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Metabolomic study of myocardial ischemia and intervention effects of Compound Danshen Tablets in rats using ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry

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ABSTRACT

Myocardial ischemia (MI) is a worldwide epidemic. Compound Danshen Tablets (CDTs), an herbal compound preparation, are widely used to treat MI in China. In this study, we aimed to explore novel biomarkers to increase the understanding of MI and investigate therapeutic mechanisms of CDT by using a metabolomic approach. Plasma extracts from sham, MI model, CDT- and western medicines (isosorbide dinitrate, verapamil, propranolol, captopril, and trimethazine)-treated rats were analyzed by ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF-MS). The orthogonal partial least square (OPLS) model was built to find metabolites expressed in significantly different amounts between MI and sham rats. Meanwhile, partial least squares discriminant analysis (PLS-DA) was used to investigate CDT's protective effects. The results showed that CDT presented protective effects on MI by reversing potential biomarkers to sham levels, especially for the four metabolites in the pathway of purine metabolism (hypoxanthine, xanthine, inosine and allantoin).

1. Introduction

Myocardial ischemia (MI) is characterized by an imbalance between the supply and demand of myocardial oxygen, causing cardiac dysfunction, arrhythmias, myocardial infarction, and sudden death [1]. Clinical treatment of MI largely depends on western medicines, such as isosorbide dinitrate (vasodilator), verapamil (calcium antagonist), propranolol (beta-adrenergic blocker), captopril (angiotensin converting enzyme inhibitor), trimethazine (fatty acid oxidation inhibitor), etc. In spite of advances in drug treatment, MI has become a leading cause of death in a worldwide epidemic. It is therefore still an area of intense research activity. According to an investigation carried out by the World Health Organization in 2005, an estimate of 17.5 million people died from cardiovascular disease, and 7.6 million of these deaths were due to MI [2]. Recently, Chinese medicines, such as *Radix Salviae Miltiorrhizae* and *Radix Notoginseng*, have been used to treat MI as well [3–5].

Compound Danshen Tablets (CDTs), an herbal compound preparation consisting of *Radix Salviae Miltiorrhizae*, *Radix Notoginseng and Borneolum Syntheticum*, are widely used to treat ischemic heart diseases in China. Early in 2003, CDT was indicated to be effective for dilating coronary artery and decreasing myocardial oxygen consumption and platelet aggregation [6]. CDT was officially recorded in the 2005's edition of Chinese pharmacopoeia [7]. However, its mechanisms of action are not completely understood, and there are three reasons for this: the complexity of active components; interactions of active components; and lack of effective methods of study.

Metabolomics is an important area of systems biology. It aims to pinpoint interesting metabolites that are related to drug toxicity, disease, and environmental or genetic variation [8–17]. Applications of multivariate analysis make it possible to simplify data of metabolite profiles [18]. In addition, recent technical advances in metabolomics have offered the possibility of biomarker identification for MI and mechanisms exploration of novel compounds. For example, Sabatine [19] pointed out, by using high-performance liquid chromatography combined with triple-quadrupole mass spectrometer (HPLC–3Q-MS) analysis, that the citric acid pathway was highly changed in MI rats; Verhoeckx's [20] study of different classification of anti-inflammatory compounds showed distinct and characteristic mRNA, protein, and metabolite expression patterns, which could be used to characterize novel anti-inflammatory compounds (western medicines).

In the present study, we applied a metabolomic strategy, based on the ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF-MS), to find

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biomarkers to increase the understanding of MI and investigate protective effects of CDT for the first time.

2. Experiment

2.1. Materials and animals

HPLC grade acetonitrile was purchased from JT Baker (NJ, USA). Spectroscopic grade formic acid, leucine enkephalin, and commercial standards were purchased from Sigma/Aldrich (MO, USA). Distilled water was purified "in-house" using a Milli-Q20 system Millipore (MA, USA). CDT was purchased from Leiyunshang Pharmaceutical Co., LTD (Shanghai, China). Isosorbide dinitrate was purchased from Forward Co., LTD (Shanghai, China). Verapamil was purchased from Shanghai Pharmaceutical (Group) Co., LTD (Shanghai, China). Propranolol was purchased from Shanghai Xinpashi Pharmaceutical Co., LTD (Shanghai, China). Captopril was purchased from Shanghai Hengshan Pharmaceutical Factory (Shanghai, China). Trimethazine was purchased from Servier Pharmaceutical Factory (Tianjin, China).

Fifty-five male Sprague-Dawley rats (200 ± 15 g) were purchased from the Slac Laboratory Animal Co., LTD (Shanghai, China). The animals were housed in stainless steel metabolic cages with free access to food and tap water under standard conditions of humidity ($50 \pm 10\%$), temperature ($25 \pm 2\,^{\circ}C$) and light (12 h light/12 h dark cycle). All animals were handled with humane care throughout the experiment.

2.2. MI model and drug administration

MI model was induced by left anterior descending coronary artery ligation. Anterior thoracotomy was performed under sterile conditions to open the pericardium. The heart was then rapidly exteriorized. The left anterior descending coronary artery was ligated approximately 2–3 mm distal from its origin with use of a 6-0 polypropylene suture [21]. Six rats died during the 24 h postoperative period because of acute pumps failure or lethal arrhythmias. 49 rats survived, including 43 MI rats and 6 sham rats (without ligation).

36 of 43 MI rats were randomly treated with six medicines ($n = 6$), which were CDT (300 mg/kg/d), isosorbide dinitrate (3 mg/kg/d), verapamil (6 mg/kg/d), propranolol (20 mg/kg/d), captopril (4 mg/kg/d), and trimethazine (6 mg/kg/d). Treated rats were consecutively oral administrated for 7 days; sham ($n = 6$) and MI ($n = 7$) rats were received 0.2 mL saline each time. Rats were fasted overnight before administrations with free access to water. In the sham, MI model, CDT-, isosorbide dinitrate-, verapamil-, propranolol-, captopril- and trimethazine-treated groups, respectively 6, 6, 6, 6, 5, 6, 6 and 6 rats survived.

All 47 survived rats were put to death after blood was collected from ophthalmic venous plexus on the 9th day. The experiment was carried out in accordance with guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of Shanghai, China. The study protocol was approved by the Animal Care and Use Committee of Second Military Medical University.

2.3. Sample preparation

1 mL blood of each rat was respectively added into 2.5 mL heparin-coated tubes and then centrifuged at $2789.1 \times g$ for 10 min at 4 °C. 100 μL of the supernatant was added to 200 μL acetonitrile and then the mixture was shaken vigorously for 30 s. After centrifugation at $9562.5 \times g$ for 10 min at 4 °C, the supernatant was stored at −80 °C until analysis.

A random blood sample (6 mL) was divided into six parts and extracted by the same method. These six samples were continuously injected to validate repeatability of the sample preparation method.

20 μL from each blood sample was pooled to generate a pooled quality control (QC) sample and aliquots of 100 μL of this pooled sample were extracted by the same method. This pooled sample was used to provide a representative "mean" sample containing all analytes that was encountered during the analysis, and it was used to validate stability of LC–MS system [22,23].

2.4. Sequence analysis

The pooled QC sample was analyzed at the beginning, the end and randomly through the analytical run to monitor the stability of sequence analysis. The typical batch sequence of plasma samples consisted of the consecutive analysis of 1 QC plasma sample (at the beginning of the study), followed by 10 unknown plasma samples, 1 QC plasma sample, before running another 10 unknown plasma samples, etc. Meanwhile, samples were analyzed in a random order for a normal good practice. An identical sequence was repeated to complete the total set of injections ($n = 53$, including QCs) analyzed in less than 1 day per mode [24].

2.5. UPLC–Q-TOF-MS conditions

Metabolomics analysis was performed on an ACQUITY™ UPLC system coupled to a Micromass Q-Tof Micro™ (Waters MS Technologies, Manchester, UK) equipped with an electrospray ionization source. A 2.1 mm i.d. × 100 mm ACQUITY™ 1.7 μm column (Waters, Milford, MA, USA) was used. The column was maintained at 45 °C. The mobile phases A and B were water with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively. Injection volume was 5 μL. The gradient duration program was: 0–1.5 min, 5% B; 1.5–9 min, 5–100% B; 9–14 min wash with 100% B; and a 3 min recycle time. The flow rate was 400 μL/min. The mass detection was operated in both positive and negative modes so as to monitor as many ions as possible, with parameters set as followed: desolvation gas, 400 L/h; cone gas, 20 L/h; desolvation temperature, 250 °C; source temperature, 100 °C; capillary voltage, 3000 V; cone voltage, 30 V; collision energy, 5 eV, while it was set at 20 eV in MS/MS mode to identify potential biomarkers. The data acquisition rate was set as 0.4 s, with a 0.1 s inter-scan delay. Lock spray was utilized to calibrate accuracy of mass. Leucine enkephalin was used as the lock mass (m/z 556.2771 in the positive mode, and 554.2615 in the negative mode). Data were collected in continuum mode, and the lock spray frequency was set at 10 s, and data were averaged over 10 scans. Full scan mass range of 50–1000 m/z was acquired.

2.6. Data processing

The LC–MS data were exported by Micromass MarkerLynx™ applications manager version 4.1 software (Waters Corporation, Milford, MA, USA). Before multivariate analysis, the data of each sample was normalized to total area to correct for the MS response shift from the first injection to the last injection due to the long duration, overnight or longer, of an LC–MS analysis in metabolomic studies. After this operation, the sum of the ion peak area within each sample was set to 10,000. Then, orthogonal partial least square (OPLS) and partial least squares discriminant analysis (PLS-DA) were used for analysis of metabolite profiles. Multivariate analysis was performed by the SIMCA-P 11 version (Umetrics AB, Umeå, Sweden). The significance was expressed by using one-way analyses of variance (ANOVAs) of the SPSS 13.0 for Windows (SPSS Inc.,

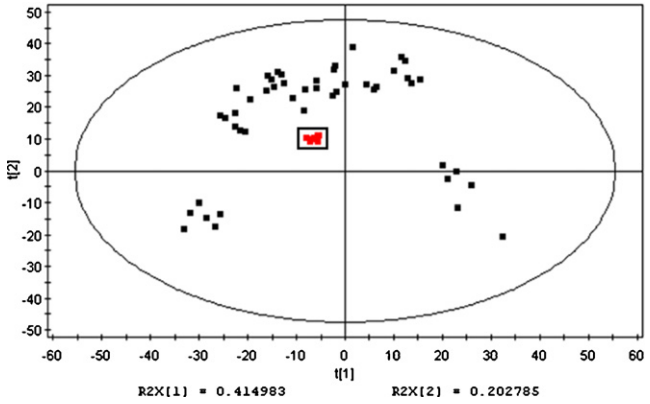

Fig. 1. PCA model of QC sample (k = 2700, n = 53, R^2 [1] = 0.415, R^2X [2] = 0.203, Q^2 = 0.267, A = 5) showing the first two principal components: 6 QC samples (■ in square), 47 plasma samples (■).

Chicago, IL, USA), followed by Duncan post hoc tests. P values less than 0.05 were considered significant.

3. Results and discussion

3.1. Sample repeatability

Extracts from six aliquots of a random blood sample were continuously injected to evaluate the repeatability. Five common extracted ion chromatograms (EICs) shared by these injections were selected according to their different chemical polarities and m/z values. The relative standard derivations (RSDs) of these peaks were 7.73–13.67% for peak areas and 0.04–0.97% for retention times in the positive mode (6.97–14.91% for peak areas and 0.02–0.99% for retention times in the negative mode).

3.2. System stability

The LC–MS system stability for the large-scale sample analysis was demonstrated by the test of pooled QC samples. The principal components analysis (PCA) score plot shows the QC samples are tight clustered (Fig. 1). Moreover, peak areas, retention times and mass accuracies of five selected EICs in six QC samples also showed good system stability. RSDs of the five peaks were 6.84–14.18% for peak areas, 0.03–1.02% for retention times and 0.15E-04%–0.92E-04% for mass accuracies in the positive mode (7.13–14.78% for peak areas, 0.04–1.05% for retention times and 0.32E-04%–1.16E-04% for mass accuracies in the negative mode). Both results indicated the large-scale sample analysis had hardly any effect on the reliability of data.

3.3. Biomarkers identification and pathways

The signals in mass spectra include metabolites, adducts, ions fragments, noises and so on. In this study, the number of signals was 1531 in the positive mode (1169 in the negative mode). By traditional statistical methods, it was hard to find the discriminating ions contributing to the classification of MI and sham groups from thousands of signals.

PCA, which is a common method to handle metabolomic data, can discriminate samples from different groups and indicate ions responsible for the discrimination. We found that much of the variations of the data contributing to the discrimination were amplified noises, not reproducible potential metabolites by PCA. Orthogonal partial least squares (OPLSs) analysis is to our knowledge a better method to pick out discriminating ions that are contributing to the classification of samples and remove non-correlated variations contained within spectra. Thus, OPLS was carried out to find biomarkers of MI in our study. Features in OPLS score plot indicate sham and MI groups are distinguished clearly (each dot or box in the plot represents a sample) (Fig. 2). The S-plot shows the distribution of potential biomarkers (Fig. 3). The more away a triangle (a retention time–m/z pair) is from the origin, the more influence it would have on the separation of samples. Top 10 ions calculated from the OPLS S-plot as contributing the greatest to the inter-lot difference are shown in Table 1. A total of 160 out of 2700 ions (sum of ions in both modes) were different between sham and MI groups. Among the perturbed ions, 39 were predicted by searching Biofluid Metabolites Database (http://metlin.scripps.edu) and Human Metabolome Database (http://www.hmdb.ca/) for MS and MS/MS information. Thereafter, 22 of them were identified by matching their fragmentation patterns with commercial standards (13 in the positive mode, 9 in the negative mode) (Table 2).

Here we present inosine (an ion at m/z 267.08) as an example to illustrate the process of biomarker identification. First the quasi-

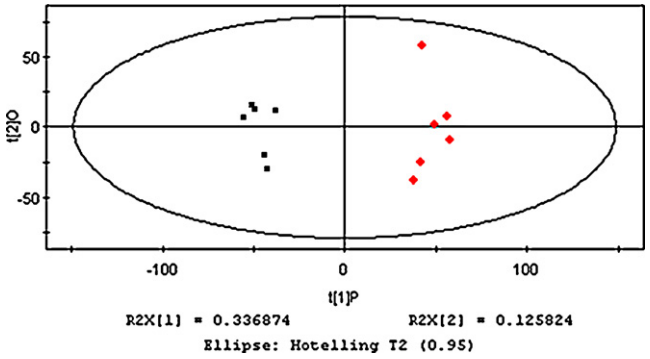

Fig. 2. OPLS model of MI (k = 2700, n = 12, R^2Y = 0.981, R^2X = 0.463, Q^2 = 0.923, A = 1 + 1; first two principal components shown): (■) sham, (◆) MI model.

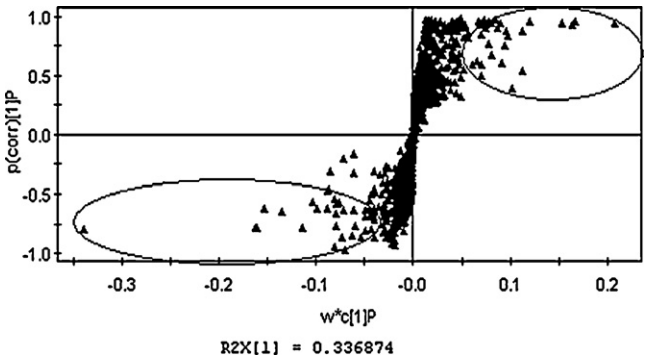

Fig. 3. S-plot of the OPLS model. Each triangle in the S-plot represented an ion. Ions in black circles were responsible for potential biomarkers.

Table 1
Top 10 mass ions contributing to differences between the sham and MI rats.

R.T.	m/z[a]	Ion mode	Trend[b]	$w^*c[1]P$	P(corr)[1]P
0.89	386.33	ESI(−)	↑	0.338943	0.977142
5.75	[9]318.30	ESI(+)	↑	0.162627	0.907213
4.02	[20]227.99	ESI(−)	↑	0.161173	0.983866
6.90	544.27	ESI(−)	↑	0.152835	0.939875
8.47	343.23	ESI(+)	↑	0.150763	0.962548
2.44	188.07	ESI(+)	↓	0.0992456	0.828042
0.33	[1]159.08	ESI(+)	↓	0.0890905	0.931826
7.23	991.65	ESI(+)	↓	0.0883186	0.959007
0.44	[5]132.09	ESI(+)	↓	0.0784007	0.953470
0.44	204.12	ESI(+)	↓	0.0747035	0.888877

[a] The superscript numbers of m/z values refer to No. in Table 2.
[b] "↑" and "↓" represent the compound is up- and down-regulated in MI rats compared with the sham, respectively.

Table 2
22 biomarkers of MI detected by UPLC–Q-TOF-MS in both positive and negative ion modes.

Mode	No.	R.T. (min)	*m/z*	Fragments	Compound	Formula	Cal. mass	Trend[a]	Related pathway
ESI(+)	1	0.33	159.08	131.08, 116.07	Allantoin	$C_4H_6N_4O_3$	158.04	↓	Purine metabolism
	2	0.40	122.09	103.96	1-Phenylethylamine	$C_8H_{11}N$	121.09	↑	Phenylalanine metabolism
	3	0.42	137.05	119.08	Hypoxanthine	$C_5H_4N_4O$	136.04	↑	Purine metabolism
	4	0.43	118.08	72.07	L-Valine	$C_5H_{11}NO_2$	117.08	↓	ABC transporters
	5	0.44	132.09	86.09	L-Isoleucine	$C_6H_{13}NO_2$	131.09	↓	Valine, leucine and isoleucine degradation
	6	0.44	120.08	102.06	L-Homoserine	$C_4H_9NO_3$	119.06	↓	Glycine, serine and threonine metabolism
	7	2.43	227.08	210.06	Carnosine	$C_9H_{14}N_4O_3$	226.11	↓	Alanine and aspartate metabolism
	8	4.09	229.14	211.13	L-Isoleucyl-L-Proline	$C_{11}H_{20}N_2O_3$	228.15	↑	Not known
	9	5.75	318.30	274.27	Phytosphingosine	$C_{18}H_{39}NO_3$	317.29	↑	Not known
	10	6.36	302.31	284.30	Dihydrosphingosine	$C_{18}H_{39}NO_2$	301.30	↑	Sphingolipid metabolism
	11	6.39	346.33	250.25	2′,3′-Cyclic GMP	$C_{10}H_{12}N_5O_7P$	345.21	↑	Purine metabolism
	12	6.98	330.34	234.24	2′,3′-Cyclic AMP	$C_{10}H_{12}N_5O_6P$	329.21	↑	Purine metabolism
	13	7.02	240.10	222.09	Dihydrobiopterin	$C_9H_{13}N_5O_3$	239.10	↓	Folate biosynthesis
ESI(−)	14	0.41	151.02	134.03	Xanthine	$C_5H_4N_4O_2$	152.03	↑	Purine metabolism
	15	0.42	267.08	135.03	Inosine	$C_{10}H_{12}N_4O_5$	268.08	↑	Purine metabolism
	16	0.43	89.02	71.01	Glyceraldehyde	$C_3H_6O_3$	90.03	↓	Pentose phosphate pathway
	17	1.31	164.07	146.05	L-Phenylalanine	$C_9H_{11}NO_2$	165.08	↓	Phenylalanine, tyrosine and tryptophan biosynthesis
	18	2.41	203.08	185.90	L-Tryptophan	$C_{11}H_{12}N_2O_2$	204.09	↓	Phenylalanine, tyrosine and tryptophan biosynthesis
	19	2.75	129.05	97.04	2-Oxoisocaproic acid	$C_6H_{10}O_3$	130.06	↓	Not known
	20	4.02	227.99	131.03	5-Phosphoribosylamine	$C_5H_{12}NO_7P$	229.04	↑	Purine metabolism
	21	7.20	319.23	301.22	15(S)-HETE	$C_{20}H_{32}O_3$	320.24	↑	Arachidonic acid metabolism
	22	8.18	369.20	341.22	Thromboxane B2	$C_{20}H_{34}O_6$	370.24	↑	Arachidonic acid metabolism

[a] "↑" and "↓" represent the compound is up- and down-regulated in MI rats compared with the sham, respectively.

molecular mass was found out to be a mass peak at *m/z* 267.08 (retention time was 0.42 min in negative mode). $C_{10}H_{12}N_4O_5$ was calculated as the most probable molecule formula, and MS/MS information was used to study its molecular structure (Fig. 4a). The above information was also searched for in Internet databases. Then considering the elemental composition, fragmentation pattern, and chromatographic retention behavior, the *m/z* of 267.08 was thought to be probably inosine. This was then confirmed by comparing with commercial standard (Fig. 4b).

Among those 22 biomarkers, 10 were decreased in MI rats, whereas 12 were up-regulated (Fig. 5). The related pathway of each biomarker was also recorded by searching KEGG PATHWAY Database (http://www.genome.jp/kegg/).

3.4. CDT protection

To evaluate protective effects of CDT, a PLS-DA model was built to analyze the top 160 significant ions. The score plot (Fig. 6) shows that sham, MI and CDT-treated groups are classified clearly, and the CDT-treated group is much closer to the sham group

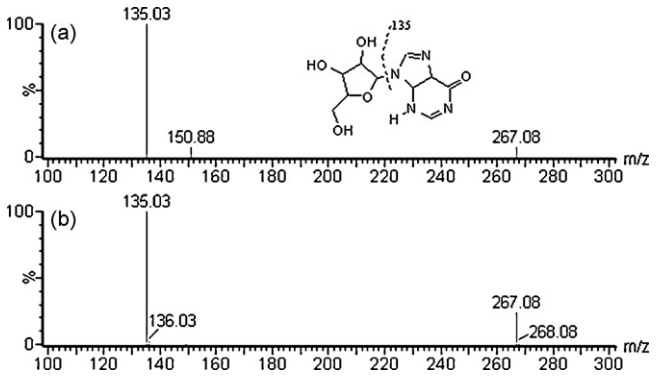

Fig. 4. MS/MS spectra of inosine (the precursor ion was *m/z* 267.08; the major fragment was *m/z* 135.03): (a) plasma, (b) standard.

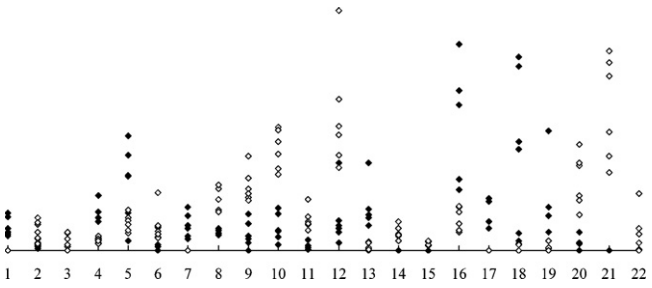

Fig. 5. Metabolomic alterations of 22 identified MI biomarkers. Each diamond represents one biomarker in one sample: sham rats (♦), MI rats (◊). The numbers of the *x*-coordinate represent 22 biomarkers numbered in Table 2. The level of 5-Phosphoribosylamine (No. 20) was reduced to 10% of real peak areas in plasma samples.

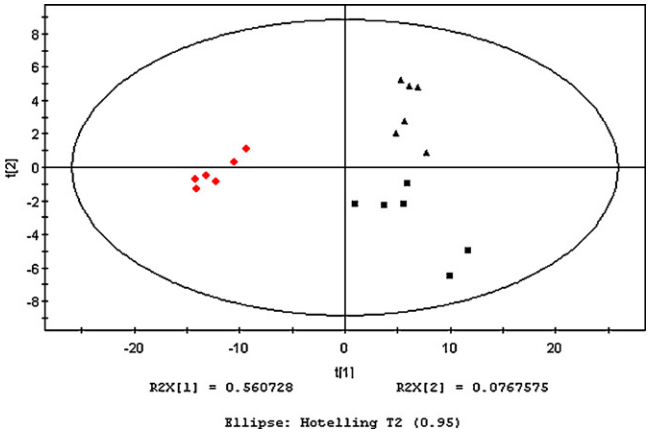

Fig. 6. PLS-DA model of CDT treatment ($k = 160$, $n = 18$, $R^2Y = 0.974$, $R^2X = 0.637$, $Q^2 = 0.716$, $A = 5$) showing the first two principal components: (■) sham, (◆) MI model, and (▲) CDT-treated groups.

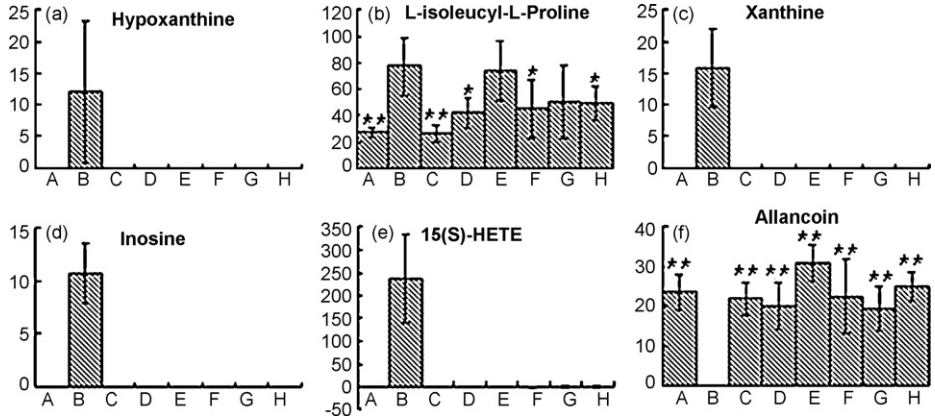

Fig. 7. Mean levels of six biomarkers reversed by CDT completely in different groups. (A) Sham, (B) MI model, (C) CDT-treated, (D) isosorbide dinitrate-treated, (E) verapamil-treated, (F) propranolol-treated, (G) captopril-treated, and (H) trimethazine-treated groups. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the MI group.

than the MI group, which might suggest CDT has a reverse effect on MI model. We also found that mean level of each biomarker (except L-Homoserine) was reversed to the sham level at different degrees after taking CDT. Among these biomarkers, hypoxanthine, L-isoleucyl-L-proline, xanthine, inosine, 15(S)-HETE and allantoin in the CDT-treated group were completely reversed to levels in the sham group (Fig. 7). Thus, these six biomarkers might serve as characters to explain mechanisms of CDT. Interestingly, hypoxanthine, xanthine, inosine and allantoin are involved in the purine metabolism. Hereby, we focused on the pathway of purine metabolism in the process of MI injury [25,26].

During ischemia, ATP is degraded and hypoxanthine accumulates. Hypoxanthine is further metabolized to xanthine and urate by xanthine dehydrogenase or xanthine oxidase and it can be converted to inosine [27]. It has been considered that xanthine oxidase generated excess oxygen-free radicals, then caused ischemia injury in the pathway [28–30], and the enhanced release of inosine in plasma was a marker of ischemic myocardium [31]. Moreover, superoxide reacts at diffusion limited rates with other free radicals, particularly with NO to form the potent oxidant peroxynitrite, which has been shown to activate metalloproteinases and degrade troponin I, contributing to depressed myocardial function [32–34]. Doehner and Yardım-Akaydın reported allantoin was a marker of oxidative stress in humans and free radical activity in chronic heart failure [35,36]. Generally, the level of allantoin should increase in MI rats; however, it decreased in our experiments. The reason is not clear yet. Therefore, a further study on the mechanism of decreased allantoin in MI rats is being carried out in our laboratory.

In summary, levels of hypoxanthine, xanthine and inosine in the purine metabolism pathway increased in MI rats, and could be reversed to sham levels by CDT, suggesting that CDT has extensive effects on the purine metabolism and that these decreases might

Table 3
38 significant ions of MI were reversed differently by the six medicines in both positive and negative modes.

R.T.	m/z[a]	Trend[b]	Significance						R.T.	m/z	Trend	Significance					
	ESI(+)		CDT	ID	V	P	C	T		ESI(−)		CDT	ID	V	P	C	T
0.33	226.95	↓	**[c]	**	**	**		**	0.37	210.84	↓	**	*	*			
0.39	162.11	↓	*[d]		*				0.37	268.80	↓	**	**	**	**		
0.39	203.05	↓	**	*	*			*	0.38	152.88	↓	**	**	*			
0.40	[2]122.09	↑			**				0.38	150.89	↓	**	*	**			
0.42	100.08	↑	*	*	*	*		*	0.43	89.03	↓	**	*	*			
0.43	[4]118.08	↓	**		*				1.31	[17]164.07	↓		**		**	**	**
0.43	130.07	↓	*	*	**				2.41	[18]203.08	↓	*					
0.44	[5]132.09	↓	**		**				6.9	544.27	↑	*	*		*	*	
0.44	204.12	↓	*		*				6.95	700.31	↓			*	*	*	
0.44	[6]120.08	↓				**	**	**	7.37	478.29	↑	**	**	**	*		*
2.43	[7]227.08	↓	**		**				7.94	636.35	↑	*					
2.44	188.07	↓	**	**	**				8.18	[22]369.20	↑			**			
2.47	239.08	↓				**	**		8.47	303.23	↑			*			
4.09	229.15	↑	**	*		*		*	8.47	439.21	↑			**			
6.95	542.31	↓	**		**	**	**	**	9.25	551.36	↓	**			**	**	**
7.02	[13]240.10	↓	*		*												
7.23	991.65	↓	**	**		*	**	*									
7.40	544.33	↑	*														
7.79	524.36	↓						**									
7.90	482.32	↓	**	*	**			**									
8.45	282.28	↑	**	**	**			*									
9.06	124.09	↑	**	**	**	**		**									
9.39	144.98	↑	*	*													

ID, isosorbide dinitrate; V, verapamil; P, propranolol; C, captopril; and T, trimethazine.

[a] The superscript numbers of m/z values refer to No. in Table 2.

[b] "↑" and "↓" represent the variable is up- and down-regulated in MI rats compared with the sham, respectively.

[c] **, $P < 0.01$ the medicine-treated group *vs.* the MI group, and $P > 0.05$ the medicine-treated group *vs.* the sham group.

[d] *, $P < 0.05$ the medicine-treated group *vs.* the MI group, and $P > 0.05$ the medicine-treated group *vs.* the sham group.

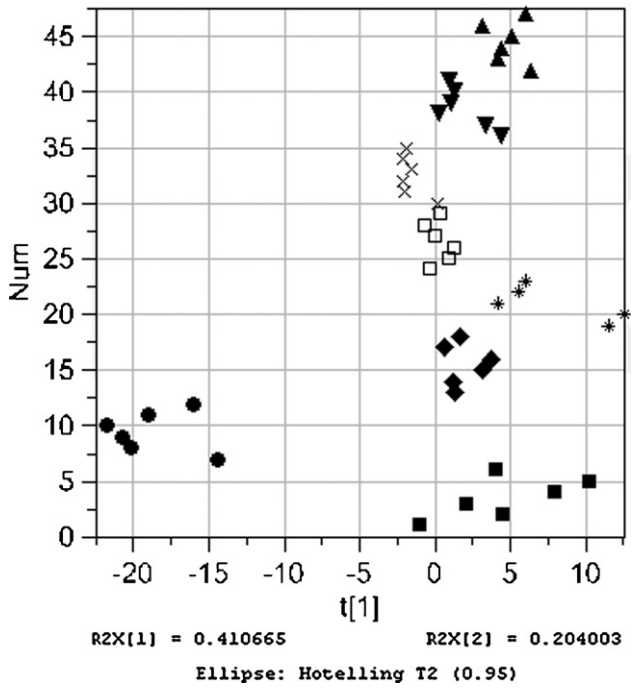

Fig. 8. PLS-DA model of all six medicines treatment (k = 160, n = 47, R^2Y = 0.941, R^2X = 0.615, Q^2 = 0.23, A = 5) showing the first two principal components: (■) Sham, (●) MI model, (♦) isosorbide dinitrate-treated, (*) verapamil-treated, (□) propranolol-treated, (×) captopril-treated, (▼) trimethazine-treated, and (▲) CDT-treated groups.

contribute to a decrease of oxygen-free radical and relieve of MI injury.

3.5. New view on mechanism research

Metabolomics offers a new view on the mechanism research of novel compounds, specifically on western medicine. For example, Verhoeckx [20] validated that metabolomics could be used to characterize mechanisms of novel anti-inflammatory compounds by referring to classic anti-inflammatory compounds. In this study, five commonly used western medicines (isosorbide dinitrate, verapamil, propranolol, captopril and trimethazine) for treatment of MI were selected as positive references to help investigating mechanisms of CDT. PLS-DA was used to investigate plasma changes of the MI model after administration (top 160 significant ions). The score plot (Fig. 8) shows five western medicine-treated groups are clearly separated, and the CDT-treated group is separated from the five western medicine-treated groups as well. Among the 160 ions, 63 were not regulated by any medicine ($P > 0.05$, each medicine-treated group *vs.* the MI group); levels of 59 ions were reversed to levels of the sham by all six medicines ($P < 0.05$, each medicine-treated group *vs.* the MI group; and $P > 0.05$, each medicine-treated group *vs.* the sham group); the last 38 ions were reversed differently by the six medicines (Table 3). The numbers of ions regulated by CDT, isosorbide dinitrate, verapamil, propranolol, captopril and trimethazine were 88, 78, 85, 73, 67 and 73, respectively, suggesting the significant ions regulated by CDT were not completely the same as any other western medicine.

Each of the referred western medicines in our study contained one component, whereas CDT was a mixture of several components, suggesting CDT might have unique characteristics for the treatment of MI. Therefore, more researches should be carried out to investigate effective components of CDT and further illustrate CDT's mechanisms in details.

4. Conclusion

In summary, 22 metabolites were identified as biomarkers of MI, and the purine metabolism (hypoxanthine, xanthine, inosine and allantoin) was the most important perturbed pathway in MI injury; CDT could reverse partial metabolic perturbations of MI, especially the perturbation of the purine metabolism. Our study indicated that the LC–MS based metabolomic strategy was a useful tool in biomarkers exploration of MI and mechanisms studies of CDT.

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