

Detection and Quantification of Neuroexcitatory Alkaloids in Modified *Da Huo Luo Dan* Prescribed for Paresis or Paralysis in Dogs

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ABSTRACT

Traditional Chinese Veterinary Medicine practitioners commonly prescribe herbal formulas. These plant-based medicines are highly complex with a number of different components sensitive to variable growing environments and processing. The development and optimization of analytical methods to identify ingredients and to assure consistent levels of biologically active molecules in these plant-based formulas is critical. A high pressure liquid chromatography (HPLC)-based method for reliable, cost efficient and consistent analysis of the Chinese herbal medicine *Da Huo Luo Dan* used in the integrative treatment of paresis or paralysis was developed. The objective of the analysis was to evaluate the levels of the clinically useful neuroexcitatory alkaloids brucine and strychnine, compounds that are also associated with toxicity at high levels. Initial screening using a gas chromatography-mass spectrometry method (GC-MS) at an analytical laboratory was performed to identify the organic plant compounds consistent with the constituent herbs of the formula along with the alkaloids of interest. The HPLC method was then performed for quantification. The samples contained 128 mg/kg as-fed (AF) brucine and 196 mg/kg AF strychnine in one batch lot, and 143 mg/kg AF brucine and 202 mg/kg AF strychnine in another. This is equivalent to a strychnine dose of 0.02 mg/kg body weight in dogs when the herbal formula is administered at recommended levels, and is well below the suggested toxic dose in dogs of 0.45-0.75 mg/kg body weight.

Key words: *Da Huo Luo Dan*, Chinese herbal medicine, paralysis, rehabilitation, strychnine, HPLC, *Ma Qian Zi*

ABBREVIATIONS	
AF	As fed
BW	Body weight
DM	Dry matter
GC-MS	Gas chromatography – mass spectrometry
HPLC	High pressure liquid chromatography

Chinese herbal medications are a component of Traditional Chinese Veterinary Medicine and have attracted substantial scientific investigation to refine potential indications and toxicities. A client-owned dog was presented to the University of Florida veterinary teaching hospital for re-evaluation of integrative treatment of suspected cervical spondylomyelopathy. The owner administered, over a period of three months, a veterinary-specific herbal combination modified from the classical formula *Da Huo Luo Dan*, with the labeled indications of improving paresis or paralysis (Table 1).¹⁻² The herbal mixture contains *Ma Qian Zi* (*Strychnos*), which is derived from the seeds of the *Strychnos nux-vomica*

tree and contains the potentially toxic alkaloids brucine and strychnine.^{2,3} Strychnine exerts neurologic effects as a glycine receptor antagonist. Competitive inhibition of glycine, an inhibitory neurotransmitter, induces a neuroexcitatory state, which at clinically therapeutic levels creates improved reflexes and a level of neuroexcitation which facilitates near immediate spinal walking in animals with complete thoracic spinal transection.⁴ Strychnine, however, has a narrow margin of safety and at higher doses may produce muscle fasciculations, seizures, respiratory failure and death.⁵ The recommended and reported doses of strychnine given to achieve clinical effects in humans and other animals range from approximately 0.05-0.2 mg/kg body weight (BW) orally or parenterally.^{4,6-8} Toxicity in the dog is suggested at 0.45 - 0.75 mg/kg BW, which is similar to other veterinary species at 0.5-1 mg/kg with the exception of the cat which is more resistant at 2 mg/kg BW.^{9,10}

A urine sample was obtained from the patient receiving the *Ma Qian Zi* (*Strychnos*) containing herbal formula. The specimen was screened by an academic equine racing laboratory^a and 1.2 µg/L of strychnine was identified in the urine sample, which was collected 3.7 hours after administration of an oral dose of 83.3 mg/kg BW of modified *Da Huo Luo Dan*. This led

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to the primary hypothesis of this study, which was that strychnine would be present in low quantities within the commercially available veterinary Chinese herbal formula administered to this patient. A modified high-pressure liquid chromatography method (HPLC) was developed to test this hypothesis and to rapidly screen and quantify the concentration of strychnine and the related alkaloid brucine in herbal medicine samples.

MATERIALS AND METHODS

Two batches of a commercially available modified *Da Huo Luo Dan* were obtained from the manufacturer as a dried mixed herbal powder for analysis (Table 1). *Ma Qian Zi* (Strychnos), a constituent herb within the formula, was also obtained from the same supplier as a dried ground powder. The products were submitted to an academic toxicology laboratory^b for initial gas chromatography-mass spectroscopy to identify potential contaminants and any naturally-occurring toxic compounds, including strychnine and brucine.

Additional samples of the herbal products were prepared for HPLC with a methanol extraction. An

extraction was performed with 1 gram of the combination formula added to 10 mL of HPLC-grade methanol, whereas 1 gram of *Ma Qian Zi* (Strychnos) powder was extracted in 15 mL of methanol. Samples were sonicated^e for 30 minutes at 10 watts. The solution was centrifuged at 7000 rpm for 10 minutes following sonication, and the liquid extract subsequently filtered using a 0.45 µm filter. An HPLC vial was filled with 1 mL of resultant solution and loaded into the autoinjector tray of the HPLC system.^d A standard amount of sample (10 µL) was injected into a solvent system using HPLC-grade acetonitrile^e and a 50:50 v/v mixture of 0.01 M 1-heptanesulfonic acid sodium salt monohydrate^e and 0.02 M KH₂PO₄^c as a buffer, with the latter adjusted to pH 2.8 with 10% H₃PO₄^e. An HPLC column (C18 25cm x 4.6mm, 5 µm) with a guard column^f (C18, 2.5 mm x 4.6 mm) was used and the UV detector was set for 235 nm. A flow rate of 0.5 mL/min, and run time of 40 minutes were used with a mobile phase of 21% acetonitrile and 79% buffer. All herbal samples were extracted in duplicate, and each extraction was assayed in triplicate.

Standard curves were prepared using anhydrous

Table 1: Labeled ingredients in Modified *Da Huo Luo Dan*²

<i>Pin Yin</i>	Common Name	TCM Indication ^{3,6}	Scientific Effects ³
<i>Ba Ji Tian</i>	Morinda	Tonify Kidney <i>Yang</i>	No relevant effects
<i>Bu Gu Zhi</i>	Psoralea	Tonify Kidney <i>Yang</i>	Urinary retention (smooth muscle)
<i>Chi Shao</i>	Paeonia	Clears Heat and <i>Qi</i> -Blood Stagnation	Sedative, antiplatelet
<i>Chuan Xiong</i>	Ligusticum	Clears <i>Qi</i> -Blood Stagnation	Antiplatelet, vasodilation
<i>Di Long</i>	Pheretima	Clears Heat, opens channels	Poorly documented
<i>Du Zhong</i>	Eucommia	Tonify Kidney <i>Yang</i> , Strengthen Bone	Sedative, anti-inflammatory
<i>Fu Zi (Shu)</i>	Aconite	Tonify Spleen, Kidney, Heart <i>Yang</i> , Eliminates Cold-Damp, opens channels	Anti-inflammatory, analgesic
<i>Gan Cao</i>	Glycyrrhiza	Tonify Spleen <i>Qi</i> , Clears Heat, Harmonizes	Mineralocorticoid effects, anti-inflammatory, gastroprotection, analgesic
<i>Gu Sui Bu</i>	Drynaria	Tonify Kidney, Strengthen Bone	Increase bone calcium deposition
<i>Hong Hua</i>	Carthamus	Clears Blood Stagnation and Stasis, opens channels	Antiplatelet, sedative, positive inotrope
<i>Huang Qi</i>	Astragalus	Tonify Spleen, Lung, and <i>Wei Qi</i>	Immunostimulant, antibiotic
<i>Ma Qian Zi</i>	Strychnos	Opens Channels, Clears <i>Qi</i> Stagnation	Neuroexcitatory
<i>Mo Yao</i>	Myrrh	Clears <i>Qi</i> -Blood Stagnation	Analgesic
<i>Quan Xie</i>	Buthus	Clear Internal Wind, opens channels	Anticonvulsant
<i>Ru Xiang</i>	Olibanum	Clears <i>Qi</i> -Blood Stagnation	Analgesic
<i>(Tian) San Qi</i>	Notoginseng	Clears <i>Qi</i> Blood Stagnation	Antiplatelet, hemostatic, immunostimulant, CNS effects
<i>Wu Yao</i>	Lindera	Tonify Kidney <i>Yang</i> , Clears <i>Qi</i> Stagnation	Hemostatic
<i>Xu Duan</i>	Dipsacus	Tonify Kidney <i>Yang</i> and Liver, Moves Blood, Opens Channels	Antioxidant, Regulator of osteoblast activity
<i>Xue Jie</i>	Draconis	Clears Blood Stagnation	Antiplatelet and hemostatic effects reported
<i>Chuan Niu Xi</i>	Cyathula	Tonify Kidney and Liver <i>Yin</i> , Moves Blood	Hemostatic, anti-inflammatory
<i>Dang gui</i>	Angelica	Tonify Blood, Clears Blood Stagnation	Anti-inflammatory, analgesic, antiplatelet, inotropic, antiarrhythmic

brucine and strychnine diluted in methanol. Serial dilutions were performed to obtain concentrations of 1000, 500, 250, 125 and 62.5 µg/mL. Concentrations of 20.8 and 3.13 µg/mL were also prepared from the 62.5 µg/mL dilution. All values were run in triplicate with mean values used to determine standard curves and equations. The standard equations were then used to calculate the concentration of brucine and strychnine in each herbal extraction.

RESULTS

A number of plant-based organic compounds were detected during initial GC-MS screening, and there was general agreement between the two samples in the occurrence of these chemicals (Table 2). Strychnine and brucine were also identified in the toxicology screen of each sample. Moreover, strychnine and brucine were successfully quantified using the HPLC method (Figure 1). The standard curves yielded reliable predictive equations. The standard equation for brucine was that the concentration (µg/mL) = $[(\text{peak area in } \mu\text{V/s}) + 138324]/33677$ ($R^2 = 0.996$). The standard equation for strychnine was concentration (µg/mL) = $[(\text{peak area in } \mu\text{V/s}) + 584517]/43522$ ($R^2 = 0.998$).

Duplicate extractions from the first batch lot of modified *Da Huo Luo Dan* yielded brucine concentrations of 133 and 122 mg/kg as fed (AF) (142 and 131 mg/kg dry matter (DM)). The second lot contained 148 and 138 mg/kg AF (163 and 152 mg/kg DM). Strychnine was present at 205 and 186 mg/kg AF (219 and 199 mg/kg DM) in the first batch and 207 and 196 mg/kg AF (227 and 215 mg/kg DM) in the second. At this concentration, a 45 kg dog would consume about 1 mg of strychnine, or 0.02 mg/kg BW, twice daily and 0.7 mg of brucine, or 0.015 mg/kg BW. *Ma Qian Zi* (Strychnos) contained a mean of

Table 2: Compounds in Modified *Da Huo Luo Dan* Detected by GC-MS

Strychnine
Brucine
Longifolene
Longicamphenylone
Tetrachlorohydroquinone dimethyl ether
Angelicin
Isopimaric acid
Copaene
Camphene
Ethylparabens
Alpha- and gamma-himachalene
Linalool oxide
3-butyldiene phthalide
Alpha guaiene
Aromadendrene
Paeonol
Alpha-cubene

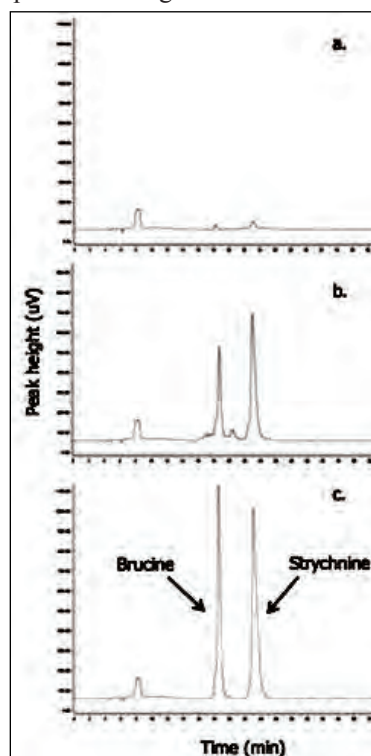
5,170 mg/kg AF brucine and 8,060 mg/kg AF strychnine in duplicate extractions. The percent difference between duplicate extractions for all samples was never greater than 10 percent.

DISCUSSION

Chinese herbal medications have been adapted for a number of suggested uses, and *Da Huo Luo Dan* is most frequently used for the treatment of paresis or paralysis caused by various disorders.¹ Modifications from classical formulations are common in many patent formulas, including those marketed for veterinary use. One such modification of *Da Huo Luo Dan* contains *Ma Qian Zi* (Strychnos), purportedly to activate the Channels, relieve pain, and to clear Wind-Damp according to traditional Chinese principles.² The stated traditional actions of most of the constituent herbs are well-described (Table 1), and ethnobotanical and scientific investigations have highlighted potential physiologic effects.³

Ma Qian Zi (Strychnos) seeds are commonly included in the medical traditions of several Asian cultures, and Chinese Medicine continues to employ the herb sparingly for paralysis and severe pain. Modern TCVM textbooks advise *Ma Qian Zi* (Strychnos) doses of 0.1-0.3 grams (100 – 300 mg) for dogs and 1.5-6 grams (1500 – 6000 mg) for horses.⁶ A historical reference text of conventional veterinary medicine suggested a dose of ¼ to 3 grain (16

Figure 1: Sample Chromatogram Peaks from HPLC Analysis



- Modified *Da Huo Luo Dan*.
- Ma Qian Zi* (Strychnos) powder.
- Standard solutions (1000 µg/mL). Chromatograms have been modified to remove other peaks for ease of peak identification.

– 195 mg) per dog and 15-30 grains (975 – 1950 mg) of *Strychnos*, or *Nux vomica*, seeds in horses for paralysis, or a “depressed state of the nerves or nervous system.”⁷⁷ Subcutaneous doses of 0.3-1 mg strychnine were advised for conditions in dogs for which improved reflexes and neuroexcitation would be beneficial.⁸ Subcutaneous injections of 0.1 mg/kg of strychnine sulfate facilitated near immediate spinal walking in dogs with complete thoracic spinal transection.⁴ The proposed therapeutic mechanism of action for both pure strychnine and the strychnine-containing *Ma Qian Zi* (*Strychnos*) is post-synaptic glycine receptor antagonism, which decreases the activity of glycine, an inhibitory neurotransmitter.⁵

Although the percentage of ingredients and the concentrations of neuroexcitatory alkaloids in the veterinary exclusive formula of modified *Da Huo Luo Dan* are proprietary, the gas chromatographic screening confirmed the presence of some of the chemical compounds present within the listed herbal ingredients. For example, *Chi Shao* (*Paeonia*) contains paeonol, and some cultivars of *Dang Gui* (*Angelica*) contain the furanocoumarin angelicin.^{11,12} However, quantification of the detected compounds, which could suggest the concentrations of individual herbs in the formula, was not performed due to budgetary restrictions and represents a limitation of the study. The GC-MS toxicological screening did detect the expected presence of the neuroexcitatory alkaloids strychnine and brucine, but quantification of these substances using GC-MS was impractical and expensive for routine use. Therefore, an inexpensive, simple HPLC quantification method for strychnine in this veterinary formula and in other *Ma Qian Zi* (*Strychnos*) containing herbal formulas was desirable.

The Pharmacopoeia of the People’s Republic of China provides testing methods for a number of Chinese herbal drugs.¹³ *Ma Qian Zi* (*Strychnos*) is described as the dried ripe seed of *Strychnos nux-vomica* which is gathered in winter and dried in the sun. An identification procedure using thin layer chromatography is described as well as a chromatographic assay. The latter suggests an identical mobile phase as that employed in the present investigation. Notable differences, however, include the recommended UV detector wavelength of 260 nm, and the extraction method, which includes chloroform, sodium hydroxide, a 2-hour heating phase, and methanol. The Pharmacopoeia gives the concentration of alkaloids in *Ma Qian Zi* (*Strychnos*) as 1.2-2.2% DM strychnine and greater than 0.8% DM brucine.¹³

A modification of the Pharmacopoeia quantification method was reported by Han and colleagues for the identification of strychnine, brucine, and loganic acid in *Strychnos* seeds.¹⁴ Their proposed method used a gradient solvent system with the UV detection set at 235 nm. Powdered seed was processed with ultrasonication and methanol extraction. In the present study, the HPLC technique used the simpler extraction and the UV wavelength reported by Han, but used the reagents and

mobile phase of the Pharmacopoeia method.

The detection of strychnine and the related alkaloid brucine confirms that *Ma Qian Zi* (*Strychnos*) was present in this herbal formula, as stated on the label. The detected concentration of around 200 mg/kg AF strychnine permits a better estimation of the safety of this patent formula. Brucine, the less toxic alkaloid, was present in concentrations about 25% less than those of strychnine, which is consistent with Pharmacopoeia standards. However, the *Ma Qian Zi* (*Strychnos*) used by the manufacturer of this veterinary formula contained strychnine concentrations no greater than 0.85% DM, which is less than the Pharmacopoeia standards of 1.2-2.2% DM.¹³ Brucine was also less than typically reported by other sources. A study with similar techniques found strychnine concentrations of 1.6-2.3% AF in crude seeds, and much less for processed seeds.¹⁴

A comparison of the concentration of strychnine in both batches and in the raw *Ma Qian Zi* (*Strychnos*) powder suggests that the seeds comprised 2.4-2.5% and 2.5-2.8%, respectively, of the total mass of the herbal formula. The manufacturer was consulted, and the formulators indicated that the herbal seeds were added to achieve 3% of total formula weight. This disparity could suggest incomplete extraction of strychnine in the modified *Da Huo Luo Dan* (about 80% recovery), but spiked samples demonstrated greater than 90% recovery when tested with the described HPLC method. It is possible other herbs in the formula may have prevented complete extraction in methanol or that the sonication was insufficient in the chosen dilution volume. This study should be repeated with the exact published Pharmacopoeia extraction for comparison, but doing so would significantly increase the labor involved. Standard curve equations could also be responsible for the disparities between calculated concentration and manufacturer-reported values. The low concentrations present in the modified *Da Huo Luo Dan* could magnify the potential for error although predictive equations were created using both the low concentrations, like those found in the formula, and higher concentrations, such as those found in seeds. Finally, inaccurate measuring or mixing during formula processing could also explain the observed values.

The present investigation is subject to several limitations. Only two batches of modified *Da Huo Luo Dan* were available for testing; additional samples would have further clarified the extent of the observed inter-batch variability. One sample of *Ma Qian Zi* (*Strychnos*) was available and factors such as processing, growing region, and any chemical alteration may have influenced strychnine concentrations. The HPLC method utilized in the investigation was novel and could produce unexpected challenges or variability when used in a greater sample of herbs. A variety of other detection methods have been reported, any one of which might produce more accurate results.¹⁵⁻¹⁷ The method discussed herein should therefore be evaluated concurrently with other such methods in

future investigations. Additional spiked samples should also be used to test the repeatability of the described findings.

In conclusion, this study identified that a modification of *Da Huo Luo Dan* marketed for veterinary use contains low levels of the neuroexcitatory alkaloids strychnine (0.02 mg/kg BW/dose) and brucine (0.015 mg/kg BW/dose). This formula, however, is unlikely to produce clinically relevant toxicity if dosed according to label instructions. Additional clinical studies are required to better evaluate the utility of this patent formula, its safety, and its optimal dose. Nevertheless, the HPLC method used in the present investigation may prove to be a simple and reliable method of screening for the strychnine content in formulas containing *Ma Qian Zi* (*Strychnos*).

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FOOTNOTES

- a. Equine Racing Laboratory, College of Veterinary Medicine, University of Florida, Gainesville, Florida
- b. Michigan State University Diagnostic Center for Population and Animal Health
- c. 60 Sonic Dismembrator, Fisher Scientific, Waltham, Massachusetts
- d. Series 200 autosampler and pump, Flexar UV/VIS detector, PerkinElmer, Waltham, Massachusetts
- e. Chemicals purchased from Fisher Scientific, Waltham, Massachusetts with the exception of 1-heptanesulfonic acid (Sigma Aldrich, St. Louis, Missouri)
- f. Zorbax SB-C18, Agilent, Santa Clara, CA

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