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Short communication

Characterization of the constituents in rat biological fluids after oral administration of Fufang Danshen tablets by ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry

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ABSTRACT

An ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry method was established to detect as many constituents in rat biological fluids as possible after oral administration of Fufang Danshen tablets (FDTs). A C18 column (1.8 μm particle size) was adopted to separate the samples, and mass spectra were acquired in both negative and positive modes. First, the fingerprints of FDTs were established, resulting in 43 components being detected within 25 min. Among these compounds, 37 were tentatively identified by comparing the retention times and mass spectral data with those of reference standards and the reference literature; the other 6 components were tentatively assigned solely based on the MS data. *In vivo*, 14 components and 8 metabolites of FDT were observed in plasma, and 12 components of FDT were detected in urine. Tanshinaldehyde, danshexinkun B, a glycine conjugate of danshensu and a methylated conjugate of danshexinkun B were newly detected in rat biological fluids. This study developed a high-speed and sensitive method that was successfully utilized for screening the active ingredients of a Chinese medical formula and provided helpful chemical information for further pharmacology and active mechanism research on Chinese medicine.

1. Introduction

Fufang Danshen tablets (FDTs), an herbal preparation consisting of Radix salviae miltiorrhiza (450 g), Radix notoginseng (141 g), and Borneolum syntheticum (8 g), are widely used in China [1]. FDT carries many biological activities, including dilation of coronary arteries, improving coronary circulation and decreasing myocardial oxygen consumption, and it has been used to treat coronary heart disease, cardiac angina and atherosclerosis in the clinic [2,3]. However, the active ingredients and metabolites of FDT are not well understood.

Recently, there was one published paper [4] reporting the chemical and metabolic components of a Fufang Danshen prescription. The "common prescription", a mixture of Radix salviae miltiorrhiza and Radix notoginseng (1:1), however, did not accurately reflect the proportion found in FDTs used in the clinic. The proportion of these compounds in the FDTs is about 3:1, and FDT is not a simple mixture of the three crude drugs, but prepared through a complicated process [1]. Furthermore, using this "common prescription", only 9 components were observed in rat plasma, and all 23 metabolites of saponins were found in rat feces, except protopanaxatriol, which was found in plasma. In addition, the method employed required long analysis times of approximately 80 min.

In order to get as many active ingredients and metabolites as possible from a FDT in less time, it was necessary for us to set up an effective and reliable analytical method. Fortunately, a high-speed and sensitive technique with shorter analysis times and greater accuracy of the *m/z* value, ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF/MS), has attracted ever-increasing attention [5] and has been successfully applied to identify active ingredients and metabolites in Traditional Chinese Medicine (TCM) [6]. In this study, the UPLC–Q-TOF/MS system using a C18 column (1.8 μm particle size) was adopted to characterize the constituents of FDT *in vitro* and *in vivo*.

2. Experimental

2.1. Chemicals and materials

HPLC grade acetonitrile and formic acid were purchased from Merck (Merck, Darmstadt, Germany). Ultrapure water was purified by a Milli-Q50 SP Reagent Water System (Millipore, Bedford, MA, USA). FDT was purchased from Leiyunshang Phar-

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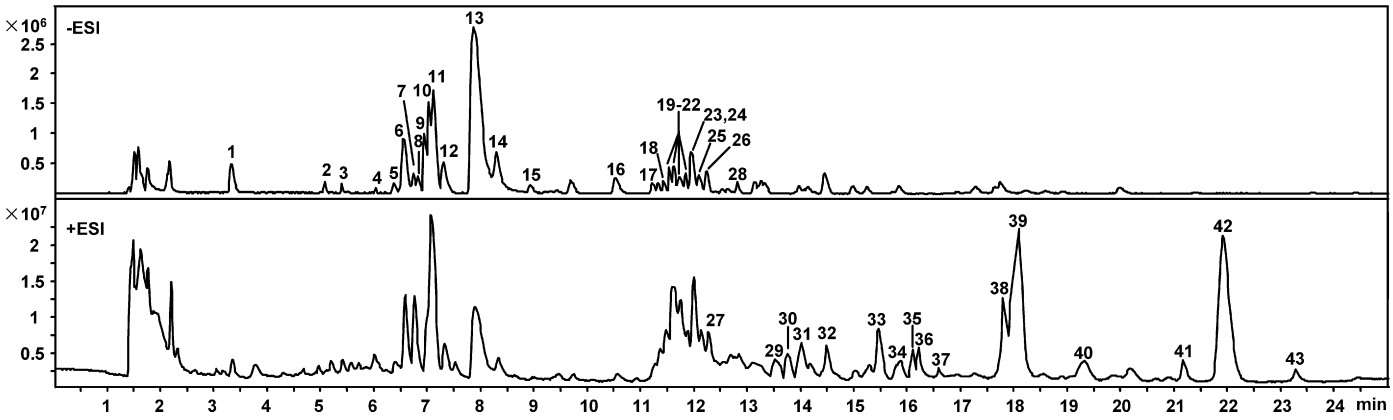

Fig. 1. TIC chromatograms of FDT extracts in negative and positive modes. 43 peaks represent 43 components of FDT.

maceutical Co., Ltd. (Shanghai, China). Crude drugs of Radix salviae miltiorrhiza and Radix notoginseng were purchased from Hengfengtai Ginseng Co., Ltd. (Hong Kong, China). The reference standards of danshensu, protocatechuic aldehyde, caffeic acid, notoginsenoside R1, ginsenoside Re, Rg1, Rf, Rb1, F1, F2, dihydrotanshinone I, tanshinone I, cryptotanshinone, tanshinone IIA, protopanaxatriol and protopanaxadiol were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Salvianolic acid B was isolated in our laboratory (purity >99.0%).

2.2. Sample preparation

2.2.1. Preparation of samples for analysis in vitro

0.5 g of FDT powder was accurately weighed into a 5 mL volumetric flask and subjected to ultrasonic treatment at room temperature with 70% methanol for 30 min. The methanol extraction was centrifuged at *ca.* 10,000 × *g* for 10 min at 4 °C. The supernatant was collected and filtered through a 0.20 μm membrane prior to UPLC–Q-TOF/MS analysis. Radix salviae miltiorrhiza and Radix notoginseng were extracted with the same method described above, which was used to confirm identification of compounds in FDT extracts.

2.2.2. Preparation of samples for analysis in vivo

12 male Sprague–Dawley rats (200 ± 15 g) were purchased from the Slac Laboratory Animal Co., Ltd. (Shanghai, China). The animals were acclimatized to the facilities for 5 days and then fasted with free access to water for a 12-h period prior to the experiment. FDT was ground into a fine powder and dissolved in a 0.5% carboxymethyl cellulose sodium salt (CMC-Na) aqueous solution. The prepared suspension was orally administered to 6 rats at a dose of 300 mg/kg, and 0.5% CMC-Na aqueous solution was orally administered to 6 additional rats as a control. 4 h after drug administration, the animals were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (0.15 mL/100 g body weight). Blood was collected from the hepatic portal vein and then centrifuged at *ca.* 10,000 × *g* for 5 min at 4 °C to separate plasma. Urine was collected for 4 h after drug administration as well. The plasma and urine were processed for UPLC–Q-TOF/MS analysis by the previously described method [4]. All procedures were in accordance with the National Institute of Health's guidelines regarding the principles of animal care (2004).

2.3. Instrumentation and conditions

Analysis was performed on an Agilent-1200 LC system coupled with an Agilent-6520 accurate-mass Q-TOF mass spectrometry equipped with an electrospray ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA). The separation of all samples was performed on an Eclipse plus C18 column (1.8 μm, 3.0 mm × 100 mm, Aglient) at a column temperature of 35 °C. The flow rate was 0.3 mL/min and the mobile phase consisted of 0.1% (v/v) formic acid (A) and acetonitrile (B). The following gradient program was used: 0–1 min, 10–15% B; 1–3 min, 15–28% B; 3–9 min, 28–30% B; 9–11 min, 30–66% B; 11–15 min, 66–66% B; 15–25 min, 66–80% B. The sample injection volume was 3 μL, and the mass detection was operated in both positive and negative ion modes with parameters set as follows: drying gas (N_2) flow rate, 10 L/min; gas temperature, 330 °C; pressure of nebulizer, 10 psig;

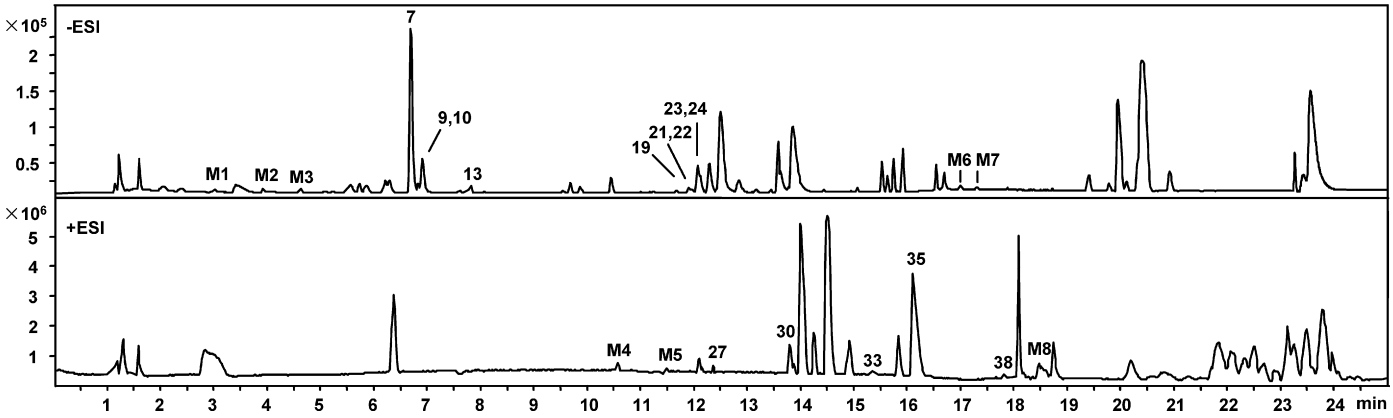

Fig. 2. TIC chromatograms of dosed rat plasma in negative and positive modes. Peak numbers (7, 9, 10, 13, 19, 21, 22, 23, 24, 27, 30, 33, 35 and 38) refer to Fig. 1. M1–8 represent 8 metabolites of FDT.

Table 1
UPLC–Q-TOF/MS data of 9 newly detected constituents in FDT extracts. Peak number refers to Fig. 1.

Peak	RT (min)	MS (*m*/*z*)		MS/MS		Formula	Exact mass (calc.)	Diff (ppm)	Compound	Origin[a]
		Negative	Positive	Negative	Positive					
20	11.65	1123.5881		945.5503, 783.5021, 621.3768, 459.3532		$C_{53}H_{90}O_{22}$	1123.5906	2.54	Ginsenoside Rc	PG
29	13.52		329.1391		311.1165	$C_{19}H_{20}O_5$	329.1384	−2.44	–[b]	SM
30	13.76		311.1290		283.1062	$C_{19}H_{18}O_4$	311.1278	−3.85	Tanshinaldehyde	SM
31	13.99		327.1242		309.1165	$C_{19}H_{18}O_5$	327.1227	−4.61	–	SM
32	14.47		297.1499		282.1291, 254.1187	$C_{19}H_{20}O_3$	297.1485	−4.48	Isocryptotanshinone	SM
34	15.87		315.1604		287.1492	$C_{19}H_{22}O_4$	315.1591	−4.08	–	SM
36	16.24		339.1239		309.1069, 91.0867	$C_{20}H_{18}O_5$	339.1227	−3.55	Methyltanshinonate	SM
37	16.63		295.1339		280.1229, 252.1031	$C_{19}H_{18}O_3$	295.1329	−3.43	Isotanshinone II	SM
41	21.21		281.1541		263.1437, 248.1304	$C_{19}H_{20}O_2$	281.1536	−1.61	–	SM

[a] SM: salvia miltiorrhiza and PG: panax pseudo-ginseng.
[b] Not known, but their optimal formula and calc. exact mass were given by the software.

HV voltage, 3500 V and a scan range of *m*/*z* 50–1500. The MS/MS analysis was acquired in targeted MS/MS mode with a fixed collision energy of 20 V.

3. Results and discussions

3.1. UPLC–Q-TOF/MS analysis of FDT extracts

In our study, 43 components of FDT were separated and detected using a UPLC–Q-TOF/MS system (Fig. 1 and Table 1). The chromatograms showed that phenolic acids and saponins gave better signals in negative mode than in positive mode, while diterpenoid quinones showed better signals in positive mode.

Peaks 1, 2, 3, 7, 9, 10, 13, 18, 19, 24, 28, 33, 38, 39 and 42 were respectively attributed to danshensu, protocatechuic aldehyde, caffeic acid, notoginsenoside R1, ginsenoside Re, Rg1, salvianolic acid B, ginsenoside Rf, Rb1, F1, F2, dihydrotanshinone I, tanshinone I, cryptotanshinone and tanshinone IIA, by comparison with the retention times and mass spectral data of the reference standards. Utilizing Agilent MassHunter Qualitative Analysis software and searching the Spectral Database for Organic Compounds (SDBS) [7], or comparing with the literature data [8–16], 22 peaks were identified. They were notoginsenoside R3 (peak 4), notoginsenoside R6 (peak 5), salvianolic acid D (peak 6), salvianolic acid E (peak 8), rosmarinic acid (peak 11), salvianolic acid H (peak 12), salvianolic acid A (peak 15), salvianolic acid C (peak 16), ethyllithospermate (peak 17), ginsenoside Rc (peak 20), notoginsenoside R2 (peak 21), ginsenoside Rg2 (peak 22), ginsenoside Rd (peak 23), notoginsenoside K (peak 25), ginsenoside Rh1 (peak 26), tanshinaldehyde (peak 30), isocryptotanshinone (peak 32), danshexinkun B (peak 35), methyltanshinonate (peak 36), isotanshinone II (peak 37), methylenetanshiquinone (peak 40) and miltirone (peak 43). The identification of peaks 14, 27, 29, 31, 34 and 41 is still in progress.

3.2. UPLC–Q-TOF/MS analysis of plasma and urine samples

3.2.1. Identification of prototype components in dosed rat plasma and urine

By comparing the chromatograms of dosed rat plasma with control plasma in both negative and positive modes, 22 peaks were observed in dosed rat plasma that did not appear in control plasma. Among the 22 peaks, 14 peaks (peaks 7, 9, 10, 13, 19, 21, 22, 23, 24, 27, 30, 33, 35 and 38) were indicated as prototype components of FDT by comparison with the chromatograms of FDT extracts (Fig. 2).

In negative mode, eight target peaks at *m*/*z* 977.5324, 991.5439 (2 peaks at different retention times), 845.4891, 1107.5986, 815.4772, 829.5002 and 683.4354 were identified as notoginsenoside R1, ginsenoside Re, Rd, Rg1, Rb1, notoginsenoside R2, ginsenoside Rg2, and F1, respectively, by comparison with the extracted ion chromatograms of target peaks in TIC chromatograms of FDT extracts. In addition, another component (*m*/*z* 717.1452) in dosed rat plasma exhibited the same fragmentation pattern as salvianolic acid B. In the positive mode, 5 peaks, at *m*/*z* 313.1486, 311.1301, 279.1043, 281.1189 and 277.0849, were respectively identified as an unknown diterpenoid quinone (peak 27), tanshinaldehyde, dihydrotanshinone I, danshexinkun B and tanshinone I. Similarly, 12 peaks in dosed rat urine were identified as notoginsenoside R1, ginsenoside Re, Rg1, Rb1, Rg2, Rd, F1, F2, tanshinaldehyde, danshexinkun B, tanshinone I, and methylenetanshiquinone.

3.2.2. Identification of metabolites in dosed rat plasma

As 14 peaks in dosed rat plasma were identified as prototypes, the other 8 of the 22 peaks (M1–8) were tentatively predicted to be

Table 2
UPLC–Q-TOF/MS data of 8 metabolites of FDT. Peak number refers to Fig. 2.

No.	RT (min)	MS (*m*/*z*)		MS/MS		Formula	Exact mass (calc.)	Diff (ppm)	Metabolite
		Negative	Positive	Negative	Positive				
M1	3.02	254.0659		210.0719, 195.0603, 153.0458, 109.0276		$C_{11}H_{13}NO_6$	254.0670	−4.33	Glycine conjugate of danshensu
M2	3.93	329.0527		175.0421, 153.0317, 109.0297		$C_{13}H_{14}O_{10}$	329.0514	−3.91	Glucuronic acid conjugate of protocatechuic acid
M3	4.64	153.0189		109.0303		$C_7H_6O_4$	153.0193	2.61	Protocatechuic acid
M4	10.62		313.1455		295.1347, 277.1297	$C_{19}H_{20}O_4$	313.1440	−4.98	18-Methyl hydroxycryptotanshinone
M5	11.49		311.1291		282.1203	$C_{19}H_{18}O_4$	311.1278	−4.01	–[a]
M6	16.98	475.3801		457.3761		$C_{30}H_{52}O_4$	475.3793	−1.41	Protopanaxatriol
M7	17.45	459.3857		441.3803		$C_{30}H_{52}O_3$	459.3844	−2.51	Protopanaxadiol
M8	18.52		295.1337		280.1207, 252.1158	$C_{19}H_{18}O_3$	295.1329	−3.12	Methylated conjugate of danshexinkun B

[a] Not known, but their optimal formula and calc. exact mass were given by the software.

metabolites of FDT (Fig. 2), all of which were tentatively identified, except M5 (Table 2).

The metabolite M1 (*m*/*z* 254.0659, eluted at 3.02 min in negative mode) exhibited a series of product ions at *m*/*z* 210.0719, 195.0603, 153.0458 and 109.0276 in the MS/MS spectrum, which indicated that M1 was a glycine conjugate, as the cleavage of –COOH, $–CH_2–$COOH and –CO–NH–CH_2–COOH moieties to lose 44, 59 and 101 Da, respectively, is characteristic [17]. Eventually, this metabolite was identified as the glycine conjugate of danshensu.

M2, a peak at *m*/*z* 329.0527 and eluted at 3.93 min in negative mode, showed product ions at *m*/*z* 175.0421, 153.0317, and 109.0297 in the MS/MS spectrum. Interestingly, another protonated ion (M3) at *m*/*z* 153.0189 eluted at 4.64 min in the negative MS spectrum and gave a product ion at *m*/*z* 109.0303 as well. Both peaks showed the same product ion at *m*/*z* 109, which was identified as that of the protocatechuic aldehyde. M3 was, therefore, tentatively considered as protocatechuic acid, since the loss of 16 Da could be assigned as a hydroxy substituent, while M2 was considered as the glucuronic acid conjugate of protocatechuic acid, because 175 Da is the product ion of glucuronide [17].

M4 (*m*/*z* 313.1455 eluted at 10.62 min in positive mode), showing product ions at *m*/*z* 295.1347 and 277.1297 in the MS/MS spectrum, was tentatively identified as 18-methyl hydroxycryptotanshinone by comparison with the literature data [18]. M5, a peak at *m*/*z* 311.1291, eluted at 11.49 min in positive mode and was hypothesized to be an oxidative product of tanshinone IIA. The identification of M5 is still in progress.

M6 (*m*/*z* 475.3801 eluted at 16.98 min) and M7 (*m*/*z* 459.3857 eluted at 17.45 min) were detected in negative mode and identified as protopanaxatriol and protopanaxadiol by comparison with reference standards.

M8, a peak at *m*/*z* 295.1337 which eluted at 18.52 min in positive mode, exhibited product ions at *m*/*z* 280.1207 and 252.1158

Fig. 3. Structures of newly detected components and metabolites of FDT: (a) newly detected components of FDT *in vitro* and (b) newly detected components and metabolites of FDT *in vivo*.

in the MS/MS spectrum. It was tentatively assigned as the methylated conjugate of danshexinkun B by analyzing MS and MS/MS spectra.

4. Conclusion

In vitro, 43 components of FDT were detected within a 25-min UPLC–Q-TOF/MS experiment. In all, 9 compounds, including ginsenoside Rc, tanshinaldehyde, isocryptotanshinone, methyltanshinonate, isotanshinone II, and four unknown diterpenoid quinones (peaks 29, 31, 34 and 41), were newly separated and detected (Fig. 3a). *In vivo*, 14 components and 8 metabolites of FDT were observed in plasma, while 12 components of FDT were found in urine. Of these, tanshinaldehyde, danshexinkun B, a glycine conjugate of danshensu (M1) and a methylated conjugate of danshexinkun B (M8) were newly detected in rat biological fluids (Fig. 3b). Although M5 and 6 components of FDT were not identified, it was demonstrated that the high-speed and sensitive UPLC–Q-TOF/MS analytical system was a useful tool to investigate active ingredients and metabolites of TCM.

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