Investigation on the spectrum-effect relationships of Da-Huang-Fu-Zi-Tang in rats by UHPLC-ESI-Q-TOF-MS method

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Canonical correlation analysis

Talatisamine (PubChem CID: 159891)
Hydroxy-chrysophanol (PubChem CID: 442731)
Aloe-emodin (PubChem CID: 10207)
Nonzoylmesaconine (PubChem CID: 24832695)
Mesoracnicine (PubChem CID: 24832657)
Benzoylmesaconine (PubChem CID: 121312)
Hypaconine (PubChem CID: 23337)
Rhein (PubChem CID: 10168)
Torachrysone (PubChem CID: 5321977)
Emodin (PubChem CID: 3220)

\textbf{A B S T R A C T}

\textbf{Ethnopharmacological relevance:} Da-Huang-Fu-Zi-Tang (DHFZT) is a crucial TCM formula commonly used for the treatment of acute pancreatitis in Chinese clinical application. Our previous work found that DHFZT could act against pancreatic injury in rats with severe acute pancreatitis (SAP). The goal of this paper was to study the underlying correlations between the chemical spectra and the protective effect of DHFZT on pancreatic acinar cell to reveal the real bioactive compounds in DHFZT.

\textbf{Materials and methods:} The fingerprint chromatograms of rat serum after oral administration of DHFZT were established by UHPLC-ESI-Q-TOF-MS technique. At the same time, the model of anti-acute pancreatitis on cells was established by adding 10\textsuperscript{−7} mol/L cerulein to AR42J cell line, and the protective effects of the serum on pancreatic acinar cell from injury was evaluated by detecting the efficacy of amylase. Then, the spectrum–effect relationships between UHPLC fingerprints and anti-acute pancreatitis activities were evaluated using canonical correlation analysis (CCA) statistical method. The chromatogram separation was performed on a C\textsubscript{18} reversed phase UHPLC column (2.1 mm × 100 mm, 3.5 μm, Agilent), the column temperature was set at 35 °C. The mobile phase consisted of 0.1% formic acid and acetonitrile with gradient elution. The serum samples were analyzed both in negative and positive ion mode. The mature and productive ions were scanned within the mass range of m/z 100–1200 and 50–1200, respectively. A thorough analysis of a great deal of information of the constituents in the rat serum was undertaken. The structure identification of the detected compounds was achieved by using high resolution MS values as well as the MS/MS fragments.

\textbf{Results:} Eighteen peaks in rat serum after oral administration of DHFZT were detected within only 30 min recorded chromatograms. The structure of the 18 compounds were then given out, of which 10 were the original form of compounds absorbed from DHFZT, 8 were the metabolites of the compounds existed in rat serum. According to the CCA results, talatisamine, rhein glucoside, rhein isomer methylation, hyaconine, hydroxyl-chrysophanol, emodin glucuronide conjugation, and chrysophanol glucuronide conjugation were finally found to be the main anti-acute pancreatitis components in DHFZT.

\textbf{Conclusions:} The model presented in this paper successfully discovered the spectrum–effect relationships of DHFZT, which showed a representative way to discover the primary active ingredients from the complicated herbal drugs.

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\textbf{Abbreviations:} CCA, canonical correlation analysis; CE, collision energy; CE–MS, capillary electrophoresis–mass spectrometry; CNKI, Chinese National Knowledge Infrastructure; DBS, dynamic background subtraction; DP, declustering potential; GC–MS, gas chromatography–mass spectrometry; IDA, information-dependent acquisition; LC, liquid chromatography; NMR, nuclear magnetic resonance; SAP, severe acute pancreatitis; TCM, traditional Chinese medicine; TEM, turbo spray temperature; UHPLC-ESI-Q-TOF-MS, ultra-high performance liquid chromatography–electrospray ion source–quadrupole–time of flight–mass spectrometry; XICs, extracted ion chromatograms

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1. Introduction

Recently, Traditional Chinese Medicine (TCM) and their preparations become more and more popular in Asian and Western countries due to their stable therapeutic effects and weak toxicity in clinic (Chen et al., 2012; Qiu et al., 2011; Su et al., 2010). As a result, there is an increasing number of research institutions and universities studying optimal and bioactive chemical composition of TCM (Zhang et al., 2012; Sun et al., 2007). To develop rapid, reliable and sensitive analytical approaches for TCM quality control and efficacy assurance is becoming more and more significant and meaningful. Owing to the limitation of study ways on material basis research of TCM, it is of great necessary and significance to develop novel models to make clear what the effective compositions are during their clinical application.

The traditional chemical research methods for identification of constitutions of TCM are usually time consuming and expensive. No matter the conventional approaches such as LC, NMR or the applications of advanced techniques such as GC–MS and CE–MS, their application are greatly limited by the time-consuming periods and the lack of corresponding standards. Moreover, the effectiveness of a TCM could not be evaluated relying on only a few compounds identification. So, a combinative and powerful method which could offer higher quality structural information and comprehensive components inside is therefore required for the extensive characterization of TCM systems. UHPLC-ESI-Q-TOF-MS, as an important analytical method, has got quick development in recent years. It can be used to determine the contents, analyze the constituents of many compounds and get the fingerprints of the drug, drug and other materials (Cabral et al., 2012; Gupta et al., 2013; Guo et al., 2013; Xiao et al., 2013; Yin et al., 2013). UHPLC-ESI-Q-TOF-MS is so powerful a tool for serum analysis also due to its high sensitivity, sound separation and identification ability to show chemical structures without standard reference (Wang et al., 2013). Alongside this increased use of spectrum techniques, there have also been significant developments in combination with chemometrics (Massart et al., 1988). To combine CCA statistical method as one of the chemometrics with UHPLC-ESI-Q-TOF-MS method can better reveal the underlying bioactive compounds in TCM, which has been applied widely (Kong et al., 2009, 2008; Nie et al., 2011).

Da-Huang-Fu-Zi-Tang (DHFZT), a crucial TCM formula commonly used for the treatment of appendicitis, acute pancreatitis, biliary colic, chronic dysentery, acute ileus and adhesive ileus (Liang et al., 2006; Wu et al., 2013a, 2013b), is composed of three herbs, Rheum officinale Baill. (Polygonaceae), Aconitum carmichaelii Debx. (Ranunculaceae) and Asarum sieboldii Miq. (Aristolochiaceae). It is first recorded in “Jin-Gui-Yao-Lue”, a classical treatise on febrile and miscellaneous diseases written by a physician Zhong-Jing-Zhang (150–219 A.D.) in Eastern Han Dynasty. Though DHFZT was believed to be one of the main formulas to treat acute pancreatitis in TCM clinic, the exact mechanism of its action is still unknown until now, and the material basis of its anti-acute pancreatitis activities has not been reported yet, neither. The goal of this paper was to study the underlying correlations between the chemical spectra and the protective effect of DHFZT on pancreatic acinar cell to reveal the real bioactive compounds in DHFZT.

2. Materials and methods

2.1. Materials and reagents

*Rheum officinale Baill.*, *Aconitum carmichaelii Debx.* and *Asarum sieboldii Miq.* were purchased from Nanjing Haichang Chinese medicine group corporation (Nanjing, China). Their species were identified by Prof. Jianwei Chen (College of Pharmacy, Nanjing University of Chinese Medicine). Acetonitrile and water were of LC–MS grade from Merk Company (Darmstadt, Germany). HPLC grade methanol was purchased from ANPEL Scientific Instrument Co., Ltd. (Shanghai, China), as well as the HPLC grade formic acid with a purity of 99% (Anqua chemicals supply, USA). All other reagents were of analytical grade and obtained from Nanjing Chemical Reagent Company (Nanjing, China). Rat pancreatic acinar AR42J cells (ATCC CRL 1492) were obtained from the American Type Culture Collection. Cerulein and 0.25% trypsin–0.1% EDTA were purchased from Sigma Chemicals Co. (Spain). Fetal bovine serum was obtained from Invitrogen. Amylase assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Animals

Male Sprague-Dawley rats (n=3), weighed 200–220 g, were supplied by the Slaccas Experiment Animal Company (Shanghai, China). Temperature, humidity, and light conditions in the rats environment were kept constant, with food and water provided ad libitum. All rats were acclimated in the laboratory for at least one week prior to the experiment. Before testing, animals were fasted overnight with free access to water. All animal experiments were carried out according to the Guidelines for the Care and Use of Laboratory Animals, and were approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine.

2.3. UHPLC-ESI-Q-TOF-MS

2.3.1. Sample collection and preparation

To prepare DHFZT, the prepared crude drugs of *Rheum officinale Baill.*, *Aconitum carmichaelii Debx.* and *Asarum sieboldii Miq.* were first mixed together in a ratio (3:4:1, w/w/w) and macerated in deionized water for 30 min, and then decocted twice with boiling water (1:8, w/v) each for 20 min, then the solution was filtered through a two-layer mesh, and was combined and concentrated to a density of 1 g/mL. A characteristic spectrum based on analyzing the features of the area and retention time of the main components in DHFZT was established to evaluate the quality of DHFZT. The fingerprint spectrum was shown in Fig. 1. A total of male Sprague-Dawley 3 rats were orally administered by gavage with a syringe, DHFZT (1.5 g/100 g body weight). Blood samples (about 0.75 mL) were collected from the orbital vein in tube without heparinized at pre-administration (0) and post-administration (0.5 h, 1 h, 2 h, 4 h, 5 h, 6 h, 7 h) and were immediately centrifuged at 4000 rpm for 5 min. The serum samples were collected and stored at −20 °C until analysis. 0.1 mL of serum was spiked in centrifuge tubes and then mixed with 500 μL of methanol by vortex mixing for 30 s. The aqueous and organic layers were separated by centrifugation at 12,000 rpm for 5 min and the organic layer was then transferred to another tube and evaporated to dryness under nitrogen at 40 °C. The residue was reconstituted with 100 μL of chromatographic methanol and vortexed for 30 s and centrifuged at 12,000 rpm for 3 min. A volume of 100 μL of the supernatant was injected for analysis.

2.3.2. Instruments and UHPLC-ESI-Q-TOF-MS conditions

2.3.2.1. Instruments. For analysis of multiple constituents in DHFZT, UHPLC system was couple with hybrid quadrupole time-of-flight tandem mass spectrometry LCMS–Q–TOF (LC/MS-Triple TOF™ 5600, AB SCIEX, Foster City, CA) equipped with an electrospray ionization (ESI) interface.
2.3.2.2. Chromatographic separation. Chromatographic separation was performed on a C18 reversed phase UHPLC column (2.1 mm × 100 mm, 3.5 μm, Agilent), column temperature was set at 35 °C. Flow rate was 0.4 mL/min and the sample injection volume was 5 μL. The gradient profile was optimized as follows: The mobile phase consisted of (A) 0.1% formic acid in water and (B) acetonitrile with the following gradient elution: 0–2 min, 2% B; 2–10 min, 2–10% B; 10–15 min, 10–20% B; 15–20 min: 20–50% B; 20–23 min, 50–100% B; 23–26 min, 100% B; 26–27 min, 100–2% B; 27–30 min, 2% B. The temperature of the autosampler was maintained at 35 °C.

2.3.2.3. Mass spectrometry. The mass spectrometer was operated both in positive and negative ion mode. The following parameters settings were used: the ion spray voltage of 7 eV; turbo spray temperature (TEM) of 450 °C; declustering potential (DP) of 70 V; collision energy (CE) of 40; nebulizer gas (gas 1) of 55 psi; heater gas (gas 2) of 55 psi and curtain gas of 35. Nitrogen was kept as the nebulizer and auxiliary gas. TOF MS and TOF MS/MS were scanned with the mass range of m/z 100–1200 and 50–1200, respectively. The experiments were run with 200 ms accumulation time for TOF MS and 80 ms accumulation time for TOF MS/MS. Continuous recalibration was carried out at each 3 h. In addition, dynamic background subtraction (DBS) trigger information-dependent acquisition (IDA) was used to trigger acquisition of MS/MS of low level constituents. The accurate mass and composition for the precursor ions and fragment ions were analyzed using the Peakview software integrated with the instrument.

2.3.3. Data acquisition and processing

By searching from such databases as PubMed of the U.S. National Library Medicine and the National Institutes of Health, SciFinder Scholar of American Chemical Society and Chinese National Knowledge Infrastructure (CNKI) of Tsinghua University, all components reported in the literatures on *Rheum officinale Baill.*, *Aconitum carmichaelii Debx.* and *Asarum sieboldii Miq.* were all summarized in a Microsoft Office Excel table to establish a in-house library, which includes the name, molecular formula, chemical structure and literatures of each published known compound. The “Find” function of Microsoft Office Excel was used to match the empirical molecular formula with that of published known compounds in the library. The empirical molecular formula was short listed by comparing the accurately measured mass value to the exact mass value of putative molecules at the mass accuracy less than 5 ppm.

Post-acquisition analyses were performed using the Peakview 1.2 program, which employs an extensive list of information of fragmentation in combination with the elemental compositions of the substrate molecules to generate a series of extracted ion chromatograms (XICs). These XICs were compared between the control and sample to eliminate those chromatographic peaks in the sample that also appear in the control.

2.4. Anti-acute pancreatitis activities experiment

2.4.1. Sample preparation

Sample preparation for anti-acute pancreatitis experiment is the same as that for UHPLC-ESI-Q-TOF-MS analysis.

2.4.2. Experimental procedure

The well-grown AR42J cell line with a density of 1 × 10^5 cells/ml was inoculated in special culture dish together with 35 mm laser. They were divided in to three groups, including control group, model group and test group with different point-in-time, and each group with three double holes was administrated with the volume of 0.2 mL. Continuing to cultivate cells 24 h after adherence, drug serum hatch with cerulein of 10^{-7} mol/L in cells after absorbing and abandoning nutrient solution (Wu, et al., 2014) and the amylase liberation was detected in liquid supernatant after continuing to develop cells 24 h.

All these pancreas acinus cells were processed with irritant in different corresponding point in time, and were immediately centrifuged separated at 800 rpm for 5 min at 4 °C, and the supernate was collected and diluted 100 times. 10 μL of supernate was spiked in centrifuge tubes and heated in water bath at 37 °C, and then mixed with iodine solution and distilled water by vortex.
mixing. The absorbance value was collected after zero calibration with distilled water.

2.5. Canonical correlation analysis

The basic idea of CCA is to study on the correlation of two sets of variations. This analysis can reveal and catch the most information between the two sets of variations. In this work, canonical correlation analysis was used for the spectrum–effect relationships between the values of peak area in UHPLC fingerprints from rat serum and the amylase parameters using SPSS statistics software (SPSS for Windows 17.0, SPSS Inc., USA).

3. Results and discussion

3.1. UHPLC-ESI-Q-TOF-MS analysis of DHFZT

The TIC chromatograms of DHFZT and rat serum by UHPLC-ESI-Q-TOF-MS in negative and positive ESI mode are shown in Figs. 2 and 3. The constituents in rat serum after administration of DHFZT were well separated and identified by using their retention time and mass spectra. By comparing the chromatograms of serum containing drug and control serum, 18 peaks were identified by using their retention time and mass spectra. By comparing the chromatograms of serum containing drug and control serum, 18 peaks were

**Fig. 3.** TIC chromatograms in negative ion mode. (a) rat serum (b) DHFZT.

**Fig. 4.** The results of amylase liberation in rat cell after adding serum sample (1 Control group; 2 Model group; 3–9 Test group: 30 min, 60 min, 120 min, 240 min, 300 min, 360 min and 420 min after drug administration).

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<th>Num.</th>
<th>Rt</th>
<th>Identified compounds</th>
<th>Negative ion (m/z)</th>
<th>Positive (m/z)</th>
<th>Element composition</th>
<th>mv (Da)</th>
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<td>6.7</td>
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<td>2</td>
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<td>14.9</td>
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<tr>
<td>5</td>
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<td>424.26962</td>
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</tr>
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<td>NA</td>
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<td>17</td>
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<td>269.06007</td>
<td>-0.1</td>
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<td>NA</td>
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</tbody>
</table>
identified. As a result, peak 2, 3, 13, 18 were the original form compounds existing in *Rheum officinale* Baill.; peak 1, 7, 9, 10, 11 came from *Aconitum carmichaelii* Debx.; and peak 4, 6, 8, 16 were metabolites of rhein, mesaconine, chrysophanol and emodin, respectively. The MS data of (+) ESI–MS spectra and (−) ESI–MS

<table>
<thead>
<tr>
<th>Peak number</th>
<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<th>9</th>
<th>10</th>
<th>11</th>
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<td>0.512</td>
<td>0.408</td>
<td>0.528</td>
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<td>0.505</td>
<td>0.269</td>
<td>−0.027</td>
<td>0.396</td>
<td>0.554</td>
<td>0.606</td>
<td>0.010</td>
<td>0.317</td>
<td>0.294</td>
<td>0.727</td>
<td>0.390</td>
<td>0.454</td>
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</tbody>
</table>

**Table 2**

The correlation coefficients between the common characteristic peaks and the results of amylase efficacy assay (√).

Fig. 5. Chemical structures and mass spectra of the compounds (A) talatisamine, (B) rhein glucoside, (C) rhein isomer methylation, (D) hypaconine, (E) hydroxyl-chrysophanol, (F) emodin glucuronide conjugation, (G) chrysophanol glucuronide conjugation.)
spectra, as well as the identification results are all shown in Table 1.

3.2. Results of anti-acute pancreatitis activity

The anti-acute pancreatitis activities were evaluated by amylase decrease that induced by active components in rats serum after oral DHFZT administration. Therefore, the model of anti-acute pancreatitis on cells was established by adding cerulein to AR42J cell line, and the control was developed by adding control serum. The amylase liberation in rat cell after adding different serum samples is shown in Fig. 4. T test statistical method has been used to evaluate the significant difference between the experimental groups. As shown in Fig. 4 the significant level of \( P < 0.05 \) between the blank group and the model group stands for a successful model building. In the same way, a significant level of \( P < 0.01 \) indicates that the serum sample added to the test groups exerted obvious efficacy.

3.2.1. Results of canonical correlation analysis (CCA)

CCA was used for the spectrum–effect relationships between the area values of 18 common peaks in the UHPLC fingerprints and the amylase liberation values of anti-acute pancreatitis activities. The results are shown in Table 2.

![Image](image-url)

**Fig. 6.** Serum intensity-time curves of the 18 compounds identified from rat serum after oral administration of DHFZT extract.
injury (Liu et al., 2013; Wu et al., 2013a, 2013b). Therefore, these results in reports mentioned above were definitely in accordance with the results in this study. According to our knowledge, talatisamine and hycaponein had not been found to exhibit pharmacological activity of anti-acute pancreatitis so far. In this study, talatisamine and hycaponein were first found to be able to reduce pancreatic acinar cell injury, meaning these compounds exhibit obvious anti-acute pancreatitis activity. All the results above demonstrating the material basis of DHFZT could be helpful for the study on its action mechanism.

4. Conclusions

In our study, UHPLC-ESI-Q-TOF-MS and determination of amylose were combined first for the study in rats to reveal the underlying bioactive compounds in DHFZT. The internal quality and the possible active components of DHFZT were clearly discovered. This research provided a available reference mode for revealing the material basis in TCM.

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References


