Assessment of the solubility and bioaccessibility of arsenic in realgar wine using a simulated gastrointestinal system

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A B S T R A C T

Consumption of arsenic (As) wine is a traditional activity during the classic Chinese festival of Duanwu, colloquially known worldwide as the Dragon Boat Day. Arsenic wine is drunk on the morning of the fifth day of the fifth lunar calendar month to commemorate the death of Qu Yuan, a famed Chinese poet who drowned himself in protest of a corrupt government, and to protect against ill fortune. Although realgar minerals are characteristically composed of sparingly soluble tetra-arsenic tetra-sulfides (As₄S₄), purity does vary with up to 10% of As being present as non-sulfur bound species, such as arsenate (AsV) and arsenite (AsIII). Despite, the renewed interest in As speciation and the bioaccessibility of the active As components in realgar based Chinese medicines, little is known about the safety surrounding the cultural practice of drinking As wine. In a series of experiments the speciation and solubility of As in a range of wines were investigated. Furthermore, a simulated gastrointestinal system was employed to predict the impact of digestive processes on As bioavailability. The predominant soluble As species found in all the wines were AsIII and AsV. Based on a common 100 mL measure of wine with a concentration of 400 mg As L⁻¹, the amount of soluble As would equate to around half of the acute minimal lethal dose for adults. This is likely an underestimate of the bioaccessible concentration, as a three-fold increase in bioaccessibility could be observed in the intestinal phase based on the results from the stimulated gastrointestinal system.

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1. Introduction

The Duanwu or Dragon Boat Festival is one of the oldest Chinese holidays and has been celebrated throughout Asia Pacific since 300 BC. Although, there are numerous interpretations and tellings of the Chu Yuan legend, all assign symbolic importance to open waters, snakes, and where snake attacks are frequent and their folklore abounds, the provinces of Hunan and Hubei where the story of Chu Yuan originates. Some of the traditions of the Duanwu festival have waned in modern times, yet in many rural parts of south-central China, such as in the provinces of Hunan and Hubei where the story of Chu Yuan originates and where snake attacks are frequent and their folklore abounds, the practice of realgar wine drinking is still common and ingrained in local habits.

Realgar, an ore composed mainly of tetra-arsenic tetra-sulfide (As₄S₄), is referred to in Chinese as “Xionghuang”, with the etiology of the word rooted in the deep aurora-red to orange-yellow coloration of the ore. Realgar is still currently being used in many traditional medicines (Chinese Pharmacopeia Committee, 2005), because it is easy to access and cheap to purchase. However, As is highly toxic, in addition to being a non-threshold carcinogen (Agency for Toxic Substances and Disease Registry, 2005; National Research Council, 1999). A number of studies have investigated the bioaccessibility of As in realgar medicines using either a simulated gastrointestinal system, animal experiments or collected data from human users of the medicines (Wang et al., 2008; Koch et al., 2007; Tang and Wang, 2005; Lu et al., 2002; Wu et al., 2002; Kwan et al., 2001). These findings showed that enhanced solubility of As in medicines containing realgar was observed in simulated body juices in comparison with the mineral itself. Most of these studies, however, were focused on realgar in non-alcohol based matrices and do not reflect the reality of As wine consumption in parallel with in vivo digestive processes. Although the As in pure realgar has very low solubility in water (7.1 μg g⁻¹), the solubility in artificial body fluids can increase substantially to 1.5 mg g⁻¹ (Beak et al., 2006). Furthermore, ethanol has been demonstrated to stimulate the absorption of As and increase its accumulation in rats.
(Flora et al., 1997). Indeed, incidences of acute renal failure after drinking realgar wine have occurred (Tsai et al., 2008).

In the present study, the assessment of the solubility of realgar in a range of common wines with different ethanol percentages was investigated. Bioaccessibility of As and its speciation in realgar wine were elucidated using a simulated gastrointestinal system.

2. Materials and methods

2.1. Chemicals

Realgar was purchased from a Chinese drugstore in Beijing. Guaranteed reagent nitric acid (HNO₃) (70%) was obtained from Beijing Beihua Fine Chemicals Co. Ltd. (China). Indium (In, 1000 μg mL⁻¹) was obtained from Agilent (Shanghai, China). All arsenic standards were of reagent grade or higher. Monosodium arsenite (Na₃H₂AsO₄) and sodium arsenite (Na₂AsO₃) were purchased from Merck; Germany, methylarsonic acid (MMA) was purchased from Chem Service MC (West Chester), and dimethylarsinic acid (DMA) was purchased from Sigma Chemicals.

Six kinds of wine with varying ethanol percentages were purchased in local supermarkets or kindly donated by local villagers just prior to the start of the Dragon Boat Festival.

2.2. Preparation of realgar wines

A range of realgar masses (0.025, 0.05, 0.25, 0.5 and 2.5 g) was mixed with 5 mL of various wines to yield a series of solutions with differing As content; defined as 0.005, 0.01, 0.05, 0.1, and 0.5 g mL⁻¹. The realgar wines were incubated at room temperature in accord with customary practices (25 °C). All samples were centrifuged at 1200 g for 30 min. The supernatants were passed through 0.45 μm filters (Millipore, Bedford, MA), diluted 1000 times with nitric acid (1%) and stored in the dark at 4 °C until analysis.

A solid to liquid ratio of 0.1 g mL⁻¹ was utilized to test the solubility of As in different kinds of wines. Realgar and wine (200 mL) were mixed, shaken vigorously for 5 min and then left to steep at room temperature (25 °C). At time intervals of 0, 12, 24, 48 and 72 h, realgar wine was homogenized by shaking and then 1 mL of wine was taken, filtered through 0.45 μm filters, diluted 1000 times with 1% HNO₃ and kept at 4 °C for analysis.

2.3. Simulated gastrointestinal system

The bioaccessibility of As in realgar wine was conducted in two sequential phases: gastric and intestinal phases. Arsenic wines (0, 0.005, 0.01, 0.05, 0.1 and 0.5 g mL⁻¹) were prepared by adding realgar powder into the wines, mixed by shaking vigorously for 5 min and kept at room temperature for 48 h. One volume of realgar wine solution was spiked into 6 volumes of simulated gastric fluid (16.4 mL HCl + 1000 mL distilled water + 10 g protease, pH 1.2) according to the Chinese Pharmacopoeia (Chinese Pharmacopoeia Committee, 2005). The mixtures were immediately put into a rotating (50 rpm) water bath and incubated for 2 h at 37 °C, and then sampled. The samples were centrifuged at 6000 rpm for 10 min; the supernatant was passed through 0.45 μm filters, and then kept at 4 °C for analysis. The residual gastric phase solution was modified to the intestinal phase solution by adjusting the pH to 6.8 with 0.1 M NaOH followed by the addition of pancreatin (10 g) and KH₂PO₄ (6.8 g) (Chinese Pharmacopoeia Committee, 2005). The intestinal phase solutions were incubated in a water-bath-shaker for 2 h at 37 °C and 50 rpm. The samples were prepared following the method for gastric solutions.

2.4. Total arsenic and its speciation determination

An inductively coupled plasma-mass spectrometry (ICP-MS) 7500 (Agilent Technologies) was used to determine total arsenic concentration as reported (Sun et al., 2008). Arsenic speciation was assayed simultaneously by high performance liquid chromatography–inductively coupled plasma-mass spectrometry (HPLC–ICP-MS) (Sun et al., 2008). Species separation was achieved using a PRP-X100 10-μm anion-exchange column (250×4.1 mm), with a HPLC mobile phase prepared using ammonium hydrophosphate (NH₄H₂PO₄) and ammonium dihydrogen orthophosphate (NH₄H₂O₄). Retention time for the As species was determined using a species mixture comprising standards of 10 μg L⁻¹ As³⁺, As⁵⁺, DMA, and MMA. For quality assurance, 3 reagent blanks were analyzed, plus all samples were duplicated and a further 3 samples were spiked with 50 μg As³⁺ L⁻¹. The recoveries were determined by subtracting the amount of endogenous As in unspiked samples from the spike values. The recovery for matrix spiked As speciation was 90.2 ± 5.5%, and the recovery for sum of species vs total As was 80.0 ± 7.6%. The same procedure of quality control, i.e., 3 matrix-spiked samples (50 μg As L⁻¹) and 3 reagent blanks, was also prepared for total As analysis. The mean recovery for total As was 105.2 ± 4.7%.

3. Results and discussion

3.1. Solubility of realgar in different wines

As can be seen in Fig. 1, the concentration of soluble As in wine increased linearly, almost doubling from 50.1 to 96.1 mg L⁻¹ within a 72 hour period (Fig. 1) and this trend was consistent across all of the wines tested. This is an important observation which adds considerably to the risk assessment for realgar wine consumption. If the wine is made at home, it is typical to mix the realgar and alcohol a few hours prior to drinking. Although we have no evidence why this practice is favored, it could be related to the continued release of As into the wine over time and previous illness or mortalities associated with the consumption of aged realgar wines. However, commercially sold realgar wines are available for purchase, and they would have been mixed for a considerable period of time. Based on our findings, it

<table>
<thead>
<tr>
<th>Name of wine</th>
<th>Ethanol (%)</th>
<th>Raw materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chundiao</td>
<td>12</td>
<td>Glutinous rice, wheat</td>
</tr>
<tr>
<td>Jingdu Erguotou</td>
<td>41</td>
<td>Wheat, sorghum</td>
</tr>
<tr>
<td>Xiaoyingjia</td>
<td>45</td>
<td>Rice, glutinous rice, sorghum, wheat, corn</td>
</tr>
<tr>
<td>Rice wine (home)</td>
<td>45</td>
<td>Rice</td>
</tr>
<tr>
<td>Jingui</td>
<td>50</td>
<td>Sorghum</td>
</tr>
<tr>
<td>Rice wine (market)</td>
<td>60</td>
<td>Rice</td>
</tr>
</tbody>
</table>

Fig. 1. The dissolution kinetics of realgar in rice wine (M) (p<0.0001). The content of realgar in wine was 0.1 g mL⁻¹. The time course was 1, 12, 24, 48 and 72 h and the arsenic concentration was analyzed by ICP-MS.
would be predicted that the proportion of soluble As in these wines would be appreciably high, although this might be tempered by a lower starting realgar to wine ratio. Unfortunately, we were unable to source any commercial realgar wines to include in our study as a comparison.

Realgar can be oxidized yielding As(III) and As(V) (Lengke and Tempel, 2003) and this would account in part for the increase in As released into solution overtime. Both As(III) and As(V) are readily soluble in aqueous solutions. It was not possible to determine if the increase in soluble As was due to speciation transformations or represented better extraction of the inorganic As impurities already present in the realgar. In this study, only one realgar powder was tested to keep a homogeneous particle size, however, when considering the safety of realgar wine drinking on a whole, the particle size of the realgar would also be an important factor, with finer grade powders exhibiting a greater surface area for As desorption. Indeed, it has been reported that the bioavailability of realgar was enhanced substantially by reducing the size of realgar to nano levels (Wu and Ho, 2006).

All the wines utilized in this study were starch/grain as opposed to fruit based products, and specifically selected to ensure that ethanol percentages varied, encompassing a range from 10 to 60% ethanol (Table 1). The percentage of ethanol in wines was found to be a crucial factor affecting the arsenic solubility of realgar in wine. There were significantly negative linear correlations between As concentration and corresponding ethanol percentage with a $R^2$ value of 0.96 ($P<0.001$) (Fig. 2). With an increase of ethanol concentration from 12 to 60%, the As levels in the realgar wine decreased sharply from 387 to 76.4 mg L$^{-1}$. The As content in realgar wine with 60% ethanol was 5 times less than that with 12% ethanol. These findings are strongly suggestive of the dangers posed of drinking realgar wines with low alcohol contents. Furthermore, the ethanol percentage had a marked impact on the As speciation in solution. In general, the As(III) concentration remains constant in all the wines but the concentrations of As(V) decreased with increasing ethanol percentage. This trend echoed that of total As, indicating that the decrease in total As solubility in realgar wine is mainly due to the decrease in As(V) solubility. No organic As or thioarsenates were detected. The amount of realgar used in the wine preparations was another major factor influencing As bioaccessibility. In all the wines tested, the As concentration increased linearly with the addition of realgar from 0.005 to 0.5 g mL$^{-1}$ (Fig. 3).

### 3.2. Bioaccessibility of arsenic in realgar wine

The bioaccessibility of As in the realgar wine was conducted with a simulated gastrointestinal system. In the simulated gastric and intestinal phases, the bioaccessible As pool was much higher than that in the corresponding original realgar wine (Fig. 4). The maximum As concentration was 824 mg L$^{-1}$ in the intestinal phase (0.5 g mL$^{-1}$, Chundiao), 1.3 times of the corresponding gastric phase (644 mg L$^{-1}$) and 3.3 times of the realgar wine (248 mg L$^{-1}$). These results show that the solubility of As in the gastrointestinal environment increased significantly the bioaccessibility of As in realgar wine.

It has been reported that about 60–90% of all soluble arsenic compounds are absorbed from the gastrointestinal tract following ingestion (Hall, 2002; ATSDR, 1990). Assuming that a typical volume of realgar wine consumed at any one time by an adult is 100 ml (0.5 g mL$^{-1}$, Chundiao), the resulting As intake would range from 49.4 to 74.1 mg based on differences in bioavailability. Therefore assuming an average body weight of 70 kg, arsenic intake through the consumption of realgar wine would be 353–529 times of the maximum tolerable daily intake (MTDI) of 2 μg kg$^{-1}$ body mass provisionally set by the World Health Organization (WHO, 1993). The acute minimal lethal dose of As in adults is estimated at around 1 mg kg$^{-1}$ body weight/day (Dart, 2004). For a 70 kg adult, drinking a common 100 mL measure of wine with a concentration of 400 mg As L$^{-1}$ the amount of soluble As exposure before gastrointestinal processes are accounted for is nearly 60% of the amount required to administer a lethal dose. Despite there being no documented human measurements of in vivo bioaccessibility of As...
from the consumption of realgar wine, our calculated bioavailability indicates that the practice of drinking realgar wine has the potential for a considerable amount of arsenic to be absorbed by the body. It is acknowledged, however, that drinking realgar wine is not a regular practice, but it is also conceivable that volumes of wine greater than 100 mL could be consumed during the merriment of the festival. An indepth assessment of the risk of realgar wine drinking is needed, especially because the toxic-kinetics of a one-time, large dose of As is not clear. Health risk assessment should therefore be conducted if the tradition of drinking realgar wine is to be practiced. It should be noted that gut microbes were not inoculated in our model system. It has been reported that gut microbes can increase As bioaccessibility from ingested mine tailings, and microbes can change the speciation of As, particularly facilitate the production of highly toxic species, such as monomethylarsonic acid (MMAIII) (Van de Wiele et al., 2010; Laird et al., 2007).

In conclusion, we have shown in this study that drinking realgar wine during the Duanwu festival may cause potential health problems. Further work is urgently needed to clarify these potential health risks.

Acknowledgments

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