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Comparison of metabolic profiles from serum from hepatotoxin-treated rats by nuclear-magnetic-resonance-spectroscopy-based metabonomic analysis

Huifeng Wu, Xiaoyu Zhang, Xiaojing Li, Zhongfeng Li, Yijie Wu, Fengkui Pei *

Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, People's Republic of China

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Abstract

Hepatotoxicities were induced in rats using α-naphthylisothiocyanate (ANIT), carbon tetrachloride (CCl_4), and hydrazine (HYD). Male Wistar rats were treated with three typical hepatotoxins, and serum samples were collected after 48 h. Biochemical effects of these toxins on plasma composition were evaluated by high-resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy of serum. The biochemical effects of CCl_4 were characterized by an elevated level of 3-D-hydroxybutyrate (HB), acetoacetate (Aca), and creatinine (Cn) in serum, and ANIT led to increases in the amounts of low-density lipoprotein (LDL), alanine, acetate, glycoprotein, succinate, Cn, acetone, 3-D-hydroxybutyrate, and Aca. For the HYD-treated group, LDL, HB, acetate, and Cn were obviously increased in serum. The region δ 0.0–10.0 of each spectrum was segmented into 0.04 ppm. The area under the spectrum was calculated for each segmented region and expressed as an integral value. After removal of the water signal (δ 4.6–5.0) the remaining 235 intensity-related descriptors were used for the pattern recognition analysis. Principal component analysis was used to visualize the similarities and differentiations in biochemical profiles of serum from the rats treated with various hepatotoxins. This work showed the power of the combination of NMR and pattern recognition for the study of biochemical effects of xenobiotics.

Keywords: NMR; Hepatotoxin; Serum; Pattern recognition; Metabolite

NMR spectroscopy of biofluids has brought new chemistry into life science and clinical medicine, and the NMR-spectroscopy-based approach has been successfully applied to studies of biochemical effects and inborn diseases with the advent of high-field NMR spectrometers [1]. In recent years, high-resolution NMR has been used for the rapid multicomponent analysis of low-molecular-weight compounds in biofluids, including urine, bile, cerebrospinal fluid, saliva, milk, and synovial fluid for clinical diagnosis of inborn diseases and investigation of biochemical effects of drugs [2–5]. ^{1}H NMR spectroscopy of biofluids presents comprehensive biochemical profiles of low-molecular-weight metabolites reflecting the biochemical effects caused by xenobiotics. These biochemical profiles can be obtained with minimal sample preparation and without destruction to the samples. Serum contains almost all of the low-molecular-weight compounds, and ^{1}H NMR spectra of serum from animals under similar physiological conditions are highly reproducible, which is useful in the diagnosis of metabolic and diseased states. Examination of ^{1}H NMR spectra of urine from rats dosed with toxins has shown that much of the information is necessary to classify and assess metabolic effects of compounds biochemically [6]. Moreover, recent developments in hardware have led to an increase in the complexity of biofluid ^{1}H NMR

* Corresponding author. Fax: +86 431 5685653.
E-mail address: peifk@ciac.jl.cn (F. Pei).

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spectra due to the improvements in the sensitivity and relative resolution of NMR spectrometers at higher magnetic field strengths [2]. As a result of the complexity of ^{1}H NMR spectra, many subtle changes in metabolite resonances might be overlooked within the natural biological variation. Thus the complexity of the ^{1}H 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 ^{1}H NMR spectra.

The application of ^{1}H NMR spectroscopy to the study of the metabolic composition of biofluids combined with pattern recognition to (PR)[1] classify the NMR-derived data has led to a "metabonomic" approach to biochemical assessment [7]. This technique can be applied to the identification of biomarkers of toxicity and disease [2,8], monitoring of time-related metabolic perturbations in biofluids and tissues following toxic insult [7,9,10], and metabolic characterization of physiological variance in humans and mild physiological stress. PR analysis is performed in a multidimensional parameter space using dimension-reduction techniques to maximize information from complex ^{1}H NMR spectra [11]. Principal components analysis (PCA) is a widely applicable dimension-reduction method in which the NMR spectra are reduced to a set of peak intensity descriptors and analyzed to identify similarities and differences between the samples from control and drug-treated animals. A PCA program is performed on a computer with each principal component (PC) being a linear combination of the original variables with appropriate weighting coefficients. 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 [11].

α-Naphthylisothiocyanate (ANIT), carbon tetrachloride (CCl_4), and hydrazine (HYD) are three known hepatotoxins [12]. Of these hepatotoxins, ANIT is a model hepatotoxin that induces intrahepatic cholestasis in a reproducible and dose-dependent manner in experimental animals [13]. HYD is a well-documented hepatotoxin that causes steatosis without necrosis in the animal liver [14], CCl_4 causes centrilobular necrosis with fatty liver [15]. The metabonomic studies on the hepatotoxicities of ANIT, CCl4, and HYD have been reported by the J.K. Nicholson group [6,16,17] based on the NMR spectroscopy of urine, plasma, and tissues. However, the integrated NMR-spectroscopic-based metabonomics analysis and comparison for these classical hepatotoxins have not been carried out. The aim of this study was to investigate and compare the biochemical profiles of the metabolites in the serum from different hepatotoxin-treated rats using ^{1}H NMR spectroscopy. Scores plots of the PCs were constructed to visualize the similarities and differences in the biochemical profiles, and the NMR spectral regions of biomarkers were identified from the values of the PC loadings (which indicated the importance of each variable to the separation).

[1] *Abbreviations used:* NMR, nuclear magnetic resonance; CPMG, Carr–Purcell–Meiboom-Gill; ANIT, α-naphthylisothiocyanate; HYD, hydrazine; HB, 3-D-hydroxybutyrate; Aca, acetoacetate; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; Cn, creatinine; PR, pattern recognition; PCA, principal components analysis; FIDS, free-induction decays; TCA, tricarboxylic acid.

Materials and methods

Samples collection and storage

Twenty Male Wistar rats (weighing 250–300 g) were divided into four groups ($n=5$) and housed individually in metabolism cages which allowed free access to food [Experimental Animal Diet Factory, Jilin, P.R. China; Production lience SCXK(JI 2003-008)] and water under controlled conditions (temperature, humidity, and a 12-h light–dark cycle). Each rat received an oral dose of either CCl_4 (1.5 ml/kg body weight), ANIT (150 mg/kg body weight), HYD (150 mg/kg body weight), or saline (0.9%, $n=5$). After 48 h, serum samples of the blood from the sacrificed rats were separated by ultrafiltration (10-kDa cutoff) and centrifugation (3000 rpm, 10 min at 4 °C) and stored frozen at −70 °C until NMR spectroscopic analysis.

^{1}H NMR measurement

Fifty microliters buffer solution (0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4, pH 7.0) was mixed with 400 μl urine to minimize variations in the pH of the urine samples.

Proton NMR measurements of serum samples (50 μl D_2O was added for locking signal) were recorded on a Bruker-Av 600-MHz spectrometer at 298 K. Water signals and the broad protein resonances were suppressed by a combination of presaturation and the Carr–Purcell–Meiboom–Gill (90°-(τ-180°- τ)$_n$-acquisition) ($\tau=200$ μs, $n=100$) pulse sequence. Thirty-two free-induction decays (FIDs) were collected into 32 K data points with relaxation delay 6 s and flip angle 90°. The FIDs were weighted by an exponential function with a 0.3-Hz line-broadening factor prior to Fourier transformation. Phase and baseline correction were made for each spectrum. A spectral width of 8992.806 Hz and an acquisition time of 0.911 s were used. All spectra were referenced to the CH_3 resonance of creatinine at δ 3.05.

Data reduction and pattern recognition analysis of ^{1}H NMR spectra

Each NMR spectrum was reduced to 245 integrated regions of equal width (0.04 ppm) corresponding to the