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

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Hydrogen sulfide (H_2S) is the second leading cause of toxin-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 48 h, while the L-valine, 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

Hydrogen sulfide is a colorless, flammable, and extremely hazardous gas that naturally occurs in hot springs, crude petroleum, and natural gas. The bacterial decomposition of organic materials and human or animal waste also generates hydrogen sulfide.

Forensic toxicology has shown that hydrogen sulfide is one of the most toxic gases.¹⁾ It has been reported that if 500 ppm of hydrogen sulfide is in the air, inhalation will rapidly lead to severe poisoning, and that 900 ppm of hydrogen sulfide in the air will cause fatality immediately.²⁾ Accidents have been reported in chemical processing plants,^{3,4)} in sewage disposal facilities,⁵⁾ and after the ingestion of sulfur products.⁶⁾ It is difficult to quantify original hydrogen sulfide levels, because the gas produced in these situations is unstable and rapidly metabolized as thiosulfate and sulfate through hepatic oxidation.⁷⁾ Toxicological analyses of sulfide and thiosulfate, a metabolite of sulfide, in blood and urine of the victims were made after fatal hydrogen sulfide poisoning in the forensic case, using the extractive alkylation technique combined with gas chromatography/mass spectrometry.^{8,9)} In our previous study, “Cocktail” probe drugs approach is used to evaluate the induction or inhibition effects of chronic hydrogen sulfide poisoning on the activities of rats cytochrome P450 isoforms such as CYP1A2, CYP2C9, CYP2B6 and CYP2D6, as a result

chronic hydrogen sulfide poisoning could induce the activity of CYP1A2 and CYP2B6 in rats.¹⁰⁾

“Metabonomics” has been defined as “the quantitative measurement of the time-related, multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification.”¹¹⁾ Metabolomics is a scientific study that aims to identify and quantify the levels of all metabolites; it has been applied widely in many areas, such as medicine, biology, and food science.^{12–15)} GC-MS is an analytic method used frequently in metabolomics studies to examine low-molecular-weight metabolites, because GC-MS provides heightened equipment stability and user-friendly tools for data analysis.^{16,17)} This paper evaluates, through the use of GC/MS-based metabolomics, the effect of acute hydrogen sulfide poisoning on the metabolic profiles of rats.

Experimental

Chemicals and Reagents *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) and trimethylchlorosilane (TMCS) were purchased from Sigma-Aldrich in Germany. Methylhydroxylamine 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 70 eV, 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 H_2S for 2 and 48 h. The rats in the control group were subjected to similar conditions, but did not experience exposure to H_2S . The rats in both groups were allowed to eat and drink *ad libitum* except during the exposure period.

Blood samples of 300 µL were drawn from the rats from the control group and acute hydrogen sulfide poisoning group after 2 and 48 h, respectively. The blood samples were placed

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in a water bath of 37°C for 0.5h and then centrifuged for 10min at 8000rpm. The serums 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 μ L of serum, kept in an ice-bath for fifteen minutes, and then 10000 $\times g$ were centrifuged for 10min 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 24h after 50 μ L of methylhydroxylamine hydrochloride (15mg/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 μ L *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)

Fig. 1. GC-MS Total Ion Chromatogram of Rat Serum

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 (Version13.0, 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-

Fig. 2. PLS-DA Score Results of Rat Serum Samples, Acute Hydrogen Sulfide Poisoning after 2h Group (PI-2h), Acute Hydrogen Sulfide Poisoning after 48h Group (PI-48h), Control Group (CON), B, D Were the Corresponding Load Diagram of A, C

Table 1. Summary of the Changes in Relative Levels of Metabolites in Rat Serum Indicated by the PLS-DA Loading Plots and Statistical Analysis

ID	Retention time	Metabolites compound	PI vs. CON	
			2h	48h
1	6.542	Lactic acid	↑**	↑**
2	7.425	L-Alanine	—	↓*
3	8.216	Ethanedioic acid	↓**	↓**
4	8.587	Butanoic acid	—	—
5	10.151	L-Valine	↓**	↓**
6	10.210	Urea	↑**	↑*
7	10.738	Phosphate	↓**	↓**
8	14.192	Pyrrolidone carboxylic acid	—	↓**
9	18.671	Galactose	↓**	↓**
10	18.775	D-Glucose	↑**	↑**
11	19.109	L-Tyrosine	↓**	↓**
12	19.174	Ribose	—	↑**
13	19.568	Palmitelaidic acid	—	—
14	19.752	Hexadecanoic acid	↓*	—
15	20.491	Myo-inositol	—	—
16	21.282	9,12-Octadecadienoic acid	↑**	↑**
17	21.322	Oleic acid	↑**	↑**
18	22.639	Arachidonic acid	↑**	↑**
19	25.169	Docosahexaenoic acid	↑*	↑**
20	26.149	Glyceryl stearate	↑**	↑*
21	28.927	Cholesterol	—	↑**

Marks indicate the direction of the change, i.e., ↓ for decrease, ↑ for increase, — for no change. * $p < 0.05$ and ** $p < 0.01$, as indicated by the statistical analysis t -test.

pared the GC-MS spectrum of PLS-DA of the rats in the acute H₂S poisoning group after 2h and after 48h with the rats in the control group (Fig. 2). Figure 2A PLS-DA score chart (R²X=0.715, Q²Y=0.998) showed that the first principal components of the rats in the acute H₂S poisoning group after 2h were distinguished from the rats in the control group. The corresponding load diagram (Fig. 2B) showed the major metabolites that separated PI–2h from control group were urea, glucose, glyceryl stearate the L-valine, galactose, L-tyrosine.

As demonstrated in Fig. 2C, the PLS-DA score (R²X=0.73, Q²Y=0.995) showed that the acute H₂S poisoning after 48h group 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–2h from control group were glucose, docosahexaenoic acid, glyceryl stearate, arachidonic acid L-valine, galactose, L-tyrosine.

Changes in Metabolite The changes of metabolites between poisoning groups and their control group were shown in Table 1. Compared to the control group, the level of urea, glucose, glyceryl 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, glyceryl 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.¹⁰⁾ In this study, we found glucose, glyceryl 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, glyceryl stearate, L-valine, galactose, L-tyrosine) were the additional evidence that supports already-known evidence sulfide and thiosulfate, 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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