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A new approach to examining the extraction process of Zhishi and Zhiqiao considering the synergistic effect of complex mixtures by PAMPA

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ABSTRACT

Zhishi (ZS) and Zhiqiao (ZQ) are two important traditional Chinese medicines (TCMs) that exert various pharmacological functions due to their active ingredients. However, the oral absorption of these ingredients requires further study. At the early drug discovery stage, the high-throughput parallel artificial membrane permeability assay (PAMPA) is one of the most frequently used to predict transcellular passive absorption in in-vitro models. This study aims to establish a new approach to examine an optimal extraction process that can take into account not only the concentration of active ingredients but also the overall absorption properties of the mixtures extracted from TCMs. A high-performance liquid chromatography triple-quadrupole mass spectrometry (HPLC-QqQ-MS/MS) method was validated for the determination of the effective permeability value (P_e) applied to the above experimental medium. The PAMPA experiment showed that certain active ingredients such as diosmin, rhoifolin, eriocitrin, narirutin, naringin, hesperidin and neohesperidin were not detected in the permeability assay of mono-constituents but were well detected and achieved a better absorption in the permeability assay of the mixture, indicating that certain unknown ingredients may act as cosolvents to improve the solubility or permeability of other ingredients. Furthermore, solid phase extraction (SPE) as an enrichment and purification process enhances absorption. In the present study, a novel in vitro approach was developed to decipher the potential role of TCMs in global absorption, and the extraction process for complex TCMs was described and systematically optimized.

1. Introduction

For centuries, traditional Chinese medicine (TCM) has been widely used in China for the prevention and treatment of various diseases [1]. Owing to its proven efficacy, large number of applications and few side effects, TCM has increasingly attracted worldwide attention. In most studies, only one or several marker components are used to control the quality of TCM [2], and these are mainly based on concentration levels. This approach is insufficient and somewhat inappropriate because the active ingredients in almost all TCMs are complex mixtures containing hundreds of different chemical constituents responsible for their therapeutic effects [3]. Furthermore, they take their effects on the premise of absorption in human beings. Thus, it is of great importance and value for the quality evaluation of TCMs that studies establish more comprehensive and efficient method that not only cover most of the active chemical constituents in TCM but also consider the overall absorptive properties of these constituents in complex mixtures. These absorptive properties may be enhanced through synergistic effects between different compounds in the mixture. However, few strategies are known for predicting and optimizing the extraction process or for allowing researchers to consider synergistic effects in TCMs.

Parallel artificial membrane permeability assay (PAMPA), introduced by Kansy et al. [4], is an in-vitro method for predicting the passive absorption process. In recent decades, PAMPA has attracted considerable interest in pharmaceutical research as a useful complement and alternative to the Caco-2 assay due to its advantages in cost

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and time effectiveness, its high throughput and high tolerance to a wider pH range. In the PAMPA model, a hydrophobic filter material is used as support. The experiment is carried out by measuring the permeability of a saturated aqueous solution of the compound after it diffuses through a membrane from the donor compartment to the acceptor compartment. The membrane is formed by a mixture of lecithin and an inert organic solvent [5]. PAMPA was adapted to mimic different biological membranes including the gastrointestinal tract (GIT), the blood–brain barrier (BBB) and the skin by changing the composition of the artificial membrane. Permeability is widely acknowledged to be important in gastrointestinal drug absorption [6]. Poor permeability and poor solubility is the source of many pharmacokinetic (PK) failures, and PK-related failures may ultimately cause drug candidate molecules to be rejected [7]. Therefore, being able to predict the in vivo performance of drug products by in vitro measurements of permeability and solubility is essential. Since Zhishi (ZS) and Zhiqiao (ZQ) are usually ingested for medicinal use as phytopharmaceuticals, the approach of the present experiment is to examine the extraction process of ZS and ZQ. This not only considers most of the active chemical constituents but also considers the overall absorptive properties of the compounds. This approach is innovative and will aid in the further investigation into optimal extraction processes for other TCMs.

ZS and ZQ are two important traditional Chinese medicines that have been officially listed in the Pharmacopoeia of the People's Republic of China (2015 Edition) due to their important therapeutic properties for humans. Modern pharmacological studies have shown that these medicines possess a variety of biological actions including anti-oxidant, anti-inflammatory, anti-cancer, anti-allergic, anti-proliferative, and anti-bacterial activities as well as cardiovascular protective, neuroprotective, and hepatoprotective effects and obesity control [8–11]. The main active constituents of the plants are flavonoids, flavanones, polymethoxylated flavones, coumarins and limonoids [10,12–16]. As oral drugs, the active constituents of ZS and ZQ must be absorbed through the gastrointestinal tract in order to reach the systemic circulation and then the site of action [17,18]. The mechanisms of drug permeation through biological membranes include active transport, passive diffusion, and paracellular pathways [6]. Previous studies have shown that the majority of constituent are absorbed primarily through passive diffusion [19]. Therefore, PAMPA can accurately predict the passive absorption process of these drugs in vivo. Combining the content of active ingredients with the overall absorptive properties of the complex mixtures extracted from ZS and ZQ, we put forward a new strategy to examine the optimal extraction process in a holistic manner, which seems more sufficient and reasonable than only considering the concentration of several active ingredients.

In this paper, a total of 43 active components from various structural types of ZS and ZQ, including 12 flavanones and their glycosyl derivatives, 12 flavones and their glycosyl derivatives, 5 flavonols and their glycosyl derivatives, 8 coumarins, 3 chalcone and their glycosyl derivatives, 2 limonoids, and 1 abscisic acid, were studied by PAMPA using HPLC-QqQ-MS/MS determination to predict the absorption mechanism in the form of a monomer. To represent different types of chemical diversity and complexity in our holistic assay of permeability, we used different extraction processes including several methanol concentrations (0, 30, 50, 75, 100%, v/v), a 75% ethanol solution, as well as solid-phase extraction (SPE)-treated samples. The aim of the present work is to find a new approach for examining the optimal extraction process for the complex-constituents found in TCMs according to their bioactive ingredients and overall absorptive properties. Here, the PAMPA method is employed to investigate the intestinal absorption of several mixture vehicles for two commonly used TCM botanical drugs, ZS and ZQ, by different extraction processes. The optimal extraction process has been selected according to the weighted Log P_e. This approach for examining the optimal extraction process of complex-constituents in TCMs is fast, efficient and reliable, thus allowing us to predict the effect of different extraction processes on the permeation mechanism of active components and providing a meaningful reference for research into the material bases and compatibility mechanisms of TCMs.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade methanol was obtained from Fisher Scientific (Fair Lawn, NJ, USA). All deionized water was redistilled. Formic acid (HPLC grade, Lot. 095224) was obtained from MREDA Technology Inc. (USA), and 7-hydroxycoumarin, bergapten, astilbin, taxifolin, kaempferol and narirutin were obtained from Chengdu Must Bio-Technology Co., Ltd. Hesperidin, naringin, apiin, kaempferitrin, diosmetin-7-O-glucoside, diosmin, isopimpinellin, naringenin chalcone, neohesperidin dihydrochalcone, naringin dihydrochalcone and sinensetin were obtained from Shanghai Source Leaf Biological Technology Co., Ltd. Eriocitrin, neohesperidin, naringenin, hesperetin, luteolin, rutin, quercetin, and tangeretin were obtained from Tianjin Mark Biological Technology Co., Ltd. 5-demethylnobiletin, nobiletin, auraptene and bergamottin were obtained from Nanjing JingZhu Biological Technology Co., Ltd. Rhoifolin, apigenin, scoparone, isorhamnetin-3-O-glucoside and diosmetin were purchased from the National Institute for Control of Biological and Pharmaceutical Products of China. Poncirin, eriodictyol, xanthotoxol, acacetin, isosakuranetin, imperatorin, limonin, nomilin and abscisic acid were purchased from Beijing fufan Biological Technology Co., Ltd. The purity of the standards was relatively high (i.e., higher than 98%). Propranolol (cat. P-0884), warfarin (cat. A-2250), carbamazepine (cat.C-8981), furosemide (cat.F-4381) were purchased from Sigma Chemical Co. Lecithin, *n*-dodecane, dimethyl sulfoxide (DMSO), fluorescein sodium are available from Aladdin; phosphate-buffered saline (PBS) is available from Beijing Biotopped life science, Co., Ltd.; 96-well polycarbonate-based filter PAMPA donor plates (Multiscreen™-IP, MAIPN4510, pore size 0.45 μm) and 96-well PVDF PAMPA acceptor plates (Multiscreen Acceptor Plate, MSSACCEPT0R) are available from Millipore Corporation. ZS and ZQ samples collected from Zhangshu, Jiangxi province in China were used in the current study.

2.2. Sample preparation

2.2.1. Mono-constituents standard solution

All the solutions of each mono-constituent were prepared in dimethylsulfoxide (DMSO) at 10 mM and then diluted with PBS (Phosphate Buffer Saline; pH = 7.4) to obtain the final nominal concentrations of 500 μM, 50 μM and 10 μM. The concentration of DMSO was kept at 5%(v/v).

2.2.2. ZS and ZQ mixture solution

ZS and ZQ samples were prepared by different extraction processes, including different methanol concentrations (0, 30, 50, 75, 100%, v/v), a 75% ethanol concentration, as well as SPE, all of the powdered samples (0.4 g) were accurately weighed and sonicated with 50 mL of the aforementioned different solvents for 30 min and then adjusted to their original weights. The extraction solutions were filtered and concentrated until dry in a rotary evaporator.

The residue for SPE-treatment was reconstituted in 10 mL water and then loaded onto an SPE column, eluent was collected after SPE-treatment and concentrated until dry in a rotary evaporator. The information of SPE procedure including pH, sorbent, breakthrough volume, washing solvent and elution solvent were as follows: Neutral environment (pH = 7) was selected for the SPE procedure. An SPE column coated with Strata-X was selected, and after sample loading, the column was washed with 2 mL of 30% methanol followed by elution with 10 mL of 100% methanol to obtain the target mixtures. The detailed optimization and validation of SPE procedure was compared and discussed in