An Evaluation of Acute Hydrogen Sulfide Poisoning in Rats through Serum Metabolomics Based on Gas Chromatography-Mass Spectrometry

Meiling Zhang, Mingjie Deng, Jianshe Ma, and Xianqin Wang*

Analytical and Testing Center of Wenzhou Medical University; Wenzhou 325035, China.

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Hydrogen sulfide (H₂S) is the second leading cause of toxic-related deaths in the operational site. Its main target organs of toxic effects are the central nervous system and respiratory system. In this study, we developed a serum metabolomic method, based on gas chromatography-mass spectrometry (GC/MS), to evaluate the effect of acute poisoning by hydrogen sulfide on rats. Pattern recognition analysis, including both principal component analysis (PCA) and partial least squares-discriminate analysis (PLS-DA), revealed that acute hydrogen sulfide poisoning induced metabolic perturbations. Compared to the control group, the level of urea, glucose, glyceryl stearate in rat serum of the poisoning group increased after two hours, and the level of glucose, docosahexaenoic acid, glyceryl stearate and arachidonic acid in rat serum of the poisoning group increased after 48h, while the L-tyrosine, galactose, L-tyrosine levels decreased. Our results indicate that metabolomic methods based on GC/MS may be useful to elucidate acute hydrogen sulfide poisoning through the exploration of biomarkers.

Key words metabolomics; GC/MS; acute hydrogen sulfide poisoning

Experimental

Chemicals and Reagents N-Methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) and trimethylchlorosilane (TMCS) were purchased from Sigma-Aldrich in Germany. Methylhydroxyamine hydrochloride and pyridine were purchased from Aladdin Industrial, Inc. in China. HPLC-grade acetonitrile and n-heptane were purchased from Tedia Reagent Company in the U.S.A.

Instrumentation and Conditions Agilent Technologies, Inc., located in the U.S.A., supplied the Agilent 6890N-5975B GC/MS. The column was HP-5MS (0.25 mm×30 m×0.25 mm, Agilent, U.S.A.). The GC oven was initially set at 80°C and was kept at this temperature for 5 min. The temperature was then gradually increased to 260°C at a rate of 10°C/min, and then kept at 260°C for 10 min. MS detection was conducted first in EI mode with electron energy of 70eV, then in full-scan mode with m/z of 50–550, and finally, by splitless mode injection.

Animal Treatment and Sample Collection Male Sprague-Dawley rats, with weights of 250±20 g, were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd. The animal license number was SCXK (Shanghai) 2012-0005. All fifteen rats were housed at Laboratory Animal Research Center of Wenzhou Medical University under the controlled conditions of a temperature of 22°C and a natural light-dark cycle. All experimental procedures were conducted according to the Institutional Animal Care Guidelines and were approved as ethical by the Administration Committee of Experimental Animals at the Laboratory Animal Center of Wenzhou Medical University.

Fifteen male Sprague-Dawley rats were randomly divided into two groups, the control group (n=8) and the acute hydrogen sulfide poisoning group (n=7). To create a model of acute hydrogen sulfide poisoning, the rats were placed in triads in an infected ark that included the hydrogen sulfide detector and then passed into a certain concentration of hydrogen sulfide gases. The rats in the acute hydrogen sulfide poisoning group were exposed to 200 ppm of H₂S for 2 and 48h. The rats in the control group were subjected to similar conditions, but did not experience exposure to H₂S. The rats in both groups were allowed to eat and drink ad libitum during the exposure period.

Blood samples of 300μL were drawn from the rats from the control group and acute hydrogen sulfide poisoning group after 2 and 48h, respectively. The blood samples were placed
in a water bath of 37°C for 0.5 h and then centrifuged for 10 min at 8000 rpm. The sera were separated and kept at a temperature of −80°C until analysis.

**Sample Preparation** Two hundred and fifty microliters of acetonitrile was added to 100 µL of serum, kept in an ice-bath for fifteen minutes, and then 10000 × g were centrifuged for 10 min at 4°C. One hundred and fifty microliters of the supernatant was transferred to a GC vial and evaporated to dryness under a stream of nitrogen gas. Methoximation was carried out at 70°C for 24 h after 50 µL of methylhydroxylamine hydrochloride (15 mg/mL in pyridine) was added. Fifty microliters of MSTFA (with 1% TMCS as the catalyst) was added and kept at 70°C for another hour, and then vortexed after adding 150 µL n-heptane.

**Data Analysis** The GC/MS data was exported into Microsoft Excel, with the peaks normalized to the total sum of spectrum prior to multivariate analyses. The resulting data was processed through principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) using SIMCA-P 12 software (Umetrics, Umea, Sweden). PLS-DA was used to revealed the differences in serum composition of two different groups, the corresponding loading plots, where each point represents a metabolite, were used to identify which variables contributed to the separation of the samples on the scores plot.

**Statistical Analysis** Statistical analysis was carried out using SPSS software (Version 13.0, SPSS). Independent samples t-test was applied in order to detect significant differences in all metabolites between two groups. A p value of <0.05 was considered statistically significant.

**Results and Discussion**

**Analytical Characteristics of Global Profiling Methods**

Figure 1 provides the typical metabolic profiles of serum acquired through GC-MS technique. Metabolic profile data pretreatment resulted in a final dataset consisting of forty-six metabolic features from GC-MS analyses. The five QC samples first investigated the reproducibility of the metabolic features. The GC-MS analysis showed that more than 70% of the forty-six metabolic features had a CV% (coefficient of variance) of no more than 30%. The endogenous metabolites in the serum were identified using the NIST 2005 mass spectrometry database.

**Metabolomics Study** Principal component analysis of the results of acute hydrogen sulfide poisoning provided an unsatisfactory separation of data between the acute hydrogen sulfide poisoning group and the control group. To improve the classification of the acute hydrogen sulfide poisoning group and control group, we subsequently used a multivariate PLS-DA classification method to maximize metabolite variations and to identify the metabolites responsible for such variations.

In order to explore the metabolic profile changes of acute H₂S poisoning in rats during different time periods, we com-
pared the GC-MS spectrum of PLS-DA of the rats in the acute H₂S poisoning group after 2 h and after 48 h with the rats in the control group (Fig. 2). Figure 2A PLS-DA score chart ($R^2=0.715$, $Q^2=0.998$) showed that the first principal components of the rats in the acute H₂S poisoning group after 2 h were distinguished from the rats in the control group. The corresponding load diagram (Fig. 2B) showed the major metabolites that separated PI–2 h from control group were urea, glucose, glycercyl stearate, l-valine, galactose, l-tyrosine.

As demonstrated in Fig. 2C, the PLS-DA score ($R^2=0.73$, $Q^2=0.995$) showed that the acute H₂S poisoning after 48 h and the control group were separated. The corresponding load diagram (Fig. 2D) better distinguishes the metabolites of the two groups. Figure 2D showed the major metabolites that separated PI–2 h from control group were glucose, docosahexaenoic acid, glycercyl stearate, arachidonic acid l-valine, galactose, l-tyrosine.

Changes in Metabolite The changes in metabolites between poisoning groups and their control group were shown in Table 1. Compared to the control group, the level of urea, glucose, glycercyl stearate in the rat serum of the acute H₂S poisoning group increased after two hours, while the l-valine, galactose, l-tyrosine level decreased. And the level of glucose, docosahexaenoic acid, glycercyl stearate and arachidonic acid in rat serum of the acute H₂S poisoning group increased after forty-eight hours group, while the l-valine, galactose, l-tyrosine levels decreased.

In previous study, it should need to pay close attention to changes in the plasma concentration to avoid drug interactions that may occur, when take drugs by intravenous that metabolized through CYP1A2 and CYP2B6 enzyme after chronic hydrogen sulfide poisoning. In this study, we found glucose, glycercyl stearate, l-valine, galactose, l-tyrosine were in different level between acute H₂S poisoning and control group. These finding may be useful for new evidences in forensic case.

Conclusion These biomarkers (glucose, glycercyl stearate, l-valine, galactose, l-tyrosine) were the additional evidence that supports already-known evidence sulfide and thiolsulfate, a metabolite of sulfide. We demonstrated that metabonomic methods based on GC/MS could provide a useful tool for exploring biomarkers to elucidate poisoning discovered in forensic toxicology.

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