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# A HILIC–MS/MS method for the simultaneous determination of seven organic acids in rat urine as biomarkers of exposure to realgar

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ABSTRACT

Realgar ($As_4S_4$) is a traditional medicine used in China and Europe for thousands of years. As an arsenical, the toxicity from realgar has raised public concern. Several organic acids in urine are found to be potential biomarkers of realgar exposure, including taurine, citric, glutamic, lactic, pyruvic, succinic and uric acid. In this study, using hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS), a rapid and sensitive method was developed to separate and quantify these compounds in urine. A ZIC®-HILIC column was used for the separation at an isocratic condition of acetonitrile and 10 mM ammonium acetate in water. Analytes were detected in multiple-reaction monitoring with negative ionization mode, using ibuprofen as internal standard. Good line arities ($R^2 > 0.996$) were obtained for all analytes with the limits of detection from 0.2 to 0.7 μg/mL. The intra-day and inter-day accuracy ranged from 89.1 to 104.4% and the relative standard deviation (RSD) did not exceed 15.0%. The recovery was more than 80%with RSD less than 14.0%. The validated method was applied to analyze the urine samples of control and reaglar treated rats, and significant changes of these organic acids were observed.



## 1. Introduction

Realgar, an ore crystal containing more than 90% tetra-arsenic tetra-sulfide ($As_4S_4$), has been used as a traditional medicine in China and Europe for more than 1500 years [1]. The therapeutic uses of realgar and realgar-containing traditional medicines range widely from common colds, tonsillitis, abdominal pains, spasms, sedation, ulcers, heat stroke, coma, and delirium to malignancies [1,2]. The daily dosage recommended by the Chinese Pharmacopoeia (2010 edition) for realgar is 0.05–0.1 g per person. Almost all registered oral realgar-containing formulae can be acquired in China without the need of prescriptions and in the absence of special warning for usage. Although realgar is poorly soluble in water and thus is considered to be less poisonous than other arsenicals, e.g. arsenic trioxide, the arsenic contained in realgar is still a potential toxic element. Indeed, a number of realgar poisoning cases have been reported in the past decades [3–5].

There have been some investigations detailing the impact of realgar or realgar-containing medicines [6–10]. Most research has evaluated the safety of realgar based on the blood or urinary arsenic levels, while less attention has been paid to the systemic metabolic consequences of realgar induction in a whole living biosystem. Recently, ametabolomic study by Wei et al. [10] has reported several organic acids as possible biomarkers for realgar exposure. Using $^1$H NMR spectra with reference to sodium-3-(trimethylsilyl)propionate, they have identified and quantified these biomarkers in urine samples of healthy control and realgar treated rats. This motivated us to develop a fast and more sensitive method to simultaneously quantify the seven organic acids (taurine, citric, glutamic, lactic, pyruvic, succinic and uric acid) in rat urine samples.

Citric, glutamic, lactic, pyruvic and succinic acid are essential components in the energy metabolism pathways (glycolysis, Kreb's cycle, lipid metabolism, etc.). These metabolic routes not only provide reducing power to the cell, but also supply important precursors for the synthesis of other biomolecules. Taurine is a final product of sulfur-containing amino acid metabolism in mammals. Its main function in the body is the conjugation of cholesterol into bile acids, changing cholesterol's solubility and enabling its excretion [11,12]. These organic acids are related to the realgar induced biochemical pathways perturbation [9]. Uric acid, which Wei et al. did not include in their study [10], was included here as an analyte. Since the uric acid concentration corresponds closely to kidney damage and realgar is able to cause injury in kidney, the level of uric acid should be monitored in urinary biomarker analysis of exposure to realgar.

So far, most of the methods developed to determine these organic acids have been based on liquid chromatography

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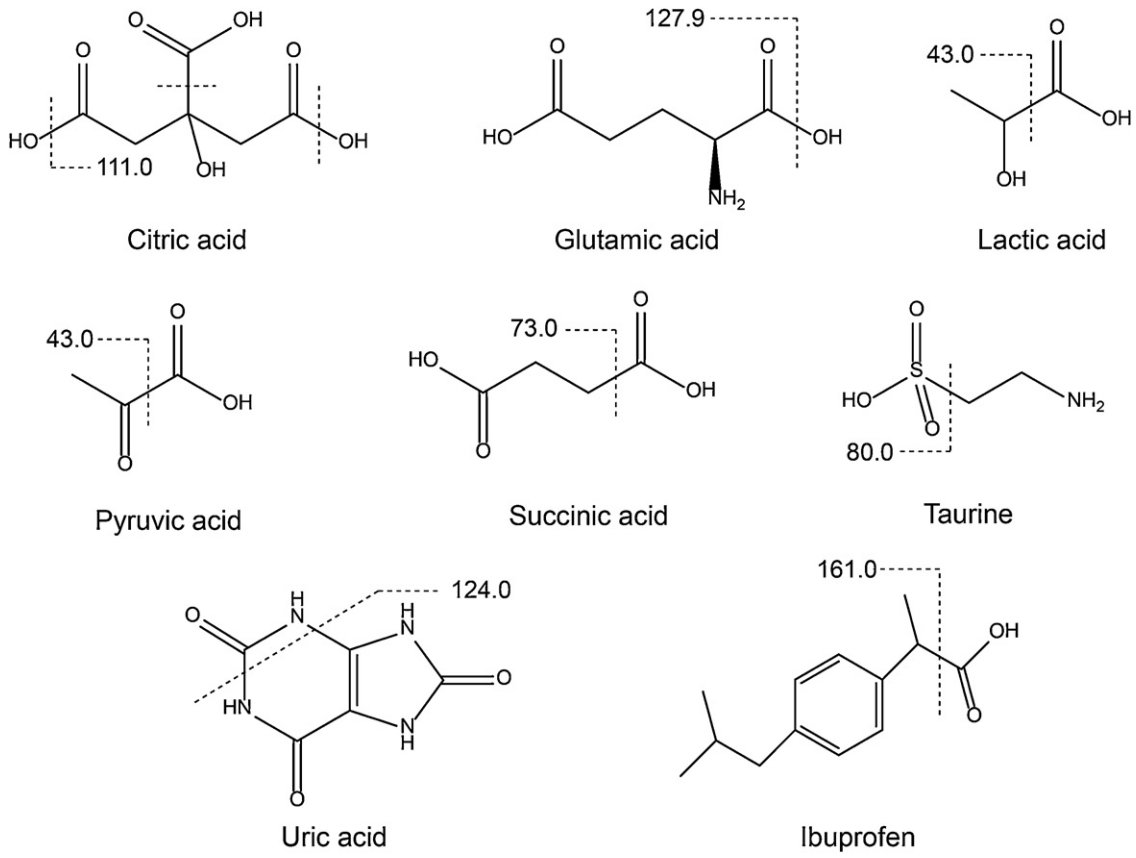


**Fig. 1.** Structures and possible fragmentations of each analyte and IS.

(LC) [13–16], capillary electrophoresis (CE) [17–20], and gas chromatography–mass spectrometry (GC–MS) [21–24]. In traditional reserved-phase HPLC, these endogenous acids are too polarto obtain a good retention as well as a good separation in chromatographic column. Although CE can achieve a good separation of the metabolites, it lacks robustness for routine analysis of biological samples. GC–MS is indeed a durable and sensitive method, but it often requires sample derivatization and extensive sample preparation. Recently, some works have employed ultra high performance liquid chromatography–mass spectrometry (UPLC–MS) to analyze metabolites in biological samples [25,26]. For example, Buescher et al. [25] presented a 25 min ion-pairing UPLC–MS/MS method for determination of 138 compounds in primary metabolism including the seven organic acids we investigated. However, UPLC–MS/MS system is rather costly and contaminations of the ion pairing agent tributy lamine are persistent and difficult to remove. Hydrophilic interaction liquid chromatography (HILIC) with an amino propyl stationary phase has exhibited good flexibility and coverage for the separation of polar metabolites [27–29]. Moreover, the HILIC column can be used on the common LC–MS/MS system without any modification. To the best of our knowledge, no work has been reported to simultaneously determine these seven urinary organic acids using HILIC–MS/MS.

In this study, we present a HILIC–MS/MS method for simultaneous quantification of seven organic acids (taurine, citric, glutamic, lactic, pyruvic, succinic and uric acid) in urine matrices as biomarkers of realgar exposure. It eliminates laborious sample preparations such as liquid–liquid extraction, and also reduces the errors introduced by the derivatization procedures. The key parameters of separation and detection were systematically investigated and optimized. The method has been successfully applied to analyze the urine samples of control and realgar treated rats.

## 2. Experimental

### 2.1. Chemicals and reagents

Realgar was obtained from Jiangsu Province Administration of Medicine, with realgar content > 90%. X-ray diffraction spectrum analysis of realgar suggested that 99% of the arsenic present in the realgar is in the form of $As_4S_4$. Seven standards (Fig. 1), including citric acid, glutamic acid, lactic acid, pyruvic acid, succinic acid, taurine anduric acid, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ibuprofen (Fig. 1), used as the internal standard (IS), was obtained from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China).

Acetonitrile and methanol of HPLC grade were purchased from Merck (Darmstadt, Germany). Analytical grade formic acid, acetic acid, ammonium formate and ammonium acetate were obtained from Nanjing Chemical Reagent Co. Ltd. (Nanjing, PR China). Sodium carboxymethylcellulose (CMC-Na) and sodium azide were obtained from the Sinopharm Chemical Reagent Co. Ltd. (Shanghai, P.R. China). Water was distilled twice before use.

### 2.2. Apparatus and analytical conditions

All analyses were performed on the Finnigan™ TSQ Quantum Discovery MAX™ LC–MS/MS system (Thermo-Fisher Scientific, CA, USA). A ZIC®-HILIC column (250 mm × 4.6 mm, 5 μm) from Merck (Darmstadt, Germany) and the Finnigan Surveyor liquid chromatography system were used for the separation. The column temperature was maintained at 45 °C with an injection of 10 μL. Mobile phase A consisted of acetonitrile while mobile phase B consisted of 10 mM ammonium acetate in redistilled water. The isocratic program was 70% A and 30% B at a flow rate of 0.5 mL/min. The run time for each injection was 14 min.

**Table 1**
MRM parameters for each analyte and IS in ESI negative mode.

| Analyte | Monoisotopic mass (amu) | Q1 mass (amu) | Q3 mass (amu) | Collision energy (V) | Tube lens (V) |
|---|---|---|---|---|---|
| Citric acid | 192.0270 | 191.0 | 111.0 | 15 | 44 |
| Glutamic acid | 147.0532 | 146.1 | 127.9 | 12 | 58 |
| Lactic acid | 90.0317 | 89.0 | 43.0 | 12 | 50 |
| Pyruvic acid | 88.0160 | 87.0 | 43.0 | 7 | 24 |
| Succinic acid | 118.0266 | 117.0 | 73.0 | 12 | 45 |
| Taurine | 125.1469 | 124.0 | 80.0 | 30 | 76 |
| Uric acid | 168.0283 | 167.0 | 124.0 | 16 | 84 |
| Ibuprofen (IS) | 206.1307 | 205.0 | 161.0 | 10 | 45 |

After chromatographic separation, column effluent was directed to a TSQ Quantum Discovery mass spectrometer equipped with an ESI interface at the rate of 0.2 mL/min via a T-union splitting. The mass spectrometer was operated in negative mode with multiple-reaction monitoring (MRM). Electrospray ionization parameters were as follows: spray voltage 4000 V, sheath gas pressure 30 arbitrary units, aux gas pressure 7 arbitrary units, ion sweep gas pressure 5 arbitrary units, capillary temperature 250 °C. Tube lens voltage, collision energy and fragment ions were optimized individually for all analytes (Table 1). Ion optics were set to 0.5 amu Q1 resolution, 0.5 amu Q3 resolution, 0.01 amu scan width and 0.1 s scan time. Mass spectra and chromatograms were acquired and processed with Xcalibur software version 2.0 (Thermo-Fisher Scientific, CA, USA).

### 2.3. Standard and working solutions preparation

Stock solutions of citric acid, glutamic acid, lactic acid, pyruvic acid, succinic acid, taurine and ibuprofen were prepared in methanol. Stock solution of uric acid was prepared in aqueous ammonia (0.002 mM) to dissolve well. All the stock solutions were stored at 4 °C for no more than 2 weeks. Working solutions were prepared by diluting the stock solutions of each standard with acetonitrile, resulting in different concentrations of 2–3000 μg/mL. Quality control (QC) samples were prepared by spiking urine with low, medium and high concentrations of standards (as referred in Table 3), respectively. All working solutions and QC samples were prepared daily.

### 2.4. 2.4.Sample preparation

Shortly before use, the urine samples were thawed at room temperature and then diluted four times using redistilled water and centrifuged at 14,000 × *g* for 10 min. A total of 400 μLof supernatant was mixed with 50 μL of IS (10 μg/mL) and 550 μL of acetonitrile containing 10 mM ammonium acetate. Then, the sample was filtered through a 0.22 μm membrane filter and analyzed by HILIC–MS/MS.

### 2.5. Method validation

Calibration curves were constructed by spiking standards on top of endogenous levels in pooled urine samples. Three samples were prepared for each concentration and averages were used to make calibration curves. Each calibration equation was fitted by the linear regression equation $y = mx + b$, where $y$ = (the peak area of analyte in spiked urine – the peak area of analyte in non-spiked urine)/the peak area of IS, $x$ = the concentration of the spiked analyte. Due to the analytes are endogenous in urine, the limit of detection (LOD) and limit of quantification (LOQ) were estimated by calculating the standard error of the intercept ($Sb$) on the calibration curves. The LOD and LOQ were expressed as 3.3 and 10 times the $Sb/m$, respectively.

The intra-day precision and accuracy were evaluated by replicate analysis ($n = 6$) of QC samples at low, medium and high concentration levels. The inter-day precision and accuracy were conducted during routine operation of the system over a period of five consecutive working days. The precision was expressed as the relative standard deviation (RSD), and the accuracy was evaluated by the percentage ratio between the measured and nominal concentrations of QC samples. The accuracy was required to be in the range of 85–115%, and the precisions not to exceed 15%.

The matrix effect was defined as the ion suppression/enhancement on the ionization of analytes. Six different rat urine samples were processed as described above and the filtrates were added with working solutions of each analyte (at low, medium and high concentrations) and IS. The analytical responses of these samples were compared with those of the working solutions after subtracting the background values. If the ratio <85% or >115%, the matrix effect was implied.

The recovery was determined by comparing the peak areas obtained from QC samples with the non-filtrated standard solutions at equivalent concentrations in the same solvent after subtracting the background values. Six replicates were run at each concentration.

The stability was assessed on three QC samples after long-term storage (1 month at −80 °C) and short-term storage (6 h at room temperature), by comparison of the results with those obtained from freshly prepared samples. In addition, post-preparative stability was assessed in the final extract by testing reproducibility in autosampler tray over a single batch period (15 °C for 24 h).

### 2.6. Method application: animals, drug administration and urine collection

A total of 20 male Sprague–Dawley rats (200 ± 20 g) from the experimental animal center of Qinglong Mountain (no. SCXK2009-0002) were employed in this study. The experimental protocol was approved by the University Ethics Committee for the use of experimental animals and conformed to the Guide for Care and Use of Laboratory Animals. Rats were acclimatized for 5 days in plastic cages and fed ad libitum with a standard rodent diet. Then they were randomly divided into two groups with ten rats in each group, and i.g. administrated with either 800 mg/kg bodyweight realgar suspended in 0.5% (w/v) CMC-Na or CMC-Na only once a day for consecutive 7 days. Urine samples of each rat were collected into ice-cooled vessels containing 1% sodium azide on the seventh day and stored at −80 °C until analysis.

### 2.7. Statistical analysis

Data processing and graphic presentation were performed with Microsoft Office Excel 2010 (Microsoft Inc.) and Sigma Plot (version 12.0, Systat Software Inc.). The significances of differences between the control and realgar treated groups were evaluated using the Student *t*-test.

**Table 2**
Linearity, LOD and LOQ of each analyte in the urine matrix ($n = 3$).

| Analyte | Linear range (μg/mL) | Slope[a] | Intercept[a] | $R^2$[a] | LOQ (μg/mL) | LOD (μg/mL) |
|---|---|---|---|---|---|---|
| Citric acid | 2–200 | 0.7463 | −0.1531 | 0.996 | 2 | 0.7 |
| Glutamic acid | 1–100 | 1.2650 | −0.1045 | 0.999 | 1 | 0.3 |
| Lactic acid | 1–200 | 0.0367 | 0.0128 | 0.998 | 2 | 0.7 |
| Pyruvic acid | 0.5–50 | 0.0182 | −0.0062 | 0.996 | 0.5 | 0.2 |
| Succinic acid | 0.5–100 | 0.4259 | 0.1492 | 0.998 | 0.5 | 0.2 |
| Taurine | 1–200 | 0.4021 | 0.1935 | 0.997 | 1 | 0.3 |
| Uric acid | 2–300 | 0.8436 | 0.1768 | 0.997 | 2 | 0.7 |

[a] The mean values of regression parameters.

## 3. Results and discussion

### 3.1. Optimization of chromatography conditions

The determination of these organic acids in urine samples required a good separation in order to eliminate matrix interference. According to the preliminary experiments, the $C_{18}$ and CN column failed to provide a good separation and all analytes were eluted quickly with the matrix peak. Therefore, HILIC separation was examined as an alternative strategy to the commonly used RPLC separation.

In HILIC, highly polar analytes are retained on the column by partitioning between a water-enriched layer attracted by the polar stationary phase and the solvent, consisting of a mixture of acetonitrile and aqueous buffer in the range of 5–50%. The choice of the aqueous solvent is crucial for HILIC separation because it has great impact on the retention time, peak shapes, sensitivity and therefore on the overall separation efficiency [28]. In this study, five different aqueous solvents were investigated, including 0.1% formic acid, 0.1% acetic acid, 10 mM ammonium formate, 10 and 5 mM ammonium acetate. While retention of the analytes was possible with all five additives, 10 mM ammonium acetate generally gave the best peak shape. Then, several trials were carried out to screen the ratio of acetonitrile to water using 10 mM ammonium acetate as the mobile phase additive. An acceptable separation efficiency and good retention time were obtained in the mobile phase containing 70% acetonitrile/30% water with 10 mM ammonium acetate. Moreover, the effects of column temperature were investigated in the range of 30–45 °C. It was found that the peak shape was improved with the temperature increasing. This might be because higher temperature results in lower viscosity and faster mass transfer. Finally, an isocratic system (acetonitrile/10 mM ammonium acetate = 70/30) with the column temperature of 45 °C was chosen as optimized HILIC conditions.

### 3.2. Selection of internal standard

It is necessary to use an internal standard (IS) to get high accuracy when LC is equipped with MS as the detector. In this study, several compounds were investigated to find a suitable IS, including ibuprofen, mycophenolic acid, parietic acid and salicylic acid. Finally,ibuprofen was selected because of its appropriate retention action, high ionization efficiency and less endogenous interference in urine.

### 3.3. Method validation

The regression parameters, such as linear range, slope, intercept, and correlation coefficients ($R^2$), are tabulated in Table 2. Good calibration linearities were obtained with $R^2$ values greater than 0.996

**Table 3**
Accuracy and precision for each analyte in urinary QC samples($n = 6$).

| Nominal concentration (μg/mL) | Intra-day | | Inter-day | |
|---|---|---|---|---|
| | Accuracy (%) | RSD (%) | Accuracy (%) | RSD (%) |
| Citric acid | | | | |
| 2 | 97.1 | 11.0 | 92.5 | 13.9 |
| 150 | 100.5 | 8.8 | 97.0 | 8.8 |
| 300 | 92.4 | 4.7 | 98.3 | 10.5 |
| Glutamic acid | | | | |
| 1 | 91.2 | 13.3 | 90.9 | 14.4 |
| 100 | 99.9 | 9.0 | 98.8 | 9.4 |
| 200 | 101.5 | 6.1 | 104.0 | 7.3 |
| Lactic acid | | | | |
| 1 | 89.9 | 13.4 | 94.9 | 14.2 |
| 100 | 93.3 | 8.1 | 97.2 | 10.1 |
| 200 | 96.3 | 8.9 | 92.7 | 11.7 |
| Pyruvic acid | | | | |
| 0.5 | 89.1 | 13.2 | 90.4 | 14.8 |
| 20 | 94.1 | 5.4 | 96.1 | 6.5 |
| 50 | 100.3 | 9.5 | 101.6 | 10.2 |
| Succinic acid | | | | |
| 0.5 | 92.3 | 11.4 | 96.1 | 13.7 |
| 100 | 96.6 | 7.6 | 98.0 | 11.1 |
| 200 | 99.2 | 8.4 | 97.4 | 10.3 |
| Taurine | | | | |
| 2 | 104.3 | 11.5 | 90.3 | 13.5 |
| 100 | 98.0 | 6.1 | 98.0 | 7.8 |
| 200 | 97.1 | 4.8 | 92.9 | 5.5 |
| Uric acid | | | | |
| 2 | 91.7 | 12.9 | 92.7 | 13.2 |
| 150 | 96.3 | 10.7 | 99.6 | 12.0 |
| 300 | 97.2 | 6.2 | 93.3 | 9.8 |

**Table 4**
Matrix effect and recovery for each analyte and IS in the urine matrix ($n = 6$).

| Nominal concentration (μg/mL) | Matrix effect | | Recovery | |
|---|---|---|---|---|
| | Mean (%) | RSD (%) | Mean (%) | RSD (%) |
| Citric acid | | | | |
| 2 | 101.7 | 10.6 | 93.1 | 8.1 |
| 150 | 105.4 | 5.1 | 91.9 | 9.5 |
| 300 | 99.7 | 4.9 | 88.8 | 5.5 |
| Glutamic acid | | | | |
| 1 | 99.0 | 11.6 | 85.5 | 7.1 |
| 100 | 107.3 | 6.9 | 84.7 | 5.3 |
| 200 | 102.8 | 3.8 | 89.6 | 5.9 |
| Lactic acid | | | | |
| 1 | 104.1 | 9.7 | 90.2 | 10.9 |
| 100 | 105.7 | 6.5 | 87.7 | 8.8 |
| 200 | 94.7 | 8.7 | 93.2 | 6.3 |
| Pyruvic acid | | | | |
| 0.5 | 101.4 | 8.6 | 89.5 | 5.4 |
| 20 | 98.5 | 12.9 | 87.9 | 7.1 |
| 50 | 95.6 | 9.9 | 83.9 | 4.5 |
| Succinic acid | | | | |
| 0.5 | 101.1 | 6.7 | 85.1 | 8.8 |
| 100 | 92.9 | 8.8 | 91.5 | 13.9 |
| 200 | 95.2 | 7.2 | 87.9 | 5.6 |
| Taurine | | | | |
| 2 | 95.3 | 9.3 | 88.7 | 7.9 |
| 100 | 102.9 | 8.5 | 91.3 | 9.5 |
| 200 | 104.3 | 3.1 | 92.3 | 6.4 |
| Uric acid | | | | |
| 2 | 97.5 | 11.8 | 92.9 | 7.9 |
| 150 | 106.2 | 7.9 | 85.2 | 5.9 |
| 300 | 102.1 | 9.9 | 91.8 | 11.1 |
| IS | | | | |
| 0.5 | 106.8 | 5.6 | 87.5 | 6.8 |

for all analytes. The LOD and LOQ of each analyte were calculated and are also shown in Table 2. They demonstrated a good sensitivity of the method, which was adequate enough to detect very low concentrations of these analytes in urine.

The accuracy, precision and recovery of the method were systematically studied and the results are shown in Table 3. For all analytes, the intra-day and inter-day accuracy were ranged from 89.1 to 104.4% and RSD was below 15.0%. This indicated that the method was accurate and precise for replicate analysis of the studied metabolites in urine samples within the same day or on different days. The matrix effect and recovery of the method were systematically studied and the results are shown in Table 4. No significant matrix effects for each analyte and IS were observed indicating that no co-eluting substance influenced the ionization of them. It can also be seen from Table 4 that more than 80% recovery was achieved for all analytes and IS.

The stability tests were designed to cover the anticipated conditions of handling of the real samples. No significant changes in concentrations (<15%) were observed in urinary QCs.

The proposed HILIC–MS/MS method overcomes most of the disadvantages of those reported methods [17–25]. Sample preparation was quick and simple avoiding the complex derivatization step. The running time was only 14 min with LOD in the range of 0.2–0.7 μg/mL which made it faster and more sensitive than most CE methods. Moreover, it was easier to access than the UPLC-MS/MS method because the normally used HPLC–MS/MS system was employed.

### 3.4. Application to rat urine samples

In this study, a very high dose of realgar (800 mg/kg) was given to the rats to make them be poisoned. The dose was determined refer to the literature [3,8,10] and based on the results of our preliminary experiments.

20 urine samples of the health control and realgar treated rats were analyzed by the validated HILIC–MS/MS method. All organic acids were well separated (Fig. 2) and the concentrations of them were determined using the internal standard based calibration curves. Since the concentrations of these metabolites are dependent on urine output, correction for creatinine concentration is necessary. Urinary creatinine was determined by the enzymatic method [30,31]. As shown in Table 5, the $p$-value of each analyte is less than 0.05, indicating that there are statistically significant differences between the two groups of rats. The exposing to realgar led to increased levels of lactic and pyruvic acid and dropped concentrations of other five organic acids in rat urine. These organic acids are essential components or final products of central metabolism. The changes of their levels indicated the disturbance of energy, choline and amino acid metabolism induced by realgar. The results obtained by this HILIC–MS/MS method were in agreement with Wei's study [10], but it was faster and more sensitive than the $^1$H NMR analysis.

The main component (>90%) of realgar is tetra-arsenic tetrasulfide ($As_4S_4$). Arsenic is notoriously poisonous to multicellular

**Table 5**
Results of HILIC–MS/MS analysis of urine samples of control and realgar treated rats ($n = 10$).

| Analyte | Urinary concentration (mean ± SD, $\mu g/mg_{creatinine}$) | | $p$-Value[a] |
|---|---|---|---|
| | Healthy control | Realgar treated | |
| Citric acid | 88.08 ± 15.62 | 133.69 ± 28.88 | 0.001 |
| Glutamic acid | 7.61 ± 1.48 | 12.65 ± 4.17 | 0.004 |
| Lactic acid | 66.26 ± 8.97 | 35.41 ± 18.9 | 0.000 |
| Pyruvic acid | 2.27 ± 0.41 | 1.83 ± 0.42 | 0.030 |
| Succinic acid | 35.98 ± 8.71 | 52.16 ± 15.3 | 0.009 |
| Taurine | 44.51 ± 20.17 | 92.85 ± 15.79 | 0.000 |
| Uric acid | 65.95 ± 16.79 | 125.09 ± 44.75 | 0.002 |

[a] $p < 0.05$ was considered statistically significant.

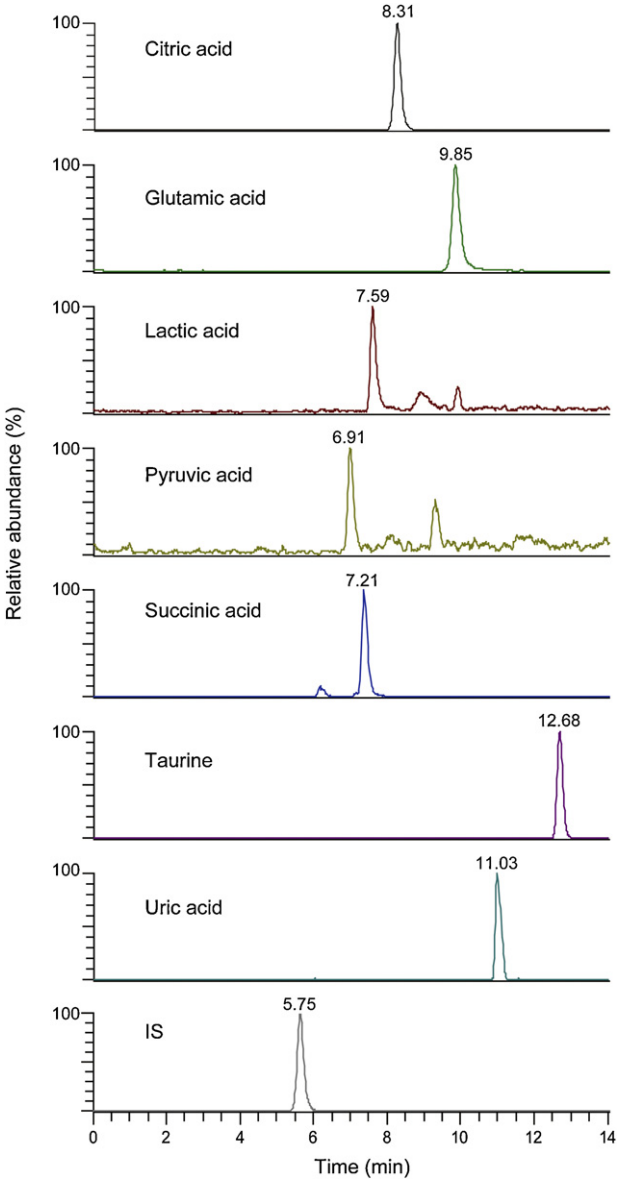


**Fig. 2.** Representative chromatogram of each analyte and IS in a urine sample of realgar treated rat (citric acid: 144.56 μg/mL; glutamic acid: 12.35 μg/mL; lactic acid: 50.42 μg/mL; pyruvic acid: 2.04 μg/mL; succinic acid: 51.90 μg/mL; taurine: 111.74 μg/mL; uric acid: 160.39 μg/mL).

life, because it disrupts ATP production through several mechanisms and the metabolic interferences lead to death from multi-systemorgan failure [32]. Thus, considering the changes of the seven pivotal organic acids, the toxicity of realgar might be caused by the arsenic contained in it. However, the metabolism of realgar in mammals is still unclear and further investigations are needed.

## 4. Conclusions

A reliable, fast and simple HILIC–MS/MS method was developed to simultaneously separate and quantify seven organic acids (taurine, citric, glutamic, lactic, pyruvic, succinic and uric acid) in urine samples. These acids can be used as potential biomarkers for the routine monitoring of exposure to realgar. Without additional time-consuming sample preparation techniques, this method can completely separate the above metabolites (using a ZIC®-HILIC column) within 14 min. Parameters affecting HILIC separation and MS/MS detection were systematically investigated and optimized. Furthermore, with simple modification, this method could potentially be used for detection of these biomarkers in other physiological fluids.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.07.038.

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