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MEASUREMENT OF ARSENIC RELATIVE BIOAVAILABILITY IN SWINE

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This study describes a method for measuring the relative oral bioavailability (RBA) of arsenic (As) in soil and other soil-like media using young swine as the animal model. Groups of animals are exposed to site soil or sodium arsenate orally for 12 d. Forty-eight-hour urine samples were collected from each animal on d 6–7, 8–9, and 10–11 and were analyzed for total As. The urinary excretion fraction (UEF) for each group was estimated by plotting the mass of As excreted in urine by each animal as a function of the dose administered, and then fitting a linear model to the data using simultaneous weighted linear regression. The RBA of a test material is calculated as the ratio of the UEF value for the test material divided by the UEF of the reference material. Uncertainty around the RBA estimate is calculated using Fieller's theorem. Application of this method to a series of test soils indicates that RBA values for As can range from 18 to 52%. This wide variability supports the conclusion that there may be important differences in RBA between sites, and that use of a site-specific RBA value is likely to increase the accuracy of risk estimates for exposure to As in soil.

Accurate assessment of the human health risks resulting from oral exposure to As requires knowledge of the amount of As absorbed from the gastrointestinal (GI) tract into the body (Orloff et al., 2009). This information on GI absorption may be described either in absolute or relative terms:

Absolute bioavailability (ABA) is the ratio of the amount of As absorbed to the amount ingested:

$$\text{ABA} = \left(\text{Absorbed dose}\right) / \left(\text{Ingested dose}\right)$$

Relative bioavailability (RBA) is the ratio of the absolute bioavailability of As present in some test material to the absolute bioavailability of As in some appropriate reference material:

$$\text{RBA} = \text{ABA (test)} / \text{ABA}\left(\text{reference}\right)$$

When measuring RBA, the form of As used as the reference material is usually an As compound dissolved in water or a readily soluble form (e.g., sodium arsenate) that is expected to completely dissolve when ingested.

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When a reliable RBA value is available for a particular site medium (e.g., soil), the RBA can be used to adjust the default reference dose (RfD) and cancer slope factor (CSF) for As to account for differences in absorption between As ingested in water and As ingested in the site medium, as follows:

$$RfD_{adj} = \frac{RfD_{default}}{RBA}$$

$$CSF_{adj} = CSF_{default} \cdot RBA$$

Alternatively, it is also acceptable to adjust the dose (rather than the toxicity factors) as follows:

$$Dose_{adj} = Dose_{default} \cdot RBA$$

This dose adjustment is mathematically equivalent to adjusting the toxicity factors as described above.

Scientists from the U.S. Environmental Protection Agency (EPA) Region 8 have been engaged in a multiyear investigation of As absorption from a variety of different environmental media, especially soils and solid wastes associated with mining, milling, and smelting sites. The protocol described here is the end product of these many years of research, and represents the current approach used by the U.S. EPA for measuring As RBA in vivo. This investigation, along with other related studies, has been described in a report prepared by the U.S. EPA (2010).

MATERIALS AND METHODS

Experimental Animals

Juvenile swine are used in these studies because their GI physiology is more similar to humans than most other animal models (Weis and LaVelle, 1991). For the studies reported here, all animals were young males of the Pig Improvement Corporation (PIC) genetically defined Line 26, and were purchased from Chinn Farms, Clarence, MO. The number of animals purchased for each study was typically six to eight more than required by the protocol. These animals were usually purchased at age 4–5 wk (weaning occurs at age 3 wk), and were held in individual stainless-steel cages. Any animals that appeared to be in poor health were excluded. To minimize weight variations between animals and groups, extra animals most different in body weight (either heavier or lighter) 4 d prior to exposure (d −4) were also excluded from the study. The remaining animals were assigned to dose groups at random. When exposure began (d 0), the animals were about 5–6 wk old and weighed approximately 7–12 kg.

Diet

Animals provided by the supplier were weaned onto standard pig chow purchased from MFA, Inc., Columbia, MO. In order to minimize As exposure from the diet, the animals were gradually transitioned from the MFA feed to a special feed (Zeigler Brothers, Inc., Gardners, PA) over a time interval from d −7 to d −3; this feed was then maintained for the duration of the study. The feed was nutritionally complete and met all requirements of the National Institutes of Health–National Research Council. Each day every animal was given an amount of feed equal to 5% of the mean body weight of all animals on study. Feed amounts were adjusted every 3 d, when pigs were weighed. Feed was administered in 2 equal portions of 2.5% of the mean body weight at 11:00 a.m. and 5:00 p.m. daily. Periodic analysis of feed samples indicated that the As level was generally below the detection limit (0.1 ppm), which corresponds to a dose contribution from food of less than 5 μg/kg-d. Drinking water was provided ad libitum via self-activated watering nozzles within each cage. Periodic analysis of samples from randomly selected drinking water nozzles indicated the As concentration was less than the detection limit (about 1 μg/L). Assuming water intake of about 0.1 L/kg-d, this corresponds to a dose contribution from water of less than 0.1 μg/kg-d.

Test Materials

Test materials were obtained from a number of sites being investigated by the U.S. EPA

TABLE 1. Test Materials

Site	Sample designation	Sample description	Arsenic concentration (ppm)	Target doses (μg/d)
Vasquez Boulevard and I-70 NPL Site, Denver, CO	VBI70 TM1	Soil composite from impacted residential property (Eastern Swansea/Elyria neighborhood)	312	500, 1250
	VBI70 TM2	Soil composite from impacted residential property (Western Swansea/Elyria neighborhood)	983	500, 1250
	VBI70 TM3	Soil composite from impacted residential property (Eastern Cole neighborhood)	390	500, 1250
	VBI70 TM4	Soil composite from impacted residential property (Western Cole neighborhood)	813	300, 600
	VBI70 TM5	Soil composite from impacted residential property (Clayton neighborhood)	368	300, 600
	VBI70 TM6	Clean site soil (from the Swansea/Elyria neighborhood) plus added PAX pesticide	516	300, 600
Silver Bow Creek/Butte Area NPL Site, Butte, MT	Butte TM1	Soil composite collected from waste rock dumps in Butte Priority Soils Operable Unit (BPSOU)	234	300, 600, 900
	Butte TM2	Soil composite collected from a residential property located adjacent to a railroad grade in Butte, MT	367	300, 600, 900
Wells G & H Superfund Site, Woburn, MA	Aberjona River TM1	Composite of sediment samples containing arsenic concentrations greater than 500 ppm, collected along the Aberjona River, Massachusetts	676	300, 600, 900
	Aberjona River TM2	Composite of sediment samples containing arsenic concentrations from 180 to 460 ppm, collected along the Aberjona River, Massachusetts	313	300, 600, 900
El Paso/Dona Ana County Metals Survey site, El Paso County, Texas, and Dona Ana County, NM	El Paso TM1	Soil sample collected approximately 1.5 miles east of the American Canal in El Paso County, Texas	74	400, 800, 1600
	El Paso TM2	Soil sample collected approximately 1.5 miles east of the American Canal in El Paso County, Texas	73	400, 800, 1600
Confidential (Study sponsored by American Chemistry Council)	ACC utility pole soil	Soil affected by chromated copper arsenate (CCA)-treated wood utility poles (poles were in place for more than 10 yr)	320	600, 1200
	ACC dislodgeable arsenic	Dislodgeable material obtained from the surface of chromated copper arsenate (CCA)-treated wood (boards from in-service residential decks, aged outdoors for 1 to 3 yr)	3,500	300, 600, 1200

where As was present at elevated levels in soil or sediment (Table 1). In general, the test materials were prepared for study by drying and sieving to <250 μm grain size. This was done because it is generally assumed that fine-grained soil particles are more likely to adhere to hands and be ingested than coarse-grained soil particles.

Dosing

Each experiment included two or three dose groups for sodium arsenate and each test soil (either two or three test soils per study). Animals (typically 5 per dose group) were exposed to sodium arsenate or test material for 12 d, with the dose for each day being administered in 2 equal portions given at 9:00 a.m. and

3:00 p.m. (2 h before feeding). This schedule was used to minimize any potential effects of GI contents on As absorption rates. Dose levels of As ranged from 300 to 1600 μg/d (Table 1). In most studies, these dose levels were held constant over the course of the experiment, even though the animals gained weight during the study. Control animals (typically 3 per study) were not exposed to As.

Dose material was placed in the center of a small portion (approximately 5 g) of moistened feed (referred to as a "doughball"). Sodium arsenate was dispensed into the doughballs from a stock solution using a micropipette, while test material was weighed and placed into the doughballs as a solid. In cases where the mass of soil was too large to fit into one doughball, the test material was distributed among two or more doughballs. Doughballs were administered to the animals by hand. Occasionally, some animals did not consume some or all of the dose (usually because the dose dropped from their mouth while chewing). All missed doses were recorded and the time-weighted average dose calculation for each animal was adjusted downward accordingly.

Collection and Preservation of Urine

Samples of urine were collected from each animal on d 6–7, 8–9, and 10–11 during the study. Collection began at about 8:00 a.m. and ended 48 h later. The urine was collected in a stainless-steel pan placed beneath each cage, and the pan drained into a plastic storage bottle. Each collection pan was fitted with a nylon screen to minimize contamination with feces, spilled food, or other debris. At the end of each collection period, the urine was well mixed, the volume was measured, and two 60-ml portions were removed for analysis. Each 60-ml sample was preserved by addition of 0.6 ml concentrated nitric acid. These samples were refrigerated until sample analysis.

Sample Digestion

As concentrations in urine were measured using a hydride generation approach. This method requires that all As exist in the form of inorganic As before hydride generation. Because some As in urine may exist in organic forms such as monomethylarsonic acid and dimethylarsinic acid (Buchet et al., 1981a, 1981b; Orloff et al., 2009), vigorous digestion of the urine prior to analysis is required. This was performed as follows. A 25-ml aliquot of acidified urine was removed and placed in a clean 100-ml beaker. To this were added 3 ml methanol, 5 drops antifoam agent, 10 ml 40% (w/v) magnesium nitrate hexahydrate, and 10 ml concentrated trace metal grade nitric acid. The beaker was covered with a watch glass and placed on a hot plate to reflux for 8–12 h at 70–80°C. After this, the temperature was increased to 200°C, and the watch glass was moved back to allow faster evaporation. The sample was then heated to complete dryness (8–12 h), covered with a watch glass, and allowed to cool. Dried samples were transferred to a cool muffle furnace that was heated at a rate of 1°C/min to a temperature of 500°C, and then held at 500°C for 3 h before cooling. Ashed samples were dissolved by adding 5 ml distilled water and 5 ml concentrated trace metal grade hydrochloric acid (HCl), and boiling gently until the white residue was completely dissolved. After cooling, the dissolved sample was diluted with distilled water to 50 ml and held until analysis.

Arsenic Analysis by Hydride Generation

Samples were prepared for hydride generation by dilution with a solution of 10% HCl, 10% potassium iodide, and 5% ascorbic acid. The samples were diluted 1/10 or 1/5 (v/v), depending on the detection limit desired. Samples were held in the diluting fluid for at least 30 min before analysis, but overnight was preferred. Analysis was performed using a Perkin-Elmer 3100 atomic absorption spectrometer (AAS) equipped with an FIAS 200 flow injection system. Calibration standards were prepared in dilution fluid (10% HCl, 10% KI, 5% ascorbic acid) at concentrations of 0, 0.2, 1, 5, 10, and 15 μg/L.

The detection limit of the method was evaluated by performing 10 replicate analyses of

a low standard (about 1 μg/L). The detection limit was defined as 3 times the standard deviation of these 10 analyses. A 1/10 dilution typically gave a detection limit of about 2 μg/L, while a dilution of 1/5 typically yielded a detection limit of about 1 μg/L. All responses below the detection limit were evaluated at one-half the detection limit.

Estimation of Urinary Excretion Fraction

Data from each study were analyzed by plotting the amount of As excreted in urine (μg/48 h) against the amount of As administered (μg/48 h) for each animal and finding the slope of the best fit straight line for each dose material:

$$\text{Mass excreted}\,(\mu g) = a + b \times \text{dose administered}\,(\mu g)$$

The slope of the line for each dose material is the urinary excretion fraction (UEF) (μg excreted/μg administered) for that dose material. Fitting was performed in Microsoft Excel using matrix functions. All of the data were evaluated simultaneously to ensure that the intercept term (*a*) is the same for all dose materials, as described by Finney (1978). Weighted regression was used because the between-sample variability tended to increase as a function of the mass of As excreted (heteroscedasticity). In this approach (Draper and Smith, 1998), the squared error for each observation is assigned a weight that is inversely proportional to the variance of the response in that group:

$$\textit{Weighted square error}_{i,j,m} = \frac{1}{\sigma_j^2}\left(x_{i,j,m} - a - b_m \cdot dose_{j,m}\right)^2$$

where:

σ^2_j = variance of responses in animals in dose group j
$x_{i,j,m}$ = mass of As excreted by animal i in dose group j of test material m
a = mass of As excreted by control animals
b_m = urinary excretion fraction for dose material m
$dose_{j,m}$ = dose of As administered to animals in dose group j of test material m

One approach for estimating the weight is to assume that σ^2_j is identical to the observed value of the sample variance (s^2_j) for each dose group. This approach was not employed because observed sample variance is a relatively unstable statistic, especially when the number of animals is only 5. That is, due to random variation, the observed sample variance may be substantially smaller or larger than the true variance, and this might result is assignment of inappropriately high or low weights to the data during the fitting process. In order to minimize this problem, σ^2_j was estimated using an "external" variance model developed from observation of the relationship between variance and mean response across many studies. Based on the combined data from multiple experiments, the variance was modeled as:

$$ln\left(\sigma_j^2\right) = k1 + k2 \cdot ln(\bar{y}_j)$$

where:

σ^2_j = expected between-animal variance in As excretion for exposure group j
$\bar{y}_j$ = average As excretion by exposure group j

Based on this analysis, the intercept and slope terms for the variance model are as follows:

$$k1\,(\text{intercept}) = -1.10$$
$$k2\,(\text{slope}) = 1.64$$

Overall goodness of fit was evaluated using analysis of variance (Draper and Smith, 1998).

Calculation of RBA

Given the UEF for each dose material, the best estimate of the RBA for each test material is calculated as:

$$RBA_m = UEF_m / UEF_{ref}$$

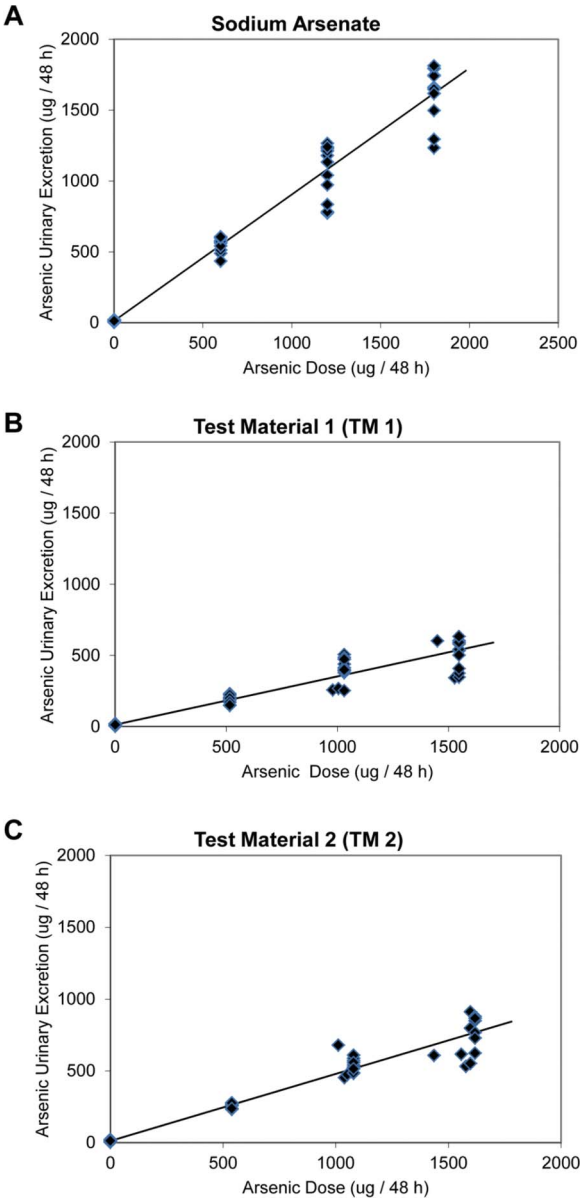

FIGURE 1. Example results for a study with two test materials. The three graphs show the raw data (solid diamonds) for (A) sodium arsenate (reference material), (B) test material 1, and (C) test material 2, along with the simultaneous best-fit variance-weighted linear regression models (color figure available online).

where RBA_m is the best estimate of the RBA for test material m, UEF_m the urinary excretion fraction for test material m, and UEF_{ref} the urinary excretion fraction for the reference material (sodium arsenate).

As described by Finney (1978), the uncertainty around this point estimate may be estimated using Fieller's theorem:

$$LB, UB = \frac{RBA - g\frac{covar_{r,t}}{var_r} \pm \frac{t}{b_r}\sqrt{W}}{1-g}$$

where LB is the lower bound of the fiduciary range on RBA, UB the upper bound of the fiduciary range on RBA, var_r the variance in the slope coefficient for the reference material, $covar_{r,t}$ the covariance in the slope coefficients for the reference and test materials, b_r the slope coefficient for the reference material, and t the t statistic for alpha (0.05) and $(n - p)$ degrees of freedom, where n is the total number of data points and p the total number of fitting parameters in the combined model,

$$g = \frac{t^2}{b_r^2} var_r$$

$$W = var_t - 2 \cdot R \cdot covar_{t,r} + R^2 \cdot var_r - g\left(var_r - \frac{covar_{r,t}^2}{var_r}\right)$$

The interval between the LB and the UB is the 95% confidence interval for the RBA.

RESULTS

Figure 1 presents the results of an example study in which two test soils were evaluated (Aberjona River sediments TM1 and TM2). As seen, the exposure-response data (mass of As excreted *vs.* mass of As administered) are well characterized by linear models. Table 2 provides the fitting statistics and calculated RBA values for this study. The slope of the line (the UEF) for sodium arsenate (0.89) is steeper than the slopes (UEF) for the two test materials (0.34 and 0.47), indicating lower excretion (and hence lower absorption) of As from the test materials than the reference material. Based on these values, the RBA values for test material 1 and 2 are 38 ± 2% and 52 ± 2%. Overall goodness of fit of the model to the data was assessed by analysis of variance (ANOVA). As indicated, the fit in this example is quite good ($R^2 = .969$, $p < .001$). Close inspection of Figure 1 reveals a tendency for increased

TABLE 2. Example Fitting Statistics

Summary of fitting	Estimate	SE	
a	12.0	1.4	
b1	0.89	0.02	
b2	0.34	0.01	
b3	0.47	0.01	
Covariance (*b1, b2*)	0.0082	—	
Covariance (*b1, b3*)	0.0063	—	
Degrees of freedom	113	—	
RBA and uncertainty	TM 1	TM 2	
RBA	38%	52%	
Lower bound	36%	49%	
Upper bound	41%	56%	
Standard error	1.6%	2.0%	
ANOVA	SSE	DF	MSE
Fit	2963.80	3	987.93
Error	92.38	112	0.82
Total	3056.18	115	26.58
Goodness-of-fit statistic	Estimate		
F	1197.7		
p	<.001		
Adjusted R^2	.969		

variance in As excretion as the average mass of As excreted rises. This is why weighted regression is judged to be appropriate. Other studies display similar attributes (data not shown).

Table 3 summarizes the RBA results for all test material analyzed using this protocol. As seen, using sodium arsenate as a relative frame of reference the estimated RBA values for these test materials range from 18 to 52%. This wide variability supports the conclusion that there may be important differences in RBA between different types of samples and that use of a site-specific RBA value is likely to increase the accuracy of risk estimates for As.

DISCUSSION

The results of this investigation indicate that juvenile swine are a useful model for quantifying GI absorption of As from different test materials, using urinary As excretion as the measurement endpoint. The approach is applicable to almost any type of As-contaminated medium that may be ingested including a wide variety of soils, sediments, and solid waste streams.

TABLE 3. Relative Bioavailability Estimates

Sample	RBA ± SE
VBI70 TM1	40 ± 4%
VBI70 TM2	42 ± 4%
VBI70 TM3	37 ± 3%
VBI70 TM4	24 ± 2%
VBI70 TM5	21 ± 2%
VBI70 TM6	24 ± 3%
Butte TM1	18 ± 3%
Butte TM2	24 ± 2%
Aberjona River TM1	38 ± 2%
Aberjona River TM2	52 ± 2%
El Paso TM1	44 ± 3%
El Paso TM2	37 ± 3%
ACC utility pole soil	47 ± 3%
ACC dislodgeable arsenic	26 ± 1%

Comparison to Other Models

Several other animal models were developed for assessing the RBA of As, including cynomolgus monkeys (Roberts et al., 2002, 2007) and mice (Bradham et al., 2011). In terms of experimental design, the swine model has several potential advantages compared to the other models. First, because of the size of juvenile swine (approximately 10 kg at the beginning of the study), it is usually possible to administer doses of test soils that are relatively close to the range thought to be of concern to humans. For example, for a soil with an As concentration of 500 ppm (500 μg/g), the amount of soil administered in the low dose group (25 μg As/d) is 500 mg/d, which corresponds to an intake of about 50 mg soil/kg body weight/d (mg/kg/d). This value is relatively close to the reasonable maximum exposure (RME) value of 13 mg/kg/d generally assumed for human children. In contrast, the monkey and mouse bioassay systems usually use soil doses that approach 1000 mg/kg/d. Thus, in the swine assay, most measurements are obtained in a portion of the dose-response curve that is more relevant to humans than is achieved in most other animal models. In addition, the swine model employed a repeated dosing protocol, with sampling not beginning until 5 d of exposure have elapsed. This allows the exposed animal to approach a quasi-steady state with regard to As intake and excretion.

An advantage of this protocol is that it reflects a more realistic human exposure scenario (continuous exposure for many months or years) than does a single dose protocol such as Roberts et al. (2002, 2007) used in the monkey model. Further, multiple measurements can be made from the same animal on different days to ensure that a steady state has been reached and to increase the number of observations upon which the fitting is performed.

Recently, the U.S. EPA (2012) compiled and compared RBA measurements from swine, monkey, and mouse studies to investigate whether the animal models yield the same or different outcomes. For swine compared to monkey, only four soil samples (all from the same site) have been analyzed in both systems. The results are too limited to draw a firm conclusion, and uncertainty bounds between paired RBA values often overlap. Nevertheless, there is a suggestion that RBA values measured in swine tend to be somewhat higher than for monkeys. For swine compared to mice, there are 11 paired samples available, and the results are qualitatively similar to monkeys. There is a tendency for swine RBA values to be somewhat higher than for mice, although the differences are not always statistically significant. At present, there is no firm basis for determining if the observed tendencies reflect authentic and significant differences between the animal models, and if so, which animal model is the most appropriate for estimating RBA in humans.

Potential Utility of RBA Data

The RBA results for different test materials investigated in the swine model support the view that absorption of As from soils and mine wastes may vary substantially, both within and between sites. The detailed chemical mechanism accounting for this variable and reduced bioavailability of As in soil-like media is not known, but almost certainly is related to variations in the chemical and physical form(s) of As in the sample.

Because As in most test materials is absorbed less extensively than soluble forms of As, and because soluble forms of As are the basis of the oral RfD and oral CSF for As, the use of the unadjusted toxicity factors for assessing human health risk (i.e., assuming an RBA value of 100%) will usually lead to an overestimate of hazard. Consequently, measurement and application of site-specific RBA values to adjust the toxicity factors to account for the lower level of absorption are expected to increase the accuracy and decrease the uncertainty in human health risk assessments for As. At sites where As is the principal source of risk from soil, this adjustment may allow for reduced remedial costs due to decreases in the estimated level of risk and hence the extent or magnitude of soil cleanup needed to protect public health.

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