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An *In Vitro* Procedure for Estimation of Lead Relative Bioavailability: With Validation

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ABSTRACT

The relative bioaccessibility leaching procedure (RBALP) is a simple, reproducible, and rapid *in vitro* procedure for estimating the *in vivo* (juvenile swine) relative bioavailability (RBA) of lead in solid media. Control of pH, temperature, and agitation are the most critical parameters of this *in vitro* procedure. The performance of the method was evaluated by triplicate analyses of each of 19 different test substances by the author and three independent laboratories, and comparison of the results to relative bioavailability (RBA) values measured *in vivo*. The results indicate that the RBALP measurements are strongly correlated with the *in vivo* RBA values ($r^2 = 0.924$, $p < 0.0001$), with an average absolute error of 10% and an average predictive error of 20%. Comparison of results within and between laboratories indicates that the procedure is highly reproducible, with inter- and intra-laboratory coefficients of variation of 4% and 6%, respectively, and within-sample precision of approximately 7%. Based on the results reported here, the RBALP can be effective in providing reliable estimates of lead RBA as predicted by the immature swine model. The method may also be valuable in evaluating site-specific differences in bioaccessibility, assessing remedial technologies intended to reduce lead RBA, providing a screening mechanism for future *in vivo* studies, and providing insight into the chemical and physical factors that control lead bioavailability.

Key Words: lead, bioaccessibility, relative bioavailability, *in vitro–in vivo* correlation, RBA, RBALP, IVBA, IVIVC.

INTRODUCTION

Estimating the exposure of children to environmental lead is improved by information on bioavailability (the degree to which lead is absorbed from the environmental medium into the blood). This absorption may be described either in absolute

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terms (absolute bioavailability, or ABA) or in relative terms (relative bioavailability, or RBA). In this study we will define absolute bioavailabity as the amount of lead entering the blood via the gastrointestinal (GI) exposure route divided by the total amount ingested, and relative bioavailability as ratio of the absolute bioavailability in the test media to that in a soluble reference material (lead acetate). For the purpose of human health risk assessment for lead, the U.S. Environmental Protection Agency (USEPA) typically assumes an RBA of 60% for lead in soil compared to lead in water. However, the solubility of lead from soil in the GI track (bioaccessibility) may be significantly influenced by a number of site-specific variations in chemical or mineral form, and/or particle-size (Ruby *et al.* 1992; Davis *et al.* 1993; Casteel *et al.* 1997; and Drexler 1997) and variations in bioaccessibility may be expected to have an effect on RBA. Significant variation in RBA values from different types of contaminated media is likely. Therefore, methods for estimating RBA are needed to improve the accuracy of human health risk assessments for lead.

Historically, lead bioavailability has been determined mainly through experimental studies in animals. However, *in vivo* assays of lead absorption are relatively slow and costly, and may have sample limitations (typical soil mass ~1–2 kg, typical concentration values >2000 mg/kg) all of which can be a deterrent to assessment of bioavailability. To help overcome these limitations of *in vivo* studies, numerous authors have attempted to develop *in vitro* techniques that are faster, easier, and less costly to perform (Miller and Schricker 1981; Ruby *et al.* 1993, 1996; Medlin 1997; Rodriquez *et al.* 1999). All of these studies measure the rate and/or extent of lead solubilization from a solid test material into an extraction fluid that is often intended to resemble human gastric fluid. However, it is important to recognize that the uptake of lead in the gastrointestinal tract is complex, involving both *physiological processes* (active and passive transport across the gastrointestinal epithelium) and *physico-chemical processes* (dissolution of metal from solid particles, which in turn depends on chemical form, particle-size and matrix of lead-bearing material) (Mushak 1991). Therefore, any *in vitro* method can only be a simplistic surrogate for all relevant metabolic and chemical interactions that occur *in vivo*, and for this reason *in vitro* bioaccessibility may not be precisely equal to *in vivo* bioavailability. However, if it can be demonstrated that an *in vitro* method yields results that are strongly correlated with reliable *in vivo* RBA values, then the *in vitro* results can be used to estimate or predict the expected *in vivo* results with good confidence. Throughout this article we will refer to this type of bioavailability estimate as IVBA (*in vitro* bioaccessibility).

This article describes the development, calibration, and round-robin characterization of a fast and simple *in vitro* extraction procedure to estimate lead RBA. The method is strongly correlative with *in vivo* measures of lead bioavailability for a wide variety of environmental lead matrices using an immature swine model as a surrogate for young children (Casteel *et al.* 2006). In addition, the method has been extensively validated by inter-and intra-laboratory testing and is highly reproducible. This *in vitro* method for lead may provide a plausible alternative to animal investigations where screening a large number of matrices is necessary or when time and cost constraints prohibit more thorough *in vivo* investigations.

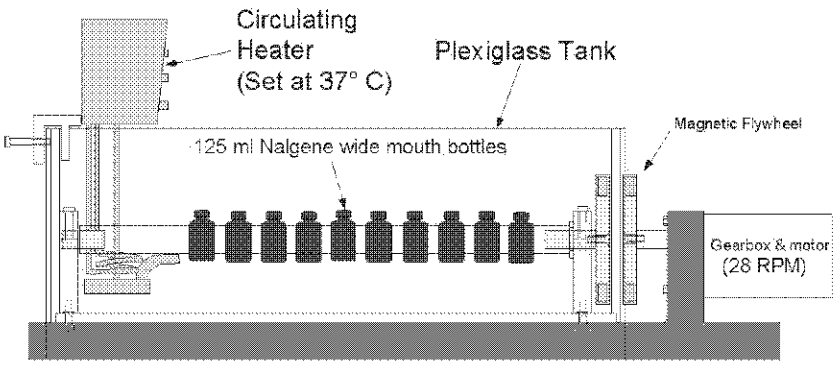


Figure 1. Schematic of RBALP rotating extraction device. Clean tap water or DI water is added to the tank to maintain a level 1″ above the Nalgene bottles. Sample bottles are filled with extraction fluid and rotated end-over-end for 1 hour at 28 RPM in the device.

MATERIALS AND METHODS

Nineteen test materials for which *in vivo* RBA values had been determined by the USEPA (Casteel *et al.* 2006) were used to evaluate the degree of correlation between the *in vitro* and *in vivo* results. These 19 test materials are listed in Table 1, and include a range of types, including residential soils, tailings, and slags from mining-related waste sites across the United States. Test materials are characterized by a broad range of lead concentrations (1270 to 11,700 mg/kg), and are composed of a diverse assemblage of relatively small (about 20 μm) mineralogical forms of lead. Detailed mineralogical data and mineralogical correlation to RBA can be found in USEPA (2006) methodology. Calculated RBA_{PE} (point estimate) values ranged from 1% to 105% (Casteel *et al.* 2006).

In Vitro Apparatus

Numerous *in vitro* testing apparatuses have been developed over the years including; rotating baskets-paddles-bottles (Smolen and Ball 1984). Simplicity, flexibility, and reproducibility are of major importance in any dissolution testing apparatus. A customized extraction device was developed in order to control temperature and mixing rate during the leaching procedure (Figure 1). The device holds ten 125-mL wide-mouth, high-density, polyethylene (HDPE) bottles. These are rotated (end over end) within a plexiglass water bath at a constant speed (28 ± 2 rpm), and the temperature in the water bath is maintained at 37 ± 2°C by means of an immersion circulatory heater.

In Vitro Procedure[1]

Test materials are prepared for the RBALP by first drying either in ambient conditions or an oven at <40°C and then sieving to <250 μm. The <250 μm size fraction

[1]A full description of the RBALP may also be obtained at http://www.colorado.edu/geolsci/legs/.

Table 1. USEPA Region VIII test materials.

Media number	Test material	Lead Conc. mg/kg	RBA_{PE} USEPA., 2006	Dominant lead mineral USEPA, 2006*	Mean particle Size (μm)**
1	Aspen Smuggler Residential	3,870	75%	$PbCO_3$/FeOOH	23/9
2	Aspen-Smuggler Berm	14,200	74%	$PbCO_3$/FeOOH/PbS	23/9/1
3	Bingham Cr. Channel	6,330	27%	$FeSO_4$/$PbSO_4$/ $PbPO_4$	6/4/12
4	Bingham Cr. Residential	1,590	27%	$PbPO_4$ /$FeSO_4$ /MnOOH	13/10/22
5	Butte	8,530	14%	$PbSO_4$/$FeSO_4$/PbS/MnOOH	13/46/9/37
6	Leadville Residential	7,510	72%	$PbPO_4$/MnOOH/$PbCO_3$/$PbSO_4$	19/43/14/9
7	Leadville Fe-Mn-PbO	4,320	105%	MnOOH/$PbPO_4$/FeOOH	45/8/20
8	Leadville AV Slag	10,600	20%	PbO/PbAsO/Slag	9/8/125
9	Leadville Oregon Gulch	1,270	6%	PbS/$PbSO_4$	3/5
10	Jasper Smelter	10,800	61%	$PbCO_3$/$PbPO_4$/FeOOH	31/7/63
11	Jasper Mill	6,940	82%	$PbCO_3$/FeOOH/MnOOH/PbO	8/30/27/17
12	Jasper Yard	4,050	90%	$PbCO_3$/PbS/$PbPO_4$	15/50/11
13	Midvale Slag	8,170	14%	PbAsO/PbMO/Slag/PbS	16/12/131/90
14	Murray Slag	11,700	40%	PbO/Slag/PbAsO/PbMO	8/73/6/18
15	Murray Soil	3,200	51%	PbAsO/PbO/$PbCO_3$	5/9/14
16	Palmerton-2	3,230	60%	MnOOH/$PbSO_4$	17/4
17	Palmerton-4	2,150	49%	MnOOH/$PbSO_4$	31/10
18	NIST SRM 2589 (paint) in soil	8,350	72%	$PbCO_3$/PbO	9/9
19	Galena in soil	11,200	1%	PbS	17

*As determined by electron microprobe analysis (EMPA).

**Average lead-bearing particle sized (based on long-axis for each particle) for each of the dominant lead forms.

is considered most representative of the grain size that adheres to children's hands and is therefore most likely to be ingested (Bornschein *et al.* 1987). Sieved samples are thoroughly mixed prior to use to eliminate particle-size segregation, which may occur during storage.

The extraction fluid is prepared by adding 60.06 g of glycine (free base, reagent grade) to 1.9 L of ASTM Type II deionized (DI) water. The solution volume is then brought to 2 L (resulting in 0.4 M glycine). Approximately 60 mL of trace-metal grade concentrated (12.1N) hydrochloric acid is then added. This buffered fluid is then placed in a water bath at 37°C until the extraction fluid reaches this temperature. The pH of the fluid is then adjusted to 1.50 ± 0.05 by drop-wise addition of HCl.

The extraction procedure begins by placing 1.00 ± 0.05 g of sieved test material (<250 μm) and 100 ± 0.5 mL of the buffered extraction fluid into a 125 mL wide-mouth HDPE bottle. The bottles are placed into the extractor and rotated in the 37°C bath for 1 hour. Following this extraction step, an aliquot of approximately 10–15 mL is removed using a disposable 20 mL syringe and filtered through an 0.45 μm cellulose acetate filter (25 mm diameter) into a clean 15-mL polypropylene centrifuge tube (or other appropriate sample vial) for lead analysis. The use of a smaller pore-size filter (0.2 μm) was found not to statistically (n = 14 pairs, t = –1.18, $t_{Critical}$ = 2.16) influence results. The pH of the remaining fluid in the extraction bottle is then measured. If the fluid pH is not within ±0.5 pH s.u. of the starting pH, the test should be repeated. If the pH changes by more than 0.5 s.u. in the re-analysis, it is apparent that the test material is buffering the solution and the test must be repeated using manual pH adjustment during the extraction process. More specifically, the extractor must be stopped at 5, 10, 15, and 30 minutes into the extraction, and the pH adjusted down to 1.5 s.u. with drop-wise additions of HCl. The final pH is checked as described above. Samples that exhibit uncontrolled rising pH values might have better success following the method of Medlin (1997).

Chemical Analyses

Lead concentrations in test materials were determined by USEPA methodology (USEPA 2006) on a split of the sample, prior to leaching. These values represent a consensus from multiple analytical methods (X-ray fluorescence XRF, inductively coupled atomic emission spectrometry ICP-AES and inductively coupled mass spectrometry ICP-MS) and it is important to note that this concentration value represents the total lead concentration in the <250 μm fraction. Filtered aliquots of extraction fluid were stored at 4°C for no more than 1 week prior to analyses by ICP-AES or ICP-MS (USEPA Method 6010 or 6020, USEPA 1986). However, repetitive analysis of multiple extracts over 18 months showed only a 10% RPD over time, so longer holding times are generally acceptable so long as microbial growth does not occur. Method detection limits (MDL) in extraction fluid are approximately 19 and 0.3 ug/L for USEPA Methods 6010 and 6020, respectively. Quality control samples run for the leaching procedure are listed in Table 2. Suggested control limits are shown, along with those observed over a 2–3 year interval at the University of Colorado, Boulder (CUB) laboratory.

Table 2. Quality assurance/quality control limits for the RBALP.

QA Sample Type	Analysis frequency	Observed	Control limits
Bottle blank	5%	0.5 μg/L $\pm$ 4.3 (N = 120)	< 25 μg/L lead
Blank spike*	5%	98.9% $\pm$ 4.0 (N = 66)	85–115% recovery
Matrix spike*	10%	89.9% $\pm$ 13.2 (N = 73)	75–125% recovery
Duplicate sample	10%	RPD** = 4.0% $\pm$ 4.9 (N = 80)	RPD= $\pm$ 20%
Control soil***	5%	CV = 7% (N = 158)	CV= $\pm$ 10%

*Spikes contained 10 mg/L lead.
**RPD = relative percent difference.
*** The National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 2711 and/or 2710 were used as a control soil.

Statistical Analyses of Data

Quantitative comparisons of IVBA data were made through analysis of variance (ANOVA) and standard *t*-tests (Cheng and Van Ness 1999). Although sample populations are not normally distributed, the authors assume if a larger population were tested they would be normally distributed. Furthermore, comparative tests (Holm-Sidak and Student-Newman-Keuls), which do not require a normal distribution, were run, and support all ANOVA conclusions. Predictive performance was evaluated by means of absolute percent error (PE_{abs}), root-mean percent error ($P_{rm}E$), and percent predictive error (%PrE) after Malinowski *et al.* (1997). All statistical comparisons were evaluated against a 5% significance level.

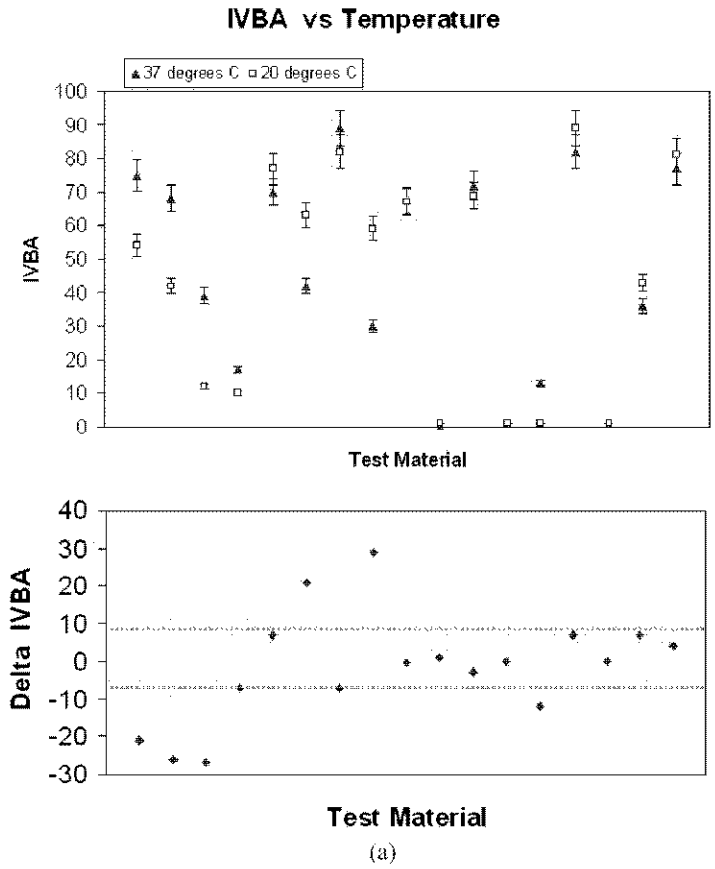
RESULTS

In Vitro Parameters

Previous *in vitro* studies (Ruby *et al.* 1993,1996; Medlin 1997; Drexler 1997) have suggested that the *in vitro* parameters most likely to affect the strength of the *in vitro-in vivo* correlation IVIVC are pH, temperature, extraction time, and sample/fluid ratios. These variables were systematically evaluated either to find the combination that yielded the highest correlation of IVBA results with RBA values or simply to quantify variable sensitivity. Because only limited amounts of material were available for some of the soils that had been tested *in vivo*, initial procedure development included only some of the samples tested *in vivo* (those with an adequate supply), along with several additional substrates chosen to ensure a broad range in lead speciation. During this initial evaluation and all subsequent work, all personnel responsible for both *in vitro* extractions and analytical results were kept blind to sample provenance. All method characterization was a result of a doubly-blind study.

Temperature

Seventeen substrates were analyzed (generally in triplicate) by means of the RBALP at 37°C and 20°C. The results are shown in Figure 2A. Although some of the samples showed little dependence on temperature, six of the samples had a variation in IVBA values that exceeded the method precision (7%). Note that the *in vitro*



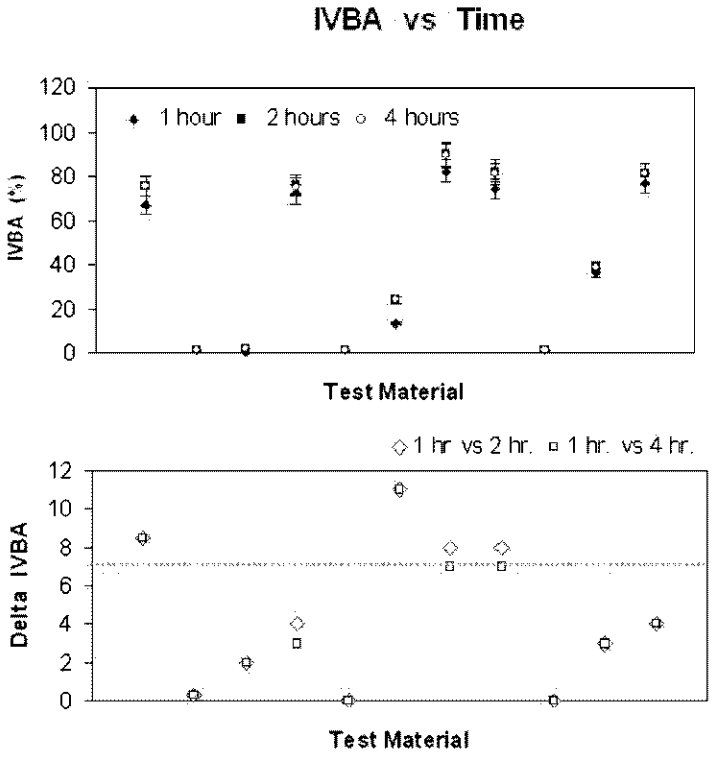
(a)

Figure 2. (A) Variation in IVBA with temperature. (B) Variations in IVBA with time at pH of 1.5. (C) Variation in IVIVC with pH. Dotted lines or error bars represent method precision of 7%. *(Continued)*

soluble lead concentrations were higher at 37°C than at 20°C for three test materials, and were lower at 37°C in the other three cases. A paired *t*-test on the two sample means indicated no significant difference ($t = -0.45$, $t_{Critical} = 1.74$). Only two of these test materials had corresponding RBA values, so it was not possible to determine which temperature provided a better IVIVC. To be consistent with previous *in vitro* studies and maintain a biological simulation, 37°C was adopted.

Time

Gastric emptying rate is reflexly dependent on smooth muscle stimulation associated with feeding. The rate of gastric emptying is a complex function of the degree of smooth muscle stimulation, humoral signaling in the gut, meal component(s), time of day, and other physiological parameters. Washington *et al.* (2001) report a half-time of stomach emptying in humans of approximately 1 hour. Eleven substrates were analyzed by means of the RBALP with varying extraction times of 1, 2, and 4 hours (Figure 2B). Only one substrate showed a variation in IVBA as a



(b)

Figure 2. (Continued)

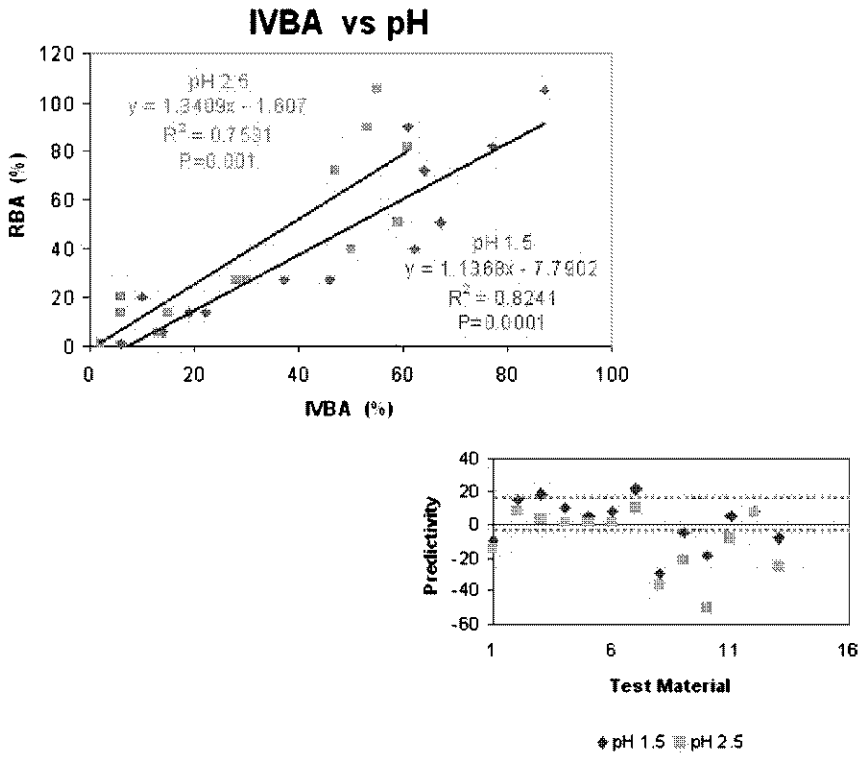
function of extraction time that exceeded the required RBALP precision, and single factor ANOVA indicated no significant difference in the two larger datasets ($F = 0.048$, $F_{Critical} = 3.32$). Therefore, an extraction time of one hour was adopted for the RBALP to minimize total testing times. In general, no significant variation (<10%) in absolute soluble lead concentration is observed if the HDPE bottles are allowed to stand for up to 4 hours after rotation.

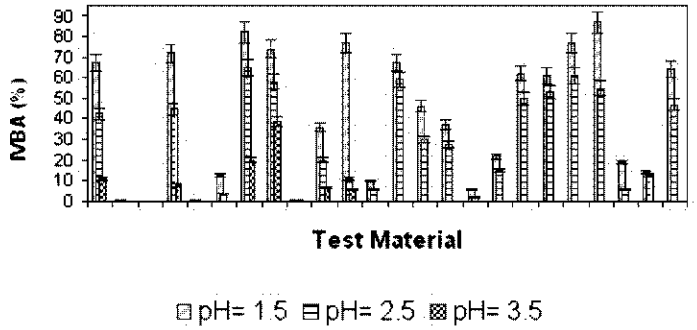
Agitation

The degree of agitation of an *in vitro* system can influence the rate and extent of solubilization of lead from the test material. If the material is allowed to accumulate at the bottom of the apparatus, the effective surface area is reduced. Agitation may affect either the diffusion layer thickness or the rate of production of fresh surface or both (Nernst and Brunner 1904 or Dankwerts 1951). An end-over-end method of agitation was chosen to best maximize sample/fluid contact while minimizing potential contamination from additional devices (*e.g.*, paddles, or stirring bars) or particle-size reduction.

Sample/Fluid Ratio

A sample/fluid ratio of 1/100 was used in the RBALP. This was chosen to reduce effects of metal dissolution that have been noted in previous studies (Sorenson





(c)

Figure 2. (Continued)

et al. 1971) when low ratios (1/5 and 1/25) were used. To improve reproducibility, however, the mass of material and volume of extract fluid were each increased in order to reduce variability due to small-scale sample heterogeneity and/or errors in test material weighting. Tests performed with a variety of standard reference materials showed no significant variation (within ±1% of control means) in IVBA with soil masses as low as 0.2 g per 100 mL of fluid (a sample/fluid ratio of 1/500). Although this consistency demonstrates that the test material to fluid ratio may not be critical in some cases, actual test materials may not be as homogeneous as the

standard reference materials. Therefore, low (<0.4 g per 100 ml) sample/fluid ratios should be avoided to reduce possible variability in IVBA.

Care must also be taken choosing the sample/fluid ratio when the test material contains high concentrations of soluble lead. Highly soluble lead forms such as lead acetate, lead oxide, and lead carbonate, may saturate the extraction fluid if these forms exceed approximately 5.0 wt% (50,000 mg/kg) lead. In this case, to prevent formation of lead chloride precipitate upon cooling, reduce the mass of a test material containing greater than 5.0 wt% lead to 0.50 ± 0.01 g.

pH

Human gastric pH ranges from 1 to 4 s.u. during fasting, and may be elevated to about 5 s.u. for a few hours after ingestion of food (Washington *et al.* 2001). Previous authors have used stomach extraction fluids with pH values of between 1.0 and 2.5 for their *in vitro* experiments (Ruby *et al.* 1993; CBR 1993; Medlin 1997; Gasser *et al.* 1996; Buckley 1997; Mercier *et al.* 2000 and Rodriquez *et al.* 1999). To evaluate the effect of pH on the RBALP, 24 different substrates were analyzed at extraction fluid pH values of 1.5, 2.5, and 3.5. As expected, the RBALP results are more sensitive to pH than any other parameter. Variation in IVBA over this pH range is large (2- to 7-fold) (Figure 2C), however, significant variation only occurs above pH 2.5. The lack of test material from *in vivo* studies prevented a more complete sensitivity analysis of pH. On the basis of the strength of the IVIVC for 13 of the 24 substrates, a gastric pH of 1.5 was chosen for the RBALP, since this pH provides the greatest improvement in predictability of RBA and limits the chances of under-estimating IVBA. This pH limit is particularly important during analysis of test materials dominated by slag, which tends to exceed the buffering capacity of the glycine and raise the pH by 1.0 s. u. or more.

Based on the work of Medlin (1997), no small intestinal phase (pH ~7) is required for the RBALP because the gastric phase of the test yielded an acceptable correlation to the *in vivo* results. In the current study, it was also found that no improvement in IVIVC was observed with the addition of an intestinal step.

Food/Nutrient

The effects of food (Maddaloni *et al.* 1998) or nutrients (Ca-Fe-P) (Ziegler *et al.* 1978; Mahaffey *et al.* 1986; Marcus and Schwartz 1987) on lead absorption *in vivo* has been well documented. Although there are probably several mechanisms by which food influences lead absorption *in vivo*, one possible factor is binding between lead and food material in the gastric fluid. For this reason, the effect of added food material (banana, milk, oatmeal, or rice) on IVBA was investigated by Medlin (1997). In general, addition of food to the RBALP extraction fluid caused a reduction in IVBA, but the extent of IVBA reduction was highly variable, depending on the lead form (lead phosphate, galena, slag, or lead oxide), the type of food added, and the amount of food added (4–16% of test volume). However, because the strategy adopted by Casteel *et al.* (2006) for measuring RBA *in vivo* focuses on absorption by the fasted animal, it was decided that the *in vitro* RBALP procedure should also focus on the fasted condition, so addition of food to the extraction fluid was not included in the protocol and was not investigated further.

Within-Laboratory Reproducibility

To evaluate the reproducibility of the RBALP, 75 and 83 replicate analyses on each of 2 standard reference materials (NIST SRM 2710 and 2711, respectively) were conducted within the CUB laboratory over several years. Results on both standards indicate the procedure is highly reproducible, with a coefficient of variation of 7% and a mean IVBA of 75% ± 5% for SRM 2710 and 84% ± 6% for SRM 2711.

Intra-Laboratory Validation

Intra-laboratory validation of the RBALP was based on triplicate analysis of each of the 19 test materials at four laboratories, including: (1) CUB, (2) ACZ Laboratories Inc., Steamboat Springs, CO (ACZ), (3) the National Environmental Research Laboratory, Las Vegas, NV (NERL), and (4) the Environmental Research Chemistry Laboratory, Denver, CO (ERCL). Results are shown in Table 3.

Intra-laboratory agreement was high, with pooled coefficients of variation (CV) generally within the range of 2% to 6% (Table 3). The between-laboratory variability was slightly higher for two of the test materials (Murry soil and Murry slag), possibly due to small-scale mineralogical variability in these test materials. ANOVA indicates no significant difference ($p = 0.21$) in predictability among the four laboratories.

Intra-laboratory precision was also high, with the ERCL and ACZ laboratories producing the tightest datasets (average CVs of 0.014 and 0.037, respectively). Both NERL and CUB had slightly higher average CVs (0.057 and 0.063, respectively);

Table 3. Intra-laboratory summary statistic for the RBALP.

Test Material	Mean* IVBA	Minimum IVBA	Maximum IVBA	Pooled IVBA CV
Aspen Smuggler Residential	71+/−3.3%	65.3%	76.9%	0.036
Aspen-Smuggler Berm	65 +/−1.7%	62.8%	67.9%	0.018
Bingham Cr. Channel	38+/−1.3%	35.6%	40.3%	0.019
Bingham Cr. Residential	47+/−2.4%	42.9%	52.2%	0.030
Butte	21+/−0.8%	19.7%	22.7%	0.029
Leadville Residential	70+/−4.5%	63.5%	77.1%	0.021
Leadville Fe-Mn-PbO	90+/−3.1%	85.4%	93.2%	0.015
Leadville AV Slag	17+/−7.8%	7.6%	28.1%	0.186
Leadville Oregon Gulch	14+/−1.7%	10.5%	15.1%	0.046
Jasper Smelter	71+/−2.9%	62.9%	73.6%	0.040
Jasper Mill	86+/−4.7%	77.6%	92.2%	0.042
Jasper Yard	79+/−4.1%	72.5%	82.8%	0.039
Midvale Slag	21+/−3.6%	16.8%	27.6%	0.069
Murray Slag	73+/−6.7%	60.8%	81.3%	0.059
Murray Soil	84+/−6.9%	67.3%	90.9%	0.047
Palmerton-2	67+/−2.3%	63.2%	70.5%	0.016
Palmerton-4	69+/−2.8%	66.7%	74.7%	0.024
NIST SRM 2589 (paint) in soil	75+/−3.8%	68.9%	80.6%	0.026
Galena in soil	6+/−2.3%	3.3%	13.0%	0.297

*For each test material, n ~ 12 (triplicate analyses in three independent laboratories and CUB) +/−standard deviation .

Table 4. RBALP predictive statistics based on all four laboratories.

Test material	Absolute predictive Error (PE_{abs})	Root-mean Predictive error (PE_{rm})	Percent predictive error (%PrE)
		Point Estimate Values	
Aspen Smuggler Residential	2.2	1.5	3.1
Aspen-Smuggler Berm	5.7	2.4	8.1
Bingham Cr. Channel	9.3	3.1	32.1
Bingham Cr. Residential	15.6	4.0	50.5
Butte	3.31	1.8	18.4
Leadville Residential	12.8	3.6	15.5
Leadville Fe-Mn-PbO	22.2	4.7	19.8
Leadville AV Slag	7.2	2.7	30.0
Leadville Oregon Gulch	6.7	2.6	96.2
Jasper Smelter	8.3	2.9	13.2
Jasper Mill	2.7	1.7	3.3
Jasper Yard	13.9	3.7	15.0
Midvale Slag	3.3	1.8	18.5
Murray Slag	29.5	5.4	65.5
Murray Soil	20.2	4.5	31.5
Palmerton-2	7.1	2.7	9.6
Palmerton-4	11.4	3.4	19.6
NIST SRM 2589 (paint) in soil	6.0	2.4	7.4
Galena in soil	5.3	2.3	532.5

however, their completeness is higher and the reduced precision is based on results from a single sample that other two laboratories did not analyze (Figure 3). Additionally, the decrease in CUB's precision reflects results collected over a greater period of time, by multiple technicians and multiple (Method 6010 and 6020) analytical methods. ANOVA indicates no significant differences between replicates, replicates/labs, or replicates/soils ($p = 0.142$, 0.512, and 0.911), respectively.

As shown in Table 4, predictive statistics, based on mean values from all four laboratories, show an absolute percent error (PE) of between 1% and 29% (average = 10%), and a percent predictive error (%PrE) of between 2% and 532%. If one excludes the two samples with very high %PrE and low RBA (Galena in Soil and Leadville Oregon Gulch), the average %PrE is 20%.

IVIVC Evaluation

Several alternative statistical approaches were considered for use in characterizing the IVIVC between IVBA and RBA. Because the values of RBA are known to be heteroscedastic, fitting methods that do not account for heteroscedasticity in RBA were excluded. This included ordinary linear regression and the geometric mean functional relationship described by Till (1974) and by Draper and Smith (1998). As detailed in USEPA (2006), the first step in the evaluation was to use weighted regression to test the fit of a linear model and several nonlinear models (power, two-parameter exponential, three-parameter exponential) to the data. The weight

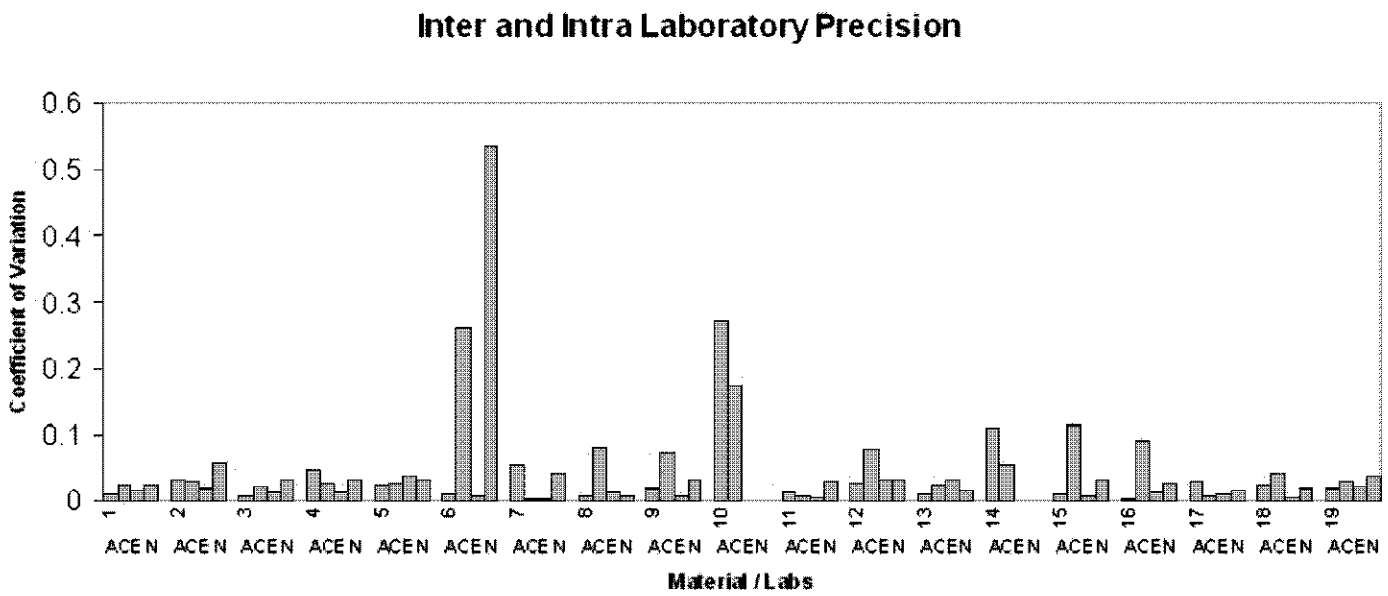


Figure 3. Inter and Intra laboratory precision. A = ACZ, C = CUB, E = ERCL, and N = NERL laboratory completeness are 98%, 100%, 89% and 89%, respectively. Test materials follow the numbering sequence in Table 1.

assigned to each data pair ($IVBA_i$ and RBA_i for test soil "i") was the inverse of the variance in each value of RBA_i. Although the exponential models fit the data slightly better than the linear model, this was judged to be an artifact of the relative lack of mid-range RBA values, so the linear model was identified as the preferred approach. Based on this, the best-fit weighted linear regression model is:

$$RBA = 0.878 \cdot IVBA - 0.028 (\text{weighted } r^2 = 0.924,\ p < 0.001)$$

However, weighted regression accounts only for errors in RBA, and does not account for measurement errors in IVBA. For this reason, the data were also evaluated using an approach that accounts for errors in both IVBA and RBA. Two techniques were considered: the bivariate linear regression techniques described by Riu and Rius (1996) and the chi-square merit function described by Press *et al.* (1992). These two methods are identical except that the method of Riu and Rius (1996) includes a covariance term in the weights, while Press *et al.* (1992) assume a value of zero for the covariance. Because multiple measurements of each of the variables (RBA and IVBA) are needed for each soil sample to compute the covariance, and because only one RBA value is available for each soil, the method of Press *et al.* (1992) was selected for use. It is considered that assuming a value of zero for the covariance term in the weight is very unlikely to significantly alter the results.

The two fits (chi-square merit function and weighted linear regression) are shown in Figure 4A. As seen, the fit resulting from the chi-square merit function (intercept = –0.028, slope = 0.883, $r^2 = 0.917$) is nearly identical to the results from weighted linear regression (intercept = –0.028, slope = 0.878, $r^2 = 0.924$). This result is not surprising because the variance in IVBA is small compared to the variance in RBA. Because the difference is so small, the results from weighted linear regression are selected as the preferred approach.

Results for weighted least squares IVIVC for CUB along with each validation laboratory are summarized in Table 5. As observed, there is relatively little difference among the datasets, with no statistical difference in slope (P values of 0.63, 0.88, and 0.54) and only a small statistical difference in intercept (P values of 0.27, 0.24, and 0.02) for one pair (CUB-NERL). It is important to recognize that use of this equation to calculate RBA from a given IVBA measurement will yield the RBA value as corresponding to the best-fit line and that the true RBA may be either higher or lower, as reflected by the 95% prediction intervals indicated in Figure 4B. Correlation of

Table 5. Between-Laboratory Variability in IVIVC.

	Slope		Intercept			Comparison* CUB vs Lab_x P-value
Laboratory	Best Est	SE	Best Est	SE	r^2	Slope/Intercept
CUB	0.878	0.061	−0.028	0.010	0.924	
NERL	0.930	0.058	−0.064	0.010	0.937	0.541/0.017
ACZ	0.838	0.056	−0.044	0.010	0.930	0.628/0.274
ERCL	0.867	0.055	−0.044	0.010	0.942	0.888/0.244
All Labs	0.883	0.058	−0.044	0.010	0.933	NA

*Based on the fact that observed slopes and intercept follow the t distribution; $t = \frac{b^1 - b^2}{\sqrt{s^2_{b1} + s^2_{b2}}}$ and $v = n_1 + n_2 - 4$ df, (Glantz 2002).

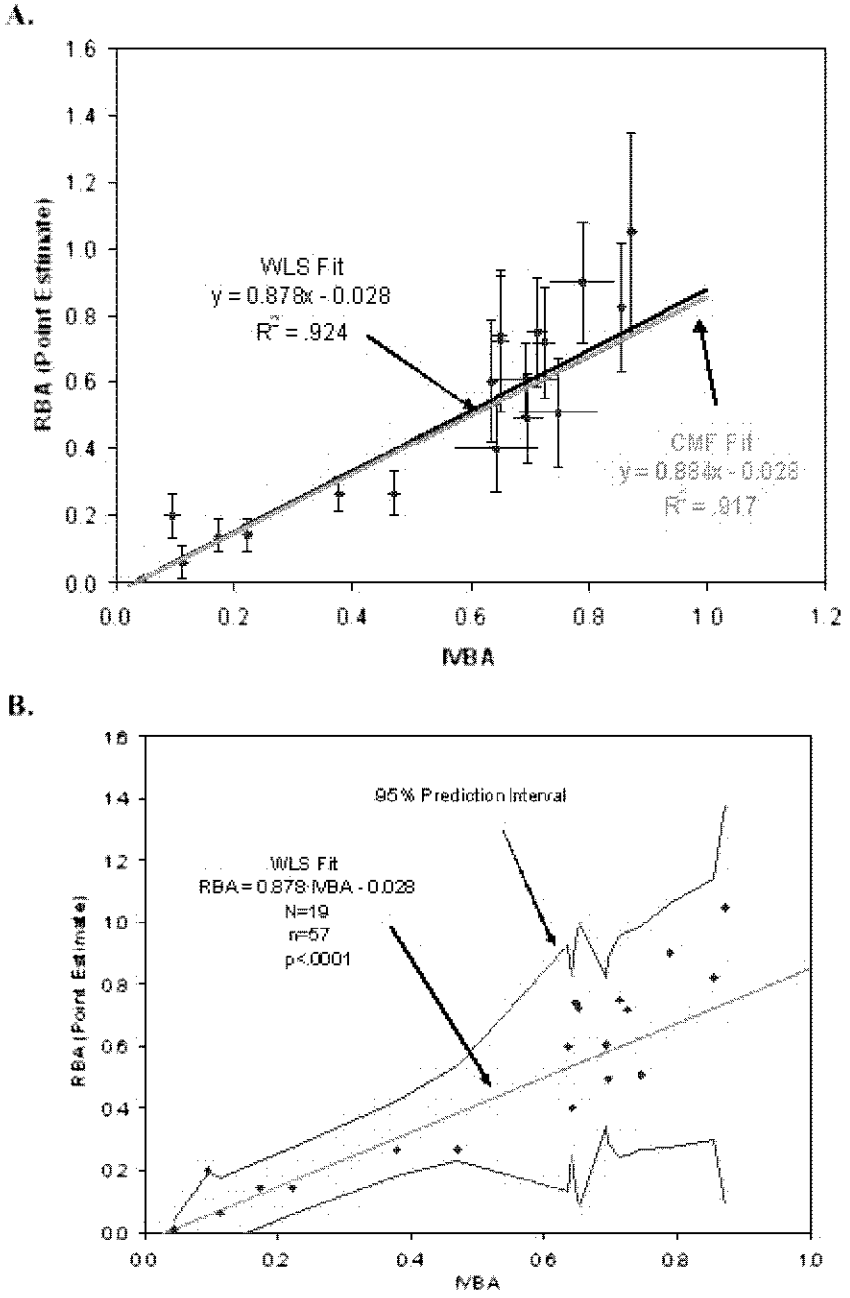


Figure 4. A.) Comparison of multiple correlation methods; using (CUB data set only) **WLS** Weighted least squares, and **CMF** ChiSq merit function along with standard error of the mean for each substrate. B.) Proposed IVIVC using weighted least squares (**WLS**), along with 95% confident prediction intervals. N = total number of substrates tested, n = total number of replicate analyses used in IVIVC.

Table 6. Overall comparison of IVBA methods for lead.

Reference	Fluid pH*	Sample/Fluid g/ml	Method Complexity	QA/QC**	R^2	Slope†	Intercept†	p	Precision "CV"	Test animal	Range in RBA	Test materials	Statistical analyses+
Ruby *et al* 1993	1.3/7.0	1/10	High	None					ND	Rabbit	9 %	1	None
CBR, 1993	2.0/7.0	1/30	High	None					ND				None
Medlin 1997	1.5	1/110	High	Extensive	0.63	0.90	−8.21	<.0001	ND	Swine		15	None
Ruby *et al* 1996	1.3/7.0	1/10	High	Minor	0.92	0.439	6.8	<.0005	ND	Rat	9–41 %	7	None
Gasser *et al* 1996	1.0/3.0	?	Simple	None					ND				None
Buckley 1997	1.8/7.1	1/100	Very High	None					ND	Rat	1 %	1	None
Mercier *et al* 2000	2.0/6.0	1/20	Simple	None					ND				None
SHIME Oomen *et al* 2002	2.0/6.5	1/60	High	None					ND	Adult Human	26 %	1	None
TIM Oomen *et al* 2002	1.8/7.2	1/2400	Very High	None					ND	Adult Human	26 %	1	None
Oomen *et al* 2003	1.1/7.8	1/100	High	None					ND	Adult Human	26 %	1	None
Schroder *et al* 2004	1.8/5.5	4/600	High	None	0.79–0.86	1.2–2.2	12.3–0.03	<.0002	ND	Swine	1–105 %	18	None
This Study	1.5	1/100	Low	Extensive	0.924	0.878	−0.028	<.0001	7%	Swine	1-105 %	19	Extensive

*Multiple pH fluids when gastric and intestinal phases modeled. **QA/QC beyond analytical (blanks, spikes (matrix and blank), duplicates, and traceability. †Slope and intercept may be recalculated if in vivo data not originally plotted on the y-axis. +Complete validation: sensitivity testing, inter and intra laboratory testing, confidence limits, predictability.

the IVBA results with endpoint-specific RBA values (blood, liver, kidney, and femur) were generally similar, with R^2 values of 0.937, 0.909, 0.876, and 0.868, respectively.

DISCUSSION

Although IVIVCs have historically been used by drug manufacturers, until now, the development of a bio-equivalent *in vitro* method for evaluating absorption of environmental lead has had limited success. In Table 6, based on an extensive literature search, are summarized the key components of earlier methods. Many of these alternative methods contain fundamental omissions and/or limitations. Commonly, they lack calibration against an appropriate *in vivo* surrogate. Others have lacked statistical strength or have tested samples with only a narrow range of RBA. Furthermore, no previous method has been independently tested.

The RBALP described in this study overcomes many of the shortcomings of other methods: the IVBA values produced by the method are strongly correlative with *in vivo* RBA_{PE} values, and the method has been characterized by extensive inter- and intra-laboratory studies. As such, the RBALP provides a valuable tool for developing site-specific estimates of relative lead bioavailability (with a predictive error of 20%). The RBALP presented here may be useful for screening materials for exposure hazards, as in the refinement of human health risk assessments or in the management of residential exposures to environmental lead. By providing the ability to rapidly and relatively inexpensively estimate RBA of lead on a medium-by-medium basis (perhaps a yard-by-yard basis), remedial strategies at contaminated sites can more effectively be developed, evaluated, and implemented. One potential use for such RBA estimates would be to incorporate site-specific data into the USEPA's IEUBK model. The use of an IVBA estimate for input into the model can be accomplished in the following manor: (1) estimate the RBA by using the following equation: $RBA_{PE} = 0.8782^{*}$ IVBA – 0.028 (from our IVIVC). (2) multiply the estimated RBA for your soil or soil-like material by 50% (0.5) to arrive at an estimate of ABA_{soil} based on the assumption that ABA for food and soil is 50% that in water (USEPA 1994). This value can then be substituted for the 30% default values (soil/dust) in the "Total Percent Accessible" window of the IEUBK model.

Continued work to improve on the RBALP includes; refining and quantifying pH sensitivity, expanding the variety of test matrices to include more media in the mid-range of RBA (30–60%), and testing of media which have lead masses dominated by lead paint, including the most common pigments (lead oxide, lead carbonate, lead sulfate, and lead chromate). In addition, we are expanding the test matrix outside of the mining/milling environment to include; shooting ranges, amended soils, dusts, and industrial sites.

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