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Research Paper

Comparing the Relative Oxidative DNA Damage Caused by Various Arsenic Species by Quantifying Urinary Levels of 8-Hydroxy-2′-Deoxyguanosine with Isotope-Dilution Liquid Chromatography/Mass Spectrometry

Jin-Zhu Wu[1,2] and Paul C. Ho[1,3]

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Purpose. To investigate the association between various arsenicals and the potential oxidative stress caused, we examined the urinary levels of 8-hydroxy-2′-deoxyguanosine (8-OH-dGuo), a biomarker of oxidative DNA damage in rats after daily oral administration of arsenic trioxide/arsenite (As_2O_3), realgar (α-As_4S_4) and orpiment (As_2S_3) over 14 days and compared the levels with control rats.
Methods. 8-OH-dGuo in urine was quantified with isotope-dilution liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) after sample cleaning with solid phase extraction (SPE). Urinary arsenic concentrations were measured by graphite furnace atomic absorption spectrometry (GFAAS).
Results. All arsenicals caused elevated urinary 8-OH-dGuo excretion in rats from day 1 after oral administration ($p<0.01$ respectively). There were significant correlations between urinary 8-OH-dGuo and urinary arsenic levels (slope=0.8164, 0.5801, 0.6582; $r^2=0.5946$, 0.7883, 0.8426 for arsenite, realgar and orpiment-treated group respectively, $p<0.001$). This illustrates that urinary 8-OH-dGuo level could be a valid biomarker for detecting the extent of arsenic exposure. Arsenite was found to cause significantly higher urinary 8-OH-dGuo levels than both realgar and orpiment ($p<0.01$) even after creatinine and dose adjustments.
Conclusions. Arsenite could cause more oxidative DNA damage than both realgar and orpiment and may be more genotoxic.

KEY WORDS: arsenite; orpiment; oxidative DNA damage; realgar; 8-hydroxy-2′-deoxyguanosine.

INTRODUCTION

Arsenic is a well-established human carcinogen based on epidemiological studies, although the underlying mechanisms of carcinogenesis remain unclear (1,2). Paradoxically, arsenic has been used therapeutically for more than 2,400 years to treat many diseases (3). Recently, the potential anticancer activity of arsenicals became a revived research focus, due to the notable success of arsenic trioxide (As_2O_3) in treating both newly diagnosed and relapsed patients with acute promyelocytic leukemia (APL) (4,5). In 2000, the USA food and Drug Administration (FDA) approved the arsenic trioxide injection, Trisenox™, for treatment of relapsed or refractory APL. In 2002, a pilot clinical study of pure realgar (α-As_4S_4) and orpiment (As_2S_3) in treatment of patients with APL was conducted in China, and impressive responses in APL were achieved and reported (6). Despite the success of arsenic trioxide and other arsenic species in treatment of APL, it is still difficult to accurately assess the toxic effects of arsenicals. It is well known that the toxicity of arsenicals depends on their chemical states (7). In addition, the toxicity of arsenicals depends on the exposure dose, frequency and duration, the biological species, age and gender, as well as on individual susceptibilities, genetic and nutritional factors (7).

Recently, oxidative stress is recognized as one of the most plausible modes of action for arsenic carcinogenesis (8–12), as proved by some *in vitro* and *in vivo* studies (8–12). In the presence of the oxidative stress, reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) generated *in vivo* can cause damage to lipid, protein, and nucleic acid (13).

There is great interest in studying the oxidative DNA damage caused by ROS and various biomarkers associated with this damage. Urinary 8-hydroxy-2′-deoxyguanosine (8-OH-dGuo) is reported to be the most accepted biomarker of the oxidative DNA damage, because of its good water-solubility, stability, non-invasive sampling, absence of artifacts as encountered in DNA extraction, relatively high abundance, and more importantly etiological role in mutations and gene expression (causing G:C to T:A transversion) (14,15). Urinary 8-OH-dGuo also represents the average rate of damage in the total body. Therefore, determination of urinary 8-OH-dGuo can be used for investigation of different types of exposure to DNA-damaging factors (14–16).

[1] Department of Pharmacy, Faculty of Science National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore.
[2] Department of Applied Chemistry, Faculty of Science, Harbin Institute of Technology, Harbin 150001, China.
[3] To whom correspondence should be addressed. (e-mail: phahocl@nus.edu.sg)

In this study, we conducted a pilot study to examine possible associations between the intakes of different arsenic species and the potential oxidative stress caused; we compared the urinary 8-OH-dGuo levels in rats after arsenite, realgar and orpiment administration with control rats. The findings would add valuable information on the relative genotoxicity of arsenite, realgar and orpiment in their clinical applications.

EXPERIMENTAL

Chemicals

8-Hydroxy-2′-deoxyguanosine (8-OH-dGuo, 100% in purity) was purchased from Berry & Associates Inc. (Ann Arbor, MI, USA). Stable heavy isotope labeled [^{15}N5]-8-OH-dGuo (98% in purity) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Formic acid of mass spectrometry grade was purchased from Sigma Chemical Corp. (St. Louis, MO, USA). Methanol of HPLC grade was obtained from Merck Corp. (Darmstadt, Germany). Milli-Q water with a resistivity of 18 MΩ·cm (Ultra-Pure Water System, MilliPore Corp., Bedford, MA, USA) was used throughout experiment. Aqueous stock standard solutions of 8-OH-dGuo and [^{15}N5]-8-OH-dGuo were prepared by dissolving them respectively in Milli-Q water to give a concentration of 10 μg/ml, and stored at –80°C. Working standard solutions of [^{15}N5]-8-OH-dGuo (0.5 to 50 ng/ml) were freshly prepared by series dilution with Milli-Q water.

Realgar and orpiment were cryo-ground into fine particles with the assistance of polyvinylpyrrolidone (PVP) and sodium dodecyl sulfate (SDS) at weight ratio of 1:3:1 (R/PVP/SDS, O/PVP/SDS) as described previously (17). In both preparations, realgar and orpiment nanoparticles with less than 200 nm in sizes accounted for about 15% of the total ground particles, and the rests were the same materials with larger particle sizes ranging from 200 nm to 15 μm (unpublished data). Despite the sizes of the test materials were not all below 200 nm, our previous study indicated that the bioavailability of the cryo-ground realgar powder (69.6%) was substantially higher than that of the original raw powder (24.9%) (17). The presence of PVP and SDS in the preparations facilitated the production of more nanosized realgar particles during the grinding procedure (17). The PVP and SDS adhered onto the surfaces of the nanosized realgar particles, providing both steric and ionic barriers to aggregation and/or agglomeration of the drug particles. In this study, the respective suspensions of realgar (R/PVP/SDS) and orpiment (O/PVP/SDS) were prepared by individually dispersing the R/PVP/SDS and O/PVP/SDS preparations into Milli-Q water at a concentration of 4.0 mg compound/ml. Arsenite solution with a concentration of 2.0 mg arsenite/ml was prepared by firstly dissolving exact amount of arsenic trioxide into 1 N NaOH followed by adjustment of pH to 7.0±0.2 with concentrated HCl.

Animal Model and Arsenic Compounds Administration

Healthy male Sprague-Dawley (SD) rats (6–7 weeks of age with average body weight of 200±20 g) were purchased from Laboratory Animals Center, Singapore. They were randomly selected and housed individually in polycarbonate metabolic cages and provided with a standard diet (Mouse pellets, Laboratory Animals Centre, Singapore) and water *ad libitum*. The housing conditions were kept on a 12/12-h light/dark cycle at a temperature of 23±1°C and relative humidity of 50±10%. At least 1 week of acclimatization period was allowed for rats prior to drug administration. The animal experiment protocol followed the guidelines for proper and humane care of animals in scientific research, were approved by the Institutional Animal Care and Use Committee (National University of Singapore, Singapore).

Rats were divided randomly into four groups of six rats each: one control group and three experimental groups. The three experimental groups received individual arsenic compounds by gavage administration for consecutive 14 days, whilst the control group received drinking water instead. Doses were 20 mg pure drug/kg body weight for realgar and orpiment suspensions, and 10 mg arsenite/kg body weight for arsenite solution. At the end of the experiment, all rats were euthanized by carbon dioxide gas inhalation.

Urine Sample Collection, Normalization and Purification

Every 24 h urine outputs were collected and stored frozen at –80°C until analysis. Prior to analysis, each sample was thawed at 37°C for 10 min to re-dissolve possible 8-OH-dGuo precipitate during freezing storage (18), vigorously mixed, and then centrifuged at 1,500×*g* for 10 min to obtain a clear supernatant.

Since the urine concentration is highly variable between different subjects and in the same subject at different time points, the volume of urine was adjusted according to its creatinine concentration level measured by Jaffe method after slight modification (19). Briefly, the formation of acid-sensitive chromogen after reduction of the urine sample with picrate was measured at UV absorbance of 500 nm. The different creatinine concentration gave different color. The urine was then normalized according to creatinine concentration by adding appropriate amount of 10% formic acid solution. Normalized urine samples were then subjected to further purification steps.

The solid phase extraction (SPE) clean-up procedure was processed and optimized with Waters Oasis® HLB Vac cartridges (Waters Corp., Milford, MA, USA) according to the standard protocol after slight modification. A volume of 1 ml of the normalized urine was loaded into a preconditioned SPE cartridge. The cartridge was then washed with 2 ml of Milli-Q water. The fraction containing 8-OH-dGuo was eluted with 1 ml of HPLC running buffer. To optimize and evaluate the recovery of 8-OH-dGuo after clean-up procedure, [^{15}N5]-8-OH-dGuo equaling to 10 ng/ml was added to each urine sample as an internal standard.

Analysis of 8-OH-dGuo by Isotope-Dilution LC/MS/MS

The HPLC system used was Agilent 1200 Series LC systems (Agilent Technologies Inc., Santa Clara, CA, USA). A Waters Symmetry300™ C18 column (150×1.0 mm i.d., 3.5 μm particle size) (Waters Ltd., Watford, UK) with an identical guard column (10×2.0 mm, 3.5 μm) was used. The isocratic mobile phase was 30% methanol with 0.1% formic acid, delivered at a flow rate of 50 μl/min. The HPLC was connected to an API 3200 QTRAP® mass spectrometer (Applied Biosystems, Foster, CA, USA) equipped with a

TurboIonSpray™ source. Electrospray ionization (ESI) was performed. Multiple reaction monitoring (MRM) mode with positive ionization was used. Optimization of mass responses (compound parameters) was achieved by infusion of the 8-OH-dGuo solution in mobile phase (1 μg/ml) at a flow rate of 10 μl/min by using a syringe pump. After optimization, a volume of 10 μl of purified urine sample was injected into the LC/MS/MS instrument for urinary 8-OH-dGuo determination.

Data were acquired and analyzed with Analyst® 1.4.2 software (Applied Biosystems, Foster, CA, USA). The urinary 8-OH-dGuo concentration was corrected by using individual urinary creatinine concentration (ng/mg creatinine).

Measurement of Urinary Arsenic Concentration By Graphite Furnace Atomic Absorption Spectrometry

Determination of urinary arsenic concentration by GFAAS was described previously (17). Briefly, an electrodeless discharge lamp (EDL) operated at 5 mA was used. Argon gas was chosen as carrier and sheath gas. Typical analytical conditions were as follow: drying at 130°C, ashing at 1300°C, atomization at 2300°C, and finally cleaning at 2600°C. Pd $(NO_3)_2$ (10.0±0.3 g/L as Pd) and $Mg(NO_3)_2$ (10.0±0.3 g/L as Mg) solutions were purchased from Sigma Chemical Co. (St. Louis, MO, USA), their combination with final concentrations of 3,000 ppm Pd and 2,000 ppm Mg was used as a matrix modifier for the arsenic determination. A 5 μl of matrix modifier was injected into graphite furnace tube, followed by 20 μl standard/sample solution. Three replicates were measured under background correction mode for each analysis.

Prior to analysis by GFAAS, pre-treated and normalized urine samples were diluted ten-fold with 70% nitric acid prepared from fuming nitric acid (extra pure, Merck kGaA, Darmstadt, Germany) overnight at room temperature. Concentrations of urinary arsenic were calculated from a standard calibration curve. Five standard arsenic solutions with arsenic concentrations of 5, 10, 20, 30, and 50 ppb were prepared from an arsenic standard solution containing 997±5 mg/l As (E. Merck, Darmstadt, Germany) after appropriate dilution for the calibration curve establishment. A correlation coefficient of 0.9984 was obtained for calibration curve by using linear regression across zero point.

Statistical Methods

The results were presented as mean ± standard deviation (SD). Differences among data were evaluated by using one-way ANOVA with the post hoc Tukey's multiple comparison test (GraphPad Prism V4.0). Linear regression model was applied to study the association of the urinary arsenic recovery levels with urinary 8-OH-dGuo levels (GraphPad Prism 4.0). A p-value<0.05 was considered a significant difference.

RESULTS AND DISCUSSIONS

Typical Mass Spectra and Chromatogram of 8-OH-dGuo and [^{15}N5]-8-OH-dGuo

Production-ion spectra of 8-OH-dGuo and [^{15}N5]-8-OH-dGuo were acquired respectively at optimal MS/MS conditions as shown in Fig. 1a and b. The most abundant fragment of 8-OH-dGuo was detected at m/z 168, resulting from cleavage of the N-glycosidic bond accompanied by transfer of a hydrogen atom, while protonated sugar moiety at m/z 117 and molecular ion $[M+H]^+$ at m/z 284 were also observed with relatively lower intensities. The corresponding fragmentation scheme was proposed in Fig. 1a, consistent with the previous reports (20,21). [^{15}N5]-8-OH-dGuo, a stable internal standard (22,23) yielded the same fragmentation pattern as 8-OH-dGuo (Fig. 1b).

Multiple reaction monitoring (MRM) mode with transition ion pairs of 284/168 for 8-OH-dGuo and 289/173 for [^{15}N5]-8-OH-dGuo was selected for the detection according to the individual production ion spectra (Fig. 1a and b). A typical chromatogram of a mixture of 8-OH-dGuo and [^{15}N5]-8-OH-dGuo in aqueous solution is shown in Fig. 2. Both 8-OH-dGuo and [^{15}N5]-8-OH-dGuo were simultaneously eluted at the same retention time of 3.6 min.

Characteristics of the SPE Isotope-Dilution LC/MS/MS Method for Quantification of Urinary 8-OH-dGuo

It is troublesome and unnecessary to completely remove the endogenous 8-OH-dGuo in the urine matrix. Therefore, for accurate determination of 8-OH-dGuo in real biological samples, its stable isotope was used as an internal standard (22,23). Furthermore, the isotope-dilution method is to correct for the losses of the analytes during the sample preparation and variations in the mass spectrometric responses, and thus is able to improve method reliability.

The developed SPE clean-up procedure removed most interferences in the urine matrix which adversely affected detection of the analytes, enabling determination of the analytes in urine. The average recovery of [^{15}N5]-8-OH-dGuo in the present SPE purification procedure from [^{15}N5]-8-OH-dGuo spiked urine was found to be 65.47±4.73% (n=6), which was used to reflect the efficiency of the SPE for purifying 8-OH-dGuo from urine.

In this study, a urinary calibration curve for 8-OH-dGuo measurement was built on [^{15}N5]-8-OH-dGuo spiked urine samples. A linear urinary calibration curve, Y=0.7126 X, with correlation coefficient better than 0.995 was obtained in the selected concentration range from 1.0 to 50.0 ng/ml of [^{15}N5]-8-OH-dGuo (calibration samples at 1.0, 5.0, 10.0, 25.0, and 50.0 ng/ml were conducted in triplicate respectively). The calibration range was chosen to match the range of concentrations actually measured. The limit of quantitation (LOQ) of the instrument for urinary [^{15}N5]-8-OH-dGuo was 1.0 ng/ml determined as the concentration that gave a signal to noise (S/N) ratio of ~10.

The accuracy and precision of the established SPE isotope-dilution LC/MS/MS method was ascertained by performing replicate determinations of urine samples spiked with [^{15}N5]-8-OH-dGuo at 1.0 and 5.0 ng/ml, respectively. Parameters in Table I indicate the accuracy and precision as the measured values against the theoretical true values and the coefficients of variation (CV) for the intra-day and inter-day measurements.

Concentrations of 8-OH-dGuo in Rats Urines Before and After Arsenic Compounds Administration

Arsenic is unusual, as it is one of the few demonstrated human carcinogens for which carcinogenicity in laboratory

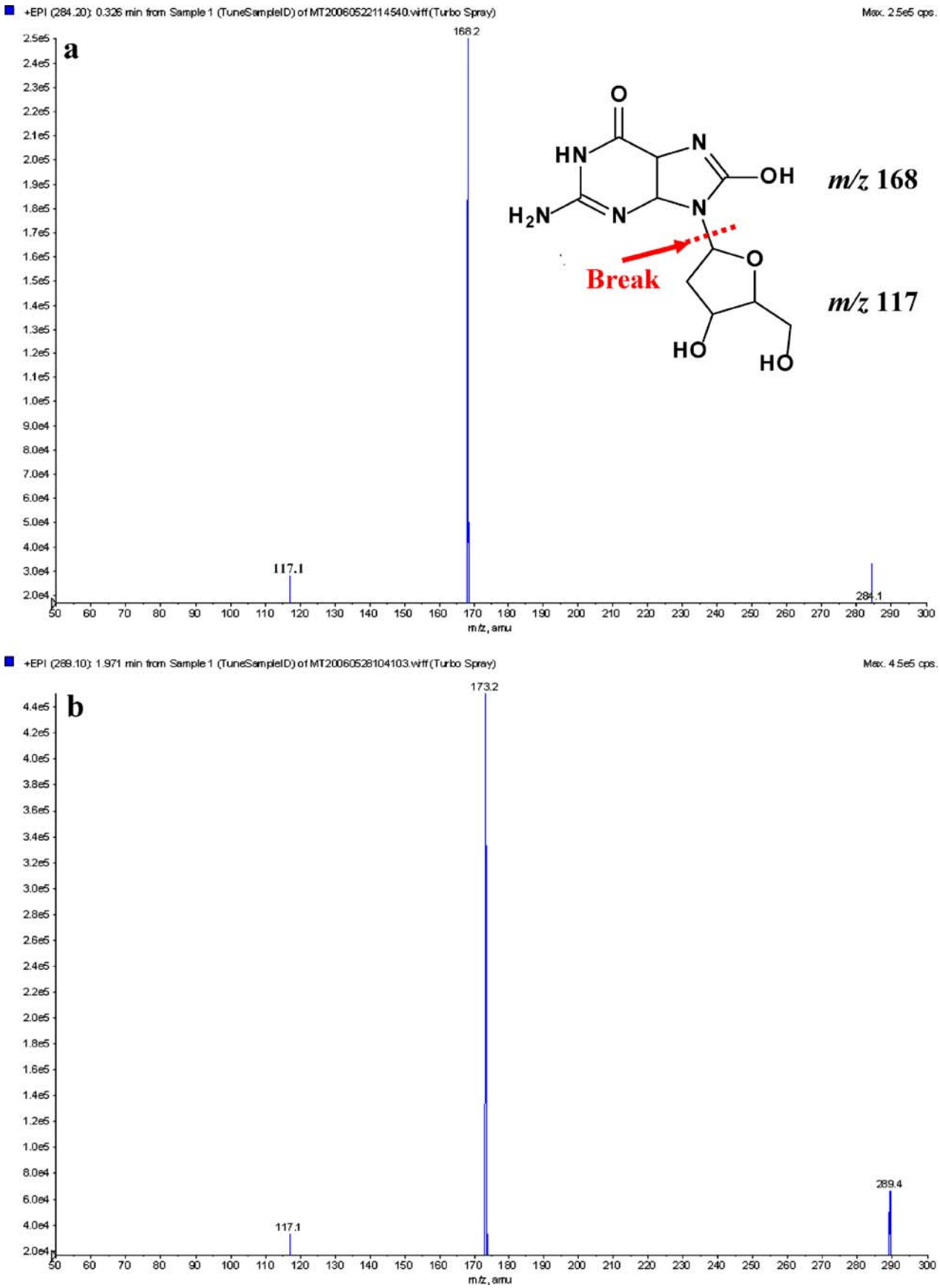

Fig. 1. Positive production-ion spectra of 8-OH-dGuo (**a** product ion scan of $[M+H]^+$ at *m/z* 284) and [^{15}N5]-8-OH-dGuo (**b** product ion scan of $[M+H]^+$ at *m/z* 289).

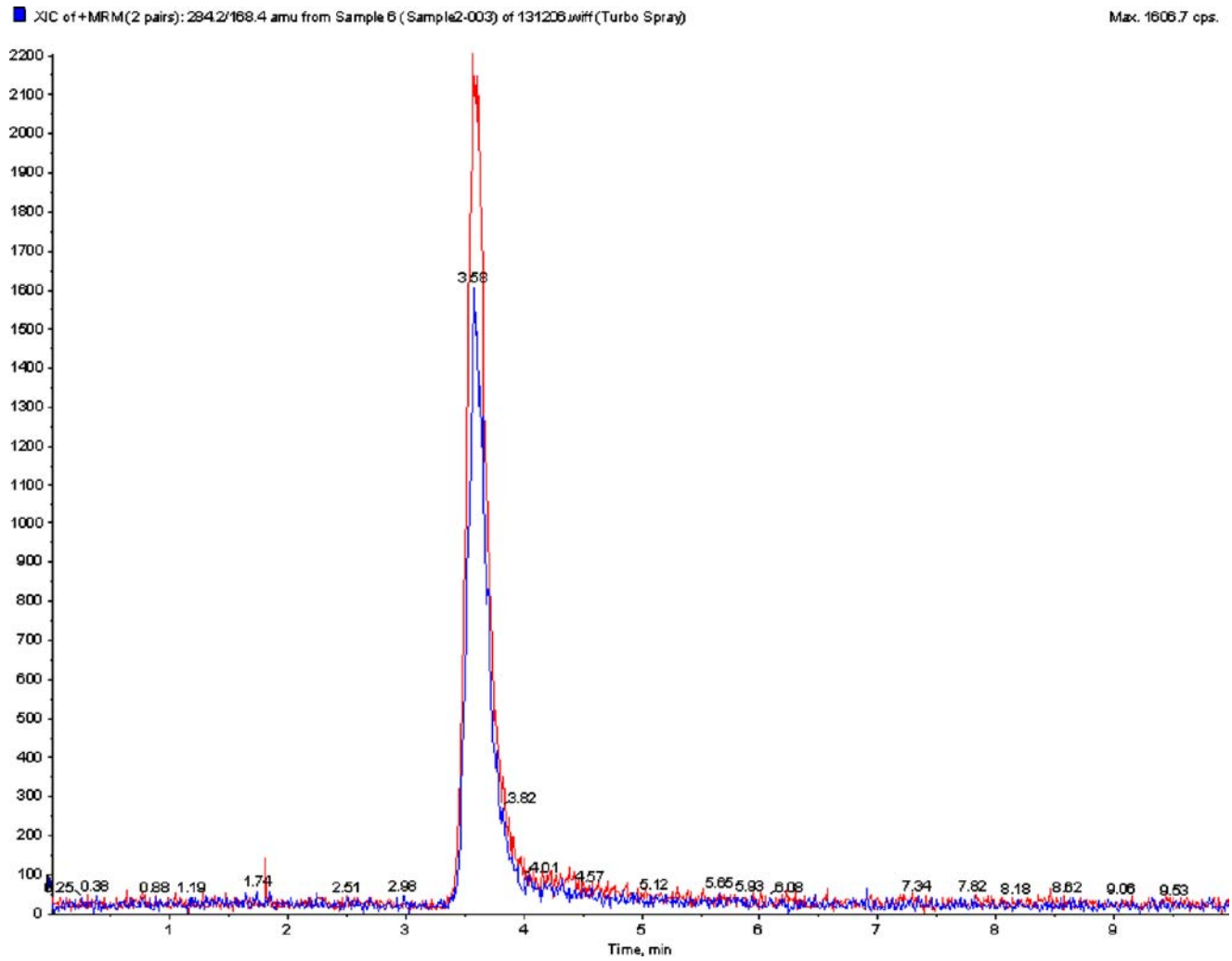

Fig. 2. MRM chromatogram for an aqueous solution of 8-OH-dGuo (4.0 ng/ml, *bottom line*) and [^{15}N5]-8-OH-dGuo (5.0 ng/ml, *top line*).

animals has not been firmly established (24,25). Nowadays, it is well recognized that increased generation of reactive species (RS) *in vivo* mainly including ROS and RNS can promote development of malignancy because many RS are powerful oxidizing agents and capable of damaging DNA and other biomolecules. Therefore, most attention has been paid to direct oxidative DNA damage by certain arsenic compounds through triggering excessive ROS (26–28).

One of the most studied DNA base oxidation products, 8-OH-dGuo was determined in this study to directly evaluate the potential damage to DNA by the target arsenicals in rats. The concentrations of urinary 8-OH-dGuo for control and treated rats were measured by the established SPE isotope-dilution LC/MS/MS method. In most studies, urinary 8-OH-dGuo levels were normalized with corresponding urinary creatinine levels (29,30), which was adopted in this study. The mean urinary 8-OH-dGuo concentrations (ng/mg creatinine) of all intact rats including the control rats and experimental rats before administration were in the range of 3.70–4.30, comparable with the reported values (31,32). Table II lists the creatinine-adjusted urinary 8-OH-dGuo concentrations in the rats studied. A large inter-individual variation in terms of mean urinary 8-OH-dGuo concentrations was found, with the CV values of up to 40%. Despite the relatively high CVs among the individuals, comparison analysis by one-way ANOVA showed that all arsenic compounds-treated rats had significantly higher mean urinary 8-OH-dGuo concentrations, more than 10 times, than the control rats

Table I. Accuracy and Precision of the SPE Isotope-Dilution LC/MS/MS Method for Analyzing Spiked [^{15}N5]-8-OH-dGuo in Urine Samples

Added [^{15}N5]-8-OH-dGuo (ng/ml)		Measured value[a] (mean ± SD ng/ml, CV (%)) (% of the theoretical true value)
1.0	Intra-day	0.96±0.06, 6.67% 96.0%
	Inter-day	0.93±0.09, 9.14% 93.0%
5.0	Intra-day	5.11±0.26, 5.01% 102.2%
	Inter-day	5.07±0.42, 8.22% 101.4%

[a] The urine samples for the inter-day and intra-day studies were spiked with 1.0 and 5.0 ng/ml [^{15}N5]-8-OH-dGuo respectively in five replicates

Table II. Urinary Creatinine Corrected 8-OH-dGuo Production in Rats Before and After Arsenic Administrations

Duration	Urinary 8-OH-dGuo (ng/mg creatinine)			
	Control group	Arsenite administration	Realgar administration	Orpiment administration
Day 0	3.86±1.06	3.94±1.19	4.00±1.23	3.73±1.19
Day 1	3.94±1.22	57.44±24.55	40.26±17.39	42.05±18.92
Day 2	4.27±1.26	60.89±20.80	43.43±17.16	44.19±17.67
Day 3	4.10±1.28	59.78±24.12	42.41±18.74	45.70±17.64
Day 4	3.75±1.31	61.23±25.83	46.89±18.19	42.40±18.28
Day 5	3.90±1.13	63.19±19.40	43.99±18.37	44.66±18.92
Day 6	3.95±1.15	58.06±25.27	46.72±17.04	45.00±18.19
Day 7	4.07±1.20	61.78±19.10	42.31±17.78	47.33±17.94
Day 8	3.94±1.18	59.93±24.38	44.64±15.52	47.80±18.30
Day 9	3.82±1.13	64.49±23.08	44.99±16.67	44.96±17.98
Day 10	4.01±1.23	60.11±26.31	47.00±18.41	43.99±18.07
Day 11	3.78±1.24	59.91±22.49	49.30±17.19	42.38±17.98
Day 12	3.86±1.32	62.30±19.40	45.09±17.74	45.80±18.55
Day 13	4.19±1.13	63.17±19.18	46.38±16.96	46.01±18.55
Day 14	4.19±1.16	60.38±22.38	49.22±18.85	48.37±17.85

Data are presented as mean ± SD (n=6)

(control *vs* arsenite/realgar/orpiment-treated group, $p<0.001$ in all cases); at current dosages, arsenite caused higher urinary 8-OH-dGuo levels than both realgar ($p<0.01$) and orpiment ($p<0.01$), but there was no significant difference between realgar and orpiment on the induction of urinary 8-OH-dGuo ($p>0.05$). All arsenic compounds induced elevated urinary 8-OH-dGuo levels immediately from day 1 ($p<0.01$ respectively), but the levels remained relatively stable thereafter over the 14-day study period ($p>0.05$ respectively).

Extensive studies demonstrate that ingested inorganic arsenic is readily absorbed into the blood and primarily taken up by cells in liver, where it undergoes a series of reduction and oxidative methylation to form various organic metabolic intermediates and metabolites mainly including monomethylarsinic acid (MMA^V), monomethylarsinous acid (MMA^{III}), dimethylarsinic acid (DMA^V), dimethylarsinous acid (DMA^{III}), and trimethylarsine oxide ($TAMO^V$) (33,34). S-adenosylmethionine (SAM) is a primary methyl donor and glutathione (GSH) serves as a main reducing agent for such metabolism (33,34). Arsenic is mainly eliminated in the feces and urine, and DMA^V is the primary metabolite excreted into the rat urine (34). Comparing with most other mammals and humans, rats possess a relatively slow urinary arsenic excretion since they preferentially retain DMA^V in red blood cells (RBCs) (34). Since inorganic arsenic is metabolized and excreted in the urine, detection of arsenic in urine has been used as a marker of recent arsenic exposure (35). Therefore, in this study, the urinary arsenic recovery in rats after the arsenic compounds administrations was measured as shown in Table III. In order to adjust the variability of urinary volume among individuals, the urinary arsenic concentrations were also corrected with corresponding urinary creatinine concentrations.

It should be mentioned that the urinary arsenic concentrations in all intact rats including the control and experimental rats before arsenic dosing were too low to be detectable under current instrumental conditions. Table III showed that similar daily urinary arsenic recoveries (~70 μg/mg creatinine) over the study period of 2 weeks were found for each study group ($p>0.05$), and no sign of arsenic accumulation was observed. Doses were 20 mg pure drug/kg body weight for realgar and orpiment suspensions, and 10 mg arsenite/kg body weight for arsenite solution. Approximately 50% of daily administered arsenite was excreted into the urine, compared to around 20% of daily administered realgar and orpiment. The relatively lower urinary arsenic recoveries for realgar and orpiment suspensions when compared to arsenite solution probably could result from the extra dissolution step involved with the suspending particles. Nevertheless, all experimental groups appeared to have similar arsenic exposure, based on their arsenic urinary recoveries (Table III).

Table II shows that consecutive administration of arsenicals for 14 days did not significantly change the production of 8-OH-dGuo, the indicator of the whole body oxidative damage rate. Data in Table II and Table III indicate that there were

Table III. Urinary Creatinine Corrected-Arsenic Recovery in Rats after Oral Administration with the Respective Arsenite, Realgar and Orpiment

Duration	Arsenic urinary recovery (μg/mg creatinine)		
	Arsenite administration	Realgar administration	Orpiment administration
Day 1	70.38±21.01	77.88±22.25	67.75±22.54
Day 2	71.62±22.69	78.21±23.04	68.21±19.47
Day 3	71.93±22.92	78.78±25.00	68.96±19.01
Day 4	72.99±21.78	78.98±25.28	68.76±20.96
Day 5	73.21±19.76	77.62±23.45	70.23±20.16
Day 6	73.27±20.64	79.01±23.10	69.89±20.45
Day 7	74.01±19.85	78.21±24.93	70.21±20.60
Day 8	74.17±19.89	79.01±25.84	70.33±21.38
Day 9	75.64±22.09	78.21±23.26	69.41±20.86
Day 10	75.71±20.14	79.37±23.77	69.21±20.08
Day 11	77.21±20.42	80.01±23.77	70.89±20.66
Day 12	77.88±26.74	78.54±23.81	69.81±19.59
Day 13	78.21±24.73	78.14±24.49	70.26±20.03
Day 14	78.32±24.56	79.61±23.28	71.77±19.21

Data are presented as mean ± SD (n=6)

likely positive correlations between urinary arsenic levels and corresponding urinary 8-OH-dGuo levels in each study group. Therefore, the urinary arsenic-adjusted 8-OH-dGuo concentrations were also calculated and listed in Table IV. Comparison analysis again showed similar results to what have been obtained with the creatinine-adjusted 8-OH-dGuo concentrations. That is, arsenite triggered more 8-OH-dGuo production than realgar ($p<0.001$) and orpiment ($p<0.001$), but there was no significant difference between realgar and orpiment on the formation of 8-OH-dGuo ($p>0.05$). It might be concluded that arsenite caused more oxidative DNA damage, and therefore, could potentially be more genotoxic than both realgar and orpiment.

In order to further examine whether there are positive correlations existing between urinary 8-OH-dGuo and urinary arsenic levels as assumed earlier, a linear regression analysis was conducted. Fig. 3 shows positive correlations between urinary 8-OH-dGuo concentrations and urinary arsenic recovery values in each experimental group (arsenite-treated group, $n=84$, $r^2=0.5946$, slope=0.8164, $p<0.001$; realgar-treated group, $n=84$, slope=0.5801, $r^2=0.7883$, $p<0.001$; orpiment-treated group, $n=84$, slope=0.6582, $r^2=0.8426$, $p<0.001$), in agreement with the observations in previous population studies that urinary 8-OH-dGuo levels correlated with urinary arsenic levels (36–38). The present study further demonstrated that arsenite induces significantly higher urinary 8-OH-dGuo levels than both realgar and orpiment on exposure to comparable amounts of arsenicals (Fig. 3).

To date, there is no information on the relative genotoxicity among arsenite, realgar and orpiment, partialy due to the absence of a suitable dosage form for realgar and orpiment. Clinically, arsenite (As^{III}) is formulated by dissolving arsenic trioxide in diluted sodium hydroxide solution and administered intravenously. In our previous study, realgar and orpiment were found converting to arsenite (As^{III}) and arsenate (As^{V}) in alkali extracts (39). Therefore, the natural effects of the two latter arsenic compounds could only be evaluated if they are formulated as nanoparticles to maintain their chemical identities (17).

Table IV. Urinary Arsenic Corrected 8-OH-dGuo Concentrations in Rats Treated with the Respective Arsenite, Realgar and Orpiment Orally

	Urinary 8-OH-dGuo (ng/μg urinary arsenic)		
Duration	Arsenite administration	Realgar administration	Orpiment administration
Day 1	0.86±0.21	0.55±0.29	0.58±0.16
Day 2	0.87±0.21	0.54±0.13	0.62±0.13
Day 3	0.83±0.21	0.51±0.12	0.64±0.12
Day 4	0.83±0.22	0.59±0.16	0.58±0.14
Day 5	0.86±0.10	0.54±0.13	0.60±0.16
Day 6	0.76±0.29	0.57±0.07	0.61±0.13
Day 7	0.83±0.15	0.52±0.12	0.65±0.10
Day 8	0.77±0.21	0.56±0.08	0.66±0.11
Day 9	0.86±0.22	0.57±0.08	0.62±0.12
Day 10	0.75±0.25	0.57±0.09	0.61±0.13
Day 11	0.77±0.14	0.61±0.11	0.58±0.22
Day 12	0.82±0.12	0.55±0.10	0.65±0.18
Day 13	0.83±0.17	0.58±0.07	0.67±0.22
Day 14	0.85±0.45	0.60±0.10	0.68±0.20

Data are presented as mean ± SD ($n=6$)

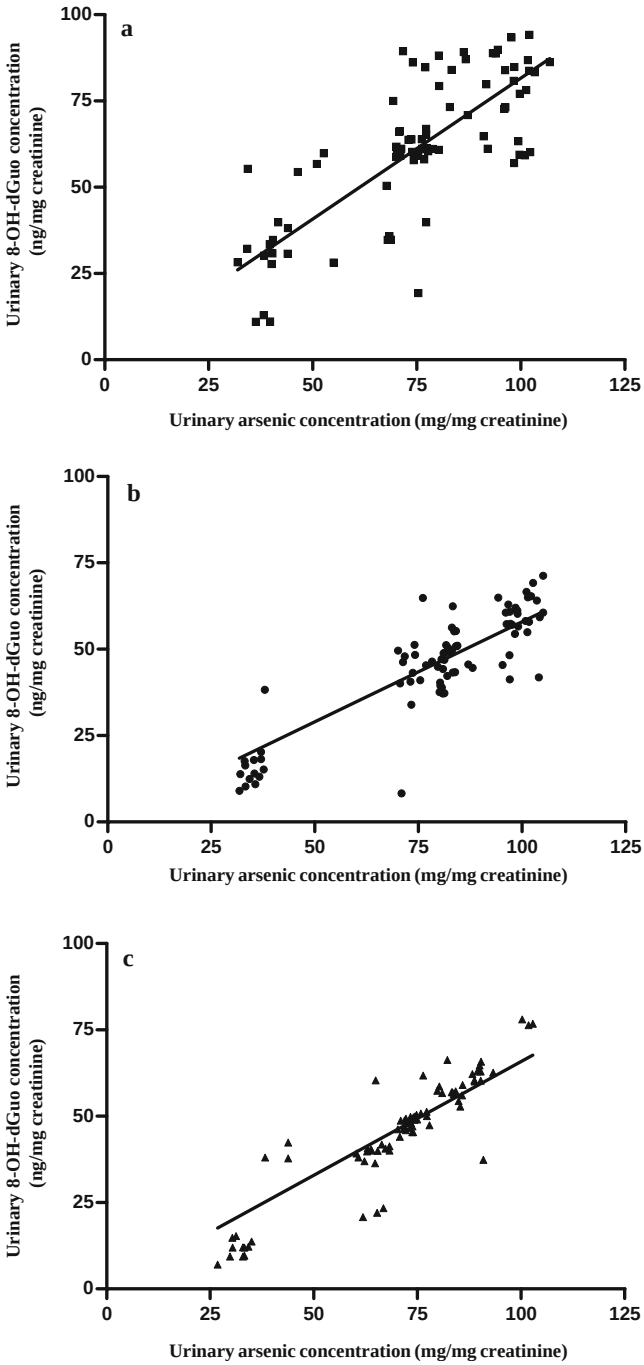

Fig. 3. Correlation between urinary 8-OH-dGuo and urinary arsenic recovery levels in **a** arsenite-treated group, **b** realgar-treated group, and **c** orpiment-treated group.

Arsenic trioxide is regarded as a toxic compound, as it is associated with adverse effects especially in long-term use (6). Furthermore, arsenic trioxide can cause severe liver damage, if given orally. As a result, the agent must be administered intravenously daily by infusion for 1 to 4 h, which limiting its usage especially in consolidation and maintenance therapy (6). Therefore, an alternative oral agent with similar efficacy

and fewer side effects would provide not only the benefits of cost-effectiveness and better quality of life but also easy access to consolidation and maintenance therapy. Realgar has been used as a traditional medicine in China and Europe for more than 1,500 years (40). Recently, researchers in China found that orally administered realgar was highly effective and safe for both remission induction and maintenance in all stages of APL (6). Patients treated with long-term oral realgar administration for 5 years encountered only moderate side effects (6). In contrast to results with other cytotoxic antineoplastic agents, no myelosuppression was observed. In addition, they observed that realgar was absorbed rapidly and excreted mostly within the first 24 h-urine. The present study also indicated that all the test arsenic compounds (arsenite, realgar and orpiment) were excreted mostly in 24 h in rats after daily oral administration, and no sign of compound accumulation was observed over the 14-day study period.

Chung *et al.* (36) compared the urinary 8-OH-dGuo levels in the respective populations (i.e., low arsenic/non-smoking; low arsenic/smoking; high arsenic/non-smoking; and high arsenic/smoking). They noticed that the populations with high total urine arsenic concentration were associated with high urinary 8-OH-dGuo levels. Our study showed a strong correlation ($p<0.001$) between urinary 8-OH-dGuo levels and urinary arsenic levels over a wide range of concentrations (Fig. 3). This illustrates that urinary-OH-dGuo level could be a valid biomarker for detecting the extent of arsenic exposure.

More developed anticancer drugs have been found to induce an enhanced formation of ROS (41). The therapeutic efficacy of these drugs may at least partially depend on ROS production, whereas their side effects may also be due to ROS generation. ROS formation is an essential mechanism of arsenic trioxide-induced apoptosis (42). Increasing evidence indicates that arsenic stimulates the formation of various types of ROS including hydrogen peroxide, superoxide anion, singlet oxygen, and hydroxyl radical in many cells through various pathways (43–46). Since the complex chemistry of arsenic, the role it plays etiologically and clinically varies accordingly.

CONCLUSIONS

The most important finding in this study is that arsenite caused significantly higher urinary 8-OH-dGuo levels than both realgar and orpiment on exposure to the same amount of compound, indicating that arsenite could potentially cause more oxidative DNA damage. This finding reflects the clinical observation on the safety of realgar (6). The slightly higher urinary 8-OH-dGuo levels in rats induced by arsenite than realgar on exposure to the same amount of compound may not explain the 100-fold difference in oral LD_{50} in mice between arsenic trioxide (33–39 mg/kg) and realgar (3.2 g/kg) (47). It is well known that mice and rats behave very differently in the extent of metabolism and toxicity (48). The purpose of toxicity study in animal models is to identify the types of toxic effects and the relative toxic effects of the test materials in the respective species. Therefore, it is not appropriate to relate the extent of effects in one species to the other species. However, the studies in the mice and rat models did show that various arsenical compounds behave differently in the levels of toxicity and oxidative stress induced. Arsenic trioxide appeared to be more toxic in mice and caused higher levels of 8-OH-dGuo in rats than the respective realgar and orpiment.

It has been recognized that the increased levels of DNA based oxidation products such as 8-OH-dGuo do not always lead to malignancy (49). The nature of the DNA damages and the effectiveness of their subsequent repair could determine the outcomes. It warrants studying the different causes, if any that would be involved in inducing the elevated levels of 8-OH-dGuo by these arsenic compounds and their effects.

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