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Determination the active compounds of herbal preparation by UHPLC–MS/MS and its application on the preclinical pharmacokinetics of pure ephedrine, single herbal extract of *Ephedra*, and a multiple herbal preparation in rats

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ABSTRACT

The herbal preparation Ma-Xing-Gan-Shi-Tang (MXGST) is a popular traditional Chinese formulation that has been used for the treatment of coughs and fevers. The potential active components of MXGST are ephedrine, amygdalin, and glycyrrhizic acid. The aim of this study was to develop a validated analytical method to measure these analytes in the herbal preparation MXGST using ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS). Multiple reaction monitoring (MRM) was used to monitor *m/z* 166.1 → 148.1 for ephedrine ($[M+H]^+$), 475.2 → 163.0 for amygdalin ($[M+NH_4]^+$), and 840.6 → 453.3 ($[M+NH_4]^+$) for glycyrrhizic acid. The analytes were separated by a reverse phase C18 column (100 × 2.1 mm, 2.6 μm). The mobile phase consisted of 5 mM ammonium acetate (0.1% formic acid) and 100% methanol (0.1% formic acid) with a linear gradient elution. Five brands of commercial pharmaceutical herbal products and a laboratory extract of MXGST were analyzed. Moreover, the modified UHPLC–MS/MS method was applied to the comparative pharmacokinetics of ephedrine in rats from the following three sources: (1) pure ephedrine, (2) an herbal extract of *Ephedra*, and (3) an herbal preparation of MXGST. Plasma samples from rats were prepared by protein precipitation, evaporation and reconstitution. The pharmacokinetic data showed that pure ephedrine was absorbed significantly faster than ephedrine of the *Ephedra* extract or the MXGST herbal preparation. However, the elimination half-life of ephedrine administered as the pure compound was 93.9 ± 8.07 min, but for ephedrine from the *Ephedra* extract and the MXGST, the half-lives were 133 ± 17 and 247 ± 57.6 min, respectively. The area under the concentration curves (AUC) did not show significant differences among the three groups. These data suggest that the rest of the herbal ingredients in the *Ephedra* extract and the MXGST may provide a compensation effect that reduces the peak concentration of ephedrine and prolongs the elimination half-life.

1. Introduction

Ma-Xing-Gan-Shi-Tang (MXGST) consists of *Ephedra* Sinica, *Armeniacae* semen, *Glycyrrhiza glabra* Linne, and gypsum (the weight ratios of ephedra, apricot kernel, licorice, and gypsum are 4:3:2:8). This preparation is a popular traditional Chinese formula used for the treatment of "heat evils congest the lung", which means cough, asthma and fever in western medication. According to the guidelines published by the Department of Chinese Medicine and Pharmacy, Taiwan Ministry of Health Welfare, and the "Treatise on Cold-Induced Diseases", the famous medical classic written 2000 years ago (150–219 A.D.) by Zhang, Zhongjing, MXGST is a basic Chinese medicine formula for treating coughs and fevers. Even now, MXGST is a popular traditional Chinese medicine in clinical application. Survey from the National Health Insurance Research Database in Taiwan, MXGST is the top third most common traditional Chinese medicine for treating adult asthma [1] and is the most frequently used for asthmatic children in Taiwan [2]. MXGST is familiar to not only Taiwanese and Chinese practitioners but also application in Kampo medicine. It is called Makyo-kanseki-to in Japan.

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Because MXGST is indicated for respiratory disorders, the pharmacological studies have focused on its anti-inflammatory, antiviral, and bronchodilation properties. A study indicated that MXGST inhibited the antigen induced immediate asthmatic responses and suppressed neutrophil infiltration into lung tissue in guinea pigs [3]. The antiviral activity of MXGST was associated with the inhibition of the TLR3- IRF3-IFN-β signaling pathway and expression of SOCS1 to reduce lung inflammation and inhibit virus replication in RSV-infected mice [4]. The pharmacological effect of each herb of MXGST is also related to respiratory physiology. *Ephedra* Sinica (Ma Huang) has been used for five thousand years in Chinese medicine, but it became a well-known herb in the early 1920s because of its active alkaloid, ephedrine. Ephedrine is an effective bronchodilator and plays an important role in the development of the theory of alpha- and beta-adrenergic receptors [5]. *Armeniacae* semen (Xin Ren) was found to have anti-asthma activity based on its inhibition of the Th2 cellular response [6]. Amygdalin, a cyanogenic glycoside, is one of the major compounds of *Armeniacae* semen. Licorice, a common herb in Chinese medicine, is often used for asthma patients [1]. The sweetness of licorice is derived from glycyrrhizic acid, which is a triterpenoid [7]. According to a recent study, triterpenic acids can protect the bronchial epithelial cells by attenuating apoptosis and inhibiting inflammation [8].

Recently, many Chinese medical doctors in Taiwan have come to prefer to prescribe commercial pharmaceutical herbal products instead of traditional decoctions because commercial pharmaceutical herbal products are convenient for patients and are produced by a cGMP pharmaceutical factory, which would monitor dry herbs source, heavy metals, pesticide residues, and etc. The commercial pharmaceutical herbal products are manufactured by industrial methods, including decoction, extraction, concentration, and packaging [9]. Because the manufacturing processes of the pharmaceutical industry differ from factory to factory, the concentrations of active compounds may vary [10]. Therefore, it is important to determine the active compounds in each MXGST commercial pharmaceutical herbal products.

The principle of Chinese medicinal combinations is followed by the basic theory of sovereign, minister, assistant and courier principles. The theory indicates that sovereign medicines is used for treatment of diseases, minister and assistant medicines support the sovereign, and courier medicines deliver the active principles to reach the target site of the body. Therefore, we are primarily interested in the pharmacokinetics of the sovereign herb, *Ephedra*, in MXGST. The sovereign herb in an herbal preparation usually directly treats the principal syndrome. High performance liquid chromatography coupled to tandem mass spectrometer (UHPLC–MS/MS) analysis provides a fast and precise way to study drug metabolism and pharmacokinetic screening [11]. *Ephedra* is a common Chinese herb, and HPLC methods have been developed for analyzing the alkaloids in *Ephedra* for qualification and pharmacokinetics in rat [12–14]. In addition, the pharmacokinetics of decoctions of the *Ephedrae-Semen Armeniacae Amarum* herb pair have been compared with those of the individual herbs [15]; however, there has been no comparison of the pharmacokinetics of pure ephedrine, the extract of the single herb *Ephedra*, and the herbal preparation of MXGST.

Because there has not been a careful analysis of the three main active compounds in MXGST or a comparison of the pharmacokinetics of the three formulations of ephedrine, the aim of study was to develop and validate a specific and sensitive analytical method using UHPLC–MS/MS to evaluate a laboratory extract and five brands of pharmaceutical industry products. Furthermore, we established a freely-moving rat model to compare the pharmacokinetics of (1) pure ephedrine, (2) an herbal extract of *Ephedra*, and (3) a multiple herbal preparation of MXGST.

2. Material and methods

2.1. Reagents and materials

Standard components were supplied by as following: Ephedrine hydrochloride, amygdalin, glycyrrhizic acid ammonium salt, carvedilol (the internal standard), and heparin were provided by Sigma–Aldrich (St. Louis, MO, USA). Pentobarbital sodium was purchased from SCI Pharmatech, Inc., (Toayuan, Taiwan). E. Merck (Darmstadt, Germany) provided all of the chemical solvents, including methanol, formic acid, sodium chloride and ammonium acetate of HPLC grade. Triple deionized water used throughout the entire experiment was purified by Millipore (Bedford, MA, USA). The five brands of MXGST commercial pharmaceutical herbal products were purchased from Sun Ten Pharmaceutical Co., Ltd., (Taipei, Taiwan), Chuang Song-Zong Pharmaceutical Co., Ltd., Kaiser Pharmaceutical Co., Ltd., (Tainan, Taiwan), (Kaohsiung, Taiwan), Sheng Chang Pharmaceutical Co., Ltd., (Taipei, Taiwan), and Koda Pharmaceutical Co., Ltd., (Taoyung, Taiwan). The Ephedra extract pharmaceutical products were purchased from Koda Pharmaceutical Co., Ltd., (Taoyung, Taiwan).

2.2. Preparation of stock and calibration standards

The stock solutions (1 mg/mL) of the standards for ephedrine, amygdalin, glycyrrhizic acid, and carvedilol were prepared in 100% methanol. The working standard solutions were diluted from the stock solutions in 50% methanol (v/v), resulting in concentrations of 250–10,000 ng/mL. The calibration curves used to quantify the components of the laboratory extract and the commercial pharmaceutical herbal products were prepared from working standard solutions (30 μL) at concentrations of 25, 50, 100, 250, 500, and 1000 ng/mL and the 270 μL internal standard solution of carvedilol (5 ng/mL).

To measure the biological sample of analyte, a series of working standard solutions were spiked into blank drug-free plasma to yield concentrations of 50, 75, 100, 250, and 500 ng/mL. All standard solutions were stored at −20 °C prior to use.

2.3. Preparation of traditional MXGST

Based on information presented in the Introduction, the weight ratios of ephedra, apricot kernel, licorice, and gypsum are 4:3:2:8. The crushed herbs were supplied by a Chinese traditional herbal medicine store in Taipei, Taiwan and confirmed by comparison with data in the literature [16]. The method for extraction by boiling was based on the "Treatise on Cold-Induced Diseases". First, 160 g of ephedra was boiled with 1.4 L water until the water was reduced to 1.2 L. Then, 45 g of apricot kernel, 30 g of licorice, and 120 g of gypsum were added. The decoction was boiled until the water was reduced to 0.6 L, and then it was filtered through gauze. The filtrate was concentrated to 0.1 L and stored at −80 °C one day for lyophilization. The lyophilized powder of the MXGST extract was used for quantitation.

The powders of the five brands of MXGST commercial pharmaceutical herbal products were randomly labeled A to E, and the laboratory extract was labeled S. A sample of 0.1 g of each preparation was carefully weighed. Each powder was suspended in 25 mL of 100% methanol and ultrasonicated for 15 min at room temperature. In this step, gypsum, $CaSO_4$, would not be extracted by the organic solvent (methanol), and it was not considered that calcium, a non-volatile salt, would cause source corrosion. The samples were centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatants were collected and filtered through 0.22 μm filters. Finally, the filtrates