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AN IN VITRO METHOD FOR ESTIMATION OF ARSENIC RELATIVE BIOAVAILABILITY IN SOIL

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This report summarizes the results of a study to develop an in vitro bioaccessibility (IVBA) extraction technique for estimating the relative bioavailability (RBA) of arsenic (As) in soil. The study was implemented in several steps. In step 1, key variables in the extraction protocol were identified. In step 2, 21 different extraction conditions were tested on 12 different soils with reliable RBA values measured in swine or monkeys to identify which yielded useful in vivo–in vitro correlations (IVIVC). In step 3, three extraction conditions were evaluated using 39 different test soils to make a final selection of the best IVIVC. In step 4, the within- and between-lab reproducibility of the extraction method was examined. The optimum IVIVC model for swine utilized a pH 1.5 IVBA extraction fluid, with an R^2 value of .723. For monkeys, the optimum IVIVC model was obtained using a pH 7 IVBA extraction fluid that contained phosphate, with an R^2 value of .755. Within-lab precision of IVBA results was typically less than 3%, with an average of 0.8% for all 4 labs. Between-lab variation in mean IVBA values was generally less than 7%, with an overall average of 3%. The principal advantages of this IVBA method compared to other in vitro methods described in the literature are that (1) the fluids and extraction conditions are simple, (2) the results have been calibrated against a larger data set than any other method, and (3) the method has been demonstrated to be reproducible both within and between labs.

Arsenic (As) is a chemical that is known to produce a range of adverse health effects in humans (ATSDR, 2007; Tsai et al., 1998; Golub et al., 1998; Orloff et al., 2009). Consequently, As is of potential concern to regulatory agencies at a number of sites. In most cases, incidental ingestion of As in soil or sediment is a primary exposure pathway. When risks to humans from ingestion of As in soil or sediment are calculated, the default assumption is that As is absorbed from soil or sediment to the same extent that it is absorbed from drinking water (the exposure medium in the studies from which the As toxicity values are derived). This ratio (absorption from soil compared to absorption from water) is referred to as the relative bioavailability (RBA). Numerous studies in animals suggest that the assumption of 100% RBA for As in soil or other soil-like media is overly conservative, with RBA estimates from As-contaminated soils at mining, smelting, herbicide, pesticide, and chemical plant

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sites generally ranging from 5 to 78% (Bradham et al., 2011; Brattin and Casteel, 2013; U.S. Environmental Protection Agency [EPA], 2010; Freeman et al., 1993, 1995; Juhasz et al., 2007; Roberts et al., 2007; Rodriguez et al., 2003). Well-performed studies in animal models that identify RBA values lower than the default are generally accepted as a basis for adjusting estimates of exposure and risk (U.S. EPA, 2007a), which often lead to substantial cost savings during site remediation compared to the use of the default RBA value. However, most animal studies of RBA tend to be relatively slow and costly, which tends to limit the application of an in vivo approach to only the largest hazardous waste sites. For these reasons, faster and less expensive methods to estimate RBA are highly desirable.

One strategy is to perform lab-based measurements of As solubilization from soil samples. In this approach, a sample of soil or sediment is extracted using a fluid that has properties that resemble a gastrointestinal (GI) fluid, and the amount of As solubilized from the sample into the fluid under a standard set of extraction conditions is measured. The fraction of As that is solubilized is referred to as the in vitro bioaccessibility (IVBA). The IVBA is then utilized to predict the in vivo RBA of As in that sample, usually through an empiric correlation model that relates IVBA to in vivo RBA (U.S. EPA, 2007b).

This study describes the development, testing, and interlab testing of an IVBA-based method for estimating the RBA of As in soil or other soil-like media. The relation between IVBA and RBA is based on 39 different As-containing test materials from mining, smelting, herbicide, pesticide, wood-treating, and chemical plant sites across the United States for which the RBA was previously measured in cynomolgus monkeys or juvenile swine.

MATERIALS AND METHODS

Basic Strategy

The starting point for the development of the As IVBA method described here was an in vitro method that was previously established for estimation of the RBA of lead (Pb) in soil (Drexler and Brattin, 2007; U.S. EPA, 2008). Method development was implemented in a stepwise fashion, as follows:

- Step 1: Identify up to three extraction fluid variables that had the largest effect on measured As IVBA values.
- Step 2: Based on the three key variables identified in step 1, test a range of different extraction fluids ($n = 21$) on an intermediate size set of test soils ($n = 12$) to see which fluids yielded potentially useful in vitro–in vivo correlations (IVIVC).
- Step 3: Based on the results from step 2, test a selected set of 3 extraction fluids on a large set of test soils ($n = 39$) to select the final extraction fluids that yield the best IVIVC.
- Step 4: Evaluate the within- and between-lab precision of IVBA results using a set of 12 different test soils extracted with 2 different extraction fluids by 4 different labs.

List of Test Soils

Table 1 provides a list and brief description of 48 soils that were used in these studies. These soils were available to the study authors in sufficient quantity to allow for multiple IVBA extraction tests, and were selected to provide a wide range of different mineralogical forms and concentrations of As. Most soils were obtained from U.S. EPA Superfund sites or other areas known to be contaminated with As, although some were uncontaminated soils that were spiked with pure mineral forms of As. Investigations of the IVIVC between IVBA and RBA values utilized only soils for which a reliable RBA value was available from studies in swine ($n = 20$) or monkeys ($n = 19$). For swine, only RBA values derived as described by Brattin and Casteel (2013) were used. That is, the in vivo study must have included dose groups to establish the sodium arsenate dose-response curve, and data must have been reduced using simultaneous weighted regression. Soils with As concentrations lower than 200 ppm were not considered due to the difficulty of accurately

TABLE 1. Description of Test Materials

Test material identifier	Site name	Site type	Sample description	Predominant mineralogy[a]	As concentration (mg/kg)[b]	RBA in swine (%)	RBA in monkeys (%)
Aberjona River TM1	Industri-plex and Wells G&H	Industrial	Aberjona River sediment	FeOOH	676	38.1	
Aberjona River TM2	Industri-plex and Wells G&H	Industrial	Aberjona River sediment	FeOOH, Fe-sulfate, $ZnSiO_4$	313	52.4	
Anaconda Flue Dust	Anaconda Smelter	Milling and Smelting	Flue dust	FeAsO	1663		
Anaconda Tailings	Anaconda Smelter	Milling and Smelting	Tailings	FeOOH	15,952		
Barber Orchard MS-1	Barber Orchard	Former apple orchard	Orchard Soil	PbAsO	290	31.0	33.0
Barber Orchard MS-4	Barber Orchard	Former apple orchard	Orchard Soil	PbAsO, MnOOH	388	40.8	28.0
Barber Orchard MS-5	Barber Orchard	Former apple orchard	Orchard Soil	PbAsO, cobaltite, FeOOH	382	48.7	38.0
Barber Orchard MS-8	Barber Orchard	Former apple orchard	Orchard Soil	PbAsO	364	52.8	25.0
BC Channel Soil	Kennecott (South Zone)	Mining	Soil from Bingham Creek Channel	FeAs sulfate, PbAsO	149		
Butte TM1	Silver Bow Creek/Butte Area	Mining, milling	Soil	Sulfosalt, FeOOH	234	17.8	
Butte TM2	Silver Bow Creek/Butte Area	Mining, milling	Soil	Fe-sulfate, FeOOH	367	23.6	
CAMT	Mesa del Oro	Mining	Residential soil containing tailings	Arsenopyrite, FeOOH	300		19.0
Clark Fork Tailings	Clark Fork River	Mining, milling	Overbank tailings	FeOOH, Fe-sulfate, Sulfosalt	181	50.7	
CORS	VBI70	Smelter	Residential soil	As_2O_3, PbAsO	1230		17.0
COSCS	Globeville	Smelter	Composite soil	AsMO, PbAsO	394		18.0
COSS	Smeltertown	Mining, milling, smelting	Soil	Fe-Sulfate, FeOOH	1492		5.0
COW-Fe	Cave of the Winds	Natural geologic formation	Natural soil	FeAsO	689		
COW-Mn	Cave of the Winds	Natural geologic formation	Natural soil	MnOOH	490		
CRM 205-225	—	Near power plant	Native soil contaminated with fly ash from the power plant	$PbMClO_4$	90		
Drexler-5	—	—	Colorado soil spiked with sodium arsenate	NaAsO	1239	100.0	
Drexler-6	Globeville	Smelting	Composite soil	AsMO, CaMO, PbAsO	1414		
Drexler-7	—	—	Colorado soil spiked with arsenic trioxide	As_2O_3	1767		
Drexler-8	—	—	Colorado soil spiked with lead arsenate	PbAsO	2961		
Enargite	—	Mining	Native CO soil spiked with crushed enargire from Butte MT	Cu_3AsS_4	191		
FLCDV	Not specified	Cattle dip site	Soil (collected by FL DEQ)	Clay, FeOOH	150		31.0
FLCPS	Near Inglis	Chemical plant	Soil (collected by FL DEQ)	Fe-sulfate, FeOOH	268		7.0

HIVS	Not specified	Volcanic	Soil (collected by HI DEH)	Clay, FeOOH, PbAsO	724		5.0
Iron King TM1	Iron King Mine/Humboldt Smelter	Mining, smelting	Soil	Fe-sulfate, sulfosalt	200	60.2	
Iron King TM2	Iron King Mine/Humboldt Smelter	Mining, smelting	Soil	Arsenopyrite, pyrite, Fe-sulfate, sulfosalt	3957	18.6	
Midvale Slag	Midvale Slag	Smelting	Slag	PbAsO	591		
MTSS	Anaconda Smelter	Smelting	Soil	FeOOH, sulfosalt, PbAsO	647		13.0
Murray Smelter Flue Dust	Murray Smelter	Smelting	Flue dust	As_2O_3	41,051		
NIST 2710	Silver Bow Creek/Butte Area	Mining, milling	NIST standard soil (from Butte)	FeOOH, sulfosalt	590 (c)	44.1	
NIST 2710A	Silver Bow Creek/Butte Area	Mining, milling	NIST standard soil (Butte soil spiked with lead oxide)	FeOOH, sulfosalt	1400 (c)	41.8	
NIST 2711	Silver Bow Creek/Butte Area	Mining, milling	NIST standard soil (from Silver Bow Creek)	FeOOH	105 (c)		
NYOS	Not specified	Apple orchard	Soil (northern NY State)	MnOOH, PbAsO	123		15.0
NYPS1	Not specified	Pesticide manufacture	Soil (northern NY State)	FeOOH, PbAsO	1000		20.0
NYPS2	Not specified	Pesticide manufacture	Soil (northern NY State)	FeOOH	549		19.0
NYPS3	Not specified	Pesticide manufacture	Soil (northern NY State)	FeOOH	339		28.0
St. Pete's	—	—	Florida soil spiked with sodium arsenate	NaAsO	514		93.0
VBI70 TM1	VBI70	Smelting, refining	Residential yard soil	As_2O_3, PbAsO	312	40.3	
VBI70 TM2	VBI70	Smelting, refining	Residential yard soil	PbAsO, As_2O_3	983	42.2	
VBI70 TM3	VBI70	Smelting, refining	Residential yard soil	As_2O_3, PbAsO, FeOOH	390	36.7	
VBI70 TM4	VBI70	Smelting, refining	Residential yard soil	As_2O_3, PbAsO	813	23.8	
VBI70 TM5	VBI70	Smelting, refining	Residential yard soil	As_2O_3	368	21.2	
VBI70 TM6	VBI70	Smelting, refining	Yard soil spiked with PAX pesticide	As_2O_3, PbAsO	516	23.5	
WAOS	WA State University experimental station	Orchard	Composite soil	PbAsO	301		24.0
WISS	Not specified (western United States)	Mining and smelting	Slag	PbAsO, FeOOH	1412		13.0

[a]As mineralogy was determined using electron microprobe analysis (EMPA) techniques. The predominant As forms are account for at least 80% of the As mass in the sample. PbAsO, Arsenopyrite, As_2O_3, NaAsO, and Sulfosalt are generally stoichiometric mineral forms with fixed As concentrations. FeOOH and MnOOH represent phases that are predominantly either iron or manganese oxides, with variable concentrations of As sorbed to their structure. Fe-sulfate is chemically similar to the common mineral jarosite ($KFe_3(OH)6(SO_4)_2$) with variable concentrations of As sorbed to the structure. $ZnSO_4$ is a non-stoichiometric zinc-rich sulfate with variable concentrations of As. Clay is an iron-rich, hydrated, alumino-silicate with variable concentrations of As sorbed to the structure. AsMO is a complex oxide with variable concentrations of As and other metals (M) including Pb, Sb, Cu, and Cd. PbMClO4 is a mixed chlorate salt with Pb and several other metals, including As.

[b]Soil concentrations measured by EPA Method 3050.

[c]As concentrations in NIST standard reference materials are based on the mean of multiple analyses by several different methods.

measuring RBA in such soils. For monkeys, all soils for which RBA values were reported by Roberts et al. (2007) were used when sufficient material was available, since the bioassay protocol used by these investigators included an internal sodium arsenate control for each test animal and the data reduction protocol was consistent across all studies.

IVBA Extraction Protocol

The extraction device used in these studies holds ten 125-ml wide-mouth high-density polyethylene bottles that are rotated end-over-end within a water bath by an electric motor with a magnetic flywheel. The water bath is filled such that the extraction bottles are fully immersed in water maintained at a temperature of 37 ± 2°C with a circulation heater. A schematic diagram of the extraction device is available online at http://www.colorado.edu/geolsci/legs/invitro1.html.

Extraction Fluids

The basic extraction fluid consisted of 0.4 *M* glycine adjusted to pH 1.5 with addition of hydrochloric acid. Other extraction fluids were created by varying the strength and/or pH of the fluid, or by addition of other components to the fluid. All extraction fluids were prepared utilizing American Society for Testing and Materials (ASTM) Type II deionized (DI) water and high-purity reagents to minimize As contamination of the fluids.

Extraction Procedure

All test substances were thoroughly mixed before use to ensure homogeneity. After mixing, 1 ± 0.05 g of test substrate was weighed and placed into a clean extraction bottle. To this was added 100 ± 0.5 ml of the designated extraction fluid. The bottles were tightly sealed, placed into the extraction device, and rotated at 30 ± 2 revolutions per minute (rpm). After 1 h, the bottles were removed and a sample of extraction fluid was withdrawn using a disposable 10-ml syringe fitted with a 0.45-μm cellulose acetate disk filter (25 mm diameter). The filtered extraction fluid was then analyzed for As using U.S. EPA Method 6020. If the final fluid pH of the extraction fluid was not within ±1 pH units of the starting pH, the test was not considered reliable. Most soils were extracted at least twice, and the mean value was used to represent the IVBA for the sample. In general, variation between replicate IVBA measurements was quite small (<3%) as described in the following (see discussion of step 4).

IVBA Quality Control

Each IVBA extraction (i.e., each set of 10 bottles) included one lab blank (a bottle containing 100 ml of extraction fluid with no added soil) and one blank-spike (a bottle containing 100 ml of extraction fluid to which was added 0.25 mg of As as sodium arsenate). Based on the results from many years of IVBA studies done by one of the authors (Drexler) at the University of Colorado, acceptance criteria for these quality control samples were set as follows: (a) blank concentration < 10 μg/L As, and (b) recovery of As from the blank spike = 85–115%.

Calculation of IVBA

The IVBA of As in the test material was calculated as follows:

$$\text{IVBA}\,(\%) = (C_{\text{fluid}} \times V_{\text{fluid}}) \,/\, (C_{\text{soil}} \times M_{\text{soil}}) \times 100$$

where C_{fluid} is the concentration of As in the extraction fluid (μg/L), V_{fluid} the volume of extraction fluid (L), C_{soil} the concentration of As in the test soil (μg/g), measured using U.S. EPA Method 3050, and M_{soil} the mass of soil placed in the extraction bottle (g). Note that results were expressed as percent total As in soil that became solubilized, and not as "bioaccessible concentration" of As in the study substrate (calculated as IVBA multiplied by C_{soil}). This is because expressing IVBA and RBA as a concentration introduces an autocorrelation between

RBA and IVBA, since both values are multiplied by the same factor (C_{soil}), which leads to a higher coefficient of determination (R^2) value for the IVIVC regression.

In Vitro–In Vivo Correlation

The IVIVC between IVBA and RBA for a set of test materials was evaluated by fitting a linear model (RBA $= a + b \times$ IVBA) using the method of maximum likelihood estimation (MLE), assuming measurement errors in RBA are normally distributed with a constant coefficient of variation. Fitting was performed using MLE rather than ordinary linear regression because measurement error in RBA tends to rise as RBA increases, at least in swine (Brattin and Casteel, 2013). Given a regression model with an adequately strong correlation, the RBA of a sample may be estimated by measuring the IVBA and substituting the value into the regression model.

Arsenic Mineralogy (Speciation)

Arsenic mineralogy in test materials was evaluated using electron microprobe analysis (EMPA). In brief, the EMPA procedure uses an electron microprobe with combined energy-dispersive spectrometer (EDS) and multiple wavelength-dispersive spectrometers (WDS) to evaluate the elemental composition of each As-bearing particle. Based on the elemental composition, each particle is assigned to an As "phase." In some cases, these phases correspond to a specific mineral with fixed stoichiometry, while in other cases, the "phase" represents a range of elemental compositions with varying stoichiometry. A detailed speciation protocol is available online at http://www.cugeology.org/legs.

RESULTS

Step 1: Identification of Up to Three Influential Variables

Extraction fluid pH Figure 1 shows the effect of pH of the extraction fluid on IVBA values for 18 test soils. The highest IVBA values were obtained at pH 1.5, with a tendency for decreasing IVBA as pH increased. However, the magnitude of the decrease was not equal for all materials. The lines in the figure are intended only to help show which data points are derived from the same soil, and do not imply a linear rate of change between measurements.

Temperature Figure 2 compares IVBA values measured at pH 1.5 at 20°C (room temperature) and 37°C. Of the 10 materials investigated, increased temperature (37°C vs. 20°C) resulted in an elevation in IVBA in 5 cases, little change in 4 cases, and a decrease in 1 case.

Extraction time IVBA was measured as a function of time over the scale of 10 min to 48 h, as shown in Figure 3. In most cases, a majority of the total As solubilization occurred rapidly (within 10–30 min), although some test materials yielded results that tended to rise slowly after the initial solubilization phase.

Addition of phosphate Because As in solution usually exists as an oxyanion, addition of other oxyanions such as phosphate to the extraction fluid may enhance the solubilization of As from soil-like materials by competition with As for cationic adsorption sites in the soil. Rodriguez et al. (2003) found that adding 0.1 *M* sodium phosphate doubled As IVBA on average compared to an extraction without sodium phosphate. Figure 4 shows the effect of adding 0.2 *M* or 0.8 *M* phosphate on the As IVBA result for various test materials. As shown, the phosphate addition tended to increase IVBA, sometimes quite substantially. However, in other cases, there was little effect.

Addition of hydroxylamine Hydroxylamine (HA) has been used to extract trace metals that are adsorbed to the surface of iron (Fe) or manganese (Mn) oxide particles in soil (Chao and Zhou, 1983; Shuman, 1982; Tessier et al., 1979). For soils where As is present mainly due to surface adsorption to Fe or Mn in soil particles, dissolution of the surface layer of Fe and Mn minerals by hydroxylamine may tend to liberate (solubilize) the As and increase IVBA. The results of adding 0.25 *M* hydroxylamine are shown in Figure 5. At pH 1.5 (Figure 5A), hydroxylamine tended to

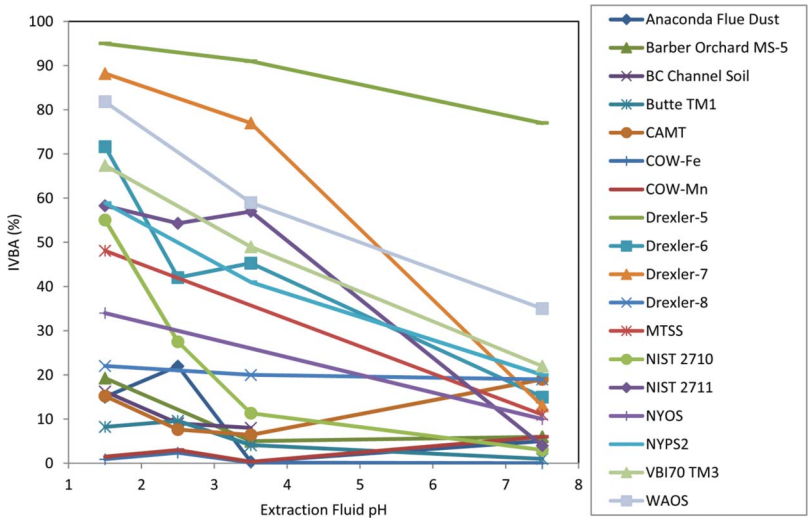

FIGURE 1. Effect of extraction fluid pH on arsenic IVBA. The IVBA of 18 different test soils was measured at pH 1.5, 2.5, 5.0, or 7.0. In most cases, the highest IVBA occurred at pH 1.5, with a general tendency for decreasing IVBA as pH increased (color figure available online).

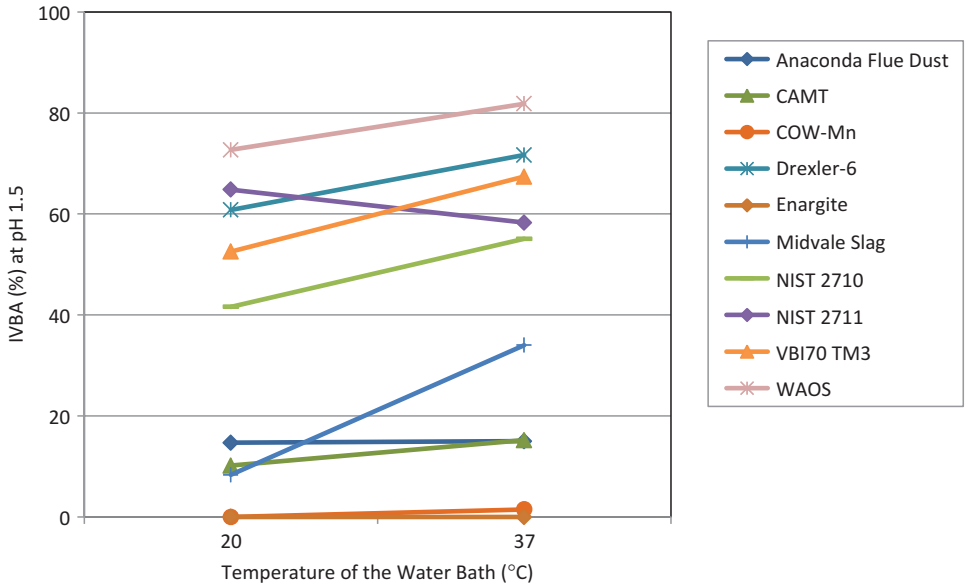

FIGURE 2. Effect of extraction temperature on arsenic IVBA. The IVBA of 10 different test soils was measured at pH 1.5 at extraction temperatures of 20°C or 37°C. In most cases, the highest IVBA occurred at 37°C, although the effect was minor for some soils (color figure available online).

elevate IVBA slightly (an average of 5%), with relatively little variation between test materials. At pH 7 (Figure 5B), the average effect was similar (an average elevation of approximately 6%), although results were more variable, with two samples showing an apparent decrease.

Other variables Several other extraction fluid variables were also investigated, including buffer strength (0.1 *M* to 0.4 *M* glycine), redox potential (modified by addition of 0.25 *M* sodium hypochlorite to create oxidizing conditions or 0.25 *M* hydroxylamine to create reducing conditions), and the mass of test material (0.2 to 1.4 g/100 ml) used in the assay. None of these variables produced marked variation in IVBA (data not shown).

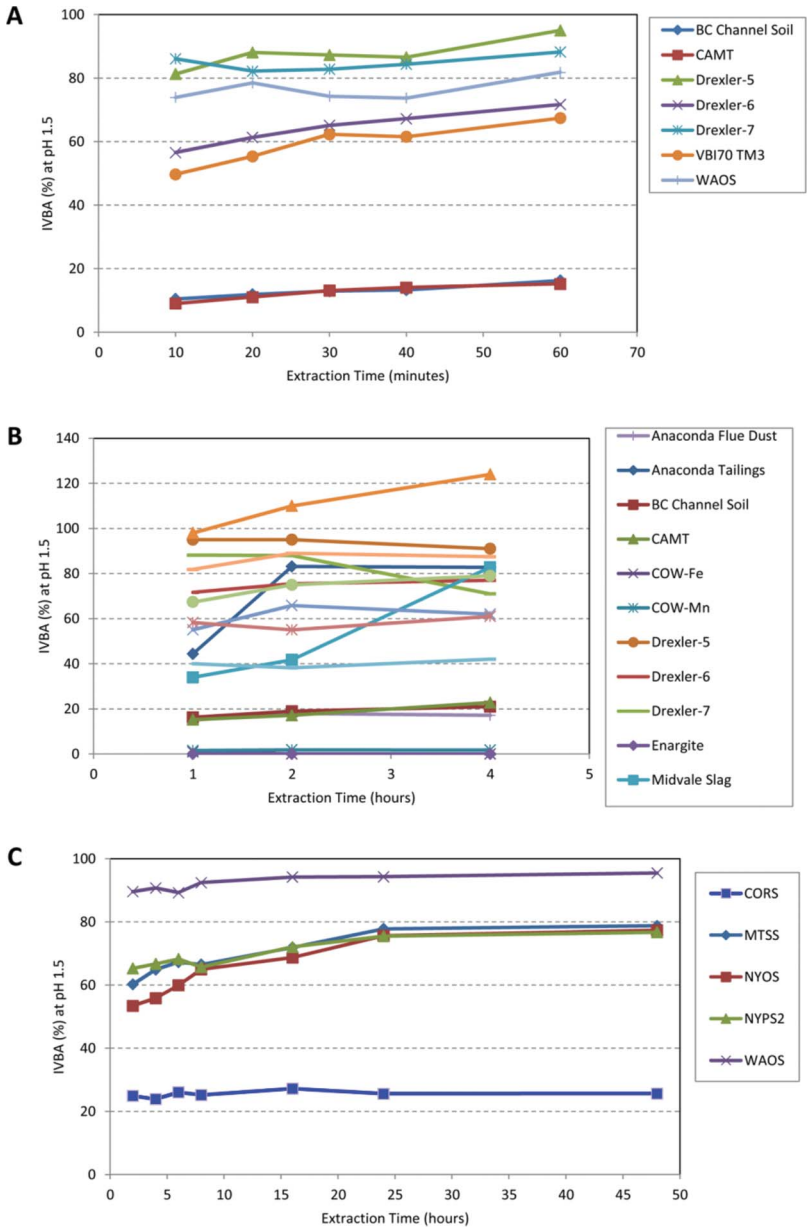

FIGURE 3. Effect of extraction time on arsenic IVBA. The IVBA of test soils was measured as a function of time over time scales of 10–60 min (A), 1–4 h (B), or 1–48 h (C). In most cases, a majority of the total arsenic solubilization occurred rapidly (within 10–30 min), although some test materials yielded results that tended to increase slowly after the initial solubilization phase (color figure available online).

Identification of influential variables The objective of step 1 was to identify up to three variables in the IVBA extraction protocol that exerted the largest effect on the IVBA of As in a variety of test soil. The results indicated that pH was clearly the most important variable tested, with phosphate and hydroxylamine affecting the results in some but not all materials. Thus, these three variables were retained for further testing. Although temperature exerted significant effects on many IVBA values, this variable was not selected for further testing because retention of 4 independent variables would have resulted in too complex a subsequent study design, and because extraction at 37°C is appropriate for simulating in vivo conditions

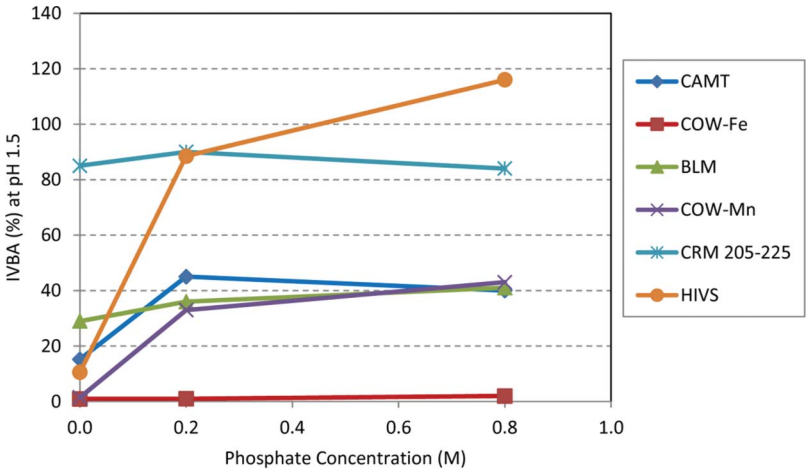

FIGURE 4. Effect of phosphate addition on arsenic IVBA. The IVBA of 6 different test soils was measured at pH 1.5 with varying levels of added phosphate (0, 0.2 *M* or 0.8 *M*). In most cases, phosphate addition tended to increase IVBA, sometimes quite substantially. However, in other cases, there was almost no effect (color figure available online).

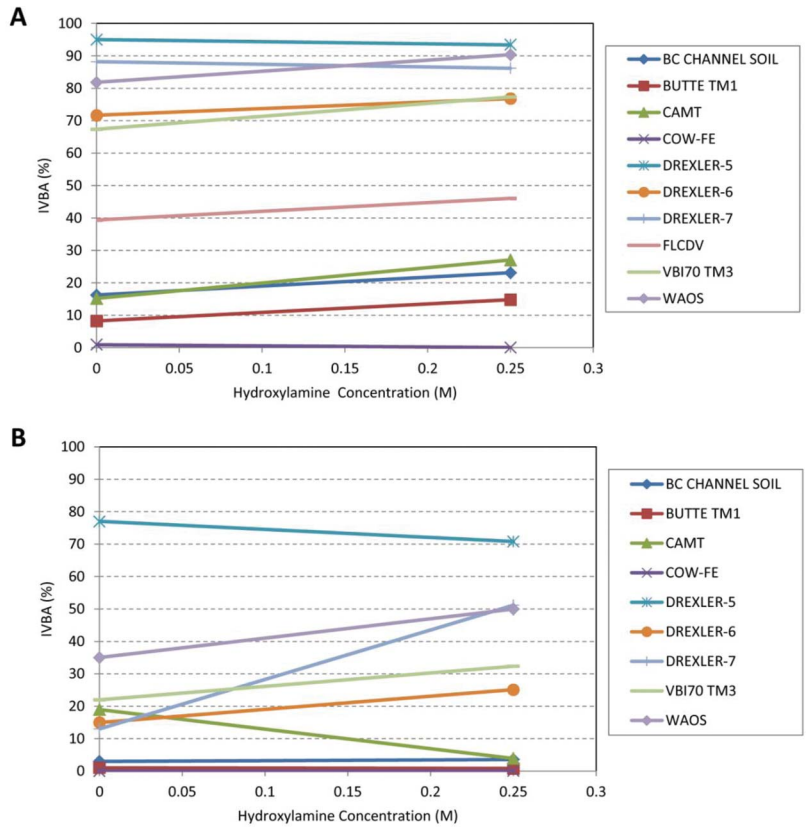

FIGURE 5. Effect of hydroxylamine addition on arsenic IVBA. The IVBA of 9–10 different test soils was measured in the absence or presence of 0.25 *M* hydroxylamine. At pH 1.5 (A), hydroxylamine tended to increase IVBA slightly (an average of 5%), with relatively little variation between test materials. At pH 7.0 (B), the average effect was similar (an average increase of about 6%), although results were more variable (color figure available online).

within the human GI tract. Extraction time, buffer strength, redox potential, and fluid-to-solid ratio exerted little effect and were not investigated further.

Step 2: Initial IVIVC Evaluation

Based on the three influential extraction variables identified in step 1 (pH, phosphate, hydroxlyamine), 21 different combinations of extraction conditions with differing pH and hydroxlyamine and phosphate concentrations were selected for initial assessment of IVIVC. These different extraction conditions were evaluated using an initial set of 12 test materials with reliable RBA values (6 measured in monkeys and 6 measured in swine). The IVBA results for each soil for each extraction condition are presented in Table 2, and the IVIVC results are shown in Table 3. As indicated by the shaded cells, a number of different extraction conditions yielded IVIVC results with R^2 values above .7, indicating a potentially useful correlation. For swine, the best correlation was obtained at pH 1.5, and addition of phosphate and hydroxylamine decreased the strength of the correlation. For monkeys and the combined data set, correlations tended to be best at pH 5 or 7, and addition of phosphate and hydroxylamine usually tended to improve the correlation slightly. However, because the number of samples used to fit the model at this step is small, it is not appropriate to draw firm conclusions regarding the strength of the correlation from this limited evaluation.

Step 3: Final IVIVC Evaluation

Based on the results of step 2, the following three IVBA extraction conditions were selected for further evaluation using a set of 39 test soils:

- pH 1.5, without phosphate or hydroxylamine additions.
- pH 7, without phosphate or hydroxylamine additions.
- pH 7, with 0.05 *M* phosphate and hydroxylamine additions (either 0.1 *M* or 0.25 *M*).

The IVBA results for each soil for each extraction condition are presented in Table 4, and the IVIVC results are shown in Table 5. For RBA values measured in swine, the best correlation ($R^2 = .72$) was obtained using pH 1.5 extraction fluid (no additions), while for RBA values measured in monkeys, the best correlation ($R^2 = .76$) was achieved using pH 7 extraction fluid containing 0.05 *M* phosphate and hydroxylamine (either 0.1 *M* or 0.25 *M*). However, no extraction condition tested yield a good correlation when the swine and monkey data sets were combined. Figure 6 plots the relation between RBA and IVBA for the best extraction conditions for swine (Figure 6A) or monkeys (Figure 6B). The solid lines represent the model fit, and the dashed lines represent the 90% prediction interval. In these regression analyses, IVBA is the independent variable (depicted on the *x* axis), since the purpose of the regression model is to predict RBA given a measured IVBA value.

Step 4: Evaluation of Precision

In order to evaluate the reliability and reproducibility of the laboratory protocols for obtaining IVBA measurements, a set of 12 test soils was provided to each of 4 labs along with a detailed standard operating procedure for performing IVBA extractions. The participating labs were (1) University of Colorado at Boulder (UCB), (2) ACZ Laboratories, Inc. (ACZ), (3) U.S. EPA Region 7 Regional Laboratory (R7), and (4) U.S. EPA Region 8 Regional Laboratory (R8). Each lab extracted each test soil using two different extraction fluids, as follows:

- pH 1.5, without phosphate or hydroxylamine additions.
- pH 7, with 0.05 *M* phosphate .

These fluids were selected because these are the fluids that yield the best IVIVC in swine and monkeys, respectively. Based on the results shown in Table 2, it was concluded that addition of hydroxylamine in the presence of phosphate at pH 7 had little effect, so hydroxylamine was not included in the pH 7 fluid.

TABLE 2. In Vitro Bioaccessibility Results for Initial In Vivo–In Vitro Correlations

	As IVBA (%)																					
pH	1.5							5.0							7.0							
PO_4 (*M*)	None	0.05		0.2		0.8		None	0.05		0.2		0.8		None	0.05		0.2		0.8		
HA (*M*)	None	0.1	0.25	0.1	0.25	0.1	0.25	None	0.1	0.25	0.1	0.25	0.1	0.25	None	0.1	0.25	0.1	0.25	0.1	0.25	RBA (%)	Species
CAMT	15.3	45.0	51.2	74.9	74.5	98.9	91.9	3.0	11.2	11.9	14.8	13.5	23.3	22.7	18.8	6.9	8.1	9.7	11.3	16.1	17.7	19.0	Monkey
COSS	7.7	11.8	12.0	14.5	13.8	16.6	16.9	1.0	2.1	2.3	2.5	2.3	4.1	4.0	1.0	1.6	1.8	2.1	2.2	3.9	3.5	5.0	Monkey
NYOS	33.7	81.8	79.7	96.5	93.7	104.3	101.3	9.0	18.8	20.8	26.3	27.0	51.2	55.0	10.4	15.3	16.3	22.9	25.3	44.6	42.5	15.0	Monkey
NYPS3	32.8	71.5	72.0	94.9	89.2	104.1	96.4	4.0	23.0	24.3	30.3	31.0	49.5	48.5	4.0	13.8	16.5	22.6	25.3	36.9	38.7	20.0	Monkey
WAOS	81.2	104.2	97.5	105.1	109.9	113.6	103.1	28.0	4.3	4.6	7.4	7.0	14.8	15.3	34.1	3.1	4.8	6.3	7.8	77.4	71.9	24.0	Monkey
St. Pete's	106.0	108.0	103.0	104.0	103.0	108.0	104.0	98.0	108.0	105.0	107.0	104.0	111.0	110.0	100.0	104.0	110.0	104.0	102.0	115.0	11.0	93.0	Monkey
BC Channel Soil	16.4	34.1	32.4	38.4	38.7	45.9	45.9	3.0	6.6	6.6	8.8	9.4	15.1	15.0	3.3	4.6	6.0	6.0	6.7	13.3	12.9	39.3	Swine
NIST 2710A	42.2	82.3	81.8	86.9	85.6	86.2	87.3	1.0	16.3	17.5	20.1	19.9	36.7	38.7	1.9	13.3	14.8	18.2	20.0	34.7	36.2	42.0	Swine
VBI70 TM1[a]	40.3	84.8	86.2	88.0	91.9	91.1	87.5	31.0	40.7	41.9	47.1	51.0	60.6	63.5	30.3	35.4	36.4	41.9	45.6	57.2	56.6	40.3	Swine
VBI70 TM3[a]	36.7	94.5	92.0	96.0	94.2	93.5	94.2	27.0	40.1	43.3	46.8	50.0	71.7	67.6	22.1	32.8	36.1	41.1	45.7	63.9	65.7	36.7	Swine
Butte TM1	8.6	30.8	32.7	39.6	39.9	49.0	49.0	0.0	4.3	4.5	5.9	6.0	11.4	10.8	0.6	3.2	3.3	4.2	4.6	9.0	9.0	17.7	Swine
Drexler-5	95.0	102.5	98.2	97.7	98.8	95.9	89.3	73.0	89.3	97.2	94.7	89.6	98.9	94.2	76.9	91.9	87.0	89.0	94.6	96.5	94.6	100.0	Swine

[a]Soil samples from the VBI70 site displayed unusual IVBA behavior. In most cases, IVBA values are reproducible and stable when measured repeatedly over time. However, IVBA values measured at pH 1.5 for VBI70 samples have tended to increase over time. The cause of this increase is not known. Because the RBA value was measured at the same time as the original pH 1.5 IVBA measurements, these original pH 1.5 IVBA values are retained as the most appropriate match to the RBA values.

TABLE 3. Initial In Vivo–In Vitro Correlation Results

	pH	1.5							5.0							7.0						
	PO_4 (*M*)	None	0.05		0.2		0.8		None	0.05		0.2		0.8		None	0.05		0.2		0.8	
Data set	HA (*M*)	None	0.1	0.25	0.1	0.25	0.1	0.25	None	0.1	0.25	0.1	0.25	0.1	0.25	None	0.1	0.25	0.1	0.25	0.1	0.25
Swine,	Slope	0.88	0.57	0.55	0.56	0.57	0.66	0.58	0.87	0.78	0.72	0.74	0.73	0.64	0.66	0.87	0.78	0.82	0.78	0.73	0.68	0.68
$n = 6$	Intercept	10.9	5.5	6.9	4.4	3.6	−4.6	1.9	26.3	20.2	20.5	18.6	18.4	14.5	13.9	26.5	22.5	20.9	19.9	19.6	14.7	14.8
	R^2	0.91	0.40	0.35	0.31	0.32	0.30	0.21	0.78	0.80	0.81	0.78	0.71	0.62	0.60	0.83	0.85	0.83	0.79	0.78	0.67	0.64
Monkeys,	Slope	0.68	0.55	0.60	0.44	0.44	0.35	0.41	0.83	0.77	0.80	0.79	0.81	0.73	0.73	0.84	0.79	0.75	0.81	0.83	0.67	−0.29
$n = 6$	Intercept	−2.2	−9.5	−12.3	−6.9	−6.2	−2.7	−5.7	9.6	7.8	6.9	4.4	4.4	−1.7	−1.6	5.9	10.4	9.6	6.8	5.3	−3.5	38.2
	R^2	0.70	0.41	0.40	0.23	0.23	0.17	0.19	0.96	0.94	0.94	0.92	0.91	0.79	0.77	0.95	0.95	0.96	0.94	0.93	0.75	0.05
Combined,	Slope	0.73	0.56	0.59	0.45	0.46	0.34	0.38	0.84	0.79	0.78	0.79	0.80	0.71	0.72	0.82	0.80	0.79	0.81	0.80	0.66	0.38
$n = 12$	Intercept	6.5	−2.3	−3.3	2.9	2.2	8.9	6.7	18.2	13.6	12.9	10.7	10.4	5.2	5.1	17.0	16.0	15.2	12.7	11.5	6.3	23.2
	R^2	0.66	0.37	0.36	0.20	0.21	0.12	0.13	0.79	0.84	0.85	0.83	0.80	0.69	0.67	0.77	0.87	0.86	0.84	0.84	0.63	0.14

Note. R^2 values greater than 0.7 are shaded in gray.

TABLE 4. Expanded Relative Bioavailability and In Vitro Bioaccessibility Calibration Data Set

Animal species	Test material	RBA (%)	IVBA (%)		
			pH 1.5 (no additions)	pH 7 (no additions)	pH 7 + PO_4 + HA
Swine	Drexler-5	100.0	96.0	76.9	89.4
($n = 20$)	Aberjona River TM1	38.1	13.0	1.0	7.0
	Aberjona River TM2	52.4	32.5	3.0	14.0
	Barber Orchard MS-1	31.0	21.0	4.3	10.0
	Barber Orchard MS-4	40.8	18.6	10.4	10.0
	Barber Orchard MS-5	48.7	19.4	6.5	11.0
	Barber Orchard MS-8	52.8	30.6	6.0	10.0
	Butte TM1	17.8	8.8	0.6	3.3
	Butte TM2	23.6	6.0	2.0	4.0
	Clark Fork Tailings	50.7	50.4	5.0	9.0
	Iron King TM1	60.2	78.0	1.0	14.0
	Iron King TM2	18.6	11.0	1.0	1.0
	NIST 2710	44.1	55.1	5.8	14.0
	NIST 2710A	41.8	42.2	1.9	14.1
	VBI70 TM1[a]	40.3	41.8	30.3	35.9
	VBI70 TM2[a]	42.2	33.2	33.9	42.0
	VBI70 TM3[a]	36.7	40.3	22.1	34.5
	VBI70 TM4[a]	23.8	22.0	32.1	43.0
	VBI70 TM5[a]	21.2	18.7	32.0	43.0
	VBI70 TM6[a]	23.5	18.6	48.0	54.0
Monkeys	Barber Orchard MS-1	33.0	21.0	4.3	10.0
($n = 17$–19)	Barber Orchard MS-4	28.0	18.6	10.4	10.0
	Barber Orchard MS-5	38.0	19.4	6.5	11.0
	Barber Orchard MS-8	25.0	30.6	6.0	10.0
	CAMT	19.0	15.7	18.8	7.5
	CORS	17.0	38.0	—[b]	—[b]
	COSCS	18.0	76.0	22.3	17.0
	COSS	5.0	8.5	1.0	1.7
	FLCDV	31.0	39.7	9.0	16.0
	FLCPS	7.0	5.7	1.0	2.0
	HIVS	5.0	10.4	1.0	6.0
	MTSS	13.0	49.8	10.6	14.0
	NYOS	15.0	34.1	10.4	15.8
	NYPS1	20.0	48.2	3.0	7.0
	NYPS2	19.0	58.3	19.9	35.0
	NYPS3	28.0	32.8	4.0	15.1
	WAOS	24.0	81.0	34.1	3.9
	WISS	13.0	48.3	—[b]	—[b]
	St. Pete's	93.0	106.0	100.0	107.0

[a]Soil samples from the VBI70 site displayed unusual IVBA behavior. In most cases, IVBA values are reproducible and stable when measured repeatedly over time. However, IVBA values measured at pH 1.5 for VBI70 samples have tended to increase over time. The cause of this increase is not known. Because the RBA value was measured at the same time as the original pH 1.5 IVBA measurements, these original pH 1.5 IVBA values are retained as the most appropriate match to the RBA values.

[b]Insufficient material was available to perform IVBA measurements under all conditions.

Each lab analyzed each soil in triplicate with each extraction fluid. Within-lab precision was evaluated by examining the magnitude of the standard deviation for each set of three replicate values. Results are presented in Table 6. Within-lab precision (panel A) was typically less than 3%, with an average of 0.7% for all 4 labs. Between-lab precision (panel B) was evaluated by examining the standard deviations in the mean IVBA values for each test soil for each extraction fluid condition. For most test soils, between-lab variation in mean values was less than 5%, with an overall average of 1.7%.

Quality control samples for all labs were within the acceptance limits identified for the project, with all blank concentrations <5 μg/L and all As spike recoveries within 98–108%.

TABLE 5. Linear Regression Parameters

		IVBA extraction fluid		
Data set	Fitting parameter	pH 1.5 (no additions)	pH 7 (no additions)	pH 7 + PO_4 + HAH
Swine	Slope	0.62	0.31	0.35
($n = 20$)	Intercept	19.68	35.45	32.55
	R^2	0.723	0.143	0.178
Monkeys	Slope	0.32	0.43	0.58
($n = 17$–19)	Intercept	11.07	17.10	14.26
	R^2	0.336	0.706	0.755
Combined	Slope	0.44	0.33	0.44
($n = 37$–39)	Intercept	16.42	27.61	23.90
	R^2	0.345	0.328	0.409

Note. Best fit model is shaded in gray.

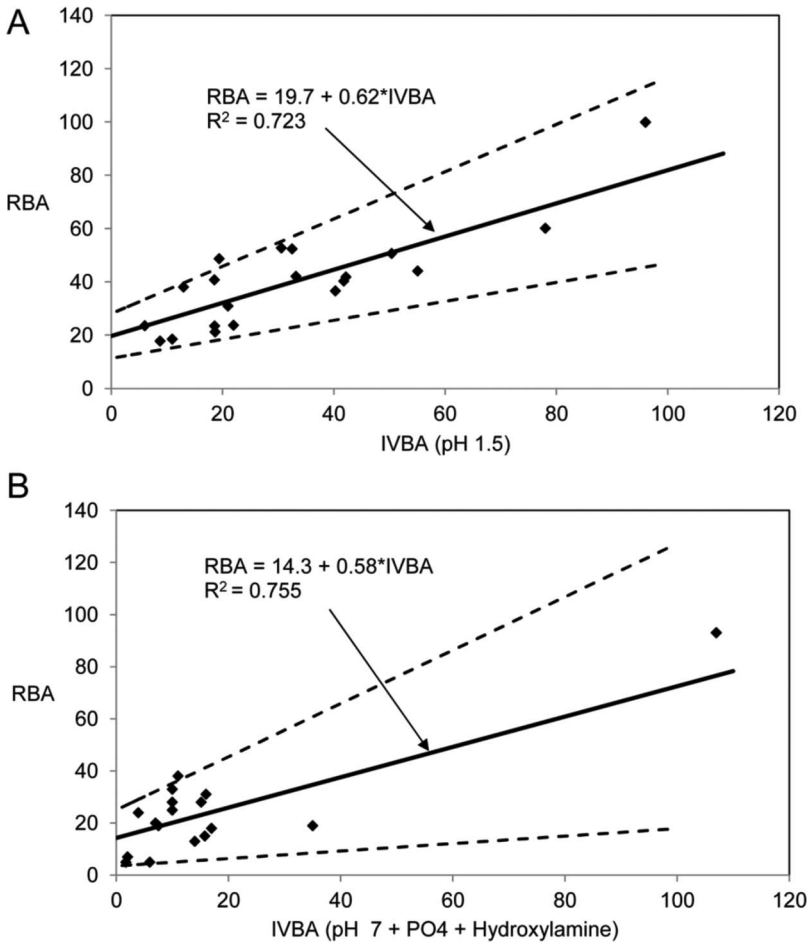

FIGURE 6. Best fit MLE linear regression models. Linear regression models (indicated by the solid lines) were fit to available RBA-IVBA data for swine (A, $n = 20$) or monkey (B, $n = 17$) by the method of maximum likelihood estimation. For swine, the IVBA data were obtained at pH 1.5, and for monkeys, the IVBA measured at pH 7 in the presence of phosphate (0.5 *M*) and hydroxylamine (either 0.1 *M* or 0.25 *M*). The dashed lines in each graph indicate the 90% predication interval.

TABLE 6. Within- and Between-Laboratory Precision

A, Within-laboratory precision[a]: Test material		pH 1.5 (no additions)				pH 7 + 0.05 *M* PO_4			
		UCB	ACZ	R7	R8	UCB	ACZ	R7	R8
1	WAOS	4.7	2.8	1.1	0.9	0.1	0.0	0.6	0.1
2	VBI70 TM1	5.9	0.5	2.4	1.5	0.3	0.6	1.0	0.8
3	NYPS2	0.7	0.6	1.7	1.8	0.2	0.3	1.3	0.3
4	COSS	0.0	0.1	0.3	0.2	0.0	0.1	0.0	0.1
5	MTSS	0.6	0.3	2.7	0.4	0.3	1.2	1.1	0.2
6	CAMT	0.4	0.1	1.6	0.1	0.1	0.3	0.8	0.2
7	NYOS	0.1	0.4	0.6	0.9	0.3	0.5	0.4	0.4
8	Barber Orchard MS-5	0.4	0.0	0.2	0.2	0.6	0.2	0.4	0.2
9	BC Channel Soil	0.5	0.6	0.6	0.8	0.2	0.1	0.0	0.1
10	Butte TM1	0.3	0.1	0.3	0.4	0.1	0.2	0.1	0.0
11	VBI70 TM3	3.0	1.0	0.4	1.0	0.3	1.3	0.6	0.5
12	NYPS3	0.8	0.6	0.3	1.0	0.3	1.6	0.4	0.4
	Mean	1.5	0.6	1.0	0.8	0.2	0.5	0.6	0.3

B, Between-laboratory precision: Test material		pH 1.5 (no additions)		pH 7 + 0.05 *M* PO_4	
		Mean[b]	SD[c]	Mean[b]	SD[c]
1	WAOS	79.9	4.2	2.8	0.3
2	VBI70 TM1	70.0	3.0	27.1	2.1
3	NYPS2	44.1	5.8	14.8	0.6
4	COSS	7.9	0.6	1.5	0.1
5	MTSS	47.9	1.2	11.4	1.2
6	CAMT	15.8	2.6	4.5	0.3
7	NYOS	32.5	4.2	10.5	1.0
8	Barber Orchard MS-5	16.9	0.7	8.7	1.0
9	BC Channel Soil	16.2	1.8	3.5	0.5
10	Butte TM1	8.2	1.4	2.5	0.2
11	VBI70 TM3	67.3	4.4	25.8	2.4
12	NYPS3	33.1	1.3	16.9	1.0

[a]Values shown are standard deviations of three replicate measurements (%).
[b]Values shown are mean (%) of means across four laboratories.
[c]Values shown are standard deviation of means (%) across four laboratories.

DISCUSSION

Recommended Model

Based on the data that are presently available, it appears that no single statistical model (that is, the same equation with the same parameters) provides a good fit to both the monkey and swine RBA values. This suggests that the RBA measurements in swine and monkeys are not equivalent. If so, these differences may be related to differences in the bioassay protocols (e.g., dosing regimen) and/or differences in GI physiology/biochemistry that determine As absorption in the two animal species.

If future data collection efforts confirm the conclusion that the monkey and swine bioassays do not yield equivalent RBA values for the same test materials, risk assessors will need to determine which animal species is a more useful predictor of RBA in humans, and use the mathematical model based on data from that species. At present, an empirical basis for determining which bioassay best predicts bioavailability of As in humans does not exist, since this would require measuring As RBA in human subjects. If new data ultimately lead to the conclusion that the apparent differences between the species are not important, then using a model that combines data sets is likely to be the best approach.

Until it is clear whether RBA values measured in swine and monkeys are similar or dissimilar, it is recommended that the statistical models based on the swine data be used as the preferred method for estimating site-specific RBA values:

$$RBA = 19.7 + 0.62 \times IVBA_{pH\,1.5}$$

This model is preferred because the data set based on measurements in swine spans a wider range of RBA values than the data set based on monkeys, has a narrower prediction interval than the monkey model, and is much less dependent on the influence of the sodium arsenate-spiked sample than the model based on monkey data.

Advantages of IVBA Methods Compared to In Vivo Methods

The approach for estimating RBA of As in test soils described in this study has a number of advantages over direct measurement in animal models, including low cost and rapid throughput. This allows for the application of the method at smaller sites where an expensive and time-consuming animal study may not be feasible, as well as the ability to evaluate a much larger set of samples from a given site to obtain a more complete understanding of within-site variability in RBA. The extraction fluids and extraction conditions are simple and the method yields highly reproducible outcomes from which in vivo RBA can be estimated with sufficient confidence to be useful for risk assessment applications.

Comparison of This Method to Other In Vitro Methods

A number of other researchers have described in vitro systems for measuring the extractability of As from soil or other soil-like materials (Basta et al., 2007; Bruce et al., 2007; Denys et al., 2012; Ellickson et al., 2001; Juhasz et al., 2007, 2009; Makris et al., 2008; Medlin 1997; Oomen et al., 2002; Rodriguez et al., 1999; Ruby et al., 1996; Wragg et al., 2011). These methods differ from each other with regard to attributes such as (a) complexity of the extraction protocol (one step or two steps); (b) complexity of the extraction fluid(s); (c) whether or not an IVIVC has been performed and if so, (d) number and diversity of samples used in the IVIVC; and (e) strength of the correlation. The principal advantages of the method described here compared to other published methods include the following:

- The current method utilizes a larger set of calibration samples ($n = 20$ for swine and $n = 17$–19 for monkeys) to establish the regression model between IVBA and RBA than most other studies. As illustrated by comparison of the apparent high correlation obtained in our preliminary studies based on a limited calibration set (Table 3) to our final correlation based on the expanded data set (Figure 6), IVIVC correlations based on a small number of samples may be misleading.
- The data set used for IVIVC is relatively diverse, with samples from multiple types of sites that contain a range of different As forms and yield a relatively wide range of RBA values. This diversity increases the confidence that the correlation is likely to be applicable across a wide range of test materials. Other studies typically do not have soils that are so diverse.
- The current method is based on a more extensive and systematic testing of extraction conditions to identify the optimal conditions than most other published methods.
- The current IVBA method has undergone interlab testing to establish within- and between-lab precision. The results of the interlab testing indicate the method yields IVBA measurements that are highly reproducible.
- The current method utilizes a single extraction step. This is in contrast to methods that utilize two or more sequential extraction steps, with each intended to represent differing parts of the GI system.
- The current method utilizes simple extraction fluids. This is in contrast to methods that seek to create extraction fluids that closely mimic complex GI fluids, including the presence of a number of biochemical constituents such as enzymes and metabolites.

TABLE 7. Comparison of Methods with IVIVCs

Reference	IVBA			IVIVC			Notes
	Extraction fluid(s)	Stomach pH	Intestinal pH	Samples (Species)	RBA Range	IVIVC	
Rodriguez et al. (1999)	Physiological	1.8	5.5	15 (Swine)	4-55	RBA=0.88*IVBA – 2.02, $R^2 = 0.69$ (a)	Stomach phase
Makris et al. (2008)	Physiological	1.0	—	5 (Mouse)	18-99	RBA=0.452*IVBA+10.04, $R^2 = 0.295$	Optimization for worst case
Ruby et al. (1996)	Physiological	2.5	7	3 (Rabbit, monkey)	34-50	RBA=0.857*IVBA+4.3, $R^2 = 0.008$	
Medlin (1997)	Physiological	1.5	6.5	6 (Swine)	8-60	RBA= 0.72*IVBA+ 19.2, $R^2 = 0.43$ (a)	
Juhasz et al. (2007)	Glycine	1.5	—	12 (Swine)	7-75	RBA=0.68*IVBA + 5.67, R^2=.69 (b)	
Wragg et al. (2011)	Physiological	1.2	6.3	11 (Swine)	4-52	RBA=1.26*IVBA + 8.6, R^2 =0.75	
Denys et al. (2012)	Physiological	1.2	6.3	14 (Swine)	3-100	RBA=1.12*IVBA-2.2, R^2=0.91 (b)	Based on RBA in urine, IVBA = stomach phase
Bradham et al. (2011)	Glycine	1.5	—	10 (Mouse)	10-55	RBA = 0.72*IVBA +5.6, $R^2 = 0.92$	
This report	Glycine	1.5	—	20 (Swine)	18-100	RBA = 0.62*IVBA + 19.7, $R^2 = 0.72$	

a. Parameters shown are based on a recalculation using updated RBA values that were not available at the time of the original publication.
b. Calculated using ordinary least squares regression using data provided in the publication.

Table 7 summarizes the attributes of the method described here in comparison to a number of other methods for which IVIVC relations have been described. As indicated, although all of the published methods have some advantages, no other method includes all of the attributes already discussed.

Influence of Sodium Arsenate-Spiked Samples

The best-fit regression models for swine and monkeys are both influenced by the inclusion of sodium arsenate spiked soil (Drexler-5 for swine and St. Pete's for monkeys). The effect of excluding these soils is to decrease the strength of the correlation. For the swine data set, the R^2 value changes from .723 to .532 with exclusion of the Drexler-5 sample, while for the monkey data set the R^2 value decreases from .755 to .057 with exclusion of the St. Pete's sample. This marked effect in the monkey data set occurs because data for all but one of the test materials evaluated in monkeys (NYPS 2) have IVBA values clustered at the low end of the range (IVBA = 0–20%). This makes it difficult to fit a reliable model without additional data points that fall outside of this narrow data range. Although the inclusion of these data points may tend to overestimate the reliability of the models, the data from these two samples are considered to be appropriate for inclusion because they represent reasonable and expected outcomes for highly bioaccessible As, and the recommended models are based on the data fits with these samples included.

RBA Predictions at Low IVBA

One feature of the linear IVBA-based modeling approach described here is that the model intercept term (obtained when IVBA = 0) is 20% (swine) or 14% (monkeys) (see Figure 6). If the physical form of As in a sample has low solubility, it is possible that the RBA for that sample might be lower than the model intercept terms. Collection of additional data pairs in the low RBA/IVBA range might lead to refined models in which the intercept term is lower.

Use of Speciation Data

Although the best-fit models described in the preceding sections are able to provide a good prediction of RBA based on IVBA data alone, additional modeling was performed to investigate whether inclusion of As mineralogy data along with the IVBA data would provide an improvement in model accuracy. The basic model was:

$$RBA = k \cdot IVBA_{\text{best}} + \sum f_i \cdot RBA_i$$

where k is the empiric fitting constant, $IVBA_{best}$ is IVBA measured at pH 1.5 (no additions) for swine or at pH 7 (with phosphate and hydroxylamine) for monkeys, f_i is the fraction of sample in phase i, and RBA_i is the phase-specific RBA (estimated by fitting)

The concept is that each unique mineralogical type of As ("phase") has an inherent phase-specific RBA, and that RBA of a soil sample containing a mixture of As phases reflects the amount-weighted average of the phase-specific RBA values. The model utilizes both the measured best IVBA value (pH 1.5 for swine, pH 7 with phosphate and hydroxylamine for monkeys) with the measured phase data to predict RBA. In total, 15 different mineral phases of As were observed in one or more samples included in the final data set of 39 samples.

The model was fitted to each data set (monkeys, swine) using MLE, assuming normal errors in RBA with a constant coefficient of variation. The quality of fit was evaluated using Akaike's information criterion (AIC), which considers both the absolute quality of the model fit to the data (as reflected in the log-likelihood value), and also the number of fitting parameters in the model. A model that included phase data was considered to be an improvement over the model that used IVBA data alone only if the R^2 value was higher and the AIC value was equal or lower. Fitting was performed in a series of steps, as follows:

1. Solve for 15 phase-specific RBA_i values using MLE.
2. Rank order the 15 RBA_i values (low to high).
3. Combine phases with similar RBA_i values into bins. Investigate a range of different binning strategies, ranging from 1 bin (all phases are assigned to the same bin) to 15 bins (each phase is assigned to a different bin).
4. Find the optimum number of bins based on minimization of AIC. In nearly all cases, the best fit was obtained by combining the 15 As phases into two or three bins.

In all cases, this approach yielded optimized models that were a substantial improvement over the models that were based on IVBA data alone. For the swine data set, the R^2 value increased from .723 to .906, and for the monkey data set, the R^2 value increased from .755 to .816. However, round-robin interlab testing of the speciation protocol (three labs, three test soils) indicated that there was poor agreement between labs (data not shown), and that the time and cost to obtain speciation data were prohibitive. Therefore, at this time, use of phase data as an input for quantitative RBA models cannot reliably be used to improve the predicted RBA. In the future, if the speciation protocol can be simplified so that it yields more reproducible data with less cost, then this strategy for model development may be worthy of reassessment.

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