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Effect of mitiglinide on Streptozotocin-induced experimental type 2 diabetic rats: A urinary metabonomics study based on ultra-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Mitiglinide is a new insulinotropic agent of the glinide class and its precise mechanism is not very clear yet. In this study, a urinary metabonomics method based on ultra-performance liquid chromatography–tandem mass spectrometry was developed to study the effect mechanism of mitiglinide on type 2 diabetes mellitus. With pattern recognition analysis of urinary metabolite profiles, a clear separation between Streptozotocin-induced type 2 diabetic rats and those treated with mitiglinide was achieved. Some significantly changed metabolites like citrate, creatinine, phenylalanine and bile acids (cholic acid, chenodeoxycholic acid and deoxycholic acid) have been identified and used to explain the mechanism. This work shows that metabonomics method is a valuable tool in drug mechanism study.

1. Introduction

The bottleneck in delivering new or improved pharmacological treatments to the patient appears to have shifted from finding new targets and producing candidate compounds (i.e."drug discovery") to choosing which candidates warrant follow-up and vital resource allocation by the pharmaceutical industry (i.e. "drug evaluation") [1]. A pivotal element which may expedite the drug evaluation process is the identification of sensitive, specific and early responding biomarkers that are closely related to the mechanism of drug action. Classical methods focused on studying drug-induced effects are essentially based on checking variations in the concentrations of specific biomarkers. An alternative method, which is recently gaining ground, considers possible effects on the metabolome as a whole.

Metabonomics, based on the analysis of entire pattern of low molecular weight compounds rather than focusing on individual metabolites, indicates a general procedure that gives information on whole organism functional integrity over time following exposition of a perturbation. It has been applied to the study of a variety of diseases [2–5] and the effects of diet [6], drugs [7], toxins [8], and stress [9]. Much of the original work of metabonomics was performed by NMR [10–13]. More recently, HPLC/MS approaches have begun to be used for metabonomic analysis, either alone or in combination with NMR analysis [14–22]. UPLC featured by small particles, has enabled better chromatographic peak resolution and increased speed and sensitivity to be obtained for complex mixture separation compared to HPLC. There have been an increasing number of applications of UPLC/MS to the analysis of biological fluids in the field of metabonomics [23–29].

Type 2 diabetes mellitus (T2DM) is a growing public health problem globally. Mitiglinide is a new insulinotropic agent of the glinide class with rapid onset [30]. It is thought to stimulate insulin secretion by closing the ATP-sensitive K^+ (K_{ATP}) channels in pancreatic beta-cells. Its early insulin release and short duration of action could be effective in improving postprandial hyperglycemia [31]. However, as a newly developed drug, the exact mechanism of mitiglinide is not clear yet. Recently, pharmacological effects of rosiglitazone and metformin on type 2 diabetes mellitus patients have been studied by metabonomics [7,32,33]. While the metabolite profiling study of mitiglinide treated type 2 diabetics has not been reported. In this study, an UPLC/MS-based metabonomic approach was utilized to study the effect mechanism of mitiglinide on STZ-induced type 2 diabetic rats.

T2DM is associated with dysfunction of many metabolic pathways, such as nutrition metabolism, tricarboxylic acid cycle and bile acids metabolism which play important physiological roles in the maintenance of glucose homeostasis and in the elimination of cholesterol, the intestinal solubilization and absorption of lipids

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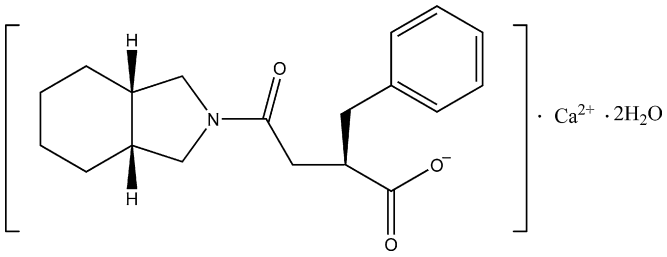

Fig. 1. Structure of mitiglinide.

[34]. The biomarkers analyzed by metabonomics are important in finding the metabolic pathways associated with mitiliglinde effect on T2DM and the action mechanism.

2. Experimental

2.1. Chemicals

Mitiglinide calcium hydrate (99.3% of purity, Fig. 1) was kindly provided by Dandong Tongyuan Medicine Co. Ltd. (Dandong, China). Streptozotocin (Fig. 2) and the reference standards of L-phenylalanine, creatinine, citrate, cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) were supplied by Sigma Corporation (St. Louis, MO, USA). Acetonitrile and formic acid (HPLC grade) were purchased from Dikma Corporation (Richmond Hill, NY, USA). Water was purified by redistillation and filtered through 0.22 μm membrane filter before use.

2.2. Animal studies

A total of 22 four-week-old male Wistar rats (165 ± 15 g) were obtained from Experimental Animal Center of Shenyang Pharmaceutical University (China). All animals were kept in a barrier system with regulated temperature (17–25 °C) and humidity (45–80%) and on a 12/12-h light–dark cycle. The rats were fed with high-sucrose and high-fat chow (10% lard, 2.5% cholesterol, 1% hydrocholate, 20% sucrose and 65.5% standard rat chow). After six-week feeding, sixteen rats were injected ip with STZ freshly prepared in citrate buffer (0.1 mol/L, pH 4.5) at a single dosage of 30 mg/kg body weight [35]. The other group (control group) was injected with citrate buffer in parallel. Two weeks 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 are defined as diabetic rats (responders) [36].

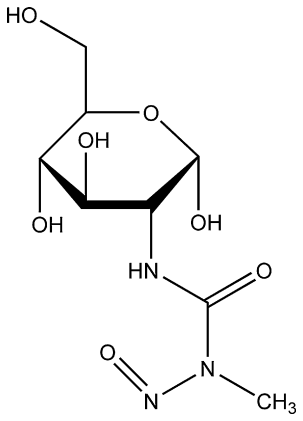

Fig. 2. Structure of Streptozotocin.

Table 1
Gradient elution program of UPLC/MS.

Time (min)	Flow rate (ml/min)	%A	%B	Curve
Initial	0.25	100	0	Initial
0.5	0.25	100	0	6
10	0.25	60	40	6
15	0.25	10	90	6
16	0.25	10	90	1

A: water (0.1% formic acid); B: acetonitrile (0.1% formic acid).

2.3. Sample collection and preparation

Twelve diabetic rats were randomly divided into two groups. One was administrated with mitiglinide 20 mg/kg body weight twice a day by gastric irrigation, the other was ig with water in parallel. After two-week continuous irrigation, 24 h urine samples from control group, mitiglinide 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 11,200 × *g* for 10 min. The supernatant was diluted at a ratio of 1:1 with water, vortex mixed and filtered through 0.22 μm membrane filter. An aliquot of 5 μL was injected for UPLC/MS analysis.

2.4. UPLC/MS analysis of urine samples

Rat urinary metabolite profiling was performed on ACQUITY UPLC™ system (Waters Corporation, Milford, MA, USA) equipped with cooling autosampler and column oven. The separation was achieved on an ACQUITY UPLC™ BEH C_{18} column (50 mm × 2.1 mm, i.d., 1.7 μm) with temperature maintained at 40 °C. Gradient elution was employed with the mobile phase composed of water and acetonitrile each containing 0.1% formic acid. The gradient elution program is shown in Table 1. After each injection, a strong/weak wash cycle was employed on the autosampler to eliminate the carryover between analyses. The weak needle wash solvent was water–acetonitrile (90:10, v/v) and the strong needle wash solvent was water–acetonitrile (20:80, v/v).

Mass spectrometric detection was carried out on a Micromass Quattro micro™ API mass spectrometer (Waters Corporation, Milford, MA, USA) with an electrospray ionization (ESI) interface. The ESI source was set in both positive and negative ion modes. The following parameters were employed: capillary voltages of 3.0 and 2.8 kV for positive and negative ion mode, respectively, cone voltage of 30 V, source temperature of 120 °C and desolvation temperature of 300 °C. Nitrogen was used as the desolvation and cone gas with the flow rate of 400 and 30 L/h, respectively. Full scan mode was employed in the mass range of 100–1000 amu. In the MS/MS experiments, argon was employed as the collision gas and the collision energy was altered between 5 and 25 eV. NaCsI was used for mass correction before the study. The data were collected in centroid mode.

For method validation study, a quality control (QC) sample was prepared by mixing equal volumes (100 μL) of urine from all the samples studied, which was used to provide a representative "mean" sample containing all the analytes that would be encountered during the analysis. The method repeatability was evaluated by five replicates analysis of QC samples on one day and the post-preparative stability of sample was tested by analyzing a prepared QC sample left at autosampler (maintained at 4 °C) for 4, 8, 12 and 24 h. The system stability was evaluated by analyzing a QC sample after each three urine samples were run.

2.5. Data analysis

The UPLC/MS data were processed using the Markerlynx applications manager within Masslynx software (version 4.0). This application manager allowed deconvolution, alignment and data reduction to give a table of mass and retention time pairs (t_R_m/z) with associated intensities for all the detected peaks. The main parameters were set as follows: retention time range 0–16 min, mass range 100–1000 amu, mass tolerance 0.01, mass per retention time 10, minimum intensity 1%, mass window 0.05, retention time window 0.20, noise elimination level 6. The resulting three-dimensional data, peak number (t_R_m/z pair), sample name and normalized ion intensity were introduced to SIMCA-P 11.5 software package (Umetrics, Umea, Sweden) for principal component analysis (PCA). Mean-centered was used for data scaling and centering. ANOVA was performed in succession to reveal the statistical differences for the variables between T2DM group and mitiglinide treated group using Excel 2003 (Microsoft, USA). For identification of potential markers, the following databases have been used: HMDB (http://www.hmdb.ca/), METLIN (http://metlin.scripps.edu/), Massbank (http://www.massbank.jp), PubChem (http://ncbi.nim.nih.gov/) and KEGG (http://www.kegg.com/).

3. Results and discussion

3.1. Streptozotocin-induced diabetic rat model and the treatment with mitiglinide

The STZ-treated animals (responders) had blood glucose levels higher than 16.7 mmol/L [36]. As expected, a small number of rats (n = 4) treated with STZ did not develop T2DM (non-responders) as reflected by their blood glucose values which did not differ from those of control group. After two weeks administration of mitiglinide, the blood glucose values of STZ-induced diabetic rats were decreased by 50%. The fasting blood sugar (FBS) of control, T2DM and mitiglinide treated group were 3.44 ± 0.62, 21.46 ± 3.82 and 10.46 ± 3.17 mmol/L, respectively.

3.2. Analysis of metabolite profiles and identification of potential biomarkers

Urine is of interest in metabonomics study, because its collection is non-invasive and it amplifies the circulating levels of metabolites by renal concentration, which consequently ensures urine a distinct representation of metabolic response. Fig. 3 shows the positive and negative ion base peak intensity (BPI) chromatograms of the urine sample from a mitiglinide treated rat.

As for method validation study, extracted ion chromatographic peaks of five ions (m/z 113.7, 165.9, 193.9, 267.0 and 409.5) in positive ion mode and five ions (m/z 190.8, 172.8, 460.9, 186.9 and 453.5) in negative ion mode were selected. The method repeatability (RSDs%) of retention times, m/z and peak areas of ten selected ions in QC samples were estimated to be 0–2.4%, 0–0.08% and 6.3–9.9%; the post-preparation stability was from −4.7% to 7.8%. The system stability (RSDs%) of retention times, m/z and peak areas of ten selected ions were 0.18–4.5%, 0.01–0.1% and 7.3–10.3%, respectively. The developed method had a good repeatability and stability.

To determine whether mitiglinide influenced the metabolic pattern of type 2 diabetic rats, two PCA models were constructed. Figs. 4 and 5 provide the PCA score and loading plots classifying T2DM group and mitiglinide treated group. Fig. 6 illustrates the 3D-PCA score plot classifying control group, T2DM group and mitiglinide treated group in positive ion mode. It can be seen from

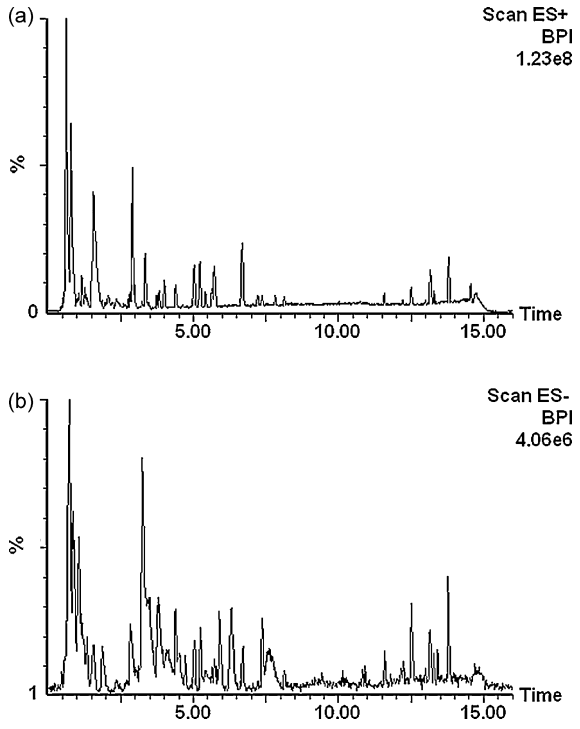

Fig. 3. (a) Positive and (b) negative ion base peak intensity (BPI) chromatograms of representative urine sample from a mitiglinide treated diabetic rat.

Fig. 4 that distinct clustering between mitiglinide treated group and untreated T2DM group was achieved in both positive and negative ion modes, which indicate urinary metabolic pattern significantly changed after the treatment of mitiglinide. The mitiglinide treated group appears to form a sparser cluster than that observed for T2DM group, due to different therapeutic effects induced by mit-

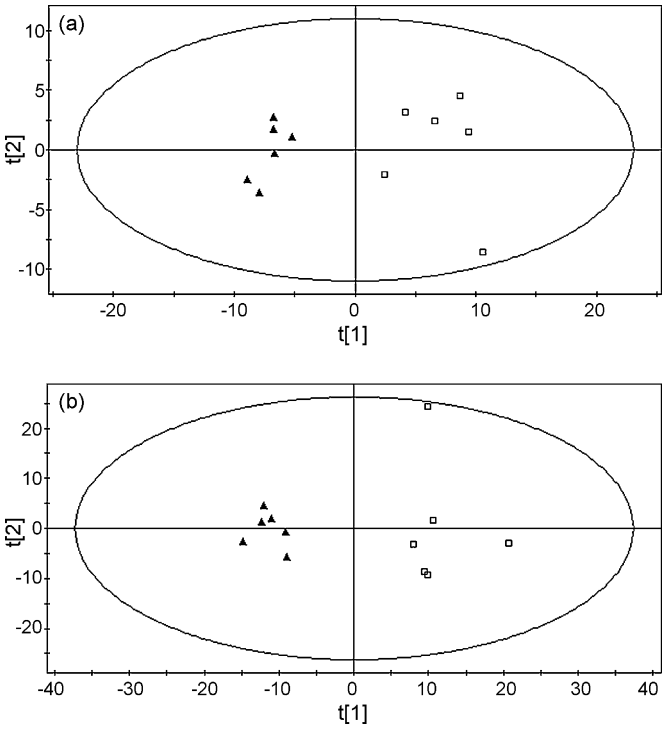

Fig. 4. Score (▲; type 2 diabetic rats, □; mitiglinide treated type 2 diabetic rats) plots (a) in positive ion mode (R2X = 0.597) and (b) negative ion mode (R2X = 0.476) from PCA model classifying type 2 diabetic rats and mitiglinide treated ones.

Table 2
Identification results of potential biomarkers detected by UPLC/MS/MS for classifying type 2 diabetic rats and mitiglinide treated group.

Positive ion mode		Negative ion mode		Identification
m/z	Quasi-molecular ion	*m/z*	Quasi-molecular ion	
113.7	$[M+H]^+$	111.7	$[M-H]^-$	Creatinine
165.9	$[M+H]^+$	163.9	$[M-H]^-$	Phenylalanine
–	–	190.8	$[M-H]^-$	Citrate
357.5	Characteristic fragment	437.5	$[M+HCOO]^-$	CDCA
373.5	Characteristic fragment	453.5	$[M+HCOO]^-$	CA
357.5	Characteristic fragment	437.5	$[M+HCOO]^-$	DCA
		783.9	$[2M-H]^-$	

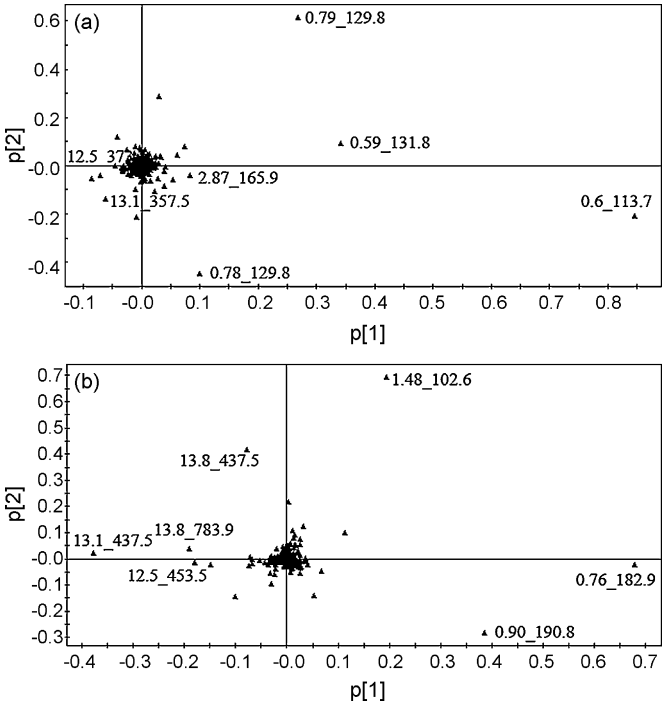

Fig. 5. Loading plots (a) in positive ion mode and (b) negative ion mode from PCA model classifying type 2 diabetic rats and mitiglinide treated ones.

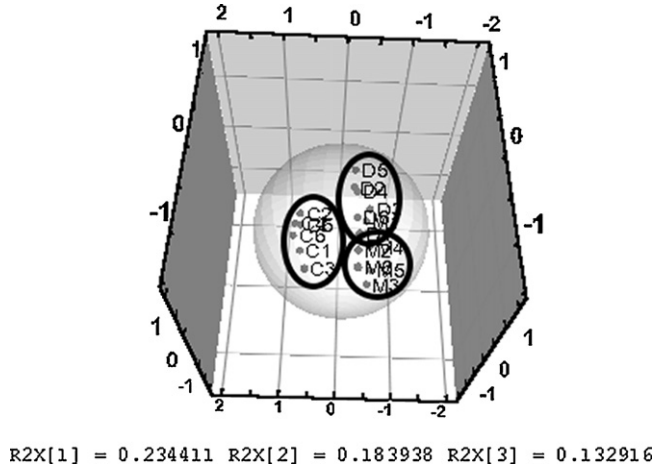

Fig. 6. Score (C; healthy controls, D; type 2 diabetic rats, M; mitiglinide treated type 2 diabetic rats) plot in positive ion mode (R2X = 0.551) from 3D-PCA model classifying healthy controls, type 2 diabetic rats and mitiglinide treated ones.

iglinide on different individuals. From Fig. 6, clear separation was also seen between control group and T2DM group.

Corresponding loading plots (Fig. 5) indicate the possible biomarkers with retention time and *m/z* pairs of 0.6_113.7, 0.59_131.8, 0.78_129.8, 0.79_129.8, 2.87_165.9, 12.5_373.5, 13.1_357.5 in positive ion mode and 0.76_182.9, 0.9_190.8, 1.48_102.6, 13.1_437.5, 12.5_453.5, 13.8_437.5, 13.8_783.9 in negative ion mode. Structure identification was performed according to their molecular ion masses and MS/MS product ion analysis comparing with authentic standards or literatures [37] and database resources. Take the variable with retention time and *m/z* pair of 13.1_357.5 in positive ion mode as an example to illustrate the identification process. The mass spectrum at retention time (t_R) 13.1 min in positive ion mode is shown in Fig. 7a. Besides the base peak ion at *m/z* 357.5, the ions at *m/z* 375.5 and 393.5 were also found. Each of them has the *m/z* differences of H_2O. Thus, we infer that there may be two hydroxyl groups in the structure. In negative ion mass spectrum (Fig. 7b), the base peak ion at *m/z* 437.5 is the adduct ion of formic acid with the metabolites at *m/z* 391.5 ($[M-H]^-$). Therefore, the quasi-molecular ions were found to be 393.5 ($[M+H]^+$) in ESI^+ and 391.5 ($[M-H]^-$) in ESI^-. The ions at *m/z* 785.9 ($[2M+H]^+$) in positive ion mode and *m/z* 783.9 ($[2M-H]^-$) in negative ion mode further validated the metabolite has a molecular mass of 392.5 Da. To define its structure, some databases like KEGG (http://www.kegg.com) and HMDB (http://www.hmdb.ca/) were searched with the molecular mass 392.5 Da, then some compounds without two hydroxyl groups were removed from the candidate list. Furthermore, its fragmentation (Fig. 7c) from tandem MS in positive ion mode was investigated. Finally, it was identified as chenodeoxycholic acid, according to the retention time and fragmentation pattern of standard reference. The possible fragment mechanism was deduced (Fig. 7d). The identification results of other potential biomarkers are list in Table 2. The identification of the variables with retention time and *m/z* pairs of 0.59_131.8, 0.78_129.8, 0.79_129.8, 0.76_182.9 and 1.48_102.6 are in progress.

3.3. *Effect of mitiglinide on the urinary metabolic pattern of STZ-induced type 2 diabetic model*

Table 3 gives the change trends of the biomarkers identified. The results effectively indicated that these metabolites may be the biomarkers of mitiglinide effects, which were related to the action mechanism of mitiglinide.

In our study, decreased phenylalanine level was observed in urinary metabolite profiles of T2DM group compared with control group and increase of phenylalanine was seen in urinary metabolite profiles of mitiglinide treated ones. It is supposed that phenylalanine can suppress appetite via stimulating the production of cholecystokinin (a peptide hormone of the gastrointestinal system responsible for stimulating the digestion of fat and protein and also acts as huger suppressant). Increase of phenylalanine in mitiglinide treated diabetic rat results in a reduction of dietary consumption,

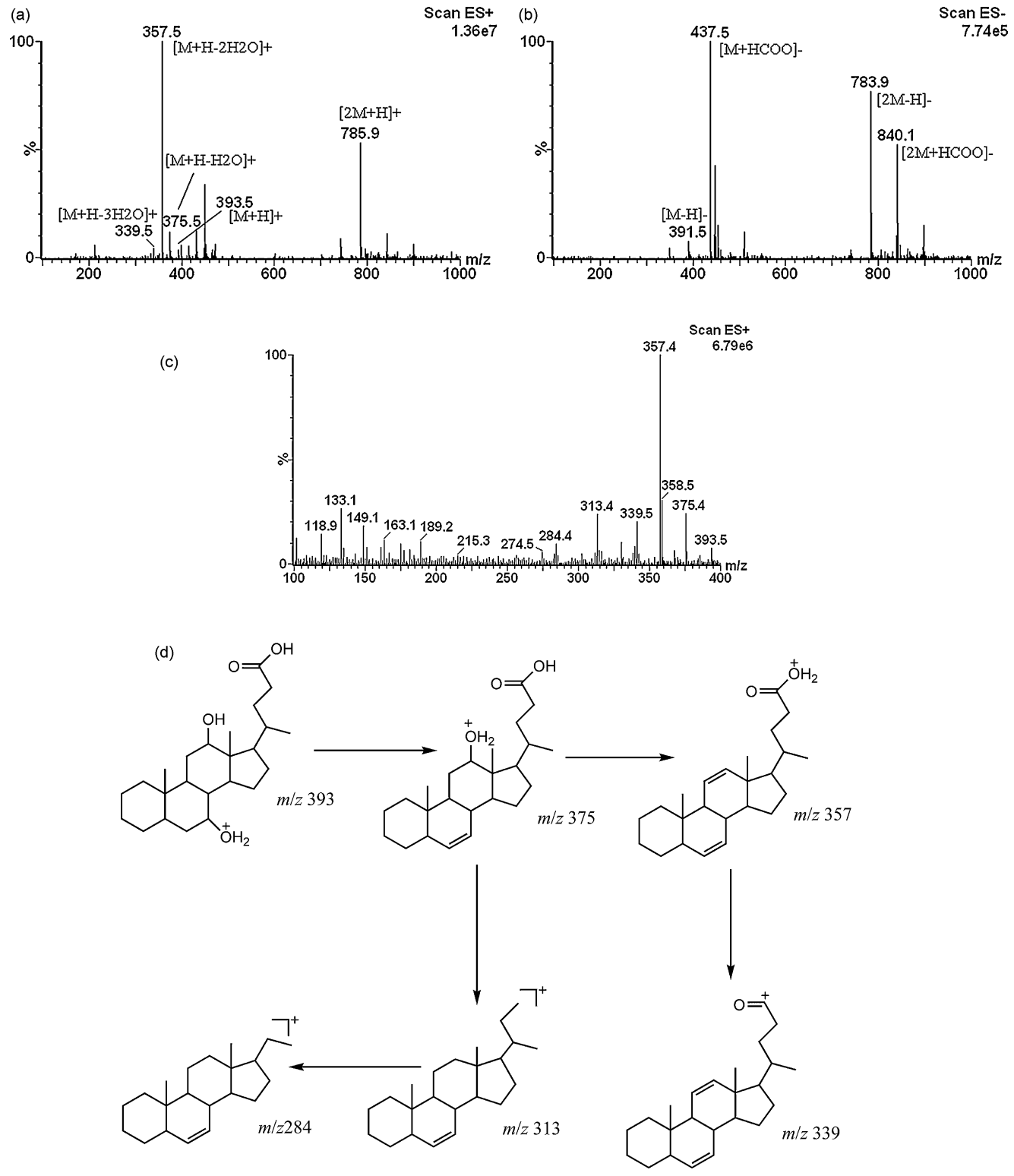

Fig. 7. Mass spectra of biomarker at (a) *m/z* 357.5 in positive ion mode, (b) *m/z* 437.5 in negative ionization mode, (c) product ion scan spectrum of the biomarker in positive ion mode and (d) possible MS fragmentation mechanism.

which is beneficial to the therapy of type 2 diabetes mellitus. The result demonstrated in our study indicates the pharmacological effects of mitiglinide on nutrition metabolism.

TCA cycle was reported to be disturbed in STZ-induced diabetic rats [3]. Citrate is a major intermediate in TCA cycle. Decreased level of citrate was observed in T2DM group compared with control group. After the treatment of mitiglinide, increase of citrate level was seen in urinary metabolite profiles of treated group. Decrease of urine citrate level suggests lower TCA cycle due to the defect in mitochondrial dysfunction observed in T2DM. Increase of citrate found in urinary metabolite profiles of mitiglinide treated diabetic rats indicates a potential effect of mitiglinide on TCA cycle. Although intestine inflammations can also lead to changes in citrate concentration in urine of rats, however, no intestine inflammation

Table 3
Change trends of biomarkers identified in metabonomics study.

Biomarkers	ANOVA analysis (*P*-Value)				Change trend of T2DM group *vs* control group	Change trend of treated group *vs* T2DM group
	T2DM *vs* Control		Treated *vs* T2DM			
	Positive	Negative	Positive	Negative		
CDCA	0.34	3.67E−02	0.19	8.21E−03	↑	↓
CA	9.63E−03	3.16E−02	1.27E−02	0.15	↑	↓
DCA	–	9.21E−03	–	1.20E−02	↑	↓
Phenylalanine	2.37E−02	–	9.26E−03	–	↓	↑
Citrate	–	3.80E−02	–	1.62E−02	↓	↑
Creatinine	1.5 E−02	–	8.78E−06	–	↓	↑

symptoms such as diarrhea and constipation were observed in the studied animals.

Creatinine is another potential biomarker for the separation of type 2 diabetic rats and mitiglinide treated group. Decrease of creatinine was observed in urinary metabolite profiles of T2DM group compared with control group and increase of creatinine was revealed in the urinary metabolite profiles of mitiglinide treated group. Creatinine is an important biomarker to evaluate renal function. It is reported that animal models with STZ-induced diabetes mellitus is associated with progressive renal disturbances [38]. Creatinine is also crucial for cellular energy transportation by participating in creatine-phosphocreatine system. Its increase in mitiglinide treated group indicates a potential effect of mitiglinide on both the early stage of nephropathy and energy metabolism of type 2 diabetes.

Significant changes of bile acids (CA, CDCA and UDCA) in mitiglinide treated diabetic rats were observed in our study. Bile acids are the major products of cholesterol catabolism. In health, only small quantities of bile acids are found in peripheral circulation and urine. However, in hepatobiliary and intestinal disease, disturbances of synthesis, metabolism, and clearance by the liver and absorption by the intestine will affect the concentration and profile of bile acids in various pool compartments (serum, liver, gallbladder, urine and feces) [39]. In our study, increased CA, CDCA and DCA levels were observed in type 2 diabetic rats, the result was consistent with that reported previously [40], which indicates a disturbances of enterohepatic circulation. Decrease of CA, CDCA and UDCA were observed in urine metabolite profiles of mitiglinide treated group, and it indicates a potential role of mitiglinide on bile acids metabolism.

4. Conclusion

A metabonomics method based on UPLC/MS has been developed to study the effects of mitiglinide on STZ-induced type 2 diabetic rats. Multivariate statistical analysis shows a clear separation between T2DM group and mitiglinide treated group. Some potential biomarkers like phenylalanine, citrate, creatinine and bile acids have been found and identified. Their changes indicated pharmacological effects of mitiglinide on nutrition metabolism, energy metabolism and bile acids metabolism. The work shows that the metabonomics method is a valuable tool in drug mechanism research.

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