— Minireview —

Elucidation of the Metabolic Pathways of Selenium and Arsenic by Analytical Toxicology

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Hyphenated analytical techniques, such as high-performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) and HPLC-electrospray ionization (ESI)-MS, are powerful tools for evaluating the elemental (HPLC-ICP-MS) and molecular (ESI-MS) speciation of metalloids, such as selenium (Se) and arsenic (As). Because the toxicity of metalloids is highly dependent upon the chemical forms that are present *in vivo*, toxicological assessment must include both the intact compound and its metabolites. We investigated the metabolic pathways of Se and As compounds and their metabolites using HPLC-ICP-MS and HPLC-ESI-MS.

Key words —— selenium, arsenic, analytical toxicology, metabolism, high-performance liquid chromatography-inductively coupled plasma-mass spectrometry, high-performance liquid chromatography-electrospray ionization-mass spectrometry

INTRODUCTION

Selenium (Se) and arsenic (As) are potentially toxic metalloid compounds. Although Se is an essential micronutrient,[1,2] in sufficient amounts it is toxic, with an extremely narrow threshold between adequate and toxic levels. Arsenic, on the other hand, is not a micronutrient, and exists in several highly toxic forms [*e.g.*, inorganic arsenite (iAs^{III}) and arsenate (iAs^{V}), both of which are well-known worldwide environmental contaminants].[3]

Both Se and As are methylated by S-adonosyl-L-methionine (SAM) with methyltransferases mediating the methyl group transfer.[4,5] Methylation was once generally believed to detoxify metalloids such as Se and As. However, recent reports indicate that methylation may not always lead to detoxification, as the trivalent arsenic metabolites monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), were shown to be more toxic than iAs.[6–8] Because cellular uptake, toxicity, and excretion of Se and As depend on the chemical forms present *in vivo*, additional research is needed to clarify the mechanisms of toxicity and detoxification mechanism of these potentially harmful metalloids. Analysis of the chemical forms of metalloids requires stable, high sensitivity analytical toxicology techniques and must include both the metalloids and their metabolites.

The combination of high-performance liquid chromatography with inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) provides a powerful tool for the elemental speciation of metalloids.[9–11] Although HPLC-ICP-MS is highly sensitive and highly selective, the technique cannot provide any molecular information. Determination of molecular speciation is possible however with another hyphenated technique, HPLC-electrospray ionization (ESI)-MS. In this minireview, we report on our recent study utilizing these techniques to more fully elucidate the metabolic pathways of Se and As.

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STUDIES ON THE METABOLIC PATHWAY OF SELENIUM

Identification of Se Urinary Metabolites for Excretion within the Required to Low-toxic Range[12)]

Se is an essential trace element. It is excreted through the urine and/or by exhalation after mono-, di-, and trimethylation. Monomethylated Se is the major urinary metabolite excreted with deficient to low-toxic doses of Se, whereas excretion of trimethylselenonium ion (trimethylated Se) through the urine and dimethylselenide (dimethylated Se) by exhalation increase with excessive Se dose in the toxic range. Although it is postulated that metabolism of Se compounds in the low-toxic range involves incorporation of a methylselenol group into the structure, the actual structure of the metabolite has not been clarified. Ogra *et al.*[13)] reported that the major urinary metabolite is a selenosugar, Se-methyl-*N*-acetylselenohexosamine. However, the structure of this metabolite, including the configuration, was not clearly resolved, even though the molecular weight and the functional group were determined by tandem mass spectrometry. We determined the chemical structure of the selenosugar using HPLC-ICP-MS, HPLC-ESI-MS/MS, and nuclear magnetic resonance (NMR) spectroscopy. The structure of 1β-methylseleno-*N*-acetyl-D-galactosamine (Fig. 1) was deduced from the spectroscopic data and confirmed by chemical synthesis.

Identification of Se Metabolites in Liver[12)]

Stable isotope labeled selenite is transformed into two metabolites in the liver, a heavier (metabolite A) and lighter (metabolite B)[14)] molecular weight form as determined by gel filtration chromatography. Metabolite A increases in the presence of the methylation inhibitor, periodate-oxidized adenosine. HPLC-ICP-MS analysis suggested that hepatic metabolite B is also the major urinary metabolite. We presumed that metabolite A is a precursor and that it is transformed into metabolite B (methylated Se metabolite) for excretion in the urine. Partially purified metabolite A was subjected to HPLC-ESI-MS analysis. Se-containing metabolites were identified from the characteristic isotope pattern for Se, Se-76 (9.36%), -77 (7.63%), -78 (23.8%), -80 (49.6%), and -82 (8.73%). Characteristic signals were detected at m/z 591 and 589 for ^{80}Se in the positive and negative-ion mode, respectively. Precursor ion m/z 591 (corresponding to $[M+H]^+$) in the first quadrupole was introduced into the second quadrupole to dissociate the precursor ion. Fragment ions were then detected in the third quadrupole. The most abundant m/z 591 fragment ion was detected at m/z 204 with a collision energy of 40 eV. This result suggests that metabolite A has the same selenosugar structure as metabolite B, because the same major fragment ion (m/z 204) was detected following metabolite B (precursor ion $- CH_3{}^{80}SeH$) fragmentation (Table 1).

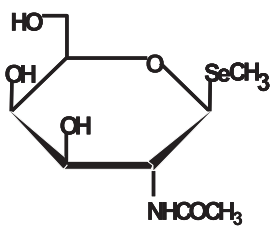

Fig. 1. The Structure of the Urinary Se Metabolite (Equivalent to Hepatic Se Metabolite B)[12)]

We determined the chemical structure of the Se metabolite using HPLC-ICP-MS, HPLC-ESI-MS/MS, and NMR by comparing the results of measurements of the selenium metabolite and the synthetic selenosugar, 1β-methylseleno-*N*-acetyl-D-galactosamine.

We then undertook to assign structures for other ions observed during HPLC-ESI-MS. We assumed that m/z 388, which can be assigned as [591-sugar]$^+$, is the fragment ion instead of $CH_3{}^{80}SeH$. As a candidate structure for the fragment ion m/z 308 (388 $-$ ^{80}Se), instead of 15 [CH_3] in metabolite B, glutathione (GSH; γ-glutamylcysteinylglycine; precursor ion = 308) was deduced from the fragment ions between the hepatic Se metabolite A (precursor ion = 591) and GSH: (462 = [591 $-$ Glu]$^+$) *vs.* (179 = [308 $-$ Glu]$^+$), (516 = [591 $-$ Gly]$^+$) *vs.* (233 = [308 $-$ Gly]$^+$) at 20 eV collision energy (Table 1). Furthermore, at 40 eV collision energy, fragment ions for precursor ion m/z 591 were detected at m/z 313 and m/z 259, corresponding to [388 $-$ Gly]$^+$ and [388 $-$ Glu]$^+$, respectively. These data suggest that metabolite A is also a selenosugar conjugated with GSH instead of methyl group, as is the case with metabolite B (Fig. 2).

STUDIES ON THE METABOLIC PATHWAY OF ARSENIC

A New Pathway for As Metabolism[15)]

In the classical pathway (Fig. 3a), As is thought to be metabolized by repetitive reduction and oxidative methylation, excreted in the urine mainly as

Table 1. Assignment of Molecular and Fragment Ions Detected by HPLC-ESI-MS/MS of Hepatic Se Metabolite A, Synthetic Selenosugar, and Reduced Glutathione

m/z	Hepatic Se metabolite A[a)]	Synthetic selenosugar[a, b)]	Reduced glutathione
591	$[M+H]^+$		
516	$[591-Gly]^+$		
462	$[591-Glu]^+$		
388	$[591-sugar]^+ = [GSSeH+H]^+$		
313	$[388-Gly]^+$		
308			$[M+H]^+$
300		$[M+H]^+$	
259	$[388-Glu]^+$		
233			$[308-Gly]^+$
204	[591−GSSeH]	$[300-CH_3SeH]$	
186	$[204-H_2O]^+$	$[204-H_2O]^+$	
179			$[308-Glu]^+$
168	$[186-H_2O]^+$	$[186-H_2O]^+$	
144	$[204-CH_3COOH]^+$	$[204-CH_3COOH]^+$	

a) Only ions containing the ^{80}Se-isotope are indicated. *b*) 1β-methylseleno-*N*-acetyl-D-galactosamine.

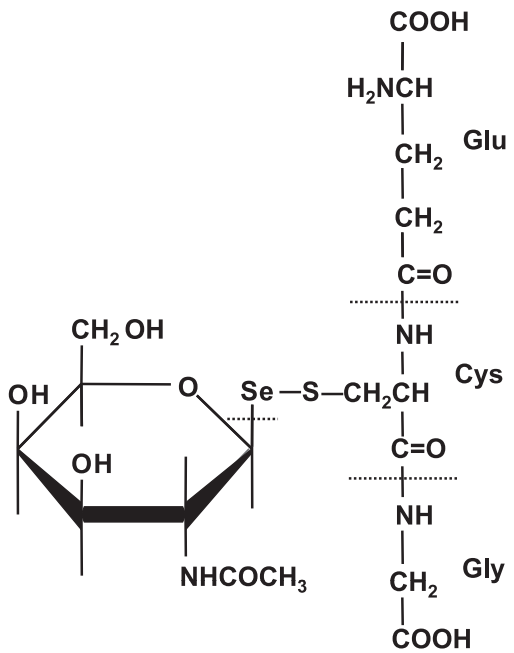

Fig. 2. The Proposed Structure for Hepatic Se Metabolite A[12)]

The structure of the urinary Se metabolite was estimated based on HPLC-ESI-MS/MS comparisons between synthetic selenosugar and reduced GSH.

dimethylarsinic acid (DMA^V). Glutathione plays an important role in the metabolism of iAs. As-GSH complexes, such as arsenic triglutathione (ATG) and methylarsenic diglutathione (MADG), were detected as major metabolites in the bile of rats intravenously injected with iAs.[16–19)] Recently, we proposed that As-GSH complexes are substrates for the SAM-dependent arsenic methyltransferase, AS3MT (previously, Cyt19) (Fig. 3b).

To confirm this hypothesis, we investigated methylation of As-GSH complexes using human recombinant arsenic methyltransferase AS3MT. Our results suggested that the metabolic pathway of arsenic should be revised. Following iAs^V reduction to iAs^{III}, iAs^{III} forms a complex with GSH, and the ATG that is generated is methylated to MADG by AS3MT. We hypothesized that MADG is further methylated to dimethylarsenic glutathione (DMAG). As-GSH complexes, such as ATG, MADG, and DMAG, are in equilibrium with As-GSH complex-related trivalent arsenicals such as iAs^{III}, MMA^{III}, and DMA^{III}, depending on the GSH concentration. Some of the trivalent arsenicals are oxidized to analogous pentavalent compounds, such as iAs^V, MMA^V, and DMA^V. In the classical metabolic pathway, pentavalent metabolites such as MMA^V and DMA^V, were thought to be generated via the more toxic trivalent arsenicals, such as iAs^{III}, and MMA^{III}. Reduction was also thought to be the means whereby DMA^V was converted to DMA^{III} in the classical pathway. The new pathway that we proposed, however, supports the suggestion of Aposhian *et al.*[20)] that oxidation is the mechanism of arsenic detoxification.

Effects of Endogenous Hydrogen Peroxide and Glutathione on the Stability of As Metabolites in Rat Bile[17)]

Recently, we demonstrated that both ATG and MADG are unstable and easily hydrolyzed to iAs^{III} and MMA^{III}, respectively. We also demonstrated that MMA^{III} is oxidized to MMA^V in bile, and exogenously added GSH stabilizes As-GSH com-

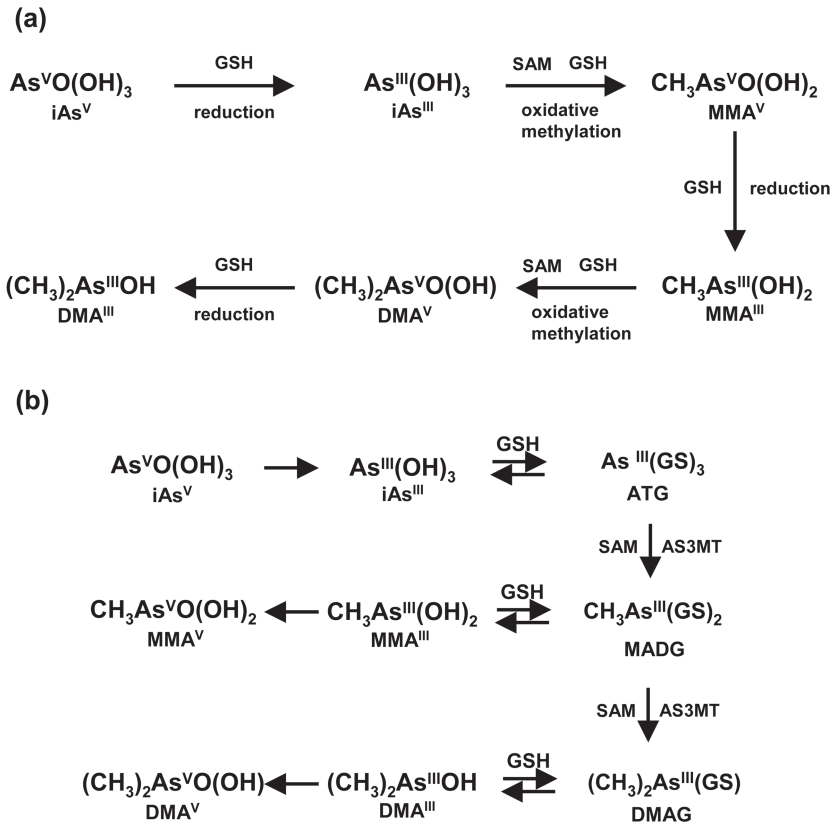

Fig. 3. (a) The Classical and (b) a New Pathways for Inorganic Arsenic Metabolism in Mammals[15)]

plexes in bile.[16)] In addition, we reported on the effects of hydrogen peroxide (H_2O_2) and GSH on the stability of As-GSH complexes in rat bile.[17)]

Male Sprague-Dawley (SD) rats were injected intravenously with saline (control group) or iAs^{III} at a dose of 0.2 (lower dose group) or 2.0 mg As/kg body weight (higher dose group), and bile fluid was collected on ice for 30 min. Although the lower dose of As did not significantly change the concentrations of biliary H_2O_2 and GSH, H_2O_2 and GSH concentrations in the higher dose group increased 12.6- and 4.5-fold, respectively, compared to the control group (Fig. 4). Approximately 9.6% of the As dose was excreted in bile within 30 min in the lower dose group, whereas 20% of the As dose was excreted in the bile within 30 min in the higher dose group. Speciation of arsenicals and sulfur compounds in this *in vivo* study was determined by HPLC-ICP-atomic emission spectrometer (AES). We detected MADG, and found that it accounted for 70.6% of the arsenic in bile from the lower dose group (Table 2). We did not detect ATG in the lower dose group, but we found that it was the major metabolite in bile from the higher dose group. Total recovery of ATG and MADG was 89.9% and 7.93%, respectively (Table 2).

Table 2. Concentrations of Arsenicals in Bile of Rats Intravenously Injected with iAs^{III}

Rats were intravenously injected with saline (control), 0.2 (lower), or 2.0 (higher) mg As/kg body weight iAs^{III}. Bile samples were collected for 30 min after injection. For the *in vivo* study, the concentration of total biliary arsenic was measured by ICP-MS. The concentrations of biliary As-GSH complexes were calculated by HPLC-ICP-AES using standard ATG and MADG (1–50 μg As/ml), respectively. Calibration curves based on peak areas were found to be linear, and the correlation coefficients were 0.9996 for ATG and 0.9997 for MADG. Data are presented as means ± S.E.

Dose (mg As/kg)	Total As[a)] (μg/ml)	ATG[b)] (μg As /ml)	MADG[b)] (μg As/ml)
0 (control)	0.018 ± 0.002	ND	ND
0.2 (lower)	13 ± 2.0	ND	9.2 ± 2.2
2.0 (higher)	189 ± 5.5*	170 ± 26*	15 ± 0.85*

Values are the mean ± S.E. (n = 4). ND; not detected. *p < 0.0001 (Bonferroni/Dunn) as compared with control group. *a*) Measured by ICP-MS. *b*) Measured by HPLC-ICP-AES.

To estimate the stability and oxidation of arsenic metabolites in bile, synthetic ATG or MADG was added to untreated, heat-treated, catalase-treated, or dialyzed bile, and then incubated at 37°C for 10 min. Complete hydrolysis of ATG to iAs^{III} was observed at 1 and 10 μg As/ml. Exogenous MADG

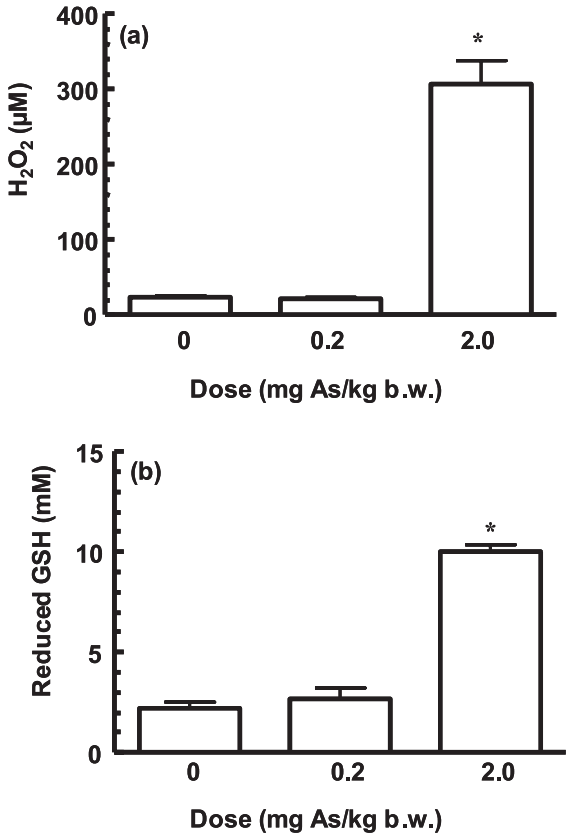

Fig. 4. *In vivo* H_2O_2 and GSH Concentrations in Bile[17)]

Rats were intravenously injected with saline or iAs^{III} at a dose of 0.2 or 2.0 mg As/kg body weight and the bile samples were collected for 30 min after injection. (a) H_2O_2 was measured using a hydrogen peroxide/peroxidase detection kit. Biliary H_2O_2 concentrations in the higher dose group increased significantly ($p < 0.0001$) compared to the control value. (b) Concentrations of biliary glutathione were calculated by HPLC-ICP-AES using standard GSH solutions (0.1–1 mM). Calibration curves based on peak areas were found to be linear, and the GSH correlation coefficient was 0.9995. Biliary GSH concentrations in the higher dose group increased significantly ($p < 0.0001$) compared to the control value.

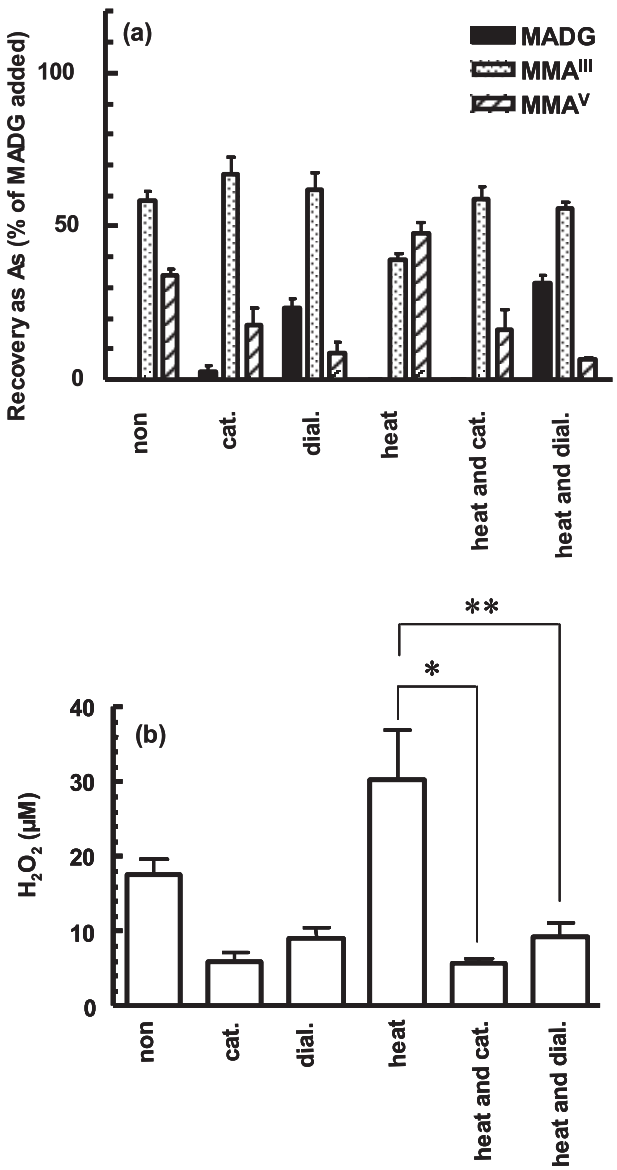

Fig. 5. *In vitro* Concentration-dependent Stability of Synthetic MADG in Bile, and Changes in Concentration of Biliary H_2O_2[17)]

(a) MADG at concentrations of 10 μg As/ml was incubated in bile at 37°C for 10 min. The samples were diluted to 10 ng As/ml with mobile phase and applied to an anion exchange column. The speciation of each arsenical was measured by HPLC-ICP-MS, and was calculated from the corresponding standard using plasma chromatographic software. (b) Control bile (non) was obtained from untreated 10-week-old male SD rats. Each bile sample was treated either by boiling for 5 min (heat) and centrifuging at 17800 *g* for 15 min, incubation with 5 U/ml catalase (cat.) at 37°C for 30 min, dialysis (dial.) against 150 mM phosphate buffer (pH 8.0) at 4°C overnight, or a combination of those treatments (heat and cat.; heat and dial.). The concentration of H_2O_2 in heat-treated bile decreased significantly upon catalase treatment (*: $p < 0.0001$) and dialysis (**: $p = 0.0004$).

was hydrolyzed and oxidized to pentavalent arsenicals depending on the biliary concentration of H_2O_2 (Fig. 5). Both catalase treatment and dialysis prevented oxidation of trivalent arsenicals to the corresponding pentavalent compounds (Fig. 5).

As-GSH complexes are decomposed by gamma-glutamyl transpeptidase (GGT).[21)] In order to elucidate the role of GGT and GSH in the metabolism of As-GSH complexes after biliary excretion, we studied GGT-mediated breakdown of As-GSH complexes with and without GSH using LC-MS. MADG, but not ATG, was decomposed by GGT, probably because ATG is unstable and rapidly hydrolyzed in solution. After biliary excretion, ATG may instead be hydrolyzed to toxic iAs^{III}. On the other hand, we detected methylarsenic cysteinylglycine-GSH and methylarsenic dicysteinylglycine-GSH complexes as a result of MADG decomposition.

In summary, GSH prevents hydrolysis of As-GSH complexes and the generation of unconjugated toxic trivalent arsenicals, while H_2O_2 converts triva-

lent arsenicals to less toxic pentavalent arsenicals.

CONCLUSIONS

Utilizing hyphenated MS analytical techniques to study the metabolic pathways of Se and As metalloids, First, we clarified the chemical structure of the urinary Se metabolite (1β-methylseleno-*N*-acetyl-D-galactosamine: selenosugar) in low-toxic doses of Se using HPLC-ICP-MS, -ESI-MS/MS, and NMR. Furthermore, HPLC-ICP-MS and HPLC-ESI-MS enabled us to determine that the hepatic Se metabolite was a selenosugar conjugated with glutathione instead of a methyl group. The hepatic metabolite was assumed to be a precursor for methylation of the urinary metabolite. Secondly, we proposed a new metabolic pathway for iAs^{III} in which As-GSH complexes are substrates for AS3MT, based on the results of HPLC-ICP-MS analyses of human recombinant arsenic methyltransferase AS3MT, GSH, arsenicals, and SAM reactions. Thirdly, we reported the effects of endogenous H_2O_2 and GSH on the stability of arsenic metabolites in rat bile by measuring a chemical form of arsenicals by HPLC-ICP-MS and HPLC-ESI-MS.

Our research indicates that fully understanding the toxicity of metalloids must include analysis of both the intact compound and its metabolites. Hyphenated mass spectrometry methods are exceptionally well suited to this type of research. Advances in the use of these analytical toxicological techniques made through studies on metalloid compounds may also be applicable to research involving other environmental toxins.

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