Chinese Science Bulletin

Metabonomics analysis of the urine of rats with Qi deficiency and blood stasis syndrome based on NMR techniques

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Metabonomics analysis of the urine of rats with Qi deficiency and blood stasis syndrome has been performed by comparison with those of normal rats based on NMR techniques. The relative contents of formate, creatinine, 2-oxoglutarate (2-OG), citrate, taurine, trimethylamine-*N*-oxide(TMAO), succinate and hippurate in the urine of the rats with Qi deficiency and blood stasis syndrome have been changed. These results have provided evidence for understanding the mechanism and the therapy of Qi deficiency and blood stasis syndrome.

metabonomics, NMR, Qi deficiency and blood stasis syndrome, urine

Recently, global analysis of the dynamic of biosystem under the physiological, pathological, pharmacological and toxicological conditions has become one hot field in biomedicine abroad; modern "-omics" approaches represented by genomics, proteomics and metabonomics have come into being[1]. Metabonomics was a technique to explore the metabolic pathway of biosystem by measuring the variation of the metabolites or time-dependent changes after physiological stimuli or disturbance, which provides a powerful experimental technique in the development of biological sciences. As one of the major techniques in metabonomics researches, NMR spectroscopy has the disadvantage of low detection limits but a lot of advantages compared with HPLC-MS. For example, NMR measurements are non-destructive, non-selective, which could be applied in physiological-like condition[2], and NMR methods are also feasible to acquire the profile of a comprehensive range of organic metabolites[3]. Therefore, NMR methods have been extensively used in metabonomics researches. Solanky et al.[4] have investigated dietary isoflavones effects of human metabolites using ^{1}H NMR-based metabonomics techniques. Wang et al.[5,6] have explored the toxicological characteristics of Z24 in rats through ^{1}H NMR spectra of urine and plasma.

"Qi deficiency" is a basic syndrome in Traditional Chinese Medicine (TCM). Patients with "Qi deficiency" in clinic exhibit the following symptoms: pale complexion, listlessness and wordlessness, fatigue, anorexia, dizziness and wheeziness after activity, desudation, liability to catching cold, pale tongue and feeble pulse. Clinical studies have manifested that "Qi deficiency" was closely correlated with many diseases including hypertension, diabetes, coronary artery disease, chronic heart failure, acute cerebral embolism and mammary adenocarcinoma[7]. Metabonomics researches on "Qi deficiency" would be beneficial to the prevention and therapy of these diseases and further quantification of clinical diagnostic signs of the syndrome.

Received January 11, 2007; accepted May 8, 2007
doi: 10.1007/s11434-007-0389-4
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1 Materials and methods

1.1 Animals, reagents and apparatus

Healthy adult Sprague Dawley (SD) rats, ♂, weight 200－220 g, SPF level, were provided by Beijing Wei Tong Li Hua Laboratory Animal Research Center, China. The animals were housed in the metabolism cages with free access to food and water at 25℃. Deuterium oxide (D_2O) was purchased from CIL Co. USA, and 3-trimethylsilyl-[2,2,3,3-*D*4]-propionate (TSP) was from Aldrich Co. USA. SGD-1 noise, light and electricity stimulating instrument was manufactured by Xi Yuan Hospital, China Academy of Chinese Medical Sciences. The NNR spectra were obtained from Switzerland Bruker AVANCE 600 spectrometer.

1.2 Modeling method and urine collection

The modeling process was based on "tire deplete Qi", a TCM theory, following the methods of sleep deprivation. Twelve rats used in this study were divided into 2 groups, Qi deficiency and blood stasis syndrome group (model group, 8 rats) and normal group (4 rats). Four processes were applied randomly onto the rats of model group housing in the SGD-1 noise, light and electricity stimulating instrument, which were ordinary light (6 AM－6 PM light, 6 PM－6 AM dark), reverse light (6 AM－6 PM dark, 6 PM－6 AM light), continuous light (24 h light) and continuous dark (24 h dark). The animals were subsequently deprived of sleep for 72 hours. The modeling process has been done until all the steps described above have been repeated for six times. Urine samples were collected into conical tubes over ice and stored at –20℃ until they were prepared for NMR measurements.

1.3 Sample preparation

Urine samples were centrifuged at 4℃ (7000 g) for 10 min in order to remove the solid debris. An aliquot of urine (400 μL) from each sample was placed in a centrifuge tube together with 200 mmol/L phosphate/D_2O buffer (200 μL). An internal reference standard, TSP, was added to the buffer, and the final concentration was 1 mmol/L. The samples were centrifuged at 4℃ (7000 g) for 10 min again after 10-min-deposition. 550 μL from each sample was then dropped into a 5-mm diameter NMR tube for final NMR test.

1.4 Acquisition of NMR urine spectra and data processing

All samples were measured on a Bruker AVANCE 600 spectrometer at 298.2 K operating at 600.13 MHz for ^{1}H observation. For each sample, a one-dimensional NMR spectrum was acquired with water peak suppression using a standard solvent pre-saturation pulse sequence (noesypr1d, Bruker), using 128 free induction decays (FIDs), 64 k data points, a spectral width of 9615 Hz, an acquisition time of 3.41 s and a total pulse recycle delay of 2 s. The FIDs were multiplied by an exponential weighting function equivalent to a line broadening of 0.3 Hz prior to Fourier transformation (SI = 32 k).

^{13}C and 2D spectra (TOCSY, HSQC, HMBC) were also acquired to identify the metabolites. The parameters used in 2D experiments are shown in Table 1.

Table 1 Parameters of 2D NMR spectra

2D experiments	TOCSY	HSQC	HMBC
Pulse program	mlevphpr	hsqcetgp	hmbcgplpndqf
Data points	2048×256	2048×256	2048×256
Number of scan	48	48	48
SI	1024×1024	1024×1024	1024×1024

The ^{1}H NMR spectra of urine samples were manually phased and baseline was corrected in XWINNMR 3.5 (Bruker) after referring to TSP (δ0.0). The spectra were subsequently reduced into consecutively integrated spectral regions corresponding to 0.04 intervals across the chemical shift range of δ 0.2－9.8. The region δ4.2－5.2 around the water signal was excluded in order to remove the effects of variations in the suppression of the water resonance. The data were then imported into Excel and the integrated spectral area was normalized to the total sum of the spectral regions.

1.5 Principal components analysis (PCA)

The matrix containing the reduced spectral data was subjected to PCA analysis using MATLAB software, and was projected into a few principal components (PCs) describing the maximum variation within the data. The metabonomics analysis of urine samples was performed subsequently.

2 Results

2.1 ^{1}H NMR spectra of urine samples

Respective ^{1}H NMR urine spectra for normal group and

model group rats are shown in Figure 1.

2.2 Results of PCA modeling

The score plot of urine samples is shown in Figure 2. As we can see, all twelve samples fell below the 95 % critical significance level, and the two groups (normal group and model group) are well discriminated (Figure 2). The first two PCs (PC1 and PC2) describe 47.2 % and 13.6 % of the ^{1}H NMR spectra variation respectively.

2.3 Identification of the metabolites

Identification of the metabolites was done according to urine ^{1}H, ^{13}C NMR spectra and the other 2D NMR spectra (TOCSY, HSQC, HMBC) as well as previous publications[8−11]. The HSQC spectrum of a urine sample is shown in Figure 3. The identification of some metabolites is shown in Figure 4 and Table 2.

3 Discussion

Urine samples from normal group and model group behaved differently in the score plot after PCA, indicating that normal and pathological samples formed their own clusters respectively. Further analysis of PC1 and PC2 showed that the relative integral areas of formate (δ 8.47) and creatinine (δ 4.05, 3.04) enhanced while those of

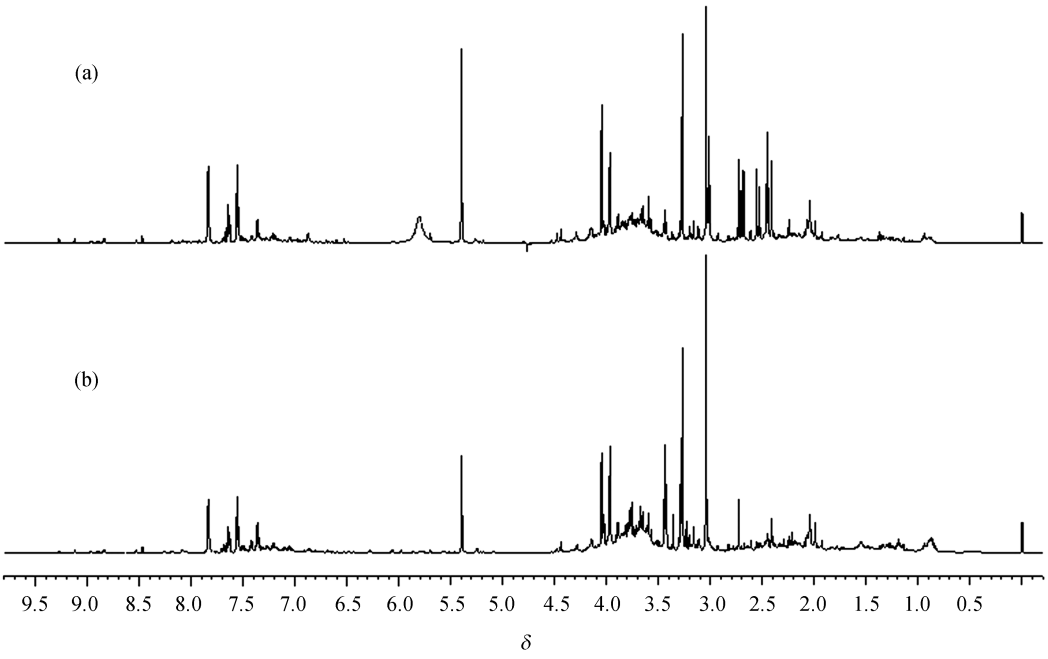


Figure 1 The ^{1}H NMR spectra of urine samples from (a) normal group; and (b) model group.

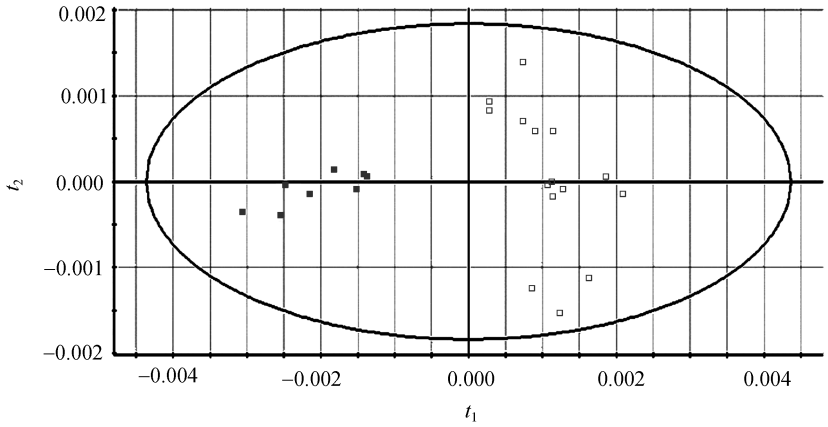


Figure 2 The score plot of normal group and model group (t_1/t_2). ■, Normal group; □, model group.

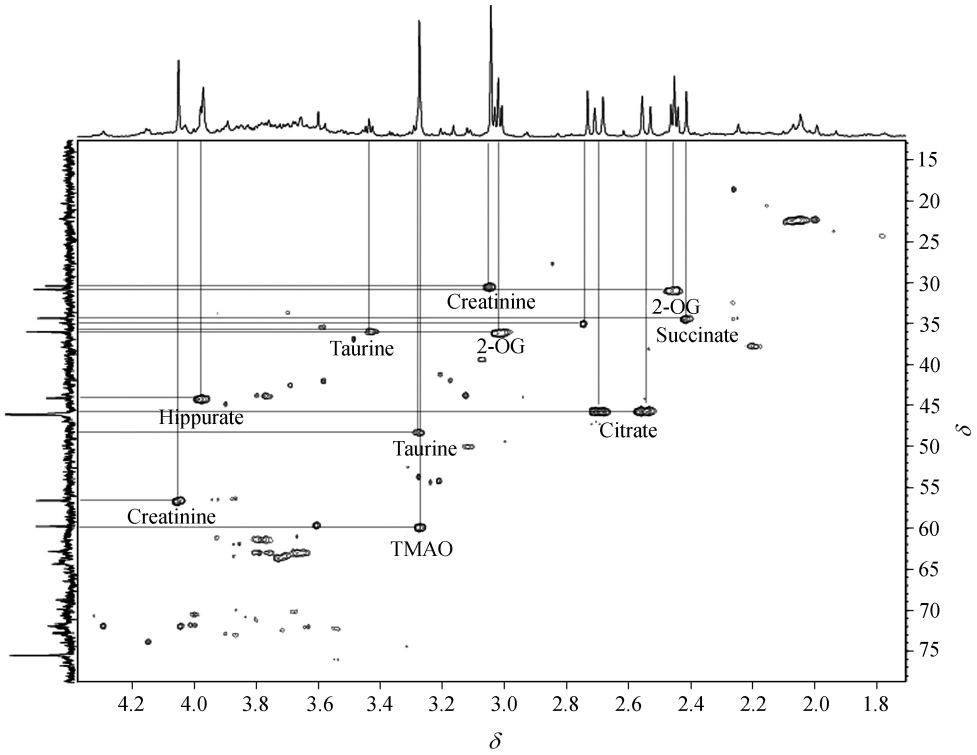


Figure 3 The HSQC spectrum of urine sample from normal group.

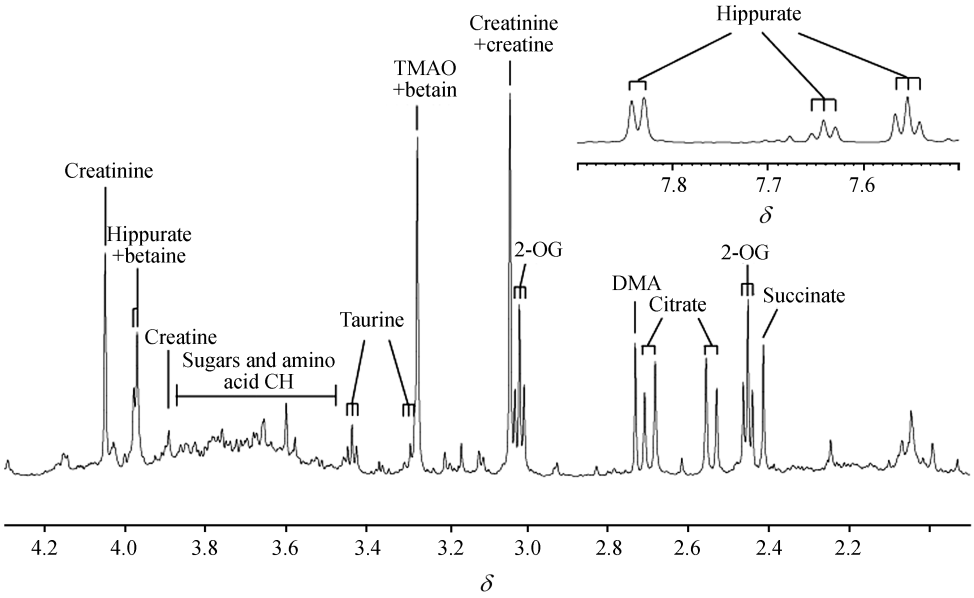
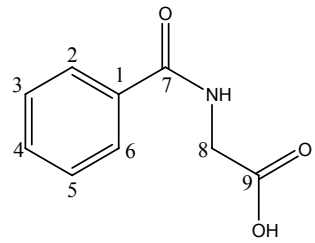
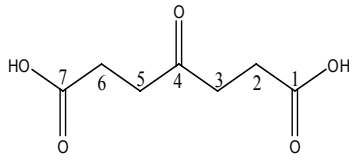
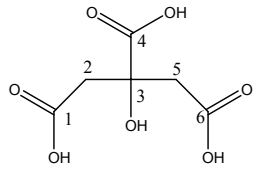
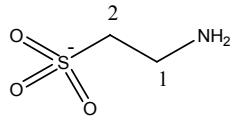
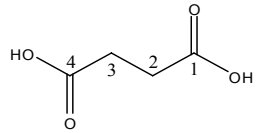


Figure 4 The ^{1}H NMR spectrum (δ1.8—4.4, δ7.5—7.9) of urine sample from normal group.

2-oxoglutarate (2-OG, δ 3.02, 2.45), citrate (δ 2.69, 2.54), taurine (δ 3.44, 3.28), trimethylamine-*N*-oxide (TMAO, δ 3.28), succinate (δ 2.41) and hippurate (δ 7.84, 7.64, 7.56, 3.97) reduced compared with the normal group samples. Among them, the increment of creatinine was the most significant, while creatinine is a biomarker indicative of renal canaliculus trauma[12] and renal toxicity[13]. Such argument indicates that Qi deficiency and blood stasis syndrome should be correlated with the abnormity of kidney functions. Variation of TMAO, citrate, 2-OG and succinate indicated possibly the existence of renal medullary toxicity[14,15]. Because citrate, succinate and 2-OG are the media of energy metabolism and glycolysis in the tricarboxylic acid cycles,

Table 2 The assignments of ^{1}H and ^{13}C chemical shifts for urine metabolites

Metabolites	Carbon	δ_H	δ_C	HMBC ($^{1}H \rightarrow ^{13}C$)	Structures
Hippurate	1	–	133.4	–	
	2, 6	7.838	127.2	2, 6→4, 2, 6→7	
	3, 5	7.555	128.9	3, 5→1	
	4	7.644	132.3	4→2, 6	
	7	–	170.6	–	
	8	3.970	44.04	8→9, 8→7	
	9	–	177.0	–	
2-Oxoglutarate	1, 7	–	181.7	–	
	2, 6	3.020	36.04	2, 6→1, 3, 4, 5, 7	
	3, 5	2.452	30.83	3, 5→1, 2, 4, 6, 7	
	4	–	205.7	–	
Citrate	1, 6	–	179.1	–	
	2, 5	2.538, 2.688	45.50	2, 5→3, 5→4	
	3	–	75.53	–	
	4	–	181.9	–	
Taurine	1	3.436	35.64	1→2	
	2	3.281	47.76	2→1	
Succinate	2, 3	2.413	34.23	2、3→1、4	
	1, 4	–	181.7	–	

such depletion of the metabolites mentioned above generally arises from mitochondrial dysfunction[16].

TCM theory holds that overstrain causes Qi deficiency, and fatigue has three categories: body fatigue, Zang and Fu fatigue, and mental fatigue. Central mental fatigue is due to sleep deprivation, belonging to mental fatigue. Sleep deprivation and day and night inversion living are intensive stressors, producing physiological and mental stresses of human body, and many negative effects that can lead to a stimulation state[17]. However, with time of stimulation state going , the neuroendocrine system and internal environment would alter consequently, resulting in the physiological and pathological changes of the body, leading to the dysfunction of multisystem and multiorgan. To some extent, this is in coincidence with the symptom of whole body hypofunction and significant disorders in the circulatory system resulting from Qi deficiency and blood stasis syndrome due to "overstrain (central mental fatigue) causing Qi deficiency" in TCM theory. In summary, metabonomics analysis of urine can demonstrate metabolic characteristics of "Qi deficiency", and provide valuable data for the further elucidation of pathological mechanism and the therapy of Qi deficiency and blood stasis syndrome.

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