Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 976–982

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Metabonomic study of biochemical changes in the serum of type 2 diabetes mellitus patients after the treatment of metformin hydrochloride

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ARTICLE INFO

Article history:
Received 5 November 2008
Received in revised form 5 January 2009
Accepted 9 January 2009
Available online 19 January 2009

Keywords:
^{1}H NMR
UPLC/MS
Type 2 diabetes mellitus
Metabolite profiles
Metformin hydrochloride

ABSTRACT

A metabonomic study on biochemical changes in the serum of type 2 diabetes mellitus patients after the treatment of metformin hydrochloride was performed. ^{1}H NMR and UPLC/MS were used to generate metabolic fingerprints for the metabonomic analysis of serum samples obtained from 20 type 2 diabetes mellitus patients without any drugs treatment and 15 type 2 diabetes mellitus patients treated with metformin hydrochloride for 3 months. The resulting data were subjected to chemometric analysis (principal component analysis and partial least squares discriminant analysis) to investigate the effect of metformin hydrochloride on serum metabolite profiles of type 2 diabetes mellitus patients. ^{1}H NMR spectroscopic analysis revealed increased trimethylamine-*N*-oxide (TMAO), 3-hydroxybutyrate (3-HB) and decreased glucose, *N*-acetyl glycoprotein (NAC), lipoprotein, lactate, acetoacetate and unsaturated lipids in serum from metformin treated patients compared to untreated ones. UPLC/MS in positive electrospray ionization detected increased tryptophan and decreased lysophosphatidylcholines (C16:0 LPC, C18:0 LPC and C18:2 LPC) as well as phenylalanine in treated group. Both analytical techniques used in this study were able to detect biochemical changes in the serum of type 2 diabetes mellitus patients after the treatment of metformin hydrochloride, which may be helpful to the understanding of action mechanism of metformin hydrochloride.

1. Introduction

Metabonomics, a novel methodology arising from the post-genomics era, is defined as the quantitative measurement of the time-related multiparametric metabonomic response of living systems to pathophysiological stimuli or genetic modification [1]. Based on the multivariate analysis of complex biological profiles, metabonomics has recently demonstrated enormous potential in many fields, such as disease diagnosis [2,3], toxicological mechanisms [4–6] and drug effects [7,8]. In clinical metabonomics study, the discovery and identification of disease biomarkers and the exploration of drug mechanism are the most important.

Much of the original work in metabonomics was performed using NMR spectroscopy [9–12]. It has been proved to be a powerful tool for characterizing the pathological states in animals and humans and can present diagnostic information and mechanistic insight into the biochemical effects of the toxins and drugs [4,7]. More recently, HPLC/MS approaches have been used for metabonomic analysis, either alone or in combination with NMR analysis [13–21]. UPLC featured by small particles of 1.7 μm, 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 [22–28].

Diabetes mellitus (DM) is a metabolic disorder characterized by a chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both [29]. Type 2 diabetes mellitus (DM-2), which accounts for 85–90% of patients with diabetes mellitus has been described as an "epidemic" of contemporary society. Therefore, the prevention and therapy of type 2 diabetes mellitus has become a major health care focus. Drug treatment is one of the therapeutic methods for type 2 diabetes mellitus. Metformin hydrochloride is an oral biguanide antihyperglycemic drug, which has been used for over 40 years in the treatment of type 2 diabetes [30]. However, the exact mechanism of metformin is not very clear yet. Many action mechanism studies on meformin hydrochloride have been performed by pharmacological methods [31–33], which may not reflect the drug-induced effects on metabolite profiles. Recently, many metabonomic studies on type 2 diabetes mellitus were performed [34–40], while the biochemical changes in the serum of DM-2 patients after the treatment of metformin hydrochloride have not been reported in detail. Here we describe both ^{1}H NMR and

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doi:10.1016/j.jpba.2009.01.008

UPLC/MS/MS methods, combined with pattern recognition techniques, to investigate the changes in endogenous metabolites in the serum of DM-2 patients after the treatment of metformin hydrochloride.

2. Experimental

2.1. Chemicals

The reference standards of L-phenylalanine and L-tryptophan were supplied by Sigma Corporation (St. Louis, MO, USA). Acetonitrile and formic acid (HPLC grade) were purchased from Dikma Corporation (Richmond Hill, NY, USA). Deuterium oxide was bought from Merck Corporation (Whitehouse Station, NJ, USA). Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$) and disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$) (analytical grade) were obtained from Yuwang Chemical Reagent Plant (Shandong, China). Water was purified by redistillation and filtered through 0.22 μm membrane filter before use.

2.2. Sample collection

Serum samples from 20 DM-2 patients without any drugs treatment and 15 age-matched DM-2 patients treated with metformin hydrochloride for 3 months were provided by the First Affiliated Hospital of China Medical University (Shenyang, PR China). Table 1 gives the patients' information. All samples were kept at −20 °C until analysis.

2.3. Sample preparation

To a 200 μL aliquot of serum samples, 400 μL of acetonitrile was added for protein precipitation. After centrifugation at 11,200 × *g* for 10 min, the supernatant was transferred and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The dried residue was then reconstituted in 100 μL of acetonitrile–water (10:90, v/v). The content was transferred to 2 mL glass vials and an aliquot of 5 μL was injected for UPLC/MS analysis. For ^{1}H NMR analysis, 50 μL of deuterium oxide was added to a mixture containing 300 μL of serum samples and 300 μL of sodium phosphate buffer (pH 7.4; 0.2 M). Then the mixture was centrifuged at 11,200 × *g* for 10 min, and the supernatant of 600 μL was placed in a 5-mm o.d. NMR tube.

2.4. Sample analysis

2.4.1. Liquid chromatography and mass spectrometry

Liquid chromatography was performed on ACQUITY™ UPLC system (Waters Corp., Milford, MA, USA) equipped with cooling autosampler and column oven enabling temperature control of column. Separation was achieved on an ACQUITY UPLC™ BEH C_{18} column (50 mm × 2.1 mm, i.d., 1.7 μm) 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 2.

Table 1
Summary of patients' information.

	DM-2 patients (n = 20)	Metformin treated patients (n = 15)
Age (mean ± S.D.)	56.8 ± 10.53	57.2 ± 10.7
Sex (M:F)	10:10	7:8
BMI (mean ± S.D.)	32.7 ± 4.61	28.8 ± 2.39
Glucose (mmol/L)	8.8 ± 1.4*	7.4 ± 1.9*
Low-density lipoprotein cholesterol	3.75 ± 0.73*	2.90 ± 0.71*
High-density lipoprotein cholesterol	0.93 ± 0.19	1.01 ± 0.42

* P < 0.05.

Table 2
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
5	0.25	60	40	6
17	0.25	6	94	6
24	0.25	0	100	6
25	0.25	100	0	1

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

Mass spectrometric detection was carried out on a Micromass Quattro micro™ API mass spectrometer (Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) interface. The ESI source was set in positive mode. The following parameters were employed: capillary voltage of 3.0 kV, 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 collision energy was set at 20 eV. NaCsI was used for mass correction before the study. The data were collected in centroid mode.

2.4.2. ^{1}H NMR analysis

^{1}H NMR analysis was carried out using a Bruker AV 600 spectrometer operating at 298.2 K. Water signals and broad protein resonances were suppressed by a combination of presaturation and the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. ^{1}H NMR spectra were measured with 64 scans into 65536 data points over a spectral width of 12019.23 Hz. The acquisition time was 2.73 s. All acquired NMR spectra were manually phased, baseline-corrected and chemical shift referenced to the doublet signal at 1.33 ppm due to methyl group of lactate [41,42] within TOPSPIN (version 2.1, Bruker Spectrospin Ltd.).

2.5. Data analysis

UPLC/MS data were processed using the Markerlynx applications manager within Masslynx software (version 4.0). The

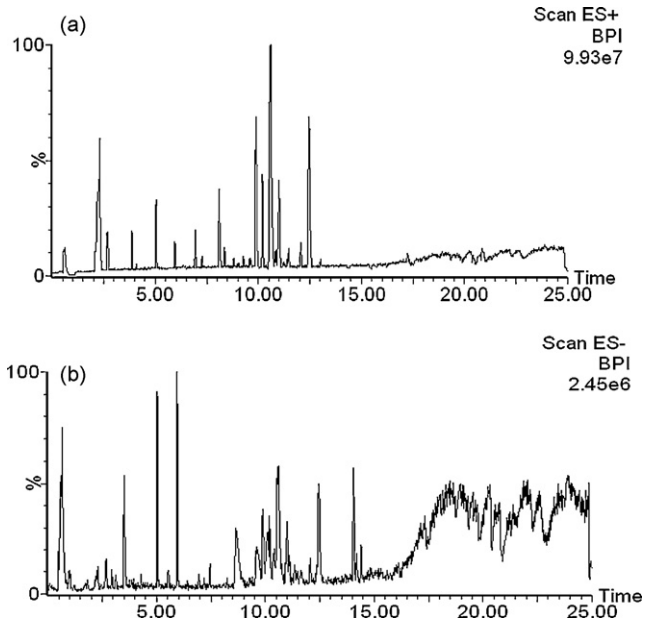

Fig. 1. (a) Positive and (b) negative ion base peak intensity (BPI) chromatograms of representative serum sample from a DM-2 patient.

retention time and *m*/*z* data pairs for each peak were determined by the software. All data were normalized to the summed total ion intensity per chromatogram. ^{1}H NMR spectra were processed using Bruker AMIX (version 3.0). Each spectrum was integrated into bins (or buckets) across the spectral regions of 0.04 ppm. The region between 4.64 and 5.26 ppm containing the resonances of water and anomeric proton of α/β-glucose was removed and the NMR spectral region 3.3–3.9 ppm that contained resonances of glucose was also removed prior to integration. The remaining spectral segments for each NMR spectrum were normalized to the total sum of the spectral intensity to partially compensate for differences in concentration of metabolites in the samples. Both the resultant data matrices were introduced to SIMCA-P 10.0 software package (Umetrics, Umea, Sweden) for principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA).

3. Results and discussion

3.1. UPLC/MS metabolite profiles

3.1.1. Method development and method validation

The preparation of serum samples for metabolic profiling analysis by UPLC/MS involved a protein precipitation step to extract low-molecular-weight compounds noncovalently bound to proteins and remove the large amounts of proteins present in the serum that would otherwise interfere with the final UPLC/MS analysis. The volume ratio of sample to precipitation reagent (acetonitrile) was investigated, no significant difference was observed in BPI chromatograms between the ratios of 1:2 and 1:3. The ratio of 1:2 was selected for easier evaporation. Full scan of serum metabolites was set in the positive ion mode because it gave more information-rich data than negative ion mode. Fig. 1 shows the positive and negative ion base peak intensity (BPI) chromatograms of representative serum sample from a DM-2 patient. No metabolites of metformin hydrochloride were found in the serum metabolite profiles of DM-2 patients treated with metformin hydrochloride. Extracted ion chromatographic peaks of seven ions (*m*/*z* 120.0, 188.3, 437.4, 520.6, 496.6, 522.6 and 524.5 in positive ion mode) were selected for method validation. Method repeatability was evaluated by five replicate analysis of a serum sample. The relative standard deviations (R.S.D.s%) of peak areas, retention times and *m*/*z* were estimated to be 2.95–10.5%, 0–0.80% and 0–0.01%, respectively. The retention time stability of selected ions in all samples for the whole run was also evaluated, the R.S.D% values were between 0.07% and 0.80%. The post-preparation stability of sample was tested by analyzing a sample left at autosampler (maintained at 4 °C) for 4, 8, 12 and 24 h. The relative errors (R.E.) of peak areas were from −5.59% to 7.87%.

3.1.2. Multivariate analysis of UPLC/MS data

To determine whether metformin hydrochloride influenced the metabolic pattern of type 2 diabetes mellitus patients and to find the metabolites with a significant change (i.e. potential biomark-

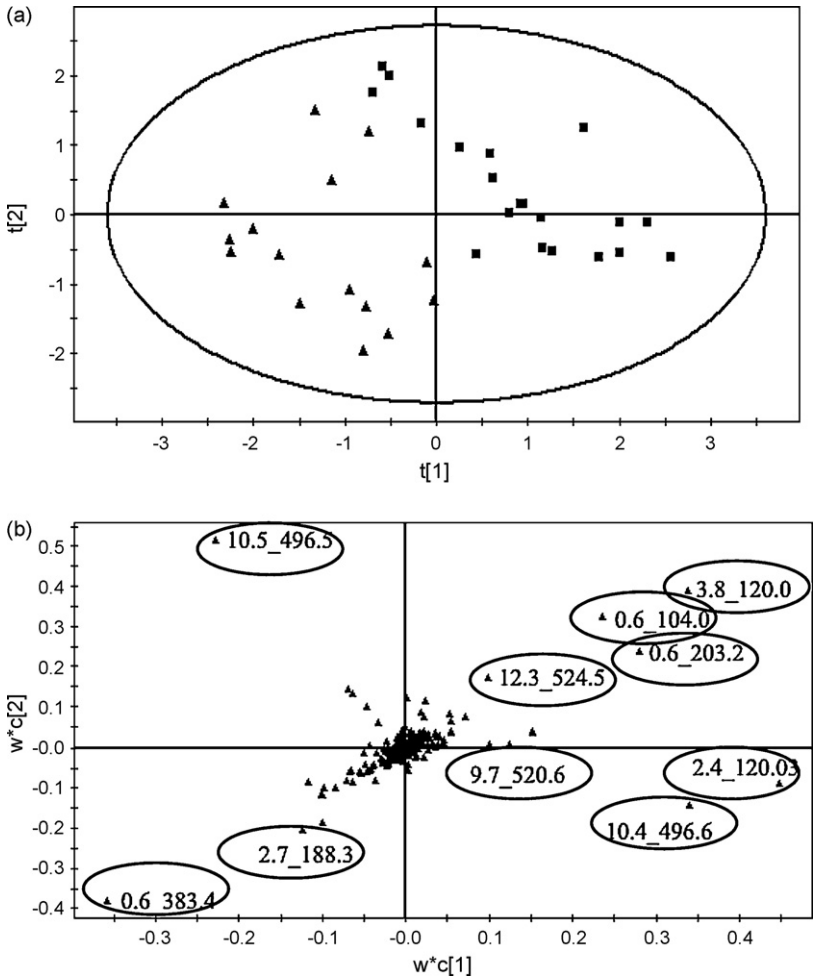

Fig. 2. (a) Score (▲: metformin treated patients, ■: untreated DM-2 patients) and (b) loading plot from PLS-DA model (Pareto-scaled) classifying DM-2 patients and patients treated with metformin hydrochloride for 3 months measured by UPLC/MS.

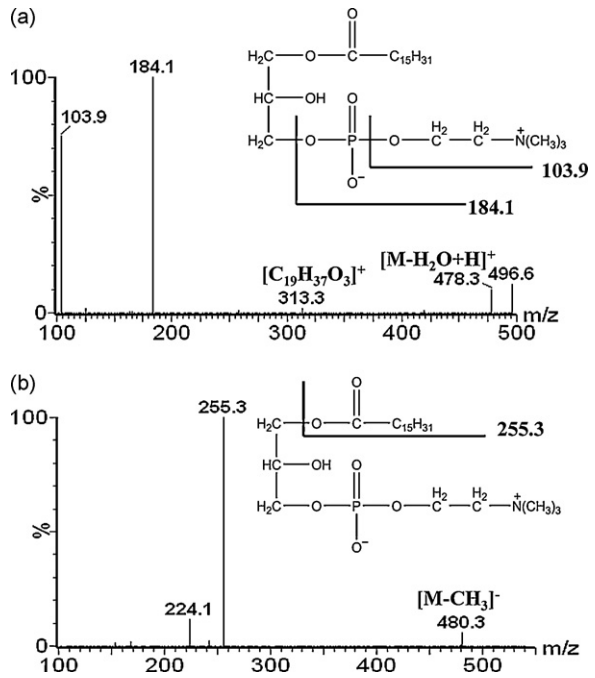

Fig. 3. Product ion scan spectra of biomarker (a) in positive ion mode (m/z 496.6) and (b) negative ion mode (m/z 540.6).

ers), a PLS-DA model was constructed. Fig. 2 shows the score and loading plots. It can be seen from the PLS-DA score plot (Fig. 2a), a separation of the metformin treated group and DM-2 group was clearly achieved, which indicates biochemical changes happened in the serum of DM-2 patients after the treatment of metformin hydrochloride. To estimate the predictive ability of our model, we used 7-fold cross-validation, the parameters for the classification from the software were R2Y = 0.775 and Q2Y = 0.611, which indicated a good fitness and prediction. A response permutation test (Y scrambling) showed no overfitting in the model (R2Y-intercept of 0.391, Q2Y-intercept of −0.33).

3.1.3. Biomarker identification

The possible biomarkers indicated by the loading plot (Fig. 2b) are those with retention time and m/z pairs of 0.6_104.0, 0.6_203.2, 0.6_383.4, 2.4_120.0, 2.7_188.3, 3.8_120.0, 9.7_520.6, 10.4_496.6 and 12.3_524.5 in the positive ion mode. The ions with retention time and m/z pairs of 2.4_120.0 and 2.7_188.3 were identified as the fragments of L-phenylalanine and L-tryptophan, respectively, by comparing with corresponding standards according to their retention times, m/z and product ion scan spectra. The biomarkers at m/z 496.6, 520.6 and 524.5 were identified by comparing with the data in literature [43] in combination with their corresponding fragments of product ion scan in the positive and negative ion mode. Take the biomarker at m/z 496.6 as an example to illustrate the identification. In positive product ion scan spectrum (Fig. 3a), the parent ion $[M+H]^+$ at m/z 496.6 contained three major fragment ions. The high-abundance fragment ions at m/z 103.9 and 184.1 represent the fragments of $[HOCH_2CH_2N(CH_3)_3]^+$ and $[H_2O_3PO–CH_2CH_2N(CH_3)_3]^+$, respectively, which provided the head group information of phosphatidylcholine class. Another major fragment ion at m/z 478.3 $[M–H_2O+H]^+$ further indicated this biomarker belonging to lysophosphatidylcholine. The fragment ion at m/z 313.3 ($[C_{19}H_{37}O_3]^+$) resulted from the loss of polar head was also observed. Negative product ion spectrum (Fig. 3b) showed a fragment ion at m/z 480.3 representing the demethylated lysophosphatidylcholine, and an intense abundant fatty acid fragment was observed at m/z 255.3 ($[C_{15}H_{31}COO]^-$). Accordingly, the biomarker

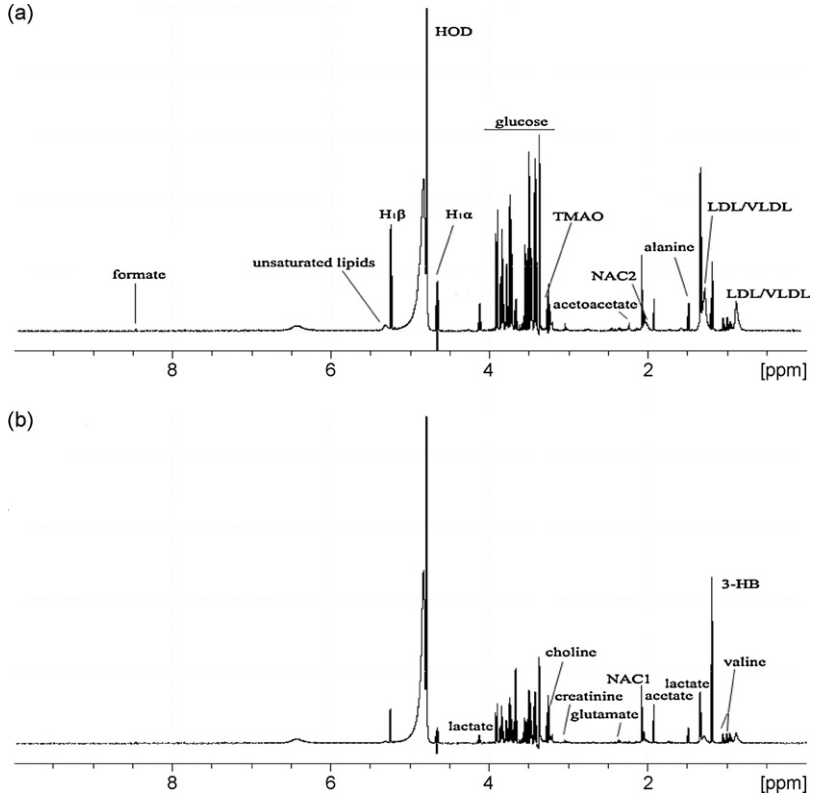

Fig. 4. Representative ^{1}H NMR spectra (CPMG) of serum samples from (a) a DM-2 patient and (b) a patient treated with metformin hydrochloride for 3 months. H1α/β, anomeric proton of α/β-glucose; NAC, *N*-acetyl glycoprotein signals; LDL, VLDL, low- and very-low-density lipoprotein; TMAO, trimethylamine-*N*-oxide; 3-HB, 3-hydroxylbutyrate; HOD, water/D_2O.

Table 3
Identification results of potential biomarkers detected by UPLC/MS/MS for classifying DM-2 patients and patients treated with metformin hydrochloride for 3 months.

Positive ion mode		Negative ion mode		Identification
m/*z*	Quasi-molecular ion	*m*/*z*	Quasi-molecular ion	
496.6	$[M+H]^+$	540.6	$[M+HCOO]^-$	16:0 LPC
524.5	$[M+H]^+$	568.5	$[M+HCOO]^-$	18:0 LPC
520.6	$[M+H]^+$	564.6	$[M+HCOO]^-$	18:2 LPC
120.0	Characteristic fragment	164.2	$[M-H]^-$	Phenylalanine
188.3	Characteristic fragment	203.3	$[M-H]^-$	Tryptophan

at *m*/*z* 496.6 is identified as C16:0 lysophosphatidylcholine. The identification of biomarkers with retention time and *m*/*z* pairs of 0.6_104.0, 0.6_203.2, 0.6_383.4 and 3.8_120.0 has not been accomplished due to lack of reference standards and related literature data. Table 3 lists the potential biomarkers identified for classifying DM-2 patients and patients treated with metformin hydrochloride.

3.2. ^{1}H NMR spectroscopy

Fig. 4a and b present representative ^{1}H NMR spectra of serum samples from a DM-2 patient and a patient treated with metformin hydrochloride for 3 months, respectively. Several differences were observed by simple visual inspection. The resonances of glucose (3.3–3.9 ppm) and low-density lipoprotein/very-low-density lipoprotein (0.86, 0.9, 1.26, 1.30 ppm) were lower in DM-2 patients treated with metformin hydrochloride compared with patients without any drugs treatment. The results are consistent with clinical parameters given in Table 1. Fig. 5 shows the expanded spectral region of 0–6 ppm.

PCA score plot of the ^{1}H NMR data (exclude the glucose resonances) obtained from DM-2 patients and patients treated with metformin hydrochloride is shown in Fig. 6a. A clear separation was observed by using the first two components, which explained 64% of the total variances. The loading plot (Fig. 6b) illustrated that the biomarkers for the separation of the two groups were lactate, lipoprotein, *N*-acetyl glycoprotein, acetoacetate, 3-hydroxybutyrate as well as trimethylamine-*N*-oxide and unsaturated lipids.

Table 4
Change trend of biomarkers identified by ^{1}H NMR in DM-2 patients after the treatment of metformin hydrochloride.

Biomarkers	Chemical shift (ppm)	Change trend of metformin treated patients *vs.* DM-2 patients
Lactate	1.34, 4.14	↓
VLDL	0.9, 1.30	↓
LDL	0.86, 1.26	↓
NAC	2.02, 2.06	↓
Acetoacetate	2.22	↓
3-Hydroxybutyrate	1.18	↑
TMAO	3.26	↑
Unsaturated lipids	5.30	↓

3.3. Biochemical interpretation

^{1}H NMR spectroscopy can detect all compounds containing hydrogen, however, it has a relatively low sensitivity for low-abundance analytes. UPLC/MS/MS is a technology with high sensitivity and is able to quantify trace levels of analytes but not those to be ionized difficultly. UPLC/MS/MS and ^{1}H NMR spectroscopy provide different analytical selectivities and their combination allows a greater insight into the biochemical changes in systems under investigation.

Tables 4 and 5 represent the change trend of biomarkers identified by ^{1}H NMR spectroscopy and UPLC/MS/MS, respectively, in the serum of metformin treated patients compared with untreated ones. ^{1}H NMR spectroscopic analysis demonstrated the

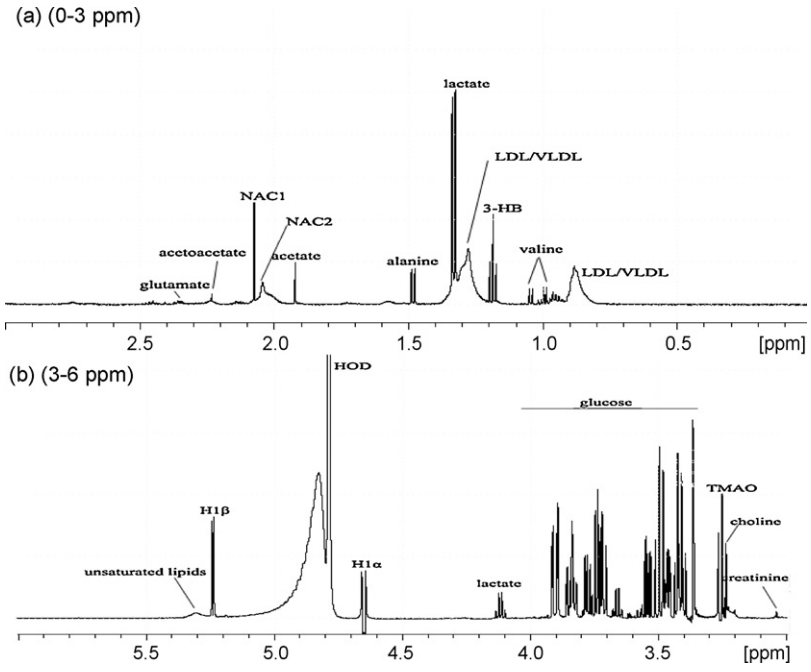

Fig. 5. Expanded ^{1}H NMR spectrum (CPMG) of serum sample from a DM-2 patient (spectral region of 0–6 ppm).

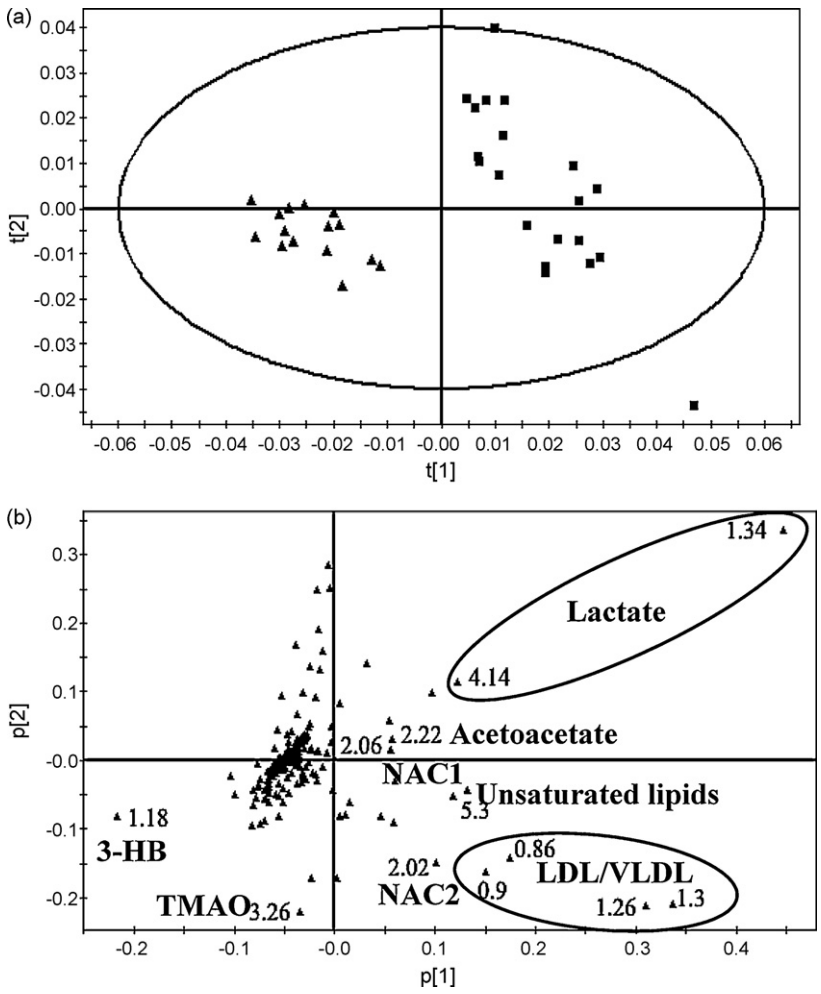

Fig. 6. PCA (a) score (▲: metformin treated patients, ■: untreated DM-2 patients) and (b) loading plot of the ^{1}H NMR data (exclude the glucose resonances) with Pareto-scaling obtained from serum samples of DM-2 patients and patients treated with metformin hydrochloride.

decrease of glucose, lipoprotein and unsaturated lipids in the serum of metformin treated patients, which may be due to the hepatic gluconeogenesis inhibition [44] and lipid regulation function [45] of metformin. It was reported that hyperglycemia- and dyslipidemia-induced oxidative stress in diabetic patients via the activation of many pathways [46,47] such as non-enzymatic glycation, polyol pathway and protein kinase C (PKC). The decrease of glucose and unsaturated lipids in DM-2 patients after the treatment of metformin hydrochloride would reduce the oxidative stress. The result from ^{1}H NMR is consistent with decreased lysophosphatidylcholine in metformin treated DM-2 patients measured by UPLC/MS, as lysophosphatidylcholine is a oxidative product of LDL and the decrease of lysophophatidylchline in metformin treated DM-2 patients also indicated a reduction of oxidative stress. In addition, ^{1}H NMR analysis indicated a decrease of lactate and aceteoacetate in the serum of DM-2 patients treated with metformin, which suggested no lactic acidosis and ketoacidosis existed. Trimethylamine-*N*-oxide, a degradation product of choline was observed to be increased in metformin treated DM-2 patients in ^{1}H NMR analysis. The pathway of choline degradation involved conversion of choline into trimethylamine, a precursor of trimethylamine-*N*-oxide, by gut microflora [48,49]. It was reported that decreased amount of trimethylamine-*N*-oxide in serum might be associated with the disruption of intestinal bacteria [50]. Increased trimethylamine-*N*-oxide in patients after metformin treatment may indicate an intestinal bacteria regulation function of metformin. Tryptophan and phenylalanine are essential amino acids which cannot be synthesized by the body. They are either incorporated into proteins or broken down for energy and metabolic intermediates. Changes of the two amino acids in metformin treated patients may indicate an alteration of the balance between nutrition intake and consumption.

Table 5
Change trend of biomarkers identified by UPLC/MS/MS in DM-2 patients after the treatment of metformin hydrochloride.

Biomarkers	ANOVA analysis (*P* value)	Change trend of metformin treated patients *vs.* DM-2 patients
C16:0 LPC	5.35E−05	↓
C18:0 LPC	1.43E−04	↓
C18:2 LPC	4.37E−03	↓
Phenylalanine	1.17E−04	↓
Tryptophan	0.03	↑

4. Conclusion

^{1}H NMR and UPLC/MS/MS were employed in the metabonomic study of biochemical changes in the serum of DM-2 patients after the treatment of metformin hydrochloride. As others have noted [38] the use of both LC/MS and ^{1}H NMR results in the collection of complementary data on biomarkers. Thus, while statistical separation was seen using for both approaches the biomarkers on which these separations depended were different.

This study demonstrates changes in endogenous metabolites in type 2 diabetes mellitus patients treated with metformin hydrochloride. In addition to its effects on glucose and lipoprotein metabolism pathways, metformin has some effects on intestinal bacteria and nutrition metabolism. The study would be helpful to further understanding of action mechanism of metformin hydrochloride.

Acknowledgement

This work is financially supported by a grant from the Ph.D. Programs Foundation of Ministry of Education of China (No. 20060163004).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2009.01.008.

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