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Effect of ginseng polysaccharide on the urinary excretion of type 2 diabetic rats studied by liquid chromatography–mass spectrometry

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ABSTRACT

Ginseng polysaccharide is known to have anti-hyperglycemic and anti-hyperlipidemic effects in vivo and its precise mechanism of action is not clear. A urinary metabolomics method based on rapid-resolution liquid chromatography/mass spectrometry (RRLC/MS) was developed to investigate the effect of water-soluble ginseng polysaccharide (WGP) on type 2 diabetes in rats. Principal component analysis (PCA) was carried out for pattern recognition and a clear separation between type 2 diabetic rats and those treated with WGP was achieved. Eight potential biomarkers were found and identified. Significantly increased inosine, serotonin, phenylpropionylglycine and dodecanedioic acid showed the effect of WGP on purine metabolism, tryptophan metabolism, fatty acid metabolism and energy metabolism. 1-Methyladenine, 4-deoxyerythronic acid, 5-hydroxyhexanoic acid and tetrahydrocortisol were significantly decreased which indicated that WGP can regulate DNA metabolism, organic acids metabolism and steroid hormone metabolism. This work is helpful in the effect mechanism study of ginseng polysaccharide.

1. Introduction

Diabetes mellitus (DM) is a group of metabolic diseases in which a sufferer has high blood glucose, either because the body does not produce enough insulin, or because cells do not respond to the insulin that is produced. Type 1 diabetes mellitus (T1DM) accounts for only a minority of the total burden of diabetes in a population but is increasing in incidence in both poor and rich countries. Type 2 diabetes mellitus (T2DM) constitutes about 90% of all diabetes in developed countries and may account for an even higher percentage in poor and developing countries [1]. Type 2 diabetes is now a common and serious global health problem which, for most countries, has evolved in association with rapid cultural and social changes, aging populations, increasing urbanization, dietary changes, reduced physical activity and other unhealthy lifestyle and behavioral patterns [2]. A variety of diabetic complications such as hypoglycemic, ketoacidosis, neuropathy, nephropathy, cardiopathy and retinopathy is caused by long-term high blood glucose.

Apart from currently available therapeutic options, many herbal medicines have been recommended for the treatment of DM [3]. Plants have been used traditionally throughout the world because of their claimed efficacy, lack of side effects and relatively low cost [4]. Investigation on such agents from traditional medicinal plants has become more important to test such claims. The antidiabetic effect of water-soluble ginseng polysaccharide has been reported in recent years. Xie et al. [5] reported that the polysaccharide fraction from American ginseng berry extract has a significant anti-hyperglycemic activity in diabetic *ob*/*ob* mice. Kwak et al. [6] reported that acidic polysaccharide from red ginseng may play an additional role in reducing hyperlipidemic conditions in rats. Yang et al. [7,8] found that ginseng polysaccharide has effects on reducing blood glucose and liver glycogen. Since the precise mechanism of water-soluble ginseng polysaccharide on T2DM is not clear, it is necessary to perform a metabolomic analysis to visualize the alteration of global circulating metabolites after water-soluble ginseng polysaccharide treatment. In our study reported here, we describe the administration of water-soluble ginseng polysaccharide and its impact on blood glucose concentration in streptozotocin-induced diabetic rats in comparison with healthy control rats. Although the therapeutic efficacy of ginseng polysaccharide in treating type 2 diabetes was confirmed by these studies, research about the glucose lowering mechanism of ginseng polysaccharide has not been established. As type 2 diabetes is a metabolic disorder with chronic perturbation of the metabolic regulatory system, more attention should be paid to the impact of ginseng polysaccharide on global metabolism.

Metabolomics is defined as 'a quantitative measurement of multi-parametric metabolic responses of multi-cellular systems to pathophysiological stimuli or genetic signaling' [9]. The

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applications of metabolomics in studying effects and mechanisms of drugs on diseases have been adopted [10–12]. These studies showed the drug effects on global metabolites and obtained significant results.

In this work, we employed a metabolomics strategy based on RRLC/Q-TOF/MS to assess the metabolic response of water-soluble ginseng polysaccharide in rats with type 2 diabetes.

2. Experimental

2.1. Chemicals

The roots of Panax ginseng were cultivated and collected from Changbai Mountain, Jilin, China. Streptozotocin (STZ) and all the reference standards were purchased from Sigma Corporation (St. Louis, MO, USA). HPLC grade acetonitrile and formic acid were purchased from TEDIA (USA) and Sigma (USA) respectively. Distilled water was filtered through a Milli-Q system (Millipore, MA, USA).

2.2. Extraction of water-soluble ginseng polysaccharide

The roots of Panax ginseng (100 g) were extracted with 1.5 L distilled water at 100 °C for 4 h and filtered through four sheets of gauze. The solid material was extracted twice again under the same conditions. The filtrates were combined, centrifuged to remove water-insoluble materials, concentrated to 100 mL with rotary evaporation and precipitated by the addition of 95% ethanol (4 volumes). After centrifugation, the precipitate was dried by solvent exchange, first using 95% ethanol, then absolute ethanol and finally ether. The crude polysaccharide was obtained (32.5 g) and a portion (30 g) was re-dissolved in distilled water (300 mL) and treated with Sevag reagent (1:4 n-butanol:chloroform, v/v, 375 mL) to remove proteins. After precipitation by ethanol and drying by solvent exchange, the deproteinated polysaccharide fraction WGP (26 g) was obtained.

2.3. Animal studies

20 four-week-old male Wistar rats (180 ± 15 g) were purchased from Experimental Animal Center of Jilin University (China). All animals were kept in a barrier system with regulated temperature (15–25 °C) and humidity (40–80%) and on a 12/12-h light–dark cycle. The rats were fed with high-sucrose and high-fat chow (18% lard, 3% cholesterol, 20% sucrose and 59% standard rat chow). After eight-week feeding, twelve rats were injected intraperitoneal with STZ freshly prepared in citrate buffer (0.1 mol/L, pH 4.5) at a single dosage of 35 mg/kg body weight. Eight rats (healthy control group) were injected with citrate buffer in parallel. One week later, tail-blood glucose value was determined with OneTouch Ultra Meter (Lifescan Inc., CA, USA). Rats presenting blood glucose levels higher than 16.7 mmol/L were defined as diabetic [13].

2.4. Sample preparation

Twelve diabetic rats were randomly divided into two groups. One was administered with WGP 1000 mg/kg body weight once a day by gastric irrigation, the other was administered with water by gastric irrigation in parallel. The healthy control group was also administered with water by gastric irrigation in parallel. After two-week continuous irrigation, 24 h urine samples from the healthy control group, WGP treated group and untreated diabetic group were collected into tubes over wet ice and stored frozen at −80 °C until analysis.

Prior to analysis, urine samples were thawed at room temperature and centrifuged at 5000 × *g* for 10 min. The supernatant liquid was filtered through a 0.22 μm membrane filter.

2.5. RRLC/MS analysis of urine samples

A 10 μL aliquot of a urine sample was injected into an Agilent 1200 series RRLC (Agilent Technologies, Santa Clara California, USA) equipped with a reversed-phase column (Eclipse Plus C18, 3.5 μm, 2.1 mm i.d. × 150 mm length; Agilent Technologies, Santa Clara California, USA). The flow rate was set at 0.3 mL/min and the column temperature was 30 °C. Mobile phase A was 0.1% formic acid in water. Mobile phase B was 100% acetonitrile. For the analysis of samples, the solvent gradient was used as follows: 0–5 min 5–20% B, 5–10 min 20–35% B, 10–15 min 35–98% B, 15–16 min 98–100% B and 16–17 min 100% B.

Mass spectrometry was performed on an Agilent 6520 Q-TOF (Agilent Technologies, Santa Clara California, USA) with an electrospray ionization source (ESI) in positive ion mode. Key operating parameters were: gas temperature 350 °C, drying gas 9 L/min, nebulizer 40 psig, fragmentor 150 V, skimmer 65 V, and capillary +3500 V. Negative mode: gas temperature 350 °C, drying gas 9 L/min, nebulizer 40 psig, fragmentor 150 V, skimmer 65 V, and capillary −3500 V. MS and MS/MS acquisition rate was 2 spectra/s and 4 spectra/s, respectively. Data were collected in centroid mode from *m*/*z* 50 to *m*/*z* 1000.

2.6. Data analysis

The RRLC/MS data were processed using the statistic software Mass Profiler Professional (Agilent Technologies, Santa Clara California, USA). The parameters were set as follows: Experiment type: Unidentified; Organism: Rattus norregicus; Minimum absolute abundance: 2000 counts; Compound alignment: RT window = 0.1% + 0.15 min, Mass window = 5.00 ppm + 2.0 mDa; Baseline Option: Z-Transform. To compare the metabolite profiles of the ginseng polysaccharide treated and untreated diabetic groups, statistical methods including *t*-test and principal component analysis (PCA) were conducted. For identification of potential markers, the following databases were used: HMDB (http://www.hmdb.ca/), METLIN (http://metlin.scripps.edu/), Massbank (http://www.massbank.jp), and KEGG (http://www.kegg.com/).

3. Results

3.1. Streptozotocin-induced diabetic rat model and the treatment with water-soluble ginseng polysaccharide

The STZ-induced rats had blood glucose levels higher than 16.7 mmol/L. After two-week administration of WGP, the blood glucose values of STZ-induced diabetic rats were decreased by 47%. The fasting blood glucose of healthy control group, T2DM group and WGP treated group was 3.6 ± 0.8, 20.2 ± 3.2 and 10.7 ± 2.3 mmol/L, respectively.

3.2. Analysis of metabolite profiles

In this study the metabolomics analysis of urine samples used RRLC/Q-TOF/MS in the positive and negative ion mode. Base peak intensity (BPI) chromatograms of urine samples for both ionization modes obtained from the same rat are shown in Fig. 1.

For method validation, a QC sample was prepared by mixing equal volumes (50 μL) of urine from all the samples studied. The system stability was evaluated by analyzing the QC sample after each of three urine samples was run. Five ions (*m*/*z*

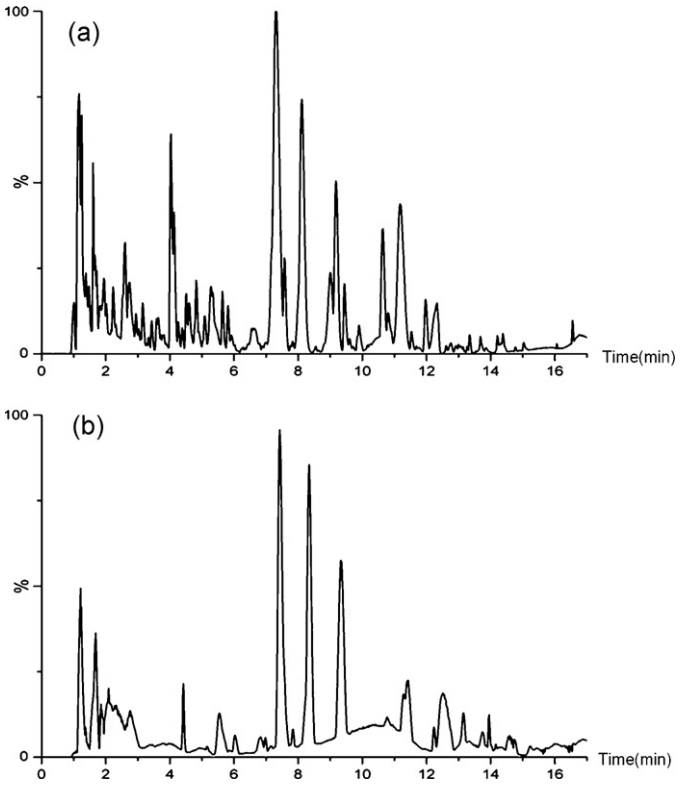

Fig. 1. (a) The positive and (b) negative ion base peak intensity (BPI) chromatograms of the urine sample from a diabetic rat.

356.2454, 238.1078, 311.1618, 180.0467 and 436.1628) in positive ion mode and five ions (*m*/*z* 175.0279, 218.1066, 187.1006, 283.0870 and 473.1527) in negative ion mode were selected. The system stability (RSDs%) of retention times, *m*/*z* and peak areas of the ten selected ions were 0.04–0.28%, 0.0002–0.0008% and 6.9–9.4%, respectively. These results demonstrated the excellent stability and reproducibility of chromatographic separation and mass measurement during the whole sequence.

The processed RRLC/MS data were investigated further using principal components analysis (PCA). A clear separation was achieved between the two groups in both positive and negative ion modes, which indicate that the urinary metabolic pattern significantly changed after the treatment with water-soluble ginseng polysaccharide. The 3D-PCA score plots classifying the control, T2DM and water-soluble ginseng polysaccharide treated groups in positive and negative ion modes are shown in Fig. 2. Separation is seen between the control and T2DM groups.

Fig. 3 shows the loading plots from the PCA in positive and negative ion modes. The further the metabolites fall from the origin, the greater their potential as biomarkers, which are responsible for the separation of T2DM group before and after treatment with water-soluble ginseng polysaccharide. Twenty-one compounds show significant differences between the two groups according to the *p* values of independent *t*-test. On this basis, the twenty-one compounds were selected as potential biomarkers for the effect of WGP on T2DM. Potential biomarkers were identified based on their accurate molecular ion masses and MS/MS product ion analysis and comparison with authentic standards or database resources. The ion of *m*/*z* 150.0793 in positive ion mode is used as an example to illustrate the biomarker identification process. The extracted ion chromatogram of *m*/*z* 150.0793 and the mass spectrum at retention time 1.563 min in positive ion mode are shown in Fig. 4(a) and (b). Because the variations of measured *m*/*z* values were below 10 ppm, the possible elemental compositions of compound were calculated, and accurate mass cutoffs were set as 10 ppm. After that, the elemental composition was compared with compounds registered in the databases used. The metabolite was tentatively identified as 1-methyladenine and mass fragmentation experiments were conducted to confirm the identification. Fig. 4(c) shows the MS/MS spectrum which shows a high degree of concordance with spectra of the standard compound. Based on all the information from the above process, the biomarker was identified as 1-methyladenine. Other compounds were also identified by this method, but several metabolites could not be identified despite searching available databases for accurate molecular weights and elemental compositions. Table 1 lists eight of the twenty-one compounds that we have successfully identified as biomarkers. ANOVA analysis was performed to reveal the statistical differences for the variables among

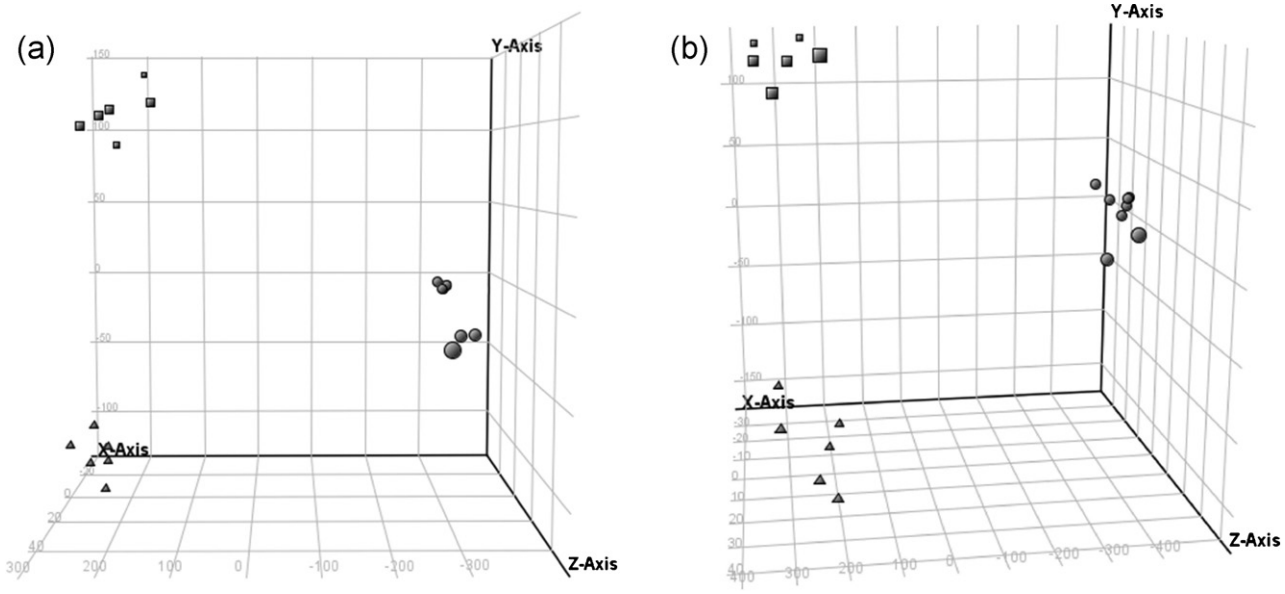

Fig. 2. Score plots (a) in positive ion mode and (b) negative ion mode from 3D-PCA model classifying healthy controls, type 2 diabetic rats and water-soluble ginseng polysaccharide treated ones (●; healthy controls, ▲; type 2 diabetic rats, ■; water-soluble ginseng polysaccharide treated type 2 diabetic rats).

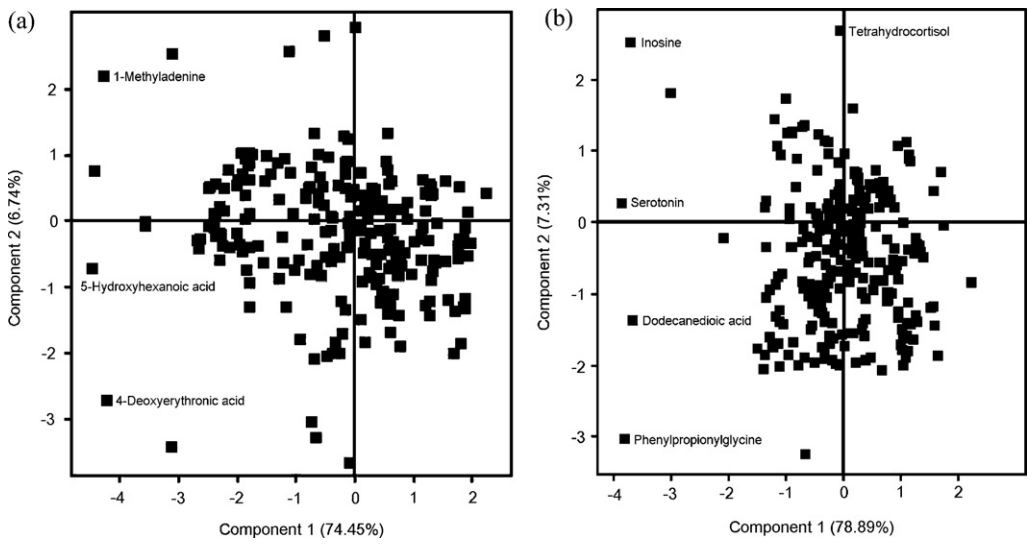

Fig. 3. Loading plots (a) in positive ion mode and (b) negative ion mode from PCA model classifying type 2 diabetic rats and water-soluble ginseng polysaccharide treated ones.

Table 1
Identification results of potential biomarkers.

Retention time (min)	Mass (*m*/*z*)	Metabolites	Deviation (ppm)	Elemental composition
Positive ion mode				
1.563	150.0793	1-Methyladenine	9.4	$C_6H_8N_5$
2.236	121.0512	4-Deoxyerythronic acid	9.2	$C_4H_9O_4$
11.507	133.086	5-Hydroxyhexanoic acid	3	$C_6H_{13}O_3$
Negative ion mode				
2.991	267.072	Inosine	3.6	$C_{10}H_{11}N_4O_5$
4.935	175.0885	Serotonin	7.9	$C_{10}H_{11}N_2O$
9.281	206.0815	Phenylpropionylglycine	1	$C_{11}H_{12}NO_3$
11.596	229.1437	Dodecanedioic acid	1.3	$C_{12}H_{21}O_4$
13.464	365.2324	Tetrahydrocortisol	1.2	$C_{21}H_{33}O_5$

healthy control group, T2DM group and WGP treated group. Table 2 shows the change in the trends of the biomarkers.

3.3. Effect of water-soluble ginseng polysaccharide on type 2 diabetic rats

In this study, we have identified eight urinary biomarkers to explain the effect of water-soluble ginseng polysaccharide on T2DM. The compounds are described in Table 2 along with the statistical significance in the change between treated and untreated rats (shown as the *p* value) and an indication of whether they were increased or decreased in concentration by the treatment.

The first, 1-methyladenine, is a product of alkylation damage to DNA [14]. Alkylating agents are abundant in the environment and are also generated inside cells. Such agents introduce a number of premutagenic and cytotoxic lesions in DNA and are often highly carcinogenic in mammals [15]. After treatment with WGP, the level of 1-methyladenine was decreased and the alkylation damage may have been limited or even repaired.

4-Deoxyerythronic acid is a normal organic acid derived presumably from L-threonine [16]. Significantly elevated level of 4-deoxythreonic acids has been detected in patients with type 1 diabetes mellitus [16] and it has also been associated with uremia [17]. In our study, a decrease in 4-deoxythreonic acid in the WGP treated group indicates a potential effect of WGP on metabolism of L-threonine.

5-Hydroxyhexanoic acid is a normal monohydroxy carboxylic acid degradation product of fatty acids. 5-Hydroxyhexanoic acid has been detected in the urine of diabetic patients with ketoacidosis [18]. In this study, the concentration of 5-hydroxyhexanoic acid was reduced in the WPG treated diabetic rats relative to the untreated animals and suggests an important role for WGP in reducing the effect of ketoacidosis in diabetes.

Dodecanedioic acid is a dicarboxylic acid which is water soluble and is involved in the metabolism of both lipids and carbohydrates [19]. Others have speculated that this compound may be a fuel substrate immediately available for tissue energy requirements, especially in conditions such as diabetes mellitus in which glucose metabolism is impaired [19]. Our use of WGP in treating diabetic rats leads to an increase in dodecanedioic acid and we speculate that this effect might be of benefit in management of type 2 diabetes mellitus.

Table 2
The change in the trends of the biomarkers.

Metabolites	*P*-value of T2DM group vs control group	*P*-value of treated group vs T2DM group	Change trend of T2DM group vs control group	Change trend of treated group vs T2DM group
Positive ion mode				
1-Methyladenine	4.31E−04	7.97E−04	Up	Down
4-Deoxyerythronic acid	3.28E−03	5.38E−04	Up	Down
5-Hydroxyhexanoic acid	1.87E−04	5.69E−04	Up	Down
Negative ion mode				
Inosine	4.23E−04	4.38E−04	Down	Up
Serotonin	3.14E−03	6.52E−04	Down	Up
Phenylpropionylglycine	3.25E−03	7.13E−04	Down	Up
Dodecanedioic acid	1.99E−04	4.35E−04	Down	Up
Tetrahydrocortisol	1.48E−04	2.87E−04	Up	Down

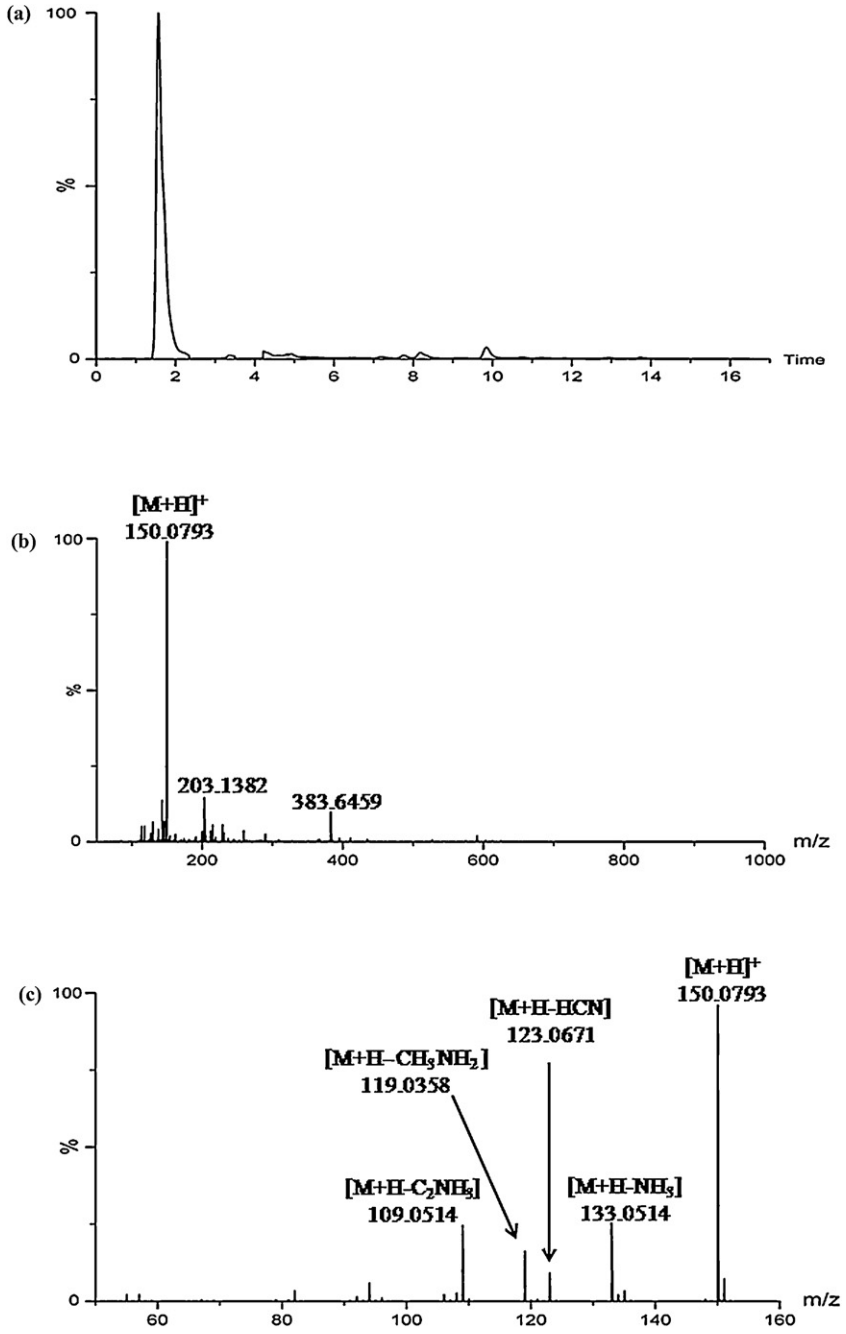

Fig. 4. Identification of a selected biomarker 1-methyladenine. (a) Peak of 1-methyladenine in extracted ion chromatogram with positive ion mode; (b) corresponding mass spectrum; (c) MS/MS spectrum, the collision energy was 20 eV, the parent isolation width is in narrow mode (~1.3 *m*/*z*).

Inosine is an intermediate in the degradation of purines and purine nucleosides to uric acid and in pathways of purine salvage. Inosine has also been shown to be an immunomodulator and anti-inflammatory agent and it protects against the development of diabetes in multiple-low-dose streptozotocin and non-obese diabetic mouse models of type 1 diabetes [20]. In our study of diabetic rats, inosine was up-regulated by WGP treatment and we suggest that this effect is likely to be positive in modulating DM.

After WGP treatment, urinary serotonin excretion was also increased. Serotonin is a biochemical messenger and regulator, synthesized from the essential amino acid L-Tryptophan. The function of serotonin includes the regulation of mood, appetite and sleep. It is reported tryptophan and serotonin concentrations are lowered in diabetic rats and increased following insulin injections [21]. The result shows that WGP may stimulate insulin excreting in diabetic rats.

Phenylpropionylglycine is an acyl glycine, one of a group of compounds known as minor metabolites of fatty acid metabolism. Phenylpropionylglycine was identified as a biomarker for proliferator-activated receptor α (PPARα) expression and activation in mice [22]. After binding to its ligands, PPARα can regulate the target genes involved in lipid, glucose and amino acid homeostasis [23,24]. Increased phenylpropionylglycine in WGP treated rats indicates that the WGP may affect lipid and glucose metabolism by regulating PPARα.

Tetrahydrocortisol is the urinary metabolite of cortisol and a decrease in the level of tetrahydrocortisol in WGP treated rats,

relative to the untreated controls, demonstrates that the level of cortisol was downregulated. Cortisol is a steroid hormone, and its primary functions are to increase blood sugar through gluconeogenesis. In type 2 diabetic subjects, cortisol secretion was found to be associated with the complications and metabolic control of diabetes [25]. WGP may affect glucose metabolism through regulating the secretion of cortisol.

4. Conclusions

A metabolomics method based on RRLC/MS has been developed to study the effect of water-soluble ginseng polysaccharide on STZ-induced type 2 diabetic rats. With multivariate statistical analysis, a clear separation between T2DM group and water-soluble ginseng polysaccharide treated group was achieved. Eight potential biomarkers have been found and identified and significant changes in their concentration in urine are indicative of the pharmacological effect of WGP on nucleic acid metabolism, lipid metabolism and glucose metabolism.

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