significantly weaker grip strength in comparison to vehicle-treated rats. No other group differences were present. It should be noted that the ANOVA showed statistically significant group differences despite the high intra-animal variability in this test and is the reason that the analysis was performed on the means of the three trials.

Weight

A one-way ANOVA showed no significant overall difference of body weight among the groups, $F(4,46) = 1.88$, n.s. (Figure 4).

Qualitative Observations

All treated animals appeared to be in good health throughout the experimental protocol. Although additional behaviors were not quantified, no obvious negative reactions were seen in any animal following any dosing with the test compound.

CONCLUSIONS

As assessed by the Straub tail test, X exhibited muscle relaxant activity at 10 mg/kg and 20 mg/kg. The lowest tested dose (2.5 mg/kg) was indistinguishable from vehicle. The positive control compound in this study (carisoprodol) also produced muscle relaxation at the dose tested, and its effect was not significantly different from the two higher doses of X tested. It should be noted that nearly half of the animals had no Straub tail response. The Straub tail test can be highly variable and the present results most likely indicate that the morphine dose was on the lower end of the effective curve. Regardless of the variability in the model, it is clear that there was a positive effect of X on these animals in reducing the most severe responses.

The locomotor activity test showed that neither the positive control nor any of the tested doses of X had a significant effect on horizontal activity.

The positive control (carisoprodol) produced significant weakening of grip strength relative to vehicle whereas no such effect was noted following X administration.

Figure Legends

Figure 1. Morphine-induced Straub tail. Figure 1A shows the frequency of each Straub tail category in the different treatment groups. The pattern of these results shows that the two higher doses of X reduced the frequency of the Straub tail response, significantly so for the more severe response (category 2). This pattern is also seen when the overall average severity of Straub tail (\pm SEM) is calculated for the different treatment groups (Figure 1B). * = $p < 0.05$ in Figure 1A based on non-parametric analysis, * = $p < 0.05$ versus carisoprodol in Figure 1B (Fisher's post-hoc test following overall significant ANOVA).

Figure 2. Average distance traveled (\pm SEM) during the 5 min open field test for different treatment groups. The overall analysis indicated no significant difference in open field activity among the treatment groups.

Figure 3. Average grip strength (\pm SEM) for different treatment groups. Carisoprodol treatment significantly decreased grip strength compared to vehicle. There was not a significant decrease in grip strength following X treatment relative to vehicle * = $p < 0.05$ versus carisoprodol.

Figure 4. Average body weight (\pm SEM) for different treatment groups. The overall analysis indicated no difference in body weight among the groups.

Figure 1

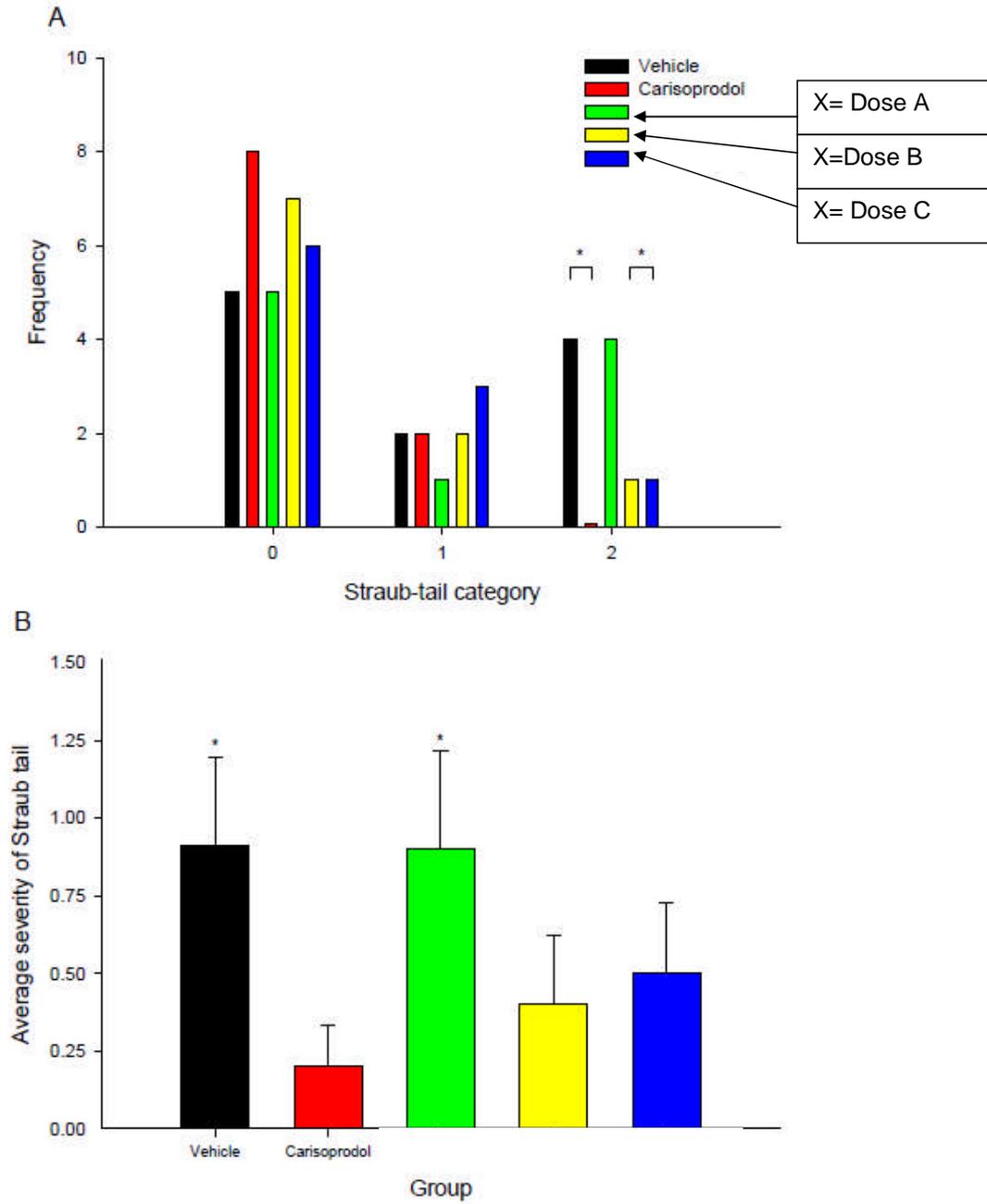


Figure 2

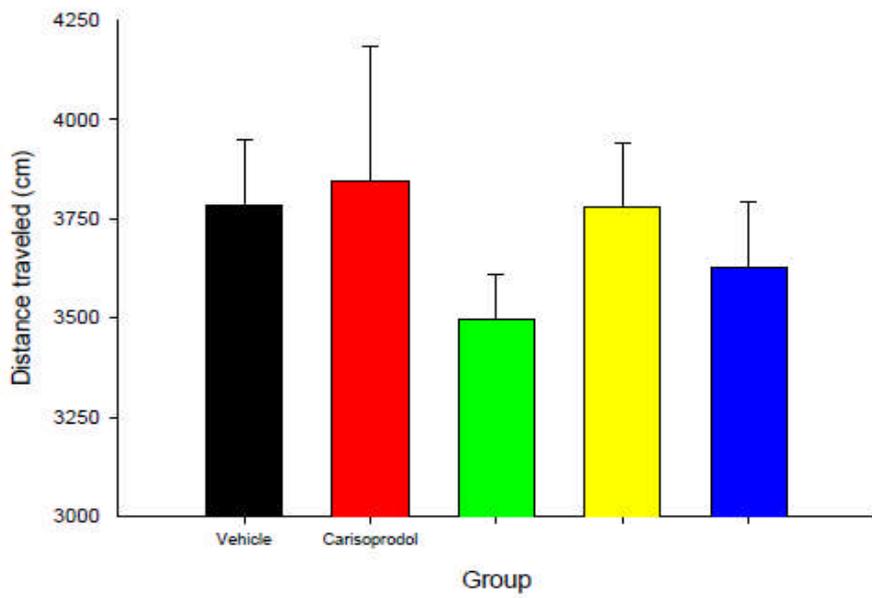


Figure 3

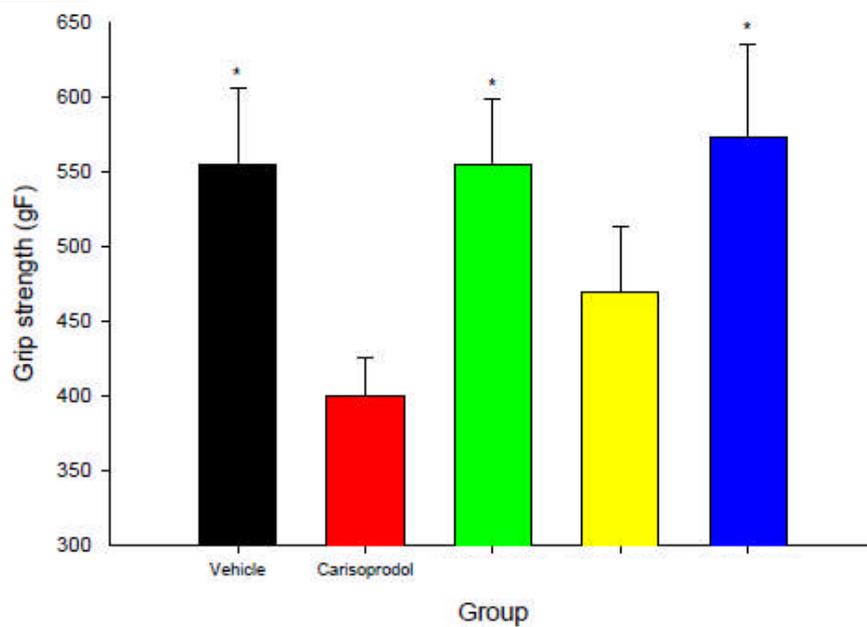


Figure 4

