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Original Article

Single-Dose Toxicity Study of Intramuscular Neuralgia-Pharmacopuncture Injection in Rats

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Objectives: Neuralgia-pharmacopuncture (NP) was recently developed as a water-soluble type of pharmacopuncture inspired by CS (care special pain)-pharmacopuncture. I aimed to evaluate the toxic response and approximate lethal dose of when NP when administered intramuscularly to Sprague Dawley rats.
Methods: The experimental group was divided into the NP test substance group and the saline control group and administered at a dose of 1.0 mL/animal to the posterior thigh muscles on both sides using a 1 mL syringe; each group consisted of five males and five females. Each rat was monitored for clinical signs and changes in body weight for 14 days after a single intramuscular injection. After completing observation, necropsy findings and localized tolerance at the injection site were assessed via gross necropsy and histopathological examination.
Results: No deaths occurred in the NP or control group, regardless of sex. During the observation period, no changes (such as general symptoms, weight change, or visual observation results at the time of autopsy) were judged to be due to the test substance. Histopathological examination showed no changes at the administration site judged to be caused by the test substance in either the male or female test substance administration groups. In addition, mononuclear cell infiltration of the outer membrane of the femoris muscle at the administration site was observed at the same frequency and extent in the control and NP groups, and was judged to be caused by physical stimulation by the injection needle; therefore, it had no toxicological significance.
Conclusion: Based on the above results, the approximate lethal dose for a single intramuscular administration of the test substance NP in Sprague-Dawley rats was judged to be > 1.0 mL/animal, and there were no findings that were judged to be due to the test substance at the administration site.

Keywords: safety, single dose intramuscular toxicity, neuralgia (singyeongtong)-pharmacopuncture, allyl isothiocyanate, CS pharmacopuncture

INTRODUCTION

Pharmacopuncture is a modern form of acupuncture therapy used in traditional medicines, such as Korean medicine (KM) and traditional Chinese medicine (TCM), to deliver herbal medicine to acupoints to regulate body function and improve pathological conditions [1, 2]. Various types of pharmacopuncture agents are used clinically, and new types of pharmacopuncture agents are continuously being developed based on evidence from the literature and clinical experiences in KM and TCM [3, 4]. It is crucial to verify the safety and effectiveness of pharmacopuncture prior to clinical use, which can be obtained through non-clinical toxicity tests on medicinal plants and herbal medicines [5-7]. Accordingly, it is important to verify the pharmacological mechanisms of action and potential toxicity of pharmacopuncture drugs at the Good Laboratory Practice (GLP) level [8, 9]. Nevertheless, few studies have conducted toxicity verification of pharmacopuncture agents in GLP-level institutions.

Neuralgia (Singyeongtong) pharmacopuncture (NP) was

developed in 2023 and is currently in use. NP consists of four drugs, including Carthami Fructus (CF), Persicae Semen (PS), Raphani Semen (RS), and Sinapis Semen (SS). SS has the effect of warming the lung to dispel phlegm, moving qi to dissipate binds, and freeing the collateral vessel to relieve pain. RS directs qi downward to resolve phlegm and promote digestion-relief distention [10]. Allyl isothiocyanate (AITC), a common ingredient in both medicines, has been traditionally used to treat rheumatic joint pain, blood circulation, and pain, and has also been shown to have anti-neuroinflammatory and neuroprotective effects [11]. CF has excellent efficacy in activating blood/unblocking the meridian and dissipating stasis/relieving pain [10]. Among its ingredients, moschamine is known for its strong anti-inflammatory effects [12]. PS has blood-activating and stasis-dispelling properties, as well as the ability to moisten the intestines and relax bowel effects [10]. Moreover, a component of amygdalin has pain and inflammation relief properties [13]. NP comprises the extracts of these herbal medicines and functions to dispel wind-cold-dampness, moves qi to relieve pain, has anti-inflammatory and analgesic effects, and is used to treat neuropathy and pain [1].

NP was inspired by care special pain (CS) pharmacopuncture (CSP) and has the same drug composition as CSP; however, the solvent and extraction methods differ from those of CSP, which is an oil-based pharmacopuncture of the cold-pressed oil extraction method. Given the difference in extraction methods, it is believed to be easier to operate and absorb into the body, can be used in larger amounts, and can reproduce a sense of qi similar to that of CSP [1].

The effects of CSP on pain relief and cervical and lumbar radiculopathy have been reported previously [14-16]. Although the therapeutic effect of NP can be predicted to some extent, there is still no relevant literature on NP developed in 2023. Therefore, in this study, we sought to evaluate the toxicity of NP through a single-dose muscle toxicity test among various toxicity evaluation methods to secure scientific evidence for the safety of NP used in KM clinical practice.

MATERIALS AND METHODS

1. Preparation of the test substances

The NP used in this study was a pharmacopuncture agent containing four herbs (CF, PS, RS, and SS) and was manufactured and provided by the Namsangcheon external herbal dispensary facility (Yongin, Korea) certified by the Ministry of Health and Welfare, which meets the Korean Good Manufacturing Practice standards. The certificate of analysis of NP was prepared by the Namsangcheon external herbal dispensary facility quality control team. After weighing CF (1,500 g), PS (600 g), RS (1,200 g), and SS (600 g), 10,000 cc of water was added as the injection solvent, and collected by circulating distillation in a distillation extractor at 110℃ (Table 1). The extract was diluted with purified water for injection, filtered, titrated to pH 7.4, placed in a sterile container, and sealed. Sterile saline solution (Dai Han Pharm. Co., Ltd., Seoul, Korea) was used as the control.

2. Experimental animals

Twenty-four Sprague–Dawley (SD) rats (7-week-old males and females) were purchased from Orient Bio Co., Ltd. (Seongnam, Korea). Rats were selected as the experimental animals for this test because they are widely used to evaluate the toxicity of

Table 1. Certificate of analysis of neuralgia-pharmacopuncture

Test items	Specifications	Results
1. Appearance	Transparent vial pharmacopuncture containing clear colorless liquid	Conform
2. pH	4.0-9.0	7.92
3. Endotoxin	< 150.15 EU/mL	Conform
4. Insoluble matter	No foreign objects visible to the naked eye	Conform
5. Insoluble particulates	10 μm or more ≤ 6,000 pieces 25 μm or more ≤ 600 pieces	Conform
6. Actual capacity	2.2 ± 0.1 mL	Conform
7. Sterile	Microorganisms not detected	Not detected
8. Confidentiality	No traces or leaks of methylene blue solution	Conform

Neuralgia pharmacopuncture: A four-herb extract consisting of Carthami Fructus, Persicae Semen, Raphani Semen, and Sinapis Semen.

various substances and have abundant basic test data.

At the time of acquisition, the animals were assessed for abnormal symptoms, and their body weights were measured. After an acclimatization period of 3 days, the animals were moved to the relevant animal room for a total of 6 days. During the acclimatization period, all animals were observed for general symptoms once daily, and their body weights were measured at the end of acclimatization. Only animals that showed no body weight abnormalities at the time of acquisition or at the end of acclimatization, and those that showed no abnormal symptoms during the acclimation period were used in the test.

The animals were housed under controlled environmental conditions at a temperature of 20.7-22.9℃, relative humidity of 55.6%-89.0%, ventilation frequency of 10-15 times/h, and free access to food and water. The study was approved by the Animal Experiment Operation Ethics Committee in accordance with the Animal Protection Act No. 18853 on April 26, 2022 (Approval No. 23R054). The experiments were conducted by Croen Inc. (Suwon, Korea) (Study No.: C23RA-161G).

3. NP single-dose intramuscular toxicity test in SD rats

1) Group composition and dosing

Animals with body weights that were far out with the average were excluded from the group separation. The 20 selected animals were divided into groups according to their ranked weights using a zigzag distribution to distribute the average weight of each group as evenly as possible. Five females and five males were assigned to each group: the control substance administration group (G1) and the test substance administration group (G2) (Table 2).

Because muscle is the clinically planned administration route of NP (0.1-1.0 mL/person), the intramuscular route was selected in this study, and an administration dose of 1.0 mL/animal was selected given that this is the maximum dose for clinical application. Administration was performed by two researchers - a corrector and an administrator - and the dose was administered to the posterior thigh muscles on both sides using a 1-mL syringe.

2) Methods of observation and examination of the animals

On the day of administration (day 0), the general condition (type of toxic signs, onset time, and recovery time) and the presence or absence of death were observed at 30 min and 1, 2, 3, and 4 h after administration. From the 1st to the 14th day after administration, general symptoms were observed once a day, and morphological degeneration, color change, and the tissue size at the administration site were observed and recorded in detail. The body weights of the rats were measured using an electronic scale (BCE2202-1SKR, Sartorius, Germany) upon animal acquisition and group separation, on the day of administration (before administration), and 1, 3, 7, and 14 days after administration (necropsy day).

3) Histopathology

After the observation period, all surviving animals were sacrificed via CO_2 gas inhalation and cutting of the posterior vena cava and abdominal aorta. To confirm the autopsy findings, all organs and tissues of the exterior, head, thoracic cavity, and abdominal cavity were observed with the naked eye. The left and right femoral muscle tissues (the injection sites) were extracted and fixed in 10% neutral-buffered formalin solution. Tissue slides were prepared for the fixed tissues of all animals. After staining with hematoxylin and eosin, histopathological examinations were performed. In addition, the presence of cell infiltration, necrosis, and edema at the administration site were evaluated.

Table 2. Group designation of a single-dose intramuscular toxicity test for neuralgia pharmacopuncture in Sprague-Dawley (SD) rats

Group		Dose of NP (mL/animal)	Injection dose amount (mL/animal)		Number of animals (object number)	
					Male	Female
G1	Control (Normal saline)	0	1.0	#Left: 0.5 #Right: 0.5	5 (1101–1105)	5 (2101–2105)
G2	Test substance (Neuralgia pharmacopuncture)	1.0	1.0	#Left: 0.5 #Right: 0.5	5 (1201–1205)	5 (2201–2205)

The substances were administered to both thigh muscles using a 1 mL syringe.
NP, neuralgia pharmacopuncture (a four-herb extract consisting of Carthami Fructus, Persicae Semen, Raphani Semen, and Sinapis Semen).

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4. Statistical analysis

Statistical analyses were performed for body weight. In the independent-group t-test, homoscedasticity was tested using the Levene test; in the case of equal variance, Student's t-test was used; and in the case of heteroscedasticity, Welch's t-test was used to compare the results to those of the control group. The significance of these results was confirmed, with statistical significance set at a p-value < 0.05. Statistical analysis was conducted using SPSS (IBM® SPSS Statistics, ver. 24), a widely used commercial statistical package.

5. HPLC analysis

AITC is the active ingredient of NP and a standard substance for RS and SS. To confirm the AITC content of NP, HPLC was conducted using NP distillate (10 μL, 50 μL, and 100 μL) and allyl isothiocyanate standard (10 μL; 0.01 mL/mL in MeOH). HPLC analysis was performed at 30℃, with a wavelength of 246 nm, a flow rate of 0.5 mL/min, and a Watchers 120 ODS-BP (4.6

Table 3. Neuralgia-pharmacopuncture-related effects on changes in clinical signs and mortality in a single-dose intramuscular toxicity study in Sprague-Dawley rats

Sex	Group	Count positives (for observable abnormality) (number of individuals with observable abnormality) Day(s) relative to start date														Total mortality
		1	1→2	1→3	1→4	1→5	1→6	1→7	1→8	1→9	1→10	1→11	1→12	1→13	1→14	
Male	G1 (n = 5)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
	G2 (n = 5)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
Female	G1 (n = 5)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
	G2 (n = 5)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5

G1: Control group administered with normal saline (1.0 mL/animal).
G2: Neuralgia-pharmacopuncture administration group (1.0 mL/animal).
Neuralgia pharmacopuncture: A four-herb extract consisting of Carthami Fructus, Persicae Semen, Raphani Semen, and Sinapis Semen.

Table 4. Effects of neuralgia-pharmacopuncture on changes in mean body weights in a single-dose intramuscular toxicity test in Sprague-Dawley (SD) rats

Sex	Group/NP dose (mL/animal)		Day 0	1	3	7	14	Day 0-14 gain (g)
Male	G1	Mean	291.09	292.54	316.05	344.39	392.62	101.53
	0	S.D.	13.59	13.12	15.08	16.12	19.60	6.69
		N	5	5	5	5	5	5
	G2	Mean	291.39	293.51	316.14	349.14	395.26	103.86
	1.0	S.D.	9.48	10.01	11.12	13.58	14.39	14.71
		N	5	5	5	5	5	5
Female	G1	Mean	219.51	219.55	231.02	247.67	267.56	48.05
	0	S.D.	8.64	8.07	6.42	8.48	16.66	12.99
		N	5	5	5	5	5	5
	G2	Mean	223.47	221.77	230.01	241.83	257.44	33.98
	1.0	S.D.	2.26	5.72	1.11	4.33	11.86	11.48
		N	5	5	5	5	5	5

Significantly different from control by Student's test.
S.D., standard deviation; N, number of animals.
G1: Control group administered with normal saline (1.0 mL/animal).
G2: Neuralgia-pharmacopuncture administration group (1.0 mL/animal).
Neuralgia pharmacopuncture: A four-herb extract consisting of Carthami Fructus, Persicae Semen, Raphani Semen, and Sinapis Semen.

× 150 mm, 5 μm) column using a 1260 infinity II LC System (Agilent Technologies, Santa Clara, USA). Chromatographic separation was performed using methanol as mobile phase B and distilled water as mobile phase A via the following gradient solvent system procedure at the retention time (RT): 0 min, 30% B; 10 min, 30% B; 20 min, 40% B; 25 min, 100% B; and 35 min, 100% B.

RESULTS

1. Observation of death and clinical signs

No deaths were observed in the male or female SD rats, nor the NP test substance-administered groups. During the observation period, no abnormalities in type of toxicity sign, onset time, or recovery time were observed in any of the animals in either group, and no abnormalities in tissues (morphological degeneration, color change, size, etc.) at the administration site were observed (Table 3).

2. Observation results for the weight change

Observations of body weight changes before administration and on days 1, 3, 7, and 14 after administration are shown in Table 4. During the observation period, no statistically significant changes in body weight were observed in the rats in the NP test substance-administered group compared to the saline control substance-administered group.

3. Macroscopic examination after necropsy

Visual observation at the time of necropsy revealed no changes that were judged to be due to the test substance in any of the rats in either group (Table 5).

Table 5. Necropsy findings in a single-dose intramuscular toxicity test for neuralgia-pharmacopuncture in Sprague–Dawley rats

Sex	Group		Organ necropsy findings	Type of sacrifice
	Dose (mL/animal)	Animal ID		
Male	G1 (n = 5)	1101	No abnormality detected	Scheduled
	0	1102	No abnormality detected	Scheduled
		1103	No abnormality detected	Scheduled
		1104	No abnormality detected	Scheduled
		1105	No abnormality detected	Scheduled
	G2 (n = 5)	1201	No abnormality detected	Scheduled
	1.0	1202	No abnormality detected	Scheduled
		1203	No abnormality detected	Scheduled
		1204	No abnormality detected	Scheduled
		1205	No abnormality detected	Scheduled
Female	G1 (n = 5)	2101	No abnormality detected	Scheduled
	0	2102	No abnormality detected	Scheduled
		2103	No abnormality detected	Scheduled
		2104	No abnormality detected	Scheduled
		2105	No abnormality detected	Scheduled
	G2 (n = 5)	2201	No abnormality detected	Scheduled
	1.0	2202	No abnormality detected	Scheduled
		2203	No abnormality detected	Scheduled
		2204	No abnormality detected	Scheduled
		2205	No abnormality detected	Scheduled

Pathological examinations of all the organs on the external surface and inside the body cavity were performed.
G1: Control group administered with normal saline (1.0 mL/animal).
G2: Neuralgia-pharmacopuncture administration group (1.0 mL/animal).
Neuralgia pharmacopuncture: A four-herb extract consisting of Carthami Fructus, Persicae Semen, Raphani Semen, and Sinapis Semen.

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4. Histopathological examination

In the control substance-administered group, one case of mononuclear cell infiltration (mononuclear cells, epimysium) was observed in the outer layer of the right femoris muscle of male rats, and two cases of mononuclear cell infiltration were observed in the outer layer of the right thigh muscle of female rats. In the NP test substance administration group, one case of mononuclear cell infiltration was observed in the outer membrane of the left thigh muscle of male rats, and one case of mononuclear cell infiltration was observed in the outer membrane of the right thigh muscle of female rats (Table 6, Fig. 1).

Table 6. Histopathological findings in a single-dose intramuscular toxicity test for neuralgia-pharmacopuncture in Sprague–Dawley rats

Group/dose (mL/animal)	Male								Female							
	G1/0				G2/1.0				G1/0				G2/1.0			
Animal ID	1101	1102	1103	1104	1105	1201	1202	1203	1204	1205	2101	2102	2103	2104	2105	2201	2202	2203	2204	2205
Type of sacrifice	Scheduled																			
Day of sacrifice	14																			
Organ/findings																				
Femoral muscle, right - Infiltrate, mononuclear cell, epimysium	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
Femoral muscle, left - Infiltrate, mononuclear cell, epimysium	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-

Grades: normal, + minimal, ++ mild, +++ moderate, ++++ marked, +++++ severe (+) present.
G1: Control group administered with normal saline (1.0 mL/animal).
G2: Neuralgia-pharmacopuncture administration group (1.0 mL/animal).
Neuralgia pharmacopuncture: A four-herb extract consisting of Carthami Fructus, Persicae Semen, Raphani Semen, and Sinapis Semen.

5. HPLC analysis

HPLC analysis confirmed the presence of AITC in the NP by performing a comparison with the RT of the authentic AITC standard (STD) (Fig. 2). After injecting 10 μL of 94% AITC STD at 0.01 mL/mL in MeOH, the AITC peak was confirmed at a RT of 20.740 min (Fig. 2A). The expected AITC peak was not found with a 10 μL volume of NP, but after injecting 50 and 100 μL NP, peaks were detected at RTs of 20.550 and 20.626 min, respectively (Fig. 2B). Accordingly, as a result of quantitative analysis of the AITC content, the concentration of AITC in NP was calculated as 2.61 ng/mL based on an injection volume of 100 μL NP.

DISCUSSION

CSP is an oil-based pharmacopuncture, the main ingredient of which is *Taxus cuspidate*. CSP is a directional pharmacopuncture, with excellent analgesic effects owing to its ability to induce a strong sense of qi; as such, CSP has been widely used in KM clinical practice [1, 17]. However, owing to difficulties in the stable supply of *Taxus cuspidata*, a modified CSP consisting of CF, PS, RS, and SS was developed and has been used since 2015. The safety of CSP has been verified by a single intramuscular administration toxicity test in SD rats [17], a reverse mutation test [18], a micronucleus test in mice [19], and a chromosomal aberration test in mammalian cultured cells [20]. Clinically, previous studies have reported the use of CSP for cervical and lumbar radiculopathy and herpes zoster [14-16].

The most frequent side effects of oil-based pharmacopuncture occur because of overdose, erroneous procedures, and spoiled pharmacopuncture. Additionally, although uncommon,

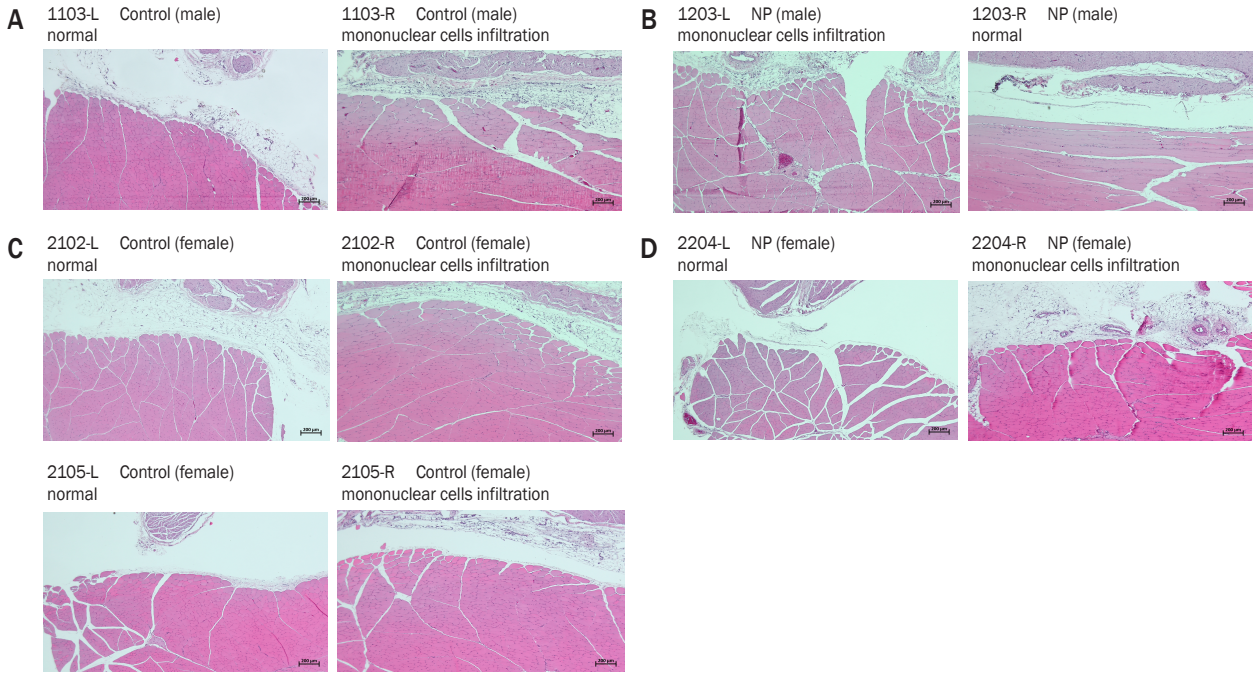

Figure 1. Individual histopathological findings showing mononuclear cell infiltration in the epimysium of the male control injection site (A), male NP group injection site (B), female control injection site (C), and female NP group injection site (D) (Original scale ×50). Mononuclear cell infiltration was observed in the epimysium of the right thigh muscle of male No. 1103 and female Nos. 2101 and 2105 in the control group and mononuclear cell infiltration was observed in the epimysium of the left thigh muscle of male No. 1203 and female No. 2204 in the NP-treated group. L, Left; R, Right; NP, neuralgia pharmacopuncture, a four-herb extract consisting of Carthami Fructus, Persicae Semen, Raphani Semen, and Sinapis Semen.

there is a possibility of allergic reactions in cases of excessive dosage [21]. Moreover, previous pharmacokinetic studies have suggested that oil-based pharmacopuncture may remain in the body longer than water-soluble pharmacopuncture [22, 23]. Accordingly, NP was developed to allow for easier injection, absorption, and excretion from the body, and is now available for use in larger doses than oil-based products because of its conversion to a water-soluble substance via hot water extraction, whilst retaining the four components of CSP.

In actual KM clinical practice, NP is used to treat neuralgia at higher doses than the existing CSP. However, there have been no related safety and efficacy reports. Therefore, to obtain scientific evidence related to the safety of NP, we sought to evaluate local tolerance through the toxic response, approximate lethal dose (ALD), and histopathological examination following a single intramuscular injection of NP to SD rats.

Toxicity studies using various experimental models, including single-dose toxicity studies, must be systematically conducted to determine the safety of pharmaceuticals and herbal medicines [5, 6]. In toxicity tests, it is necessary to select animal species with similar efficacy or metabolic patterns to humans, considering species-level and phylogenetically different biological responses to the drug being tested. In the single-dose toxicity tests, animals that were easy to handle, qualitatively uniform, and had rich background data were used, with rats being preferred among rodents. Moreover, it is recommended to assess toxicity in at least one male and one female, considering the differences in toxicological responses between the sexes [6]. Therefore, in this study, we conducted a single intramuscular toxicity test for NP using both female and male SD rats. For the test substance, a dose of 1.0 mL/animal was used in the administration group, while the control substance group were administered saline solution. The mortality, general symptoms, and body weight were measured for 14 days after the administration of the test substance, and visual and histopathological examinations were conducted at the time of autopsy after the end of the observation period. No adverse reactions, such as death or general symptoms, and no significant changes in body weight were observed during the observation period in the male and female rats in either group. In addition, visual observation at the time

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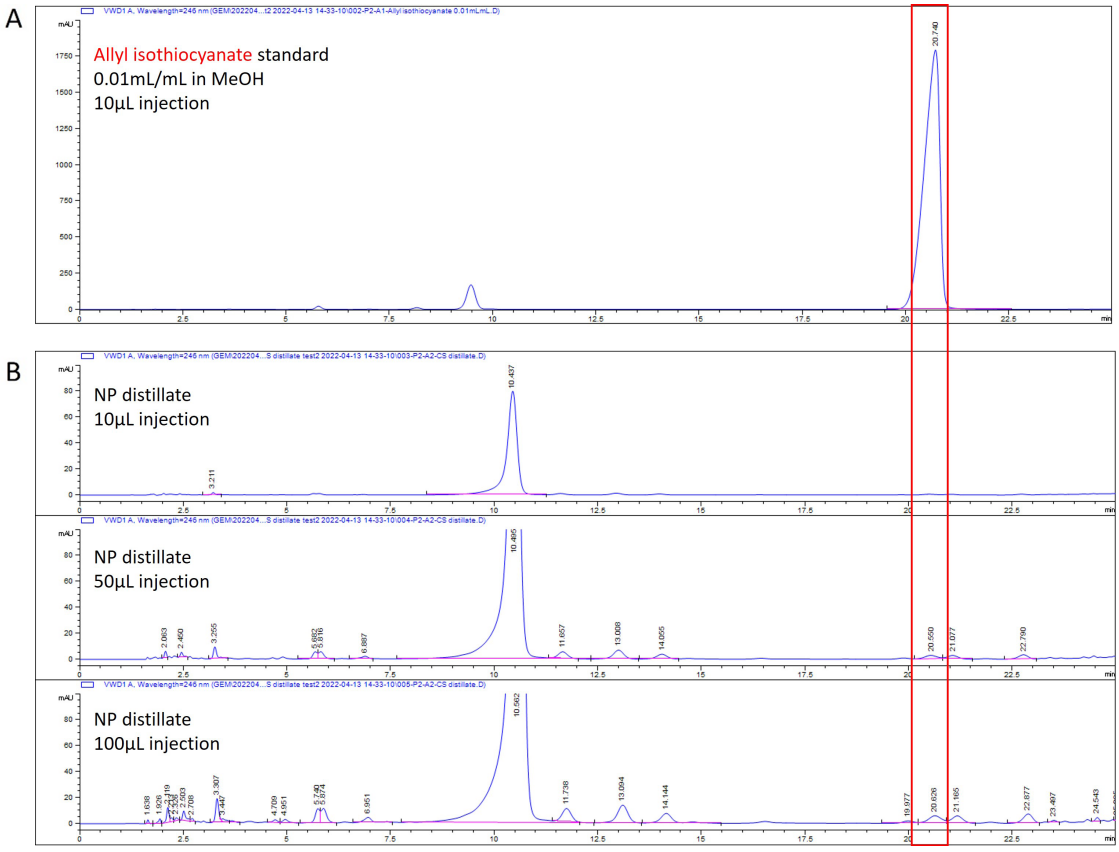

Figure 2. HPLC pattern analysis of 10 μL, 50 μL, and 100 μL injection of neuralgia-pharmacopuncture (NP) (B) and allyl isothiocyanate standard (A). NP, neuralgia pharmacopuncture, a four-herb extract consisting of Carthami Fructus, Persicae Semen, Raphani Semen, and Sinapis Semen.

of autopsy demonstrated that there were no significant changes due to the test substance. Moreover, histopathological examination demonstrated that there were no significant changes due to the test substance at the administration site in the male and female rats in either group. The extent of mononuclear cell infiltration of the outer membrane of the femoris muscle at the site of administration was observed to be similar in both groups, suggesting that the infiltration was caused by the physical stimulation of the needle during administration and does not appear to have toxicological significance.

Based on the above results, when administered intramuscularly in a single dose to SD rats, the ALD of the test substance NP was judged to be > 1.0 mL/animal, with no results that were judged to be influenced by NP at the administration site. Although the animal test results may differ from human results, our findings suggest that 1 mL of NP may be safe in humans, weighing approximately 150 times the weight of the animals used in this experiment.

This study has a limitation in that we evaluated toxicity for a relatively short period of 2 weeks, with only a single injection of NP. Additionally, multiple administrations, long-term toxicity observations, other administration routes, genotoxicity testing, and liver and kidney toxicity evaluations must be performed. Finally, various clinical reports will be necessary to clarify the safety and effectiveness of NP.

CONCLUSIONS

When NP was administered intramuscularly in a single dose to SD rats, the ALD was judged to be > 1.0 mL/animal, and no findings were judged to be due to the effect of the test substance at the administration site. Despite this, additional toxicity evaluations of NP are required.

CONFLICTS OF INTEREST

The author declares no conflict of interest.

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