*Chinese Journal of Chemistry*, **2004**, *22*, 849—853

**Article**

# NMR and Pattern Recognition Studies on the Acute Biochemical Effects of $Lu(NO_3)_3$

WU, Hui-Feng[a](吴惠丰) ZHANG, Xiao-Yu[a](张晓宇) FENG, Jiang-Hua[a](冯江华)
SUN, Guo-Ying[a](孙国英) WU, Yi-Jie[a](吴亦洁) PEI, Feng-Kui*[a](裴奉奎)
NIE, Yu-Xiu[b](聂毓秀)

[a] *Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin 130022, China*
[b] *College of Basic Medicine Science, Jilin University, Changchun, Jilin 130021, China*

Pattern recognition methods were applied to the analysis of 600 MHz $^1H$ NMR spectra of urine from rats dosed with compounds that induced organ-specific damage in the liver and kidney. Male Wistar rats were separated into groups ($n$=4) and each was treated with one of following compounds: $HgCl_2$, $CCl_4$, $Lu(NO_3)_3$ and Changle (a kind of rare earth complex mixed with La, Ce, Pr and Nd). Urine samples from the rats dosed with $HgCl_2$, $CCl_4$ and $Lu(NO_3)_3$ were collected over a 24 h time course and the samples from the rats administrated with Changle were gained after 3 months. These samples were measured by 600 MHz NMR spectroscopy. Each spectrum was data-processed to provide 223 intensity-related descriptors of spectra. Urine spectral data corresponding to the time intervals, 0—8 h ($HgCl_2$ and $CCl_4$), 4—8 ($Lu(NO_3)_3$) h and 90 d (Changle) were analyzed using principal component analysis (PCA). Successful classification of the toxicity and biochemical effects of $Lu(NO_3)_3$ was achieved.

**Keywords** biofluid, rare earth, NMR spectroscopy, principal component analysis (PCA)

## Introduction

$^1H$ NMR spectroscopy of urine offers much information on the concentrations of endogenous metabolites and on their variation in pathological states.[1] Many toxins, including $HgCl_2$, hexachlorobutadiene (HCBD), 2-bromoethanamine hydrocholoride (BEA), propyleneimine, $La(NO_3)_3$ and others have been studied by NMR method.[2-9] Examination of $^1H$ NMR spectra of urine from animals dosed with toxins has indicated that much of the information is necessary to classify and assess metabolic effects of toxins biochemically.[10] Moreover, recent development in hardware and the increasing availability of high frequency NMR spectrometers have led to an increase in the complexity of the observed biofluid $^1H$ NMR spectra due to the improvements in sensitivity at higher magnetic field strengths.[8] The complexity of the $^1H$ NMR spectra of biofluids made it necessary to use powerful methods of data reduction and analysis to gain the maximum amount of biochemical information from $^1H$ NMR spectra.[1] In the present study, we have applied computer-based pattern recognition (PR) methodology to toxicological data derived from $^1H$ NMR spectra of urine obtained from rats exposed to the toxins, $HgCl_2$, $CCl_4$, $Lu(NO_3)_3$ and Changle.

$HgCl_2$ is a typical renal toxin. It targets the terminal segment of the proximal tubule.[1] $CCl_4$ causes centribolular necrosis with fatty liver.[10] Changle was also used as a model toxin, causing main damage to kidney.[11] The toxicity of La which is a light rare earth element has been studied by NMR technique previously.[9] Lu is a heavy rare earth element, however, the toxicity has not been analyzed by this method.

PR analysis is performed in a multidimensional parameter space and displayed using dimension-reduction techniques.[12] Several approaches have been used to classify toxins on the basis of $^1H$ NMR spectra of urine. These methods have mainly included principal component analysis (PCA) and non-linear mapping (NLM) techniques.[13] Analysis utilizing automatic data reduction followed by PCA has been applied to whole spectra rather than preselecting a metabolite set.[7,8] PCA is a technique of dimension reduction,[12] 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 occupying 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.[12]

In this paper, PCA has been used to assess the classification of the urine $^1H$ NMR spectra obtained from rats following the treatment of toxins mentioned above and

* E-mail: peifk@ciac.jl.cn
Received November 24, 2003; revised February 18, 2004; accepted April 6, 2004.
Project supported by the National Natural Science Foundation of China (No. 29890280).

also applied to the analysis of the metabolic effects of $Lu(NO_3)_3$.

## Experimental

### Sample collection and storage

Thirty two male Wistar rats (weight ranging 250—300 g) were housed individually in metabolism cages and allowed free access to food and water throughout the study. Twenty eight animals were treated with a single oral dose of either $HgCl_2$ (2 mg/kg body weight, $n=4$) in saline, $CCl_4$ (1.5 mL/kg, $n=4$), $Lu(NO_3)_3$ (0.05, 2, 10, 100 mg/kg body weight, $n=16$), or saline (0.9%, $n=4$) and the other four rats were administrated with a dose of 2 mg/kg body weight Changle daily for 90 d. Urine was collected at the following time intervals: −4—0, 0—4, 4—8, and 8—24 h to the rats dosed with $HgCl_2$, $CCl_4$, $Lu(NO_3)_3$ and saline. The urine of Changle-dosed rats was gained at the 90th day.

The fresh urine samples were stored frozen at −20 ℃ until measured.

### $^1$H NMR measurement

Proton NMR measurements of rat urine (20 % $D_2O$ was added for locking signal) were made on a Bruker-Av 600 MHz spectrometer at 25 ℃. Water signals of the samples were suppressed by presaturation. Sixteen free induction decays (FID) were collected into 64 k data points with relaxation delay 5 s and flip angle 90°. The creatinine singlet at $\delta$ 3.06 was taken as the reference.

### Data analysis

Each spectral region $\delta$ 0.0—10.0 was segmented into regions of $\delta$ 0.04 width giving a total of 250 integrated regions per NMR spectrum. The area for each segmented region was used as an integral value resulting in an intensity distribution description of the whole spectrum with 250 variables prior to PR analysis. The region of the spectrum, which included water ($\delta$ 4.7—5.1), was removed from the data set for all spectra to eliminate the variation in water suppression efficiency. The region containing urea ($\delta$ 5.4—6.0) was also excluded. The rest 223 spectral segments were scaled to the total integrated area of each spectrum.[7,8] Principal component analysis (PCA) of the data was performed using the program written by ourselves.

## Results and discussion

### $^1$H NMR analysis of urine samples

$^1$H NMR spectra of biofluids provide much information, which is carried in the overall pattern of the metabolite resonances.[13] Table 1 lists the accurate assignments of important metabolites in urine samples. The 600 MHz $^1$H NMR spectra of urine samples from $HgCl_2$, $CCl_4$ and saline treated rats were shown in Figure 1.

$HgCl_2$ causes toxicity to the pars recta of the proxial tubule and produces reproducible patterns of change

**Table 1** Assignments of urinary metabolites

| Metabolite | Chemical shift $\delta$ [a] |
|---|---|
| Valine (Val) | 0.99 (d) |
| Ethanol (Eth) | 1.17 (t) |
| Lactate (Lac) | 1.35 (d) |
| Succinate (Suc) | 2.43 (s) |
| $\alpha$-Ketoglutarate ($\alpha$-Kg) | 2.46 (t) |
| Citrate (Cit) | 2.61 (AB) |
| Dimethylamine (DMA) | 2.72 (s) |
| Dimethylglycine (DMG) | 2.81 (s) |
| Creatinine (Cn) | 3.06 (s) |
| Taurine (Tau) | 3.25 (t) |
| Trimethylamine-*N*-oxide (TMAO) | 3.27 (s) |
| Glycine (Gly) | 3.57 (s) |
| Sarcosine (Sar) | 3.61 (s) |
| Glutamine (Glu) | 3.77 (t) |
| Allantoin | 5.43 (s) |
| Phenylalanine (Phe) | 7.36 (m) |
| Hippurate (Hip) | 7.87 (m) |

[a] s, singlet; d, doublet; t, triplet; m, complex multiplet; AB, second order.

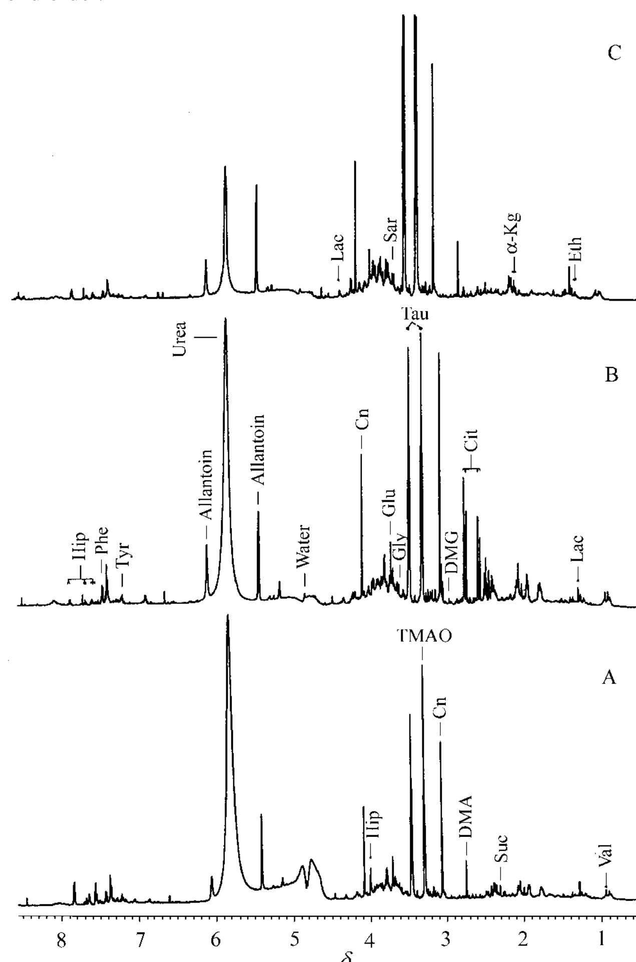


**Figure 1** A comparison of the 600 MHz $^1$H NMR spectra of the urine of 4—8 h time point from Wistar rats (A) control (0.9 % saline), (B) $HgCl_2$ (2 mg/kg body weight) and (C) $CCl_4$ (1.5 mL/kg body weight).

in urinary low molecular weight metabolites. The elevation of amino acids (valine, glutamine and glycine), glucose, and lactic acid level was observed during 0—24 h time course. The similar results were also reported by Gartland *et al.*[2] The elevation of amino acids and glucose in urine exhibited the reabsorbability decline of the renal tubule.[2,14]

Typical 600 MHz $^1$H NMR spectra of urine samples from $Lu(NO_3)_3$-treated rats (2 mg/kg body weight) were shown in Figure 2 for the period −4—0 (A) and 4—8 (B) h after dosing. Examination of the NMR spectra for the $Lu(NO_3)_3$-treated rats with different dosages (2, 10, 100 mg/kg body weight) exhibited that some metabolite levels (glutamine and glycine) varied in common with $HgCl_2$-treated rats, the levels of some amino acids (glycine and glutamine) were elevated. This result may mean that $Lu(NO_3)_3$ can damage proximal tubule.

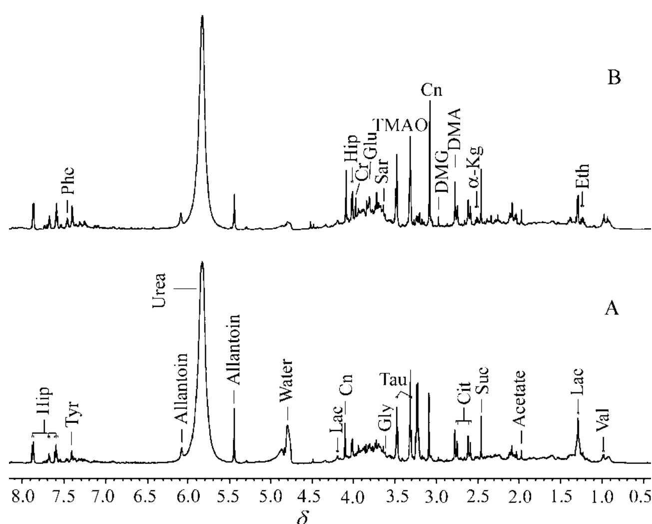


**Figure 2** A comparison of the 600 MHz $^1$H NMR spectra of the urine from a Wistar rat: (A) −4—0 h and (B) 4—8 h after the oral administration of 2 mg $Lu(NO_3)_3$/kg body weight.

$CCl_4$ is a known hepatotoxin causing centrilobular necrosis with fatty liver.[10] In this study, the most obvious change was the elevation of taurine content in the $CCl_4$-treated rats urine at all time intervals. We also found that the intensities of the three Krebs' cycle intermediates, citrate, α-ketoglutarate and succinate were decreased during the time course of 24 h.

Comparison with $CCl_4$, $Lu(NO_3)_3$ also led to an increase of taurine and a decrease in the contents of succinate and α-ketoglutarate during 4—8 h. Taurine is the most important NMR marker for liver damage.[9] Liver is the energy metabolism organ, the change of the concentration of succinate and α-ketoglutarate indicated an inhibition of mitochondria enzymes during Krebs' cycle. These alterations in the amounts of taurine, succinate and α-ketoglutarate from $Lu(NO_3)_3$-treated rat urine indicated that $Lu(NO_3)_3$ ($>2$ mg/kg body weight) treatment can cause liver damage as well as kidney damage.

For the Changle-treated rats (2 mg/kg body weight), the amino acids (glycine, glutamine and taurine) revealed an apparent increase from the control rats after 90 d. Succinate, α-ketoglutarate, dimethylamine (DMA), dimethylglycine (DMG), trimethylamine-*N*-oxide (TMAO) and creatinine (Cn) behaved similarly to the amino acids. However, citrate level was decreased. The slight increase of DMA, DMG and TMAO was observed in the rat urine dosed with $Lu(NO_3)_3$ during0—4 h time interval. DMA, DMG and TMAO were suggested as definite NMR markers to the renal papilla.[2] Therefore, these results presented renal papillary lesion to the rats administrated with higher dose of $Lu(NO_3)_3$ ($>2$ mg/kg body weight).

No detectable changes were observed for the metabolite excretion of the urine from the rats dosed with 0.05 mg of $Lu(NO_3)_3$/kg body weight during the whole experimental period. It may mean that this dose was not high enough to cause any obvious bioeffects in animal body.

## PCA analysis of urine samples

The PR methods provide a novel means of analyzing the data obtained from $^1$H NMR urinalysis after the induction of organ lesions using model toxins.[7,8,15,16] PCA was chosen as a preliminary method of analyzing the $^1$H NMR data for the reason that PCA produced a tighter clustering of points and hence good classifications.[17]

In this paper, the $^1$H NMR spectral profiles of the rats dosed with higher dosage of $Lu(NO_3)_3$ ($>2$ mg/kg body weight) during 4—8 h time interval exhibited the most drastic changes to rats. Therefore, the NMR spectral data of 4—8 h time interval were chosen as PCA input data. From the calculation, the principal components 1 and 2 involved about 80% data variance in each PCA. Thus, a plot of PC1 versus PC2 provided the most efficient two-dimensional representation of the information contained in the data set.

To the acute toxins, we chose the urine spectral data of the period of 0—8 h ($Lu(NO_3)_3$-treated rats, 4—8 h) as PCA data set, while the urine NMR spectral data of Changle-treated rat urine after 90 d were used by PCA.

Each spectral data set using PCA included the data of urinary NMR spectra from $HgCl_2$-, $CCl_4$-, Changle-, and each dosage $Lu(NO_3)_3$-treated rats.

From Figure 3, the tight cluster and clear classification were found to every kind of toxin. Figure 3A showed that $Lu(NO_3)_3$ (0.05 mg/kg) cluster was closer to the control cluster than to other toxins. This result also supported the suggestion that the dosage of 0.05 mg $Lu(NO_3)_3$/kg body weight was not high enough to cause obvious bioeffects in animal body.

From the $^1$H NMR spectral analysis by the NMR markers, it was seen that the dosage of 2 mg/kg caused lesions to rat organs. In the PC map (Figure 3B), $Lu(NO_3)_3$ cluster of 2 mg/kg body weight was discrete from control group. This made a consistent result between PCA and $^1$H NMR spectral analysis.

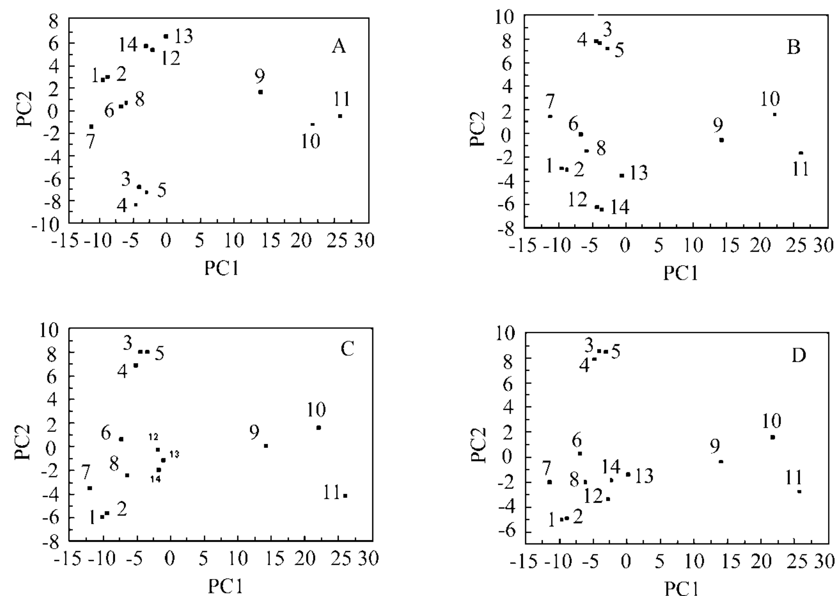


**Figure 3** PC map based on all the $^1H$ NMR descriptors for the urine samples. (A) The $^1H$ NMR descriptors for $HgCl_2$, $CCl_4$, Changle and $Lu(NO_3)_3$ (0.05 mg/kg body weight); (B) the $^1H$ NMR descriptors for $HgCl_2$, $CCl_4$, Changle and $Lu(NO_3)_3$ (2 mg/kg body weight); (C) the $^1H$ NMR descriptors for $HgCl_2$, $CCl_4$, Changle and $Lu(NO_3)_3$ (10 mg/kg body weight); (D) the $^1H$ NMR descriptors for $HgCl_2$, $CCl_4$, Changle and $Lu(NO_3)_3$ (100 mg/kg body weight). 1, 2: control group; 3, 4, 5: $HgCl_2$ (2 mg/kg body weight); 6, 7, 8: $CCl_4$ (1.5 mL/kg body weight); 9, 10, 11: Changle (2 mg/kg body weight after 90 d); 12, 13, 14: $Lu(NO_3)_3$.

However, the higher dosage $Lu(NO_3)_3$ (10 and 100 mg/kg) clusters in the corresponding PC maps (Figures 3C and 3D) were very close to $CCl_4$ cluster. It implied that high dosage of $Lu(NO_3)_3$ can cause similar toxicity to $CCl_4$. $Lu(NO_3)_3$ caused an elevation of taurine level and decrease of succinate, citrate and $\alpha$-ketoglutarate. On the other hand, as a typical liver toxin, $CCl_4$ also led to an elevation of taurine and a decrease in urinary succinate, citrate and $\alpha$-ketoglutarate. The results from $^1H$ NMR spectral analysis and PCA possibly reflected that high dosage of $Lu(NO_3)_3$ (10 and 100 mg/kg body weight) caused more apparent hepatotoxicity than nephrotoxicity and suggested that taurine, succinate, citrate and $\alpha$-ketoglutarate were the good NMR markers for liver damage.

Though some urinary metabolites (DMA, DMG and TMAO) in $Lu(NO_3)_3$-treated rats had similar $^1H$ NMR spectral pattern alterations to those of Changle-treated rats, the distance between $Lu(NO_3)_3$ and Changle clusters in each PC map was still large. To this result, one reason may be that there exist prominent mechanism differences between acute and chronic bioeffects. Another reason possibly was that the concentration change of taurine, succinate, citrate and $\alpha$-ketoglutarate was the dominant alteration, which covered up the slight changes of DMA, DMG and TMAO.

## Conclusions

These results clearly show that the combination of $^1H$ NMR spectroscopy with PR technique is a powerful approach to further the understanding of the biochemical processes associated with different types of toxic injury. With the development of NMR field strengths, 600 MHz will produce increased spectral complexity and sensitivity and hence yield greater metabolic information. Therefore, the considerable care is required to extract meaningful biological data for the interpretation and analysis steps. The data analysis methods used here provided a good classification in terms of site of toxicity, severity of organ lesion and difference between acute and chronic toxicity.

The reduction of whole $^1H$ NMR spectral data sets to high-dimensionality metabolite descriptors may provide additional discrimination in the toxicological and clinical studies in the future.

## References

1 Nicholson, J. K.; Wilson, I. D. *Prog. Nucl. Magn. Reson. Spectrosc.* **1989**, *21*, 449.

2 Gartland, K. P. R.; Bonner, F. W.; Nicholson, J. K. *Mol. Pharmacol.* **1989**, *35*, 242.

3 Nicholson, J. K.; Higham, D. P.; Timbrell, J. A.; Sadler, P. J. *Mol. Pharmacol.* **1989**, *36*, 398.

4 Foxall, P.; Bending, M.; Gartland, K. P. R.; Nicholson, J. K. *Hum. Toxicol.* **1989**, *9*, 491.

5 Sanins, S. M.; Timbrell, J. A.; Elcombe, C.; Nicholson, J. K. *Methodol. Surv. Biochem. Anal.* **1988**, *18*, 375.

6 Bales, J. R.; Bell, J. D.; Nicholson, J. K.; Sadler, P. J.; Timbrell, J. A.; Hughes, R. I.; Bennet, P. N.; Williams, R. *Magn. Reson. Med.* **1988**, *6*, 301.

7 Waters, N. J.; Holmes, E.; Williams, A.; Waterfield, C. J.; Farrant, R. D.; Nicholson, J. K. *Chem. Res. Toxicol.* **2001**, *14*, 1401.

8 Holmes, E.; Nicholis, A. W.; Lindon, J. C.; Ramos, S.;

Spraul, M.; Neidig, P.; Conner, S. C.; Connelly, J.; Damment, S. J. P.; Haselden, J.; Nicholson, J. K. *NMR Biomed.* **1998**, *11*, 235.
9 Feng, J.-H.; Li, X.-J.; Pei, F.-K.; Chen, X.; Li, S.-L.; Nie, Y.-X. *Anal. Biochem.* **2002**, *301*, 1.
10 Recknagel, R. O. *Pharmacol. Rev.* **1967**, *19*, 145.
11 Feng, J.-H.; Li, X.-J.; Pei, F.-K.; Nie, Y.-X. *Chin. J. Chem.* **2003**, *21*, 320.
12 Xu, L. *Methods of Chemometrics*, Science Press, Beijing, **1995** (in Chinese).
13 Lindon, J. C.; Nicholson, J. K.; Everett, J. R. *Annu. Rep. NMR Spectrosc.* **1999**, *38*, 1.
14 Nicholson, J. K.; Timbrell, J. A.; Sadler, P. J. *Mol. Pharmacol.* **1985**, *27*, 644.
15 Gartland, K. P. R.; Beddell, J. C.; Nicholson, J. K. *Mol. Pharmacol.* **1991**, *39*, 629.
16 Anthony, M. L.; Sweatman, B. C.; Beddell, C. R.; Lindon, J. C.; Nicholson, J. K. *Mol. Pharmacol.* **1994**, *46*, 199.
17 Holmes, E.; Bonner, F. W.; Sweatman, B. C.; Lindon, J. C.; Beddell, C. R.; Rahr, E.; Nicholson, J. K. *Mol. Pharmacol.* **1992**, *42*, 922.

(E0311245 LI, L. T.)