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ToxStrategies

Inter-Laboratory Validation of Bioaccessibility Test for Metals

Henderson R[1], Verougstraete V[2], Anderson K[3], Arbildua JJ[4], Brock TO[5], Brouwers T[6], Cappellini D[7], Delbeke K[8], Herting G[9], Hixon G[1], Odnevall Wallinder I[9], Rodriguez PH[4], Van Assche F[10], Wilrich P[11], Oller AR[12]

[1] ToxStrategies Durham, NC. [2] Eurometaux Brussels, Belgium. [3] OSU Corvallis, OR. [4] CECM, Adolfo Ibañez University Santiago, Chile. [5] Duke University Durham, NC. [6] ECTX-Consult Hasselt, Belgium. [7] KMHC,Wilkes-Barre, PA. [8] ECI Brussels, Belgium. [9] KTH Stockholm, Sweden. [10] IZA Brussels, Belgium. [11] Freie Universität Berlin Berlin, Germany. [12] NiPERA Durham, NC

Background

In vitro bioaccessibility studies have been increasingly used as an alternative to *in vivo* testing to meet the demands of new and evolving regulatory programs in recent years. Bioaccessibility refers to the amount of metals released from a material in fluids designed to mimic those of the human body and provides a conservative estimate of bioavailability[1,2]. Bioavailability of the metal at the target site in an organism is generally the most important factor in determining toxicity of metals. As such, measures of relative bioaccessibility are often used in grouping and read-across approaches for hazard and risk assessment of metals, metal substances, and complex materials. Bioaccessibility is already incorporated in some standard test methods and regulatory frameworks[3-5] and some groups have sought to standardize specific methods[6-8]. However, standardized fluid compositions and testing protocols are limited and existing studies demonstrate that sample characteristics and methodological differences can affect the absolute amount of metal released[9,10]. Defined protocols that yield reproducible bioaccessibility results are needed.

Objective

We performed a cross-laboratory bioaccessibility study of six metal-containing materials in simulated biological fluids representing oral, inhalation, and dermal routes of exposure. The resulting bioaccessibility data were evaluated by characterizing within-laboratory repeatability and between-laboratory reproducibility.

Methods

Bioaccessibility Assays

Each of 5 laboratories (coded A-E) performed bioaccessibility testing according to a Standard Operating Procedure (SOP) in the following four simulated biological fluids: gastric, lysosomal, interstitial, and perspiration. Laboratories measured the release of seven different metals depending on the composition of the 6 test materials (**Table 1**). In brief, test materials were added to simulated fluids and extracted for a set period of time under given conditions. The compositions and general testing conditions of each of the simulated fluids are described in **Table 2**. Following a filtration step, extracts were analyzed and the amount of metal release was reported as µg metal/g sample. All test materials were powders with a median particle size <60 µm. A comparison between the SOP and the 5 laboratory reports resulted in the exclusion of some datasets from statistical analysis due to methodological differences. In addition, datasets with 2 or more labs reporting results below the limit of detection (LOD) were excluded.

Statistical Analyses

The statistical analysis of retained measurements was based on ISO 5725-2[11]; based on this method, outliers were discarded and stragglers retained. The ratio of the repeatability standard deviation (s_r; within-lab) and reproducibility standard deviation (s_R; between-labs) of the log concentration, s_R:s_r, was determined and used as an indicator of the agreement between laboratories. Relative standard deviation (RSD) of the log concentration was also used to assess the fluctuations in the data relative to the data mean; RSD values <20% and <10% were used to assess agreement for reproducibility and repeatability, respectively[7,8].

Table 1. Description of test materials used in this study.

Test Material	Formula	Metal Content (%)[1]	$D_{x,3}$ (µm)[2]
Cobalt oxide	Co_3O_4	Co (73.43)	2.7
Cobalt metal	Co	Co (99.98)	3.4
Copper concentrate	N/A	Cu (23.58)	59.2
Inconel alloy	N/A	Cr (18.3), Fe (14.6), Ni (67.1)	6.1[3]
Leaded brass alloy	N/A	Cu (58.45), Pb (3.22), Zn (37.75)	56.2
Nickel sulfate hexahydrate	$NiSO_4 \bullet 6H_20$	Ni (23.07)	12.4[3]

[1] Composition information from Certificate of Analysis as provided by supplier.
[2] Particle size measured with laser diffraction as reported by supplier unless otherwise noted; $D_{x,3}$ corresponds to the median particle diameter from the volume (mass) distribution.
[3] Analysis conducted by Particle Technology Labs, Ltd.

Results

Repeatability (within laboratory variation) was good for 3 of 4 fluids

The within-lab variability was found to be generally acceptable for all treatment conditions (**Figure 1a**). The average s_r among all treatment conditions varied only slightly, with the exception of interstitial fluid at 24 h (data not shown). There were 5 instances where the RSD_r exceeded 10% out of a potential 70 treatment+metal/test substance analyses combinations. All fluids, except interstitial, had fairly low within-lab variability at 24 h (<4%).

Reproducibility (between laboratory variation) was not satisfactory overall

The s_R:s_r ratios demonstrate that the between-lab agreement relative to the within-lab agreement was not satisfactory overall (**Table 3**). The perspiration treatment conditions were poorly reproduced between labs and need improvements (ratios > 6). There was fair agreement under the gastric and long-term lysosomal treatments (ratios between 3 and 6), while the average s_R:s_r ratios for interstitial fluids and the short-term lysosomal treatment indicated good agreement in variability between labs (ratios < 3). While RSD analysis shows more favorable reproducibility outcomes for some data sets, overall results still varied more between than within-laboratories (**Figure 1b**).

Figure 1a. Within laboratory variability (%RSD_r) at 24 h

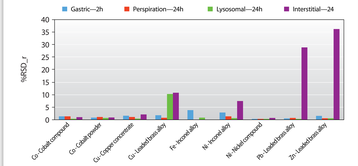

Figure 1b. Between laboratory variability (%RSD_R) at 24 h

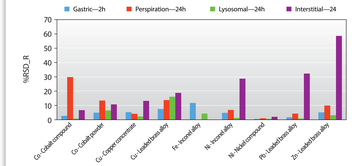

Table 3. s_R:S_r ratio results.

	Gastric 2 hrs	Perspiration 24 hrs	Perspiration 168 hrs	Lysosomal 24 hrs	Lysosomal 168 hrs	Interstitial 24 hrs	Interstitial 168 hrs
Metal - Test Substance	**s_R:s_r ratio**						
Co - Cobalt compound	2.0	**24.0**	**10.4**	2.1	**6.4**	**7.1**	2.9
Co - Cobalt powder	**6.2**	**12.7**	*5.5*	**10.0**	*4.7*	**13.2**	*4.0*
Cr - Inconel alloy 718	-	-	**42.0**	-	2.1	-	-
Cu - Copper concentrate	*3.3*	*4.0*	2.4	*5.5*	*3.5*	**6.5**	**8.3**
Cu - Leaded brass alloy	*4.3*	**19.9**	*4.1*	1.6	*3.3*	1.7	*3.2*
Fe - Inconel alloy 718	*3.1*	-	-	*5.4*	**11.5**	-	-
Ni - Inconel alloy 718	1.7	*5.4*	*4.7*	1.9	**10.6**	*3.9*	2.4
Ni - Nickel compound	2.7	*4.0*	2.3	2.3	*4.0*	3.0	-
Pb - Leaded brass alloy	*3.6*	**6.6**	-	2.8	*3.8*	1.1	-
Zn - Leaded brass alloy	*3.5*	**19.0**	*4.1*	*5.7*	*4.2*	1.6	1.9
Treatment Averages	*3.4*	**13.0**	**6.9**	2.5	*5.3*	2.2	2.3

s_R:s_r indicates agreement between the variability in repeatability and reproducibility; <3 = good, 3-6 = fair, >6 = poor
Shaded values exceed a ratio of 6

Table 2. General description of bioaccessibility fluids and protocols.

	Gastric		Lysosomal		Interstitial		Perspiration	
Composition of fluid	**Reagent**	**g/L**	**Reagent**	**g/L**	**Reagent**	**g/L**	**Reagent**	**g/L**
	Hydrochloric acid	2.55	Sodium chloride Sodium hydroxide Citric acid Calcium chloride dihydrate Sodium phosphate heptahydrate Sodium sulfate Magnesium chloride hexahydrate Glycine Sodium citrate dihydrate Sodium tartrate dihydrate Sodium lactate Sodium pyruvate Formaldehyde	3.21 6.00 20.8 0.097 0.179 0.039 0.106 0.059 0.077 0.090 0.085 0.086 1.0 mL	Magnesium chloride hexahydrate Sodium chloride Potassium chloride Sodium phosphate Sodium sulfate Calcium chloride dihydrate Sodium acetate trihydrate Sodium bicarbonate Sodium citrate dihydrate	0.203 6.02 0.298 0.142 0.071 0.368 0.953 2.60 0.097	Sodium chloride Urea Lactic acid	5.0 1.0 1.06
pH	1.5 ± 0.1		4.7 ± 0.2		7.4 ± 0.2		6.5 ± 0.1	
Temp (°C)	37 ± 1		37 ± 1		37 ± 1		30 ± 1	
Loading (g/L)	0.2		2		2		2	
Time (hours)	2		24, 168		24, 168		24, 168	
Protocol Overview	Ten (10.0 ± 0.5) mg of test material was weighed in triplicate into three separate 250 mL Erlenmeyer flasks. Subsequently, 50 mL of extraction fluid was added to each test vessel flask and to one blank control flask. After adjusting for pH, the flasks were covered with a stopper or parafilm, placed into shaker bath, and agitated for one hour. Flasks were allowed to sit without agitation for one additional hour before sampling.		One hundred (100.0 ± 5.0) mg of test material was weighed in triplicate into three separate 250 mL Erlenmeyer flasks for each sampling time. Subsequently, 50 mL of extraction fluid was added to each test vessel flask and to two blank control flasks. After adjusting for pH, the flasks were covered with a stopper or parafilm, placed into shaker bath, and agitated for 24 or 168 hours. After the appropriate extraction time, the test vessels were left to settle for 3 to 5 minutes.		One hundred (100.0 ± 5.0) mg of test material was weighed in triplicate into three separate 250 mL Erlenmeyer flasks for each sampling time. Subsequently, 50 mL of extraction fluid was added to each test vessel flask and to two blank control flasks. After adjusting for pH, the flasks were covered with a stopper or parafilm, placed into shaker bath, and agitated for 24 or 168 hours. To maintain the pH throughout the extraction at 7.4 ± 0.2, 5% CO_2 was introduced in the test vessel during the test. After the appropriate extraction time, the test vessels were left to settle for 3 to 5 minutes.		One hundred (100.0 ± 5.0) mg of test material was weighed in triplicate into three separate 250 mL Erlenmeyer flasks for each sampling time. Subsequently, 50 mL of extraction fluid was added to each test vessel flask and to two blank control flasks. After adjusting for pH, the flasks were covered with a stopper or parafilm, placed into shaker bath without agitation for 24 or 168 hours. After the appropriate extraction time, the test vessels were left to settle for 3 to 5 minutes.	
Filtration	A syringe was used to remove a 10 mL aliquot from each test vessel at a depth of two third of the supernatant. The samples were filtered through a 0.2 µm syringe filter and transferred to tubes for storage of less than one month.							

Discussion

In the current study, within-laboratory variability was generally satisfactory for all treatment conditions with the exception of some metals in interstitial fluid. However, variability between laboratories was found to exceed accepted criteria, the extent of which varied depending on whether the s_R:s_r ratios or the RSD approaches were used.

Several lessons can be learned from this exercise. For example, substances that are being compared should always be tested side-by-side or at least in the same lab. Limiting longer exposure times when complicating factors such as CO_2 incorporation and precipitation phenomena are introduced may reduce interlaboratory variability. Refinements to the SOP are clearly needed to improve upon both within and between laboratory agreement; recommendations include better defining pH control measures, a defined solution buffer technique, and ways to minimize evaporation. In addition, streamlined LODs are needed as the wide variation in the laboratories' detection limits greatly impacted the study due to a number of values (e.g., those <LOD) having to be excluded from the analysis.

In the context of some other studies of similar characteristics it is possible that the criteria used here may be too stringent. For example, in one study using a saliva migration test for organic plasticizers, an RSD of 30% was found to be the best obtainable reproducibility[12]. Similarly, in an interlaboratory study to validate a method for environmental assessment of metals[13], only 15/37 measurements had CV_R values <25%. If an RSD of 30% or 40% had been used as the standard for the current study, all between laboratory reproducibility would have been deemed acceptable for most metals and treatment conditions, with the exception of Cr from Inconel alloy in 168h perspiration fluid and Zn from leaded brass alloy in 24h interstitial fluid.

For most applications, only measures of relative bioaccessibility are needed, diminishing the requirement for satisfactory interlaboratory reproducibility in absolute metal releases as discussed above. The high within-laboratory repeatability supports the use of these bioaccessibility methods for the assessment of relative metal release and calculation of effective concentration of metals in complex materials where a matrix effect can be present. This application can permit more toxicologically relevant classifications when effective concentrations are compared to classification cut-off limits for mixtures.

Conclusions

- Within-laboratory variability for synthetic gastric, alveolar, and perspiration fluids for all treatment conditions was satisfactory.
- Interlaboratory variability was generally higher than within-laboratory suggesting that the absolute bioaccessibility results in some fluids fluctuate between laboratories.
- A better-defined SOP, stricter adherence to the SOP, and consistent LODs could help achieve better concordance in absolute metal releases.
- However, for hazard and risk assessment applications, the use of these methods to generate relative release data and calculate effective concentration of metals in complex materials appears to be acceptable.

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