Chinese Chemical Letters Vol. 16, No. 11, pp 1507-1510, 2005 1507
http://www.imm.ac.cn/journal/ccl.html

NMR Studies on the Subacute Biochemical Effects of Aristolochic Acid on Rat Serum

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Abstract: The subacute effect of aristolochic acid (AA) on rat serum was studied by NMR method. The biochemical effects induced by AA were characterized by an increase in the amounts of creatinine, trimethylamine N-oxide, acetoacetate, acetate and 3-D-hydroxybutyrate and lactate in serum from ^{1}H NMR spectra. Principal component analysis was used for further comparing the similarities of ^{1}H NMR spectral profiles of serum from rats treated with AA and model toxins.

Keywords: Aristolochic acid, NMR, serum, principal component analysis, metabolite.

Some of Chinese traditional medicines also show toxic effect[1]. Aristolochic acid, the main component of *Aristolochia* species of Chinese herb, is considered to be related to progressive renal fibrosis so-called 'Chinese-herb nephropathy' (CHN) [2]. The nephrotoxicity of AA was tested in rat model [3]. It might form DNA adducts[4]or cause p53 and H-ras genes mutations[5, 6]. However the mechanism of the toxicity is still not clear. Thus, it is important to investigate the subacute effect of AA.

^{1}H NMR spectroscopy of biofluids or tissues presents comprehensive biochemical profiles of metabolites reflecting the biochemical effects caused by xenobiotics[7]. ^{1}H NMR spectra of serum from rat under similar physiological conditions are highly reproducible, which is favorable in investigating toxicological process and diseased states. Principal components analysis (PCA) is a widely used dimension-reduction method, with each PC being a linear combination of the original variables with appropriate weighting coefficients. All PCs are calculated such that they are orthogonal with all other PCs. The first PC contains the largest proportion of variance in the data set, with subsequent PCs involving progressively smaller proportion of total variance. Therefore, a plot of the first and second PCs may contain a significant proportion of the information content of the original data set [8]. The application of PCA for data process of NMR spectroscopy can provide maximum amount of biochemical information[8, 9]. The biochemical profiles of the metabolites in the serum from AA treated rats were studied using ^{1}H NMR spectroscopy combined with PCA.

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Experimental

Twenty male Wistar rats (200-250 g) were divided into 4 groups (n=5) and housed individually in metabolism cages with free access to food and water under controlled condition (temperature, humidity, and a light-dark cycle). Control and model groups were injected intraperitoneally with saline, $NaCrO_4$ (20 mg/kg body weight), $HgCl_2$ (1mg/kg body weight), and sacrificed after 48 h. AA (Sigma Aldrich Co.) group was dosed intraperitoneally with 10 mg /kg body weight each day for 5 days, and sacrificed in day 6. Blood was get by decollation and serum samples were separated by centrifugation and stored frozen at -70˚C. Each serum sample (400 μL) was mixed with 50 μL D_2O and 50 μL buffer solution (0.2mol/L Na_2HPO_4-NaH_2PO_4, pH=7.0) and recorded on Bruker-Avance 600 MHz spectrometer at 298 K. Water signals and broad protein resonances were suppressed by a combination of presaturation and the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. Thirty-two free induction decays (FIDs) were colleted into 32 k data points with relaxation delay 6 s and flip angle 90^o. All spectra were referenced to resonance of creatinine (CH_3) at δ 3.06.

Each spectrum δ 0.0-10.0 was segmented into region of 0.04 ppm width using MestRe-c 2.3 (http://qobrue.usc.es/jsgroup/MestRe-c). The region (δ 4.6-5.0) was removed *prior to* statistical analysis to avoid water suppression variation. The remaining 235 spectral segments were scaled to the total integrated area[9]. PCA of the data was performed using a program written by ourselves.

Results and Discussion

Table 1 provides assignments of important metabolites in serum samples. **Figure 1** illustrates the typical 600 MHz ^{1}H NMR spectra of serum samples from control, model and AA-treated rats.

The alteration of the level of metabolites in ^{1}H NMR spectra of serum from AA treated rat was similar to that from $NaCrO_4$ and $HgCl_2$ treated rat. The increases of creatinine, trimethylamine N-oxide (TMAO), acetoacetate, 3-D-hydroxybutyrate (HB) lactate, and acetate were observed by ^{1}H NMR spectral analysis of serum samples from

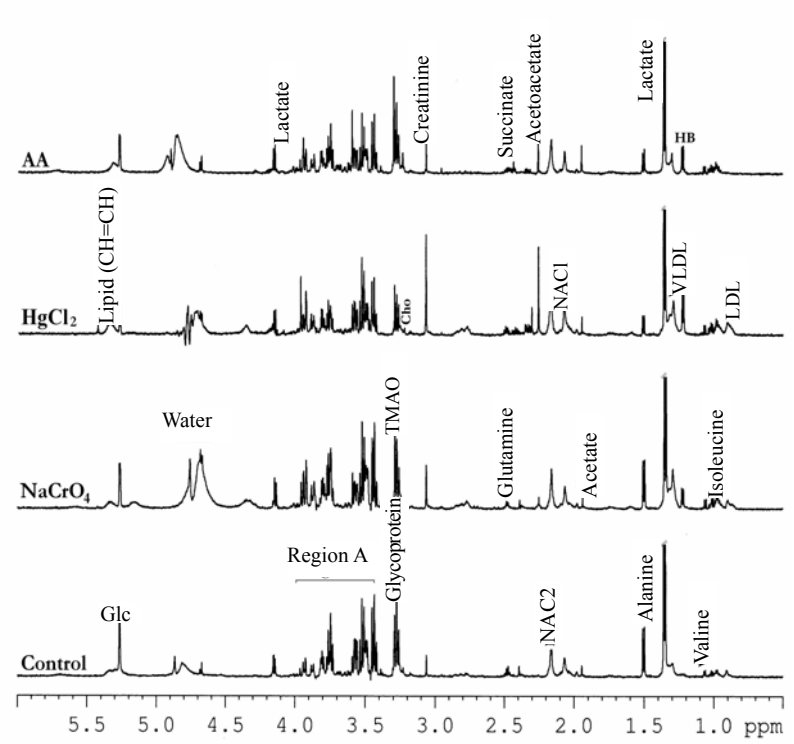
Table 1 Assignments of the metabolites in serum from male Wistar rats

Metabolite	Chemical shift (δ ppm)	Metabolite	Chemical shift (δ ppm)
Lipoprotein CH_3	0.84	Acetoacetate	2.30 (s)
Valine	0.97 (d)	Succinate	2.40 (s)
3-D-hydroxybutyrate	1.20 (d)	Glutamine	2.48 (m)
Lactate	1.32 (d)	Lipid	2.72
Alanine	1.48 (d)	Creatinine	3.06 (s)
Acetate	1.92 (s)	TMAO	3.27 (s)
Glycoprotein	2.00	Region A	3.5-4.0
NAC1	2.06	α-Glucose	5.22 (d)
NAC2	2.16	Lipid	5.32

[a] s, singlet; d, doublet; m, complex multiplet; NAC1 and NAC2, the composite N-acetyl signals from glycoproteins; Region A, glucose and amino acid CH resonances

AA-treated group. The increase of TMAO in NMR spectra appears to be associated with renal failure[10]. The higher level of creatinine and acetate in the serum is a sign of renal insufficiency[11]. The elevation of lactate levels in the serum of rats receiving AA was consistent with reduced the utilization of pyruvate in the citric acid cycle and an increase in anaerobic cell respiration. This could also account for the increased level of HB, acetate and acetoacetate[11], which was associated with a possible liver lesion. High creatinine and lactate level could also occur indirectly due to the reduction in renal cortical blood flow and lower rates of glomeruler filtration caused by perturbation of the rennin-angiotension system[12]. The decrease of low molecular glucose and amino acid (such as alanine) was also observed in the ^{1}H NMR spectra of serum from AA, which might relate to the disorder of reabsorption of renal tubule[12]. On the other hand, the waste product in metabolism could not be excreted on time result in an impairment of homeostasis and bring metabolism disorders. The increase of VLDL level in serum might also be consistent result with the disturbance of fat metabolism[13].

Figure 1 600 MHz high-resolution ^{1}H NMR spectra of Serum

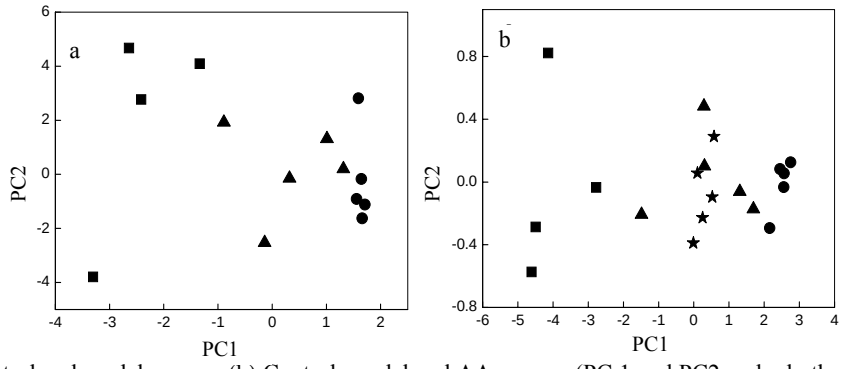


PCA was first performed to the control and model groups to remove abnormal points, which might account for the individual difference of rats. PC1 *vs.* PC2 stand for the first two PCs. A tight cluster and clear classification of PCs from control and model groups were obtained (**Figure 2a**). **Figure 2b** showed the PC plots (PC1 *vs.* PC2) of data from ^{1}H NMR spectra of serum samples from control, model and AA-treated groups respectively. It was seen that the control sample showed greater within-group

variability than the model groups and AA-treated samples. For the serum samples of AA-treated rats, all the points of AA clusters were classified to $NaCrO_4$ clusters, and close to $HgCl_2$ clusters, which are two important model toxin compound causing damage of proximal tubule. It might mean that renal tubule lesion was the main feature of renal damage caused by AA. It was also consistent with the visual comparisons of the ^1H NMR spectra of serum mentioned above.

In conclusion, NMR-PCA analysis of rat serum suggests the renal injury by AA together with a possible liver lesion. The PCA analysis suggested the cluster of AA-treated rats was similar with $NaCrO_4$ cluster, and near $HgCl_2$ cluster. This study illustrated the power of the combination of NMR technique and pattern recognition method for the analysis of biochemical effects of toxic compounds.

Figure 2 A plot of PC1 *vs.* PC2 based on the ^1H NMR spectral descriptors for the serum



(a) Control and model groups, (b) Control, model and AA groups. (PC 1 and PC2 embody the first two PCs.). ■, Control; ▲, $NaCrO_4$;●, $HgCl_2$; ★,AA

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Received 21 February, 2005