



Original Article

Establishment and Selection of Indicator Materials for *Cervi Parvum Cornu* Pharmacopuncture



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ABSTRACT

Background: Recently, *Cervi Parvum Cornu* pharmacopuncture has been widely used. But no studies on the indicator materials for *Cervi Parvum Cornu* pharmacopuncture have been conducted. The aim of this study was to select indicator materials that would aid in the uniform preparation of standardized *Cervi Parvum Cornu* pharmacopuncture.

Methods: Three lots of *Cervi Parvum Cornu* pharmacopuncture were analysed. Each lot was prepared using the same methods and materials. Chondroitin sulfate, alanine, and leucine were selected as the indicator materials for *Cervi Parvum Cornu*. For standardization, chondroitin sulfate analysis was performed using the colorimetric method, while alanine and leucine were analyzed using liquid chromatography–mass spectrometry (LC–MS).

Results: Analysis of the three lots of *Cervi Parvum Cornu* pharmacopuncture found chondroitin sulfate levels of 108.9 ± 17.3 ug/ml, 118.8 ± 5.0 ug/ml and 112.3 ± 11.9 ug/ml. Alanine levels were 44.9 ± 2.8 ug/ml, 44.6 ± 0.3 ug/ml, and 43.9 ± 0.2 ug/ml. Leucine levels were 29.6 ± 0.7 ug/ml, 29.0 ± 0.1 ug/ml, and 29.4 ± 0.1 ug/ml.

Conclusion: These results suggest that chondroitin sulfate, alanine, and leucine may be useful for the standardization of *Cervi Parvum Cornu* pharmacopuncture.

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Introduction

Cervi Parvum Cornu is the dried deer velvet from the unboned horns of young bucks. Depending on the section, the bone is classified as tip, upper section, midsection, or base. There are many amino acids, proteins, lipids, polyamines, and saccharides in the tip of *Cervi Parvum Cornu*. The midsection and the base are ossified, and the amount of the substances mentioned above is small.

In oriental medicine, the nature of *Cervi Parvum Cornu* is warm, the taste is sweet and salty, and it enters the kidney and liver. It has purported efficacy in nephrotic syndrome, hyperplasia, hypertrophy, and stiff muscles. Traditionally, it has been used for impotence, lassitude of spirit, fear of cold, dizziness, tinnitus, deafness, vaginal discharge, and lumbar vertebrae disease. Recent studies reported that *Cervi Parvum Cornu* seemed to have effects on accelerating body weight and neuromuscular development,

reducing muscle fatigue, improving musculoskeletal function, promoting neural cell growth, increasing bone strength and weight, and improving arthritis symptoms, immune function, anticancer function, and wound healing [1–3].

In recent years, the use of *Cervi Parvum Cornu* pharmacopuncture based on acupuncture, meridians, and herbalism has increased among oriental medicine doctors. In *Cervi Parvum Cornu* pharmacopuncture, extracts of *Cervi Parvum Cornu* obtained by distillation are injected into tender points or acupuncture points for an expected result similar to that from acupuncture and drug treatment.

Several studies of *Cervi Parvum Cornu* pharmacopuncture have reported it to be effective in reducing the symptom severity of arthritis [4–14] and osteoporosis [15–19], in aging [20,21] and growth disorders [22,23]. Other studies have shown that *Cervi*

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Parvum Cornu pharmacopuncture increased levels of antioxidant enzymes [20,21], promoted hormone production [24], helped to regulate the autonomic nervous system [25,26], had anticancer effects [27,28], and enhanced immune function [29].

The clinical application of *Cervi Parvum Cornu* pharmacopuncture is also increasing and related research is actively underway. However, there is a lack of consideration of *Cervi Parvum Cornu* pharmacopuncture formulation and preparation. There is also risk that the active components decompose or are removed during preparation and purification. There is insufficient standardization for the process of *Cervi Parvum Cornu* pharmacopuncture. It is necessary to set the indicator materials for the quality control and standardization of *Cervi Parvum Cornu* pharmacopuncture. Therefore, chondroitin sulfate, alanine and leucine—which are easy to analyze without precipitation during the preparation process of *Cervi Parvum Cornu* pharmacopuncture—were set as indicator materials of *Cervi Parvum Cornu* pharmacopuncture. This study was conducted to establish the method of analyzing the indices for standardization.

Materials and Methods

Instruments and reagents

Glucuronolactone, alanine and leucine were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA); their purity were 99%, 98% and 98.5%, respectively. Carbazole used in the pretreatment was purchased from Daejung Chemical and Metal Co. Ltd. (Shiheung City, Gyeonggi-do, South Korea). Sodium borate and trifluoroacetic acid were purchased from Junsei Chemical Co. Ltd. (Tokyo, Japan). Anhydrous ethanol and sulfuric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA), as were the water and acetonitrile (ACN; HPLC grade) used for quantitative analysis. Liquid chromatography–mass spectrometry (LC–MS) was used for component analysis (LCMS-2020; Shimadzu Corp., Kyoto, Japan). Sunrise RC from TECAN (Männedorf, Switzerland) was used for absorbance measurement.

How to prepare *Cervi Parvum Cornu* pharmacopuncture

The *Cervi Parvum Cornu* pharmacopuncture used in the analysis was from Namyangju outpatient department of Jaseng Oriental Medicine Hospital, Korea, which had been prepared according to Korean Traditional Medicine Standardization Project guidelines on pharmacopuncture. *Cervi Parvum Cornu* (400 g) was put through an extraction process using purified water at 105°C for 2 hours, for a total of seven times. Thereafter, it was concentrated under reduced pressure at 38°C. The concentrated extract was stirred with 90% ethyl alcohol seven times. The solution was filtered to remove the fibrous substance. The filtrate was adjusted to have an alcohol concentration of 70% using ethyl alcohol and filtered again. The filtrate was concentrated under reduced pressure at 38°C, and the extract was diluted with purified water, then sterilizing filtrated and lyophilized. The lyophilized powder was diluted to a concentration of 2 mg/ml in a clean room. The pH of the diluted solution was adjusted to 7.0–7.4, and the salinity to 0.9%. After this process, the pharmacopuncture was sterilized at 121°C for 25 minutes. It was then ready to be used in the clinic (Fig. 1).

Setting the indicator materials of *Cervi Parvum Cornu* pharmacopuncture

In order to establish suitable indicator materials for *Cervi Par-*

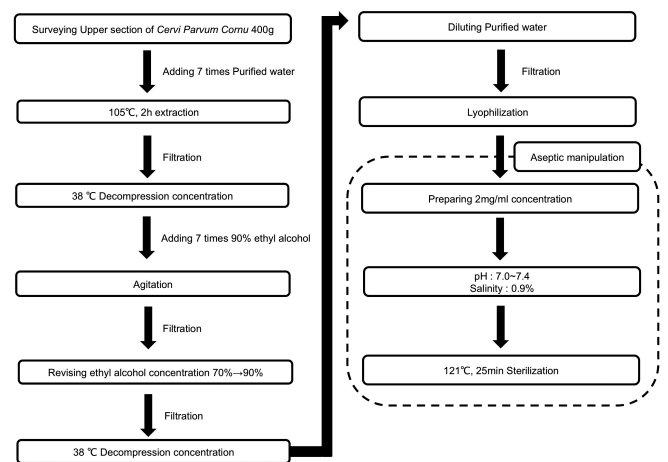


Fig. 1. Manufacturing process of *Cervi Parvum Cornu* pharmacopuncture.

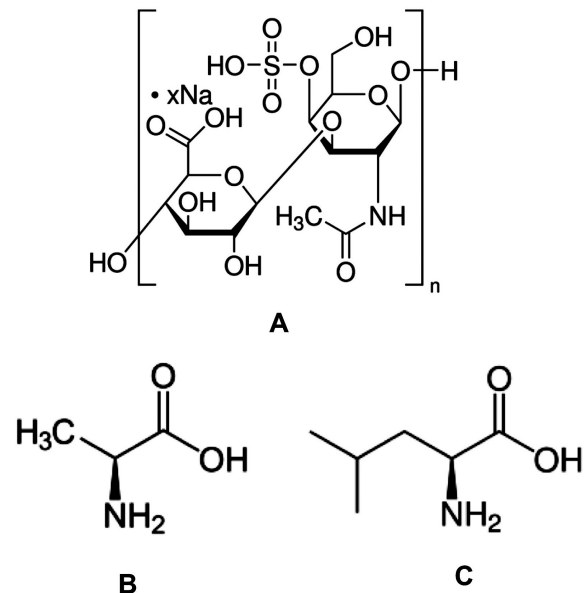


Fig. 2. Structural formula of the index components of *Cervi Parvum Cornu* pharmacopuncture: (A) chondroitin sulfate; (B) alanine; (C) leucine.

vum Cornu pharmacopuncture, we looked at the literature. Various components such as glucosamine sulfate, chondroitin sulfate, prostaglandins, collagen, proteoglycans, amino acids and linoleic acid were identified [30,31]. We looked for components that would be easy to analyze without sedimentation in the preparation process. Chondroitin sulfate, alanine and leucine were chosen as indicator materials (Fig. 2). Chondroitin sulfate is a polysaccharide composed of N-acetylgalactosamine, uronic acid and sulfuric acid, which are the main components of cartilage. Chondroitin sulfate is also found in various connective tissues such as skin and umbilical cord, and is related to the osteolysis of cartilage. Alanine can be easily metabolized with lactic acid and is involved in producing glucose from protein through the alanine circuit. Leucine is an amino acid that makes up muscles. It is involved in blood sugar regulation, bone and muscle tissue growth, hormone production,

healing of damaged tissue, and energy generation.

Analysis of chondroitin sulfate levels

The level of chondroitin sulfate in *Cervi Parvum Cornu* pharmacopuncture was analyzed by the colorimetric method which is used in the Health Functional Food Test[32]. The analytical sample was obtained by filtering the *Cervi Parvum Cornu* pharmacopuncture with a 0.2 μm polyvinylidene difluoride syringe filter. There are three groups : blank (water, reference solution), standard solution, test solution. The standard solution was glucuronolactone at a concentration of 20 μg/ml. After adding 5 ml of sodium borate sulfate solution to the colorless tube and cooling thoroughly with ice water, 1 ml of the test solution and 1 ml of the standard solution were carefully added into the tube separately. It was then heated for 10 minutes and immediately cooled with ice water. Then, 0.2 ml of carbazole solution was added and mixed separately; the mixture was heated for 15 minutes and then cooled with ice water. 1 ml of water was used as a blank in the same manner as above. Absorbance was measured at a wavelength of 530 nm and the level of chondroitin sulfate was determined according to the following equation.

$$\text{Contents of Chondroitine sulfate}(\mu\text{g/ml}) = \frac{\frac{\text{Absorbance of the test solution}}{\text{Absorbance of the standard solution}} \times \text{Concentration of Standard substance} \times 1.1023}{\text{Concentration of Sample}} \times 2.593$$

2.593 = Molecular weight of chondroitin sulfate / Molecular weight of glucuronic acid

1.1023 = Molecular weight of glucuronic acid / Molecular weight of glucuronolactone

Conditions for analysis of alanine and leucine

Analysis of alanine and leucine was based on a previously described method [33]. We developed a simultaneous method that could detect both indicator materials at the same time using LC-MS. The column used for the analysis was Shim-pack XR-ODS

Table 1. Composition of the Mobile Phase Employed in the Gradient LC System

Time (min)	Composition of mobile phase(%)	
	0.1% TFA in Water	0.1% TFA in Acetonitrile
0	95	5
19	36	64
20	95	5
30	95	5

Table 2. Mass Detector Condition of Alanine and Leucine

Analytes	Condition
Interface	ESI(+)
Nebulizer gas flow	1.5
DL temp (°C)	250
Heat block temp (°C)	400
Dry gas flow (L/min)	15
selected ion monitoring(SIM)mode(m/z)	Alanine : 90, Leucine : 132

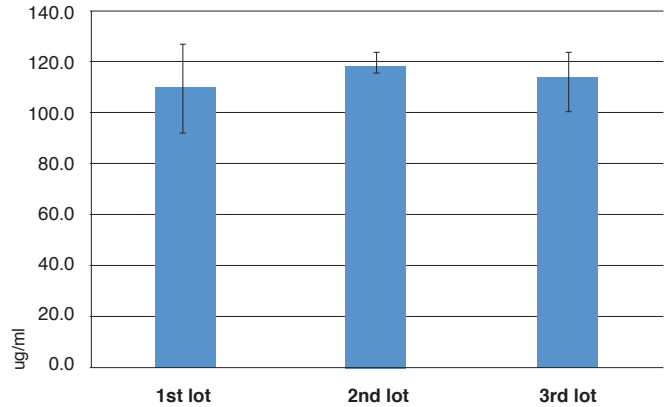


Fig. 3. Chondroitin sulfate levels in the three lots of *Cervi Parvum Cornu* pharmacopuncture.

(3.5 μm, 4.6 I.D. × 150 mm; Shimadzu Corp.). Temperature was maintained at 35°C, the injection volume was 1 μl, and the flow rate was 0.4 ml/min. (A) 0.1% trifluoroacetic acid in water and (B) 0.1% trifluoroacetic acid in acetonitrile were degassed using ultrasound and analyzed by gradient system (Table 1). Electrospray ionization–mass spectrometry was used for the detector, and the detection conditions are shown in Table 2.

Results

Chondroitin sulfate levels in Cervi Parvum Cornu pharmacopuncture

When the three lots of *Cervi Parvum Cornu* pharmacopuncture prepared in the Namyangju outpatient department of Jaseng Oriental Hospital were analyzed, chondroitin sulfate levels were 108.9 ± 17.3 μg/ml, 118.8 ± 5.0 μg/ml, and 112.3 ± 11.9 μg/ml, respectively (Fig. 3).

Analysis of alanine and leucine levels in Cervi Parvum Cornu pharmacopuncture

The coefficient of correlation between the calibration curve and linearity was calculated by analyzing the indicator materials, alanine and leucine, at five concentrations each (5, 10, 50, 100, 500 μg/ml). For alanine, a calibration curve Y = 2241X + 152,447 was obtained, while for leucine, a calibration curve Y = 66,583X + 155,241 was obtained. Both were in the range of 5–500 μg/ml, and linearity was obtained with a correlation coefficient of 0.999 or more.

After the three lots of *Cervi Parvum Cornu* pharmacopuncture prepared in the Namyangju outpatient department of Jaseng Oriental Hospital were analyzed, the results were added to the calibration curves. Alanine levels were found to be 44.9 ± 2.8 μg/ml, 44.6 ± 0.3 μg/ml, and 43.9 ± 0.2 μg/ml, while leucine levels were found to be 29.6 ± 0.7 μg/ml, 29.0 ± 0.1 μg/ml, and 29.4 ± 0.1 μg/ml (Figs. 4 & 5).

Discussion

Cervi Parvum Cornu is the cut and dried horn of young bucks, including *Cervus nippon Temminck*, *Cervus elaphus Linné*, and *Cervus canadensis Erxleben*. The horns of the young bucks are not

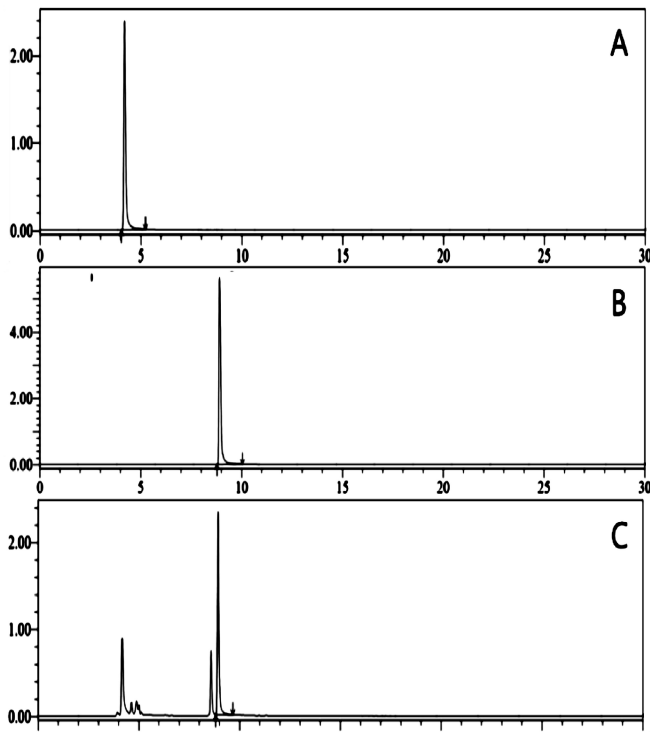


Fig. 4. Liquid chromatography-mass spectrometry chromatogram of *Cervi Parvum Cornu* pharmacopuncture and standard compounds: (A) alanine standard (SIM mode: m/z 90 [M+H]⁺); (B) leucine standard (SIM mode: m/z 132 [M+H]⁺); (C) *Cervi Parvum Cornu* pharmacopuncture (SIM mode: m/z 90, 132).

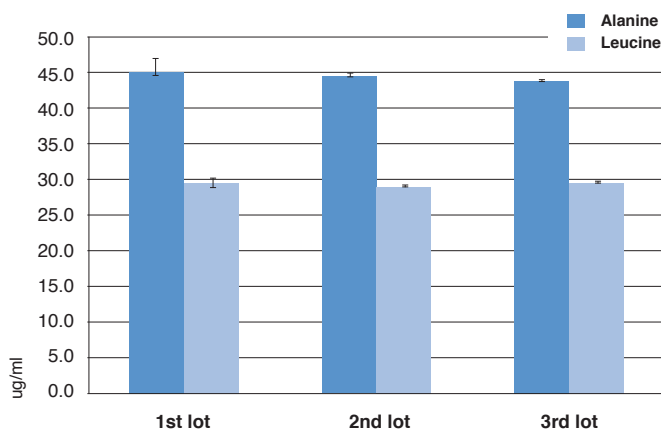


Fig. 5. Alanine and leucine levels in the three lots of *Cervi Parvum Cornu* pharmacopuncture.

ossified or slightly osseous, and are hairy. Because it is in a state of growth, there are various growth factors present. For example, it contains many components such as amino acids, proteins, lipids, polyamines, and sugars [34].

Donguibogam is a book that describes the effects of *Cervi Parvum Cornu* and prescriptions containing it. *Cervi Parvum Cornu* was believed to be efficacious in harmonizing the thoroughfare and conception vessels, so it was used in the treatment of dizziness, tinnitus, deafness, vaginal discharge, spermatorrhea, profuse menstruation, uterine bleeding, and hemophilia. It was believed to have a kidney yang-tonifying effect for the treatment of symptoms such

as impotence, seminal emission, enuresis, lassitude of spirit, fear of cold, lumbar vertebrae disease, and consumptive disease. It was even used to treat delayed development of pediatric age or weak bone and muscle because of its apparent action on the musculo-skeletal system. It also appeared to be effective in neutralizing sores and ulcers, so it was used to promote ulcer and wound healing, as well as to strengthen immune function.

Studies on components that incorporate *Cervi Parvum Cornu* have found that they contain various cell growth factors like vascular endothelial growth factor, epidermal growth factor, neuron growth factor, fibroblast growth factor, and insulin-like growth factor (IGF-1, IGF-2) [35,36]. Minerals, amino acids, polypeptides, proteins [37], fatty acids [38], and various polysaccharides are also present.

To utilize these substances within *Cervi Parvum Cornu* more efficiently and conveniently, oriental medicine doctors make use of *Cervi Parvum Cornu* pharmacopuncture. It has been reported that *Cervi Parvum Cornu* pharmacopuncture can be applied to arthritis [4-14], osteoporosis [15-19], growth disorder [22,23], and the symptoms of aging [20,21]. In addition, some studies have shown that *Cervi Parvum Cornu* pharmacopuncture has the effects of antioxidant enzymes [20,21], can promote hormone production [24], regulate the autonomic nervous system [25,26], and has anticancer activity [27,28].

As clinical use of *Cervi Parvum Cornu* pharmacopuncture increases and studies of it are actively proceeding, it has become clear that research on its formulation is insufficient and a standardization process is lacking. There is also a risk that components may be degraded or removed during the preparation and purification of *Cervi Parvum Cornu* pharmacopuncture. Therefore, it was considered necessary to select indicator materials that would aid in the uniform preparation of standardized *Cervi Parvum Cornu* pharmacopuncture and quality control. Chondroitin sulfate, alanine and leucine, which are easy to analyze without precipitation during the preparation of *Cervi Parvum Cornu* pharmacopuncture, were found to be suitable indicator materials.

Chondroitin sulfate is a suitable indicator as it has been acknowledged to be present at high levels and demonstrate good activity in *Cervi Parvum Cornu* according to the Health Functional Food Test method of the Korea Food and Drug Administration.

Though *Cervi Parvum Cornu* contains many kinds of amino acids, alanine and leucine were selected because they can be analyzed simultaneously, which means that it is easy to compare the patterns. The linearity showed a correlation coefficient of over 0.999 in the concentration range of 5-500 $\mu\text{g/ml}$. Therefore, it was confirmed that both alanine and leucine were analyzed within the normal range. Using molecular weight, the specific substance is analyzed, so experimental error due to interference from other peaks can be minimized. Indeed, it was confirmed that there was no interference with other peaks.

Conclusion

Chondroitin sulfate, alanine, and leucine are three substances that are found in high concentrations in *Cervi Parvum Cornu* pharmacopuncture and that are easy to analyze, making them suitable indicator materials. The results of this study suggest that they are useful for the standardization of *Cervi Parvum Cornu* pharmacopuncture so that the reliability of this treatment may be improved.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgments

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