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Determination of *in vitro* bioaccessibility of Pb, As, Cd and Hg in selected traditional Indian medicines

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Abstract

In vitro bioaccessibility of Pb, As, Cd and Hg in five traditional Indian medicine samples was measured as a determinant of bioavailability. The method is based on simulation of human digestion in the passage of material from the gastric to intestinal portions of the gastrointestinal tract. Total concentration and concentration in extracts from gastric and intestinal phases were analyzed for Pb, As and Cd by dynamic reaction cell inductively coupled plasma mass spectrometry (DRC-ICP-MS) and for Hg by direct mercury analyzer (DMA). Total lead ranged from 1.9 to 36000 $\mu\text{g g}^{-1}$. In each of the samples bioaccessibility of lead was significantly higher (range 28–88%) in the gastric phase than in the intestinal phase (range 1.4–75.4%). Only Ekangvir Ras had measurable arsenic (304 $\mu\text{g g}^{-1}$). Its bioaccessibility in the gastric phase and intestinal phase was 82.6% and 78.1%, respectively. Only Ayu-Nephro-Tone had measurable cadmium (14.4 $\mu\text{g g}^{-1}$). Its bioaccessibility in the gastric phase and intestinal phase was 80.5% and 2.2%, respectively. Three samples had measurable mercury (range 37 $\mu\text{g g}^{-1}$ –10000 $\mu\text{g g}^{-1}$). Mercury in these samples was not bioaccessible. For the samples with measurable amount of metal, the estimated daily amount of bioaccessible (EDAB) metal was calculated. When compared with the most liberal published safety guideline, EDAB-Pb in Mahayograj Guggulu and Ekangvir Ras were 37 and 45 fold greater. When compared with the most conservative published safety guideline, all samples had higher EDAB-Pb or EDAB-As than the suggested limits. The EDAB-Cd and EDAB-Hg were acceptably below published safety limits.

Introduction

Traditional Indian systems of medicine (TISM) including Ayurveda, Siddha, and Unani are used by a majority of India's 1.1 billion population.¹ TISM is also used by millions of South Asians and others living in North America, Europe, and Australia.¹ TISM is strongly supported by the Government of India through federal and state-funded medical colleges, hospitals, and research.¹ Multiple therapeutic modalities are used in TISM including herbs, massage, and nutrition. A large industry within India has developed for the manufacture of

TISM herbal medicines for domestic and foreign consumption.² Although TISM is widely considered to be safe, recent safety concerns have arisen due to clinical reports of heavy metal toxicity, particularly lead, associated with the use of TISM.^{3–5} For example, we recently reported three patients who presented to hospitals in Bangalore, India with elevated blood lead levels (BLLs) of 122.4 $\mu\text{g dl}^{-1}$, 115 $\mu\text{g dl}^{-1}$ and 42.8 $\mu\text{g dl}^{-1}$, respectively. At the time of evaluation, each patient was consuming traditional medicines with high lead content (Case 1 – Mahayograj Guggulu 41182 $\mu\text{g g}^{-1}$ and Puspadhanva Ras 124557 $\mu\text{g g}^{-1}$; Case 2 – Gulkand 11799 $\mu\text{g g}^{-1}$; Case 3 – Ekangvir Ras 36044 $\mu\text{g g}^{-1}$). Cessation of the medicines along with chelation, nutritional intervention and education reduced the BLLs to 27.4 $\mu\text{g dl}^{-1}$, 21.1 $\mu\text{g dl}^{-1}$, and 18.2 $\mu\text{g dl}^{-1}$, respectively.⁶ Other laboratory analyses of TISM medicines purchased in local US markets and *via* the Internet have further found that approximately 20% contain varying levels of lead, mercury, and/or arsenic.^{7,8} In fact, there is a subset of TISM products called *Rasa Shastra* where herbs are deliberately combined with *bhasmas* made from metals, minerals, or gems.⁹ Elaborate protocols to prepare *bhasmas* are described in ancient TISM texts. *Shodhana*, or ‘purification,’ of the metals is claimed to occur through the addition of different liquid solvents followed by multiple cycles of heating and cooling. TISM purveyors claim that the state of the metals is transformed such that properly prepared *bhasmas* are rendered not only nontoxic but also therapeutic.⁹

Can medicines containing *bhasmas* made from lead, mercury, cadmium and arsenic be rendered safe for consumption? If this is true, how does it occur? One plausible hypothesis is that after the complex preparation process the metal is converted to a species that is no longer available for absorption. Total concentrations alone may lead to an overestimate of potential toxicity unless bioaccessibility (fraction potentially available for absorption) is considered. The amount of a metal absorbed is based on total amount ingested and its bioavailability (the fraction which reaches the systemic circulation). Bioavailability is dependent on multiple factors. Studies which compared bioavailability (*in vivo*) and bioaccessibility (*in vitro*) have indicated that bioaccessibility could be used as a determinant of bioavailability.^{10,11} Since *in vivo* studies are time consuming, costly and relatively complicated, *in vitro* extraction tests using simulated body fluids have been used to model bioaccessibility of toxicants.^{10–13} Some of these tests model the entire passage through the gastrointestinal tract (salivary phase, gastric phase and the intestinal phase), while some tests model gastric phase only or gastric and intestinal phases. Most of the studies were based on bioaccessibility of metals and some organic toxicants in soil and food.^{10–15} Bioaccessibility studies reported were based on either the batch method developed by Ruby *et al.*¹⁰ or the online leaching method developed by Chu and Beauchemin.¹⁶ In the batch method, the sample was introduced to a fixed volume of artificial gastric fluid maintained at 37 °C in anoxic conditions. Some batch studies sequentially continued to simulate the intestinal phase of digestion. Hamel *et al.*¹² developed two batch methods (mass balance and soil recapture) to estimate bioaccessibility of Pb, Cr, As and Cd in soil using artificial biofluids. Their results indicated that each metal had a bioaccessible fraction less than its total metal content. Instead of analyzing extracts of the intermediate steps in the *in vitro* sequential extraction technique, they proposed calculating the bioaccessibility by measuring total metal, the non-extracted portion of metal, and any residual matter precipitated during the extraction (recaptured metal fraction). In the online leaching method samples placed in a column were successively leached by artificial saliva, gastric fluid, and intestinal fluid. Chu and Beauchemin¹⁶ proposed that their method was more efficient for removal of the elements from the food than the batch method and could be used to accurately estimate the maximum bioaccessibility of metals. Most of the metal analyses in these published studies were done by using ICP-MS or ICP-OES (ICP-optical emission spectroscopy).^{12,16,17} Concentration, bioaccessibility and speciation of arsenic in traditional Chinese medicines were studied by Koch *et al.*¹⁷ using hydride generation atomic absorption spectroscopy and ICP-MS. They

also studied the extraction of As and their species in urine and total As in hair after one time ingestion.¹⁷

To test whether lead, arsenic, cadmium and mercury in five commercially available TISM products are bioaccessible or not, we used an *in vitro* model adapted from previous studies^{10,18} to simulate the gastric and intestinal phases of digestion. To the best of our knowledge bioavailabilities or bioaccessibilities of metals in these TISM products have not previously been reported. Lead, arsenic and cadmium were measured by dynamic reaction cell inductively coupled plasma mass spectrometry (DRC-ICP-MS) and mercury by direct mercury analyzer (DMA). Metal concentration in the intestinal phase was used to estimate daily amounts of bioaccessible metals. Results were compared with available safety guidelines.

Experimental

Instrumentation

Samples were analyzed for Pb, As and Cd using the DRC-ICP-MS (Elan 6100, Perkin Elmer, Norwalk, CT, USA) with Nickel skimmer and sampler cones. A sample introduction system consisted of a Tracey spray chamber (cyclonic spray chamber) (Glass Expansion, West Melbourne, Australia) and a Meinhard concentric nebulizer (TQ-30-A3) (J.E. Meinhard Associates, Inc., Santa Ana, CA, USA). The Pb and Cd concentrations in high pressure asher (HPA, Anton Paar, Graz, Austria) digested samples, gastric phase and small intestinal phase extracted samples were measured using the external calibration method with Tl as the internal standard for Pb and In as the internal standard for Cd. The internal standardization method was used to effectively correct for changes in analyte sensitivity caused by the variation of matrix components in the sample and the instrument drift. Samples were pre-screened for presence of Tl and In. Arsenic in samples were measured using external calibration in DRC mode using oxygen as the reaction with In as the internal standard. Since most of the samples were diluted more than 1000 fold, the matrix effect from the gastric and intestinal phase components were minimal on the ICP-MS analysis. Samples with very low concentrations of the analyte of interest needed less dilution and were analyzed with the standard addition method. The ICP-MS instrument operating conditions are given in Table 1.

Mercury was measured using a direct mercury analyzer (DMA 80, Milestone Inc., Monroe, CT). Samples were analyzed by the external aqueous calibration method using 7 calibration points. Standards and samples were processed under the following instrument parameters: sample-drying 120 s at 300 °C; sample decomposition 180 s at 850 °C; waiting 60 s.; and amalgam heating 12 s.

Reagents and materials

Artificial gastric fluid (AGF) was prepared by adding the following compounds to 1 liter of deionized water (≥ 18 M Ω) (Milli-Q system, Millipore, Bedford, MA, USA) while stirring continuously on a stir plate: 1.25 g of pepsin (activity: 3000 units mg⁻¹) (Mallinckrodt Baker Inc., NJ, USA), 0.5 g of sodium citrate salt (Mallinckrodt Baker Inc., NJ, USA), 0.5 g of malate (DL-dimethyl) (Toshima, LU, Tokyo, Japan), 420 μ L of lactic acid (Mallinckrodt Baker Inc., NJ, USA), and 500 μ L of glacial acetic acid (Seastar Chemical Co., Pittsburgh, PA, USA). The pH of the gastric solution was adjusted to 2.0 by adding ~2–3 mL of concentrated HCl (Seastar Chemical Co., Pittsburgh, PA, USA).^{10,18}

Artificial intestinal fluid (AIF) solution was prepared by dissolving 2.60 g of bile salts (Hi-Media Laboratories, Marg, Mumbai, India) and 0.75 g of pancreatin (EMD Chemicals, Gibbstown, NJ, USA) in 100 mL of deionized water.^{10,18}

Multielement (Pb, As and Cd) calibration standards for ICP-MS analysis and Hg calibration standards for DMA analysis were prepared using 1 mg mL⁻¹ single element standards from High Purity Standards (Charleston, SC, USA). Mixed internal standard solution (3 ng mL⁻¹) was prepared using 1 mg mL⁻¹ single element standards indium (In) and thallium (Tl) from Baker-Instra-Analysed (Phillipsburg, NJ, USA). Nitric acid was from Optima grade (Seastar Chemical Co., Pittsburgh, PA, USA).

Quality control standards used were National Institute of Standards and Technology (NIST) standard reference materials (SRMs, Gaithersburg, MD, USA) 1643e (trace elements in water), 1515 (apple leaves), 1400 (bone ash) and certified reference material (CRM) GBW 07601 (human hair, Institute of Geophysical and Geochemical Exploration, China). The calibration verification standard was a 1 ng mL⁻¹ mixed standard solution.

Samples

Five commercially available traditional Indian medicine samples were purchased *via* the internet (Table 2) as previously described.⁸ Of the five, two were *Rasa Shastra* medicines made with *Naga* (lead) *bhasma* (Mahayograj Guggulu and Ekangvir Ras). Shilajit (also known as *Shilajatu* or mineral pitch) is also considered a member of the *Rasa Shastra* class. However it is made from scraping the dried exudate of the plant *Asphaltum punjabinum* found on rock. Hingwastika is an herbal-only, non-*Rasa Shastra* medicine. Ayu-Nephro-Tone is a proprietary *Rasa Shastra* medicine consisting of herbs and *bhasmas* made from *vanga* (tin), *jasad* (zinc), and *yavakshar* (potash). The average weight of the pill or capsule and recommended adult dosage per day were recorded. Ten pills or the contents of the capsules from each were pulverized using agate mortar and pestle to obtain a fine powder as homogenous as possible while taking measures to avoid cross-contamination.

Procedures

In order to obtain semi-quantitative information on sample composition, these samples were measured by the X-ray Fluorescence (XRF) method (Handheld NITON XRF Analyzer, Thermo Fisher Scientific, Rockford, IL, USA). These data were used as a guide to estimate dilution factors and to avoid possible lab and equipment contamination. Due to the high concentration of the elements of interest in the sample and to prevent contamination, preparation was not carried out in the class-10000 clean room. All the glass-ware and plastic-ware were pre-cleaned by soaking in 10% HNO₃ for 24 h and rinsing several times with deionized water. Traditional medicine samples were analyzed for total Pb, As, Cd and Hg and for the bioaccessible fraction of Pb, As, Cd and Hg in gastric and intestinal phases as summarized in Fig. 1.

Total Pb, As and Cd content by ICP-MS—Powdered traditional medicine samples and quality control samples were digested with nitric acid in a high pressure asher. Approximately 0.05 g of the sample was weighed and digested with 2 mL of HNO₃ acid in a 50 mL HPA-Quartz digestion vessel using the temperature time program published previously.¹⁹ Digested samples were diluted to 25 mL with deionized water and then further diluted several fold to bring Pb, As and Cd concentrations into an appropriate range for the ICP-MS analysis while maintaining 5% acid strength.

Total Hg content by DMA—Approximately 0.1 g of each pulverized sample was weighed and digested overnight at room temperature with 1 mL of concentrated nitric acid in a 15 mL falcon tube. The digestate was then diluted to 10 mL with deionized water and stored until analysis for Hg by DMA. Samples were analyzed for mercury as soon as possible to prevent any losses. On the day of analysis digestates were further diluted several fold to bring the Hg concentration into an appropriate range for the DMA analysis. The HPA

digestate was not used for Hg analysis due to possible loss of Hg at high digestion temperatures (230 °C).

Simulated gastric phase digestion—Artificial gastric fluid (150 mL) was added to a 250 mL wide mouth high-density polyethylene (HDPE) bottle. Argon gas was sparged for 10 min into each reaction vessel to purge off atmospheric oxygen to simulate the anoxic environment in the gastrointestinal tract. Bottles were capped tightly after adding 1 g of the pulverized sample. Measures were taken to maintain anoxic conditions. The reaction vessels were kept on a shaker which was kept in an incubator maintained at 37 °C. The reaction mixtures were allowed to stand for 10 min without agitation. Bottles were then agitated at 150 rpm for 1 h. After 1 h, bottles were removed from the shaker and the content was allowed to settle for 2 min. Ten millilitres of the digestate was extracted using a 20 mL syringe with a Luer-lock attachment. The extract was filtered in order to remove any insoluble material using a 0.45 µm cellulose acetate disc filter (25 mm diameter, Whatman Inc., Florham Park, New Jersey, USA) to a 15 mL falcon tube. An aliquot of 50 µL of concentrated nitric acid was added to the extract before being stored in the refrigerator at 4 °C until analysis for Pb, As, Cd and Hg.

Simulated intestinal phase digestion—Immediately after extracting 10 mL of digestate to analyze for gastric phase digestion, the pH of the remaining mixture was allowed to change to 7.0 by adding a 5-inch long dialysis tube (8000 MWCO, cellulose ester tubing, Spectrum Laboratories Inc., Rancho Dominguez, California, USA) containing 2 g of NaHCO₃ to each reaction vessel. An aliquot of 10 mL of the artificial intestinal fluid was added to the reaction mixture. Argon gas was sparged for 10 min into each reaction vessel. Bottles were capped and agitated (to simulate the movement of the digestate in the intestine and to prevent settling) at 100 rpm in the incubator for 3 h. Ten millilitres of the digestate were extracted using a 20 mL syringe with a Luer-lock attachment. The extract was filtered in order to remove any insoluble material using a 0.45 µm cellulose acetate disc filter (25 mm diameter, Whatman Inc., Florham Park, NJ, USA) to a 15 mL falcon tube. An aliquot of 50-µL of concentrated nitric acid was added to the extract before being stored in the refrigerator at 4 °C until analysis for Pb, As, Cd and Hg. Each sample was analyzed in triplicate.

Results and discussion

Total Pb, As, Cd and Hg content

Although XRF values could be used for the sample total concentrations, the higher detection limit (10 µg g⁻¹) for this technique would preclude detection of lower metal concentrations in some samples. In addition, previous studies have shown that HPA digestion results in near complete destruction of the organic matrices.¹⁹ Even though metal concentration measured by HPA digestion with HNO₃ acid (without HF) will not include any metal bound to silica, measured total metal concentration of samples with high metal concentration values were comparable to the concentrations obtained by the XRF method. Therefore results obtained by HPA digestion were used as the total metal concentration.

Metal concentrations varied widely from sample to sample (Table 3). Data given are the average of triplicate measurements. Lead concentrations ranged from 1.9 to 36000 µg g⁻¹. The two *Rasa Shastra* medicines containing lead *bhasmas* (Ekangvir Ras and Mahayogaraj Guggulu) had the greatest Pb concentrations (25000 µg g⁻¹ and 36000 µg g⁻¹ respectively). Ekangvir Ras was the only sample which had a reliably measurable amount of As (304 µg g⁻¹). Only Ayu-Nephro-Tone had measurable amounts of Cd (14.4 µg g⁻¹).

Even though 0.05 g may not be a representative sample size, we were compelled to use this amount for measurement of total metal content in order to avoid contamination to laboratory vessels when using higher weights (0.3–0.5 g) as previously experienced. Since there are no available standard reference materials that are compatible with the matrix of traditional medicines, we decided to use apple leaves (NIST SRM 1515) and bone ash (NIST SRM 1400) to represent the herbal and *bhasma* fractions of traditional *Rasa Shastra* medicines. Recovery of the analysis of QC standard NIST SRM 1643e, NIST SRM 1400, NIST SRM 1515, CRM GBW 07601 and 1 ng mL⁻¹ mixed standard solution were calculated using measured and certified values given in Table 4. Recovery rates ranged from 90 to 110%. The detection limit of the method for Pb, As and Cd was 0.01 µg g⁻¹.

We chose to analyze Hg in these samples using DMA instead of ICP-MS since the carry over effect of Hg in DMA is minimal. This is in contrast to the ICP-MS sample introduction system, which would require long washout times after running samples with high Hg concentrations. Even though one could analyze Hg in these samples directly by DMA without prior dissolution, we decided to dissolve samples with HNO₃ at room temperature and dilute to a concentration appropriate for DMA analysis. This was due to previous experience with analyzing one such sample without prior dissolution and subsequent contamination of the catalyst tube, the gold amalgam, and other parts in the DMA. Out of five samples tested, three samples had measurable mercury concentrations (range 37 to 10000 µg g⁻¹). Mahayogaraj Guggulu had the highest mercury concentration. Recovery of the analysis of QC standard CRM GBW 07601 (human hair) by this procedure was 90%–110% and precision (given as %RSD) was <10%. Detection limit of the method for mercury was 0.4 µg g⁻¹.

Simulated gastric and intestinal phase digestion

To assess the bioaccessibility of metals, concentrations of metals in two phases, representing gastric and intestinal conditions, were measured. We used 150 ml of artificial gastric fluid to approximately 1 g of sample (liquid to solid ratio 150 : 1 mL g⁻¹). Ruby *et al.*¹⁰ had justified using 10 : 1 mL g⁻¹ based on the experiment of rabbit stomach and small intestine. The studies done by Hamel *et al.*²⁰ on the bioaccessibility of metals (Pb, Ni, As, Cd and Cr) in soils for different liquid to solid ratios in synthetic gastric fluid have shown that the solubility of metals in the synthetic gastric juice did not alter substantially within the range 100 : 1 to 5000 : 1 mL g⁻¹.

In our study we did not include the bioaccessibility in the salivary phase since all the medicines are recommended by the manufacturer to be swallowed without chewing. Major constituents in the salivary phase that can influence metal absorption are α -amylase, salivary lipase, HCO₃⁻ and Cl⁻. If any starch is present in the pill it could act as a stimulator to secrete α -amylase, which has a pH optimum of 7.4. Since the pills are swallowed, however, the secreted α -amylase would have very limited time to act before it gets inactivated in the stomach due to the pH change. Swallowed salivary lipase (optimum pH 4) could act in the stomach phase on any fatty acids in the pill, if present. Fatty acid digestion could be continued by pancreatin (enzymes: amylase, lipase and protease) in the intestinal phase. The salivary HCO₃⁻ and Cl⁻ were added in the next two phases. Due to the above, we assumed that the effect of omitting the salivary phase would likely be minimal to the final estimation.

Metal concentrations in each phase varied considerably (Table 5). This may have been due to the differences in the composition and pH (gastric pH = 2 and intestinal pH = 7) of the two phases in addition to the composition of the sample itself. Both *in vitro* and *in vivo* conditions, such as the pH, sorption and precipitation reactions in the medium, impact the extractability of a metal. In addition, *in vivo* conditions, such as residence time, mixing rate, type of food ingested and other physiological factors, will influence the metal extractability.

Bioaccessibility was calculated for each phase using metal concentration *in vitro* gastric or intestinal phase extract ($\mu\text{g g}^{-1}$) $\times 100$ /metal concentration in the original sample ($\mu\text{g g}^{-1}$). In a number of instances, bioaccessibility was not calculated due to the concentration of the particular analyte being below the detection limit of the *in vitro* extraction method [DL (Pb, As and Cd) = $0.03 \mu\text{g g}^{-1}$ and DL Hg = $3 \mu\text{g g}^{-1}$].

The bioaccessibility of lead in the five samples ranged from 28–88% in the gastric phase and 1.4–75.4% in the intestinal phase. Bioaccessibility of lead was significantly higher in the gastric phase than in the intestinal phase (HW $p = 0.04$; MG $p = 0.0006$; ER $p = 0.06$; SH $p = 0.0001$ and ANT $p = 0.002$) (Table 5). The H^+ concentration and complex ion formation with Cl^- controls dissolution of Pb in the stomach. Both gastric enzymes and organic acids maintain Pb in the soluble form in the stomach.²¹ Decrease in the Pb concentration in the intestinal phase may be due to precipitation or sorption reactions that occur with the increase of the pH in the intestinal phase.²² Some studies have indicated that using only gastric phase extraction is reasonable to estimate the bioaccessibility of a metal.^{20,23} Although using only gastric phase extraction provides the most conservative estimate of bioaccessibility, our study illustrates the importance of carrying out both gastric and intestinal phase extractions sequentially to get a better picture of what is happening in the gastro-intestinal tract. There are no data reported on bioaccessibility for lead in traditional medicine samples for comparison. The only data available in the literature for lead bioaccessibility is for food and soil. Hamel *et al.*¹² reported that lead in soil samples tested in their study had a bioaccessible fraction less than its total Pb content (39–70%). Differences in experimental conditions, soil properties, and variations in Pb speciation may have resulted in the varying bioaccessibility. Differences in the results may also be due to the absence of a gold standard method, certified reference materials, and certified bioaccessible levels at each phase.

Bioaccessibility of arsenic in Ekangvir Ras in the gastric phase and intestinal phase was 82.6% and 78.1%, respectively. Arsenic in traditional medicines typically comes from deliberate addition of mineral arsenicals including orpiment (As_2S_3), realgar (As_4S_4), and arsenolite (contains As_2O_3). In the literature there are conflicting observations on the solubility of these arsenic compounds under *in vitro* conditions. Marafante and Vahter²⁴ showed that solubility of As_2S_3 increased with increasing pH. However Liu *et al.*²⁵ demonstrated the opposite of that solubility of As_2S_3 and As_4S_4 was low and poorly absorbed by the GI tract, whereas the bioavailability of As_2O_3 is similar to inorganic arsenic salts. Wu and Ho²⁶ have shown the bioavailability of realgar increased when administered in nanoparticle form when compared to crude powder. A study on traditional Chinese medicine containing 7% As ($70000 \mu\text{g g}^{-1}$) found only 4% of the total was soluble in the gastric and intestinal fluid phases.¹⁷ Although 4% is relatively low, the absolute amount of As that was bioaccessible was still considerable.

Cadmium was measurable only in Ayu-Nephro-Tone ($14.4 \mu\text{g g}^{-1}$). Bioaccessibility in the gastric and intestinal phases was 80.5% and 2.2%, respectively (Table 5). Mercury was measurable in three of the samples and ranged from 37 to $10000 \mu\text{g g}^{-1}$. Bioaccessibility of mercury in these three samples were <1% in both phases (Table 5). Cinnabar, which contains mercury sulfide, is the common form of mercury added to mercury-containing traditional Indian medicines. Mercury or *parad* is considered the most important metal in TISM.⁹ Cinnabar is known to have very low solubility at both acid and neutral pH.^{27 & 28} This may be an explanation for the very low bioaccessibility of mercury found in these medicines.

Estimated daily amounts of bioaccessible (EDAB) metal based on intestinal phase metal concentration

Estimated daily amounts of bioaccessible metals were calculated based on the manufacturer's recommended dosage using the following equation:

$$\text{Estimated daily amounts of bioaccessible metal} = A \times B \times C$$

A = Weight of the pill (g), B = Average dosage/day, C = Metal concentration in the intestinal phase ($\mu\text{g g}^{-1}$).

For instances where the recommended dosage was stated as a range, the average dosage was used for calculations. Since metal absorption occurs in the small intestine,²⁹ EDAB-metal was calculated using the metal concentration in the intestinal phase. Table 6 shows the estimated daily amounts of bioaccessible Pb, As, Cd and Hg compared to published regulatory standards or safety guidelines for acceptable metal intake. Since there is no one universal standard for acceptable levels of metal bioaccessibilities, various standards and guidelines are presented for comparison. The EDAB-Pb for all 5 medicines tested was higher than at least one standard. The EDAB-As in ER was higher than the most conservative regulatory standard. The EDAB-Cd in ANT was lower than the only available standard for Cd. Although cadmium levels were below the regulatory standards even at low concentrations, possible long term safety concerns need to be considered as Cd tends to accumulate in the human body. Even though the total mercury concentration was excessively high in MG and ER, the EDAB-Hg was negligible due to negligible amounts extracted into the intestinal phase.

Metal absorption in humans depends on the transport of metal *via* the mucosal cells and reaching the target tissue or the blood stream. It is postulated that the bioavailable fraction would be less than the bioaccessible fraction as it needs to pass through the intestinal–blood barriers. Since we did not take into account the determinants at the cellular level for the absorption of metals in our EDAB calculations, our estimates present a worst case scenario. Furthermore, the metal uptake in the gastrointestinal tract is affected by many factors such as presence of nutrients like calcium, phosphate, iron, vitamin D, fats, and fiber. Thus, any estimate of bioaccessibility or bioavailability will vary from person to person based on individual diet and nutrition. In addition, whether the human host is fed or fasting also impacts bioavailability. For example, studies in humans by Maddaloni *et al.*³⁰ reported that the lead bioavailability of the soil was $26.2 \pm 8.1\%$ in the fasting state compared to $2.52 \pm 1.7\%$ in the fed state.

Conclusion

Standardized reliable *in vitro* models can be used to estimate bioaccessibility of heavy metals from traditional Indian medicines. Lead in all five medicines tested in our experiments appears to be highly bioaccessible. In particular, the two *Rasa Shastra* medicines made with lead *bhasma* are estimated to yield 9–11 mg of bioaccessible lead per day, substantially exceeding all regulatory guidelines by one to four orders of magnitude. Arsenic also appears to be highly bioaccessible when present in these medicines with some amounts potentially above safe levels. In contrast, despite high levels of total mercury in several of the medicines, their bioaccessibility measures were negligible. Lastly, cadmium was uncommon in these samples and when present was not significantly bioaccessible. The bioaccessibility of metals in different products vary substantially. Our results raise considerable concern for the possibility of metal poisoning, in particular lead and arsenic, when these medicines are ingested. If the maximum suggested dosage was used instead of

the average dosage, estimated amount of bioaccessible metal would be higher than what we have used for comparison with guidelines.

There are several important limitations to our findings. It is possible that there could be small differences in our estimates by not accounting for the salivary phase of digestion in our experimental procedure. Furthermore, in our study we assumed that the bioaccessible fraction is the same as the biosoluble fraction. However, Plumlee *et al.*³¹ has reported that earth materials can contain bioaccessible toxicants in the sorbed or loosely bound to the surface of relatively bioinsoluble particles. The medicines studied are commercially manufactured, not necessarily prepared according to the classical ancient TISM texts, and represent a small subset of TISM medicines. However, our ability to find high bioaccessibility of some toxic metals is concerning and could have potential public health implications. Whether our bioaccessibility results are generalizable to classically prepared medicines or other types of TISM medicines still needs to be studied. In addition, bioaccessibility is only one determinant of bioavailability and toxicity. To determine true bioavailability and potential toxicity, appropriate animal experiments and observational studies on human subjects who are taking TISM medicines are needed. If future studies demonstrate a strong correlation between *in vitro* bioaccessibility and *in vivo* bioavailability, then the bioaccessibility method described here has the potential to be an inexpensive efficient method to screen traditional medicines for potential metal toxicity.

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Fig. 1.
Schematic diagram of experiments.

Table 1

ICP-MS operation parameters

Standard and DRC mode	
RF power	1100 W
Plasma gas flow rate	15.00 L min ⁻¹
Auxiliary gas flow rate	1.20 L min ⁻¹
Nebulizer gas flow rate	0.95–1.05 L min ⁻¹
Scan mode	Peak Hoping
Dwell time	Standard mode: 100 ms
	DRC mode: 500 ms
Sweeps/reading	10
Replicates	5
Isotopes analyzed	Standard mode: ²⁰⁸ Pb, ¹¹⁴ Cd with Sn correction, ¹¹⁵ In and ²⁰⁵ Tl
	DRC mode: ⁷⁵ As ¹⁶ O and ¹¹⁵ In
DRC Mode	
Cell gas	Oxygen (99.996% purity)
Cell gas flow rate	0.325 mL min ⁻¹
RPq	0.65
RPa	0

Table 2

Traditional Indian medicine samples tested

Sample	Manufacturer	Recommended dose/day^a	Recommended use^a
Hingwastika (HW)	Bazaar of India	3	Indigestion and nervous disorders, pneumonia, and bronchitis of children
Mahayograj Guggulu (MG)	Baidyanath	2–4	For nervous diseases, gout, rheumatism, asthma, intestinal colic, bronchitis and seminal disorders
Ekangvir Ras (ER)	Baidyanath	2–4	For paraplegia, sciatica & arthritis
Shilajit (SH)	Banyan Botanicals	1–4	Promoting rejuvenation & detoxification
Ayu-Nephro-Tone (ANT)	Sharangdhar Pharmaceuticals	4	Supports healing of kidney, bladder and urinary tract.

^aRecommended dosage and use as stated on the internet and bottle.

Table 3

Total metal concentration in traditional Indian medicine samples

Sample	Pb/ $\mu\text{g g}^{-1a}$	As/ $\mu\text{g g}^{-1a}$	Cd/ $\mu\text{g g}^{-1a}$	Hg/ $\mu\text{g g}^{-1a}$
Hingwastika	1.9 ± 0.2	<DL	<DL	37 ± 2
Mahayograj Guggulu	36000 ± 3400	<DL	<DL	10000 ± 800
Ekangvir Ras	25000 ± 3600	304 ± 61	<DL	7880 ± 1786^b
Shilajit	7.96 ± 0.02	<DL	<DL	<DL
Ayu-Nephro-Tone	331 ± 25	<DL	14.4 ± 1.7	<DL

^aMean $\pm$ SD ($n = 3$).^b($n = 5$).

DL = Detection limit of the method ($3 \times \text{SD}$ for the 10 measurements of the method blank), DL for Pb, As, Cd = $0.01 \mu\text{g g}^{-1}$, DL for Hg = $0.4 \mu\text{g g}^{-1}$.

Table 4

Quality Control (QC) sample analysis

QC Sample	Pb/ $\mu\text{g g}^{-1a}$	As/ $\mu\text{g g}^{-1a}$	Cd/ $\mu\text{g g}^{-1a}$	Hg/ $\mu\text{g g}^{-1a}$
NIST SRM 1515 Apple Leaves	0.431 ± 0.007	0.038 ± 0.003	0.0131 ± 0.0001	NA
Certified value	0.470 ± 0.24	0.038 ± 0.007	0.013 ± 0.024	
NIST SRM 1400 Bone Ash	9.29 ± 0.15	0.49 ± 0.02	0.034 ± 0.009	NA
Certified value	9.07 ± 0.12	NCV	NCV	
GBW 07601 Human Hair	NA	NA	NA	0.395 ± 0.027
Certified value				0.360 ± 0.080
Detection limit (DL)	0.01	0.01	0.01	0.4

^aMean $\pm$ SD ($n = 3$).DL = Detection limit of the method ($3 \times$ SD for the 10 measurements of the method blank), NA = Not analyzed, NCV = No certified value.

Table 5

Bioaccessibility of lead, arsenic, cadmium and mercury^a

Bioaccessibility (%)									
Sample	Pb		As		Cd		Hg		Intestinal Phase
	Gastric Phase	Intestinal Phase	Gastric Phase	Intestinal Phase	Gastric Phase	Intestinal Phase	Gastric Phase	Intestinal Phase	
Hingwastika	83.1 ± 14.0	60.0 ± 11.0	NC	NC	NC	NC	<1	<1	<1
Mahayograj Guggulu	71.0 ± 7.3	27.4 ± 5.9	NC	NC	NC	NC	<1	<1	<1
Ekangvir Ras	87.8 ± 4.4	75.4 ± 9.7	82.6 ± 2.2	78.1 ± 2.6	NC	NC	<1	<1	<1
Shilajit	28.4 ± 0.7	22.4 ± 0.5	NC	NC	NC	NC	NC	NC	NC
Ayu-Nephro-Tone	44.9 ± 4.9	1.4 ± 0.01	NC	NC	80.5 ± 8.7	2.2 ± 0.08	NC	NC	NC

^a Bioaccessibility (%) = [Concentration *in vitro* gastric or intestinal phase extract ($\mu\text{g g}^{-1}$)] / [Concentration in the original sample, ($\mu\text{g g}^{-1}$)] × 100,

NC – Not calculated, HW = Hingwastika, MG = Mahayograj Guggulu, ER = Ekangvir Ras, SH = Shilajit, ANT = Ayu-Nephro-Tone.

Table 6

Estimated daily amount of bioaccessible (EDAB) Pb, As, Cd and Hg based on intestinal phase metal concentration^a

Sample	Pb/ $\mu\text{g day}^{-1}$	As/ $\mu\text{g day}^{-1}$	Cd/ $\mu\text{g day}^{-1}$	Hg/ $\mu\text{g day}^{-1}$
Hingwastika	1.8	NC	NC	0.0
Mahayograj Guggulu	9473	NC	NC	0.0
Ekangvir Ras	11375	142.2	NC	0.0
Shilajit	3.2	NC	NC	0.0
Ayu-Nephro-Tone	10.0	NC	0.67	0.0
Safety Guideline ^b	252	150	70	16
Safety Guideline ^c	0.5	NS	NS	NS
Safety Guideline ^d	20	10	NS	20
Safety Guideline ^e	NS	21	NS	21
Safety Guideline ^f	NS	21	7	NS

NC – Not calculated, NS – Not stated.

^a Estimated daily amount of bioaccessible metal = Weight of the pill (g) $\times$ Average dosage per day $\times$ Metal concentration in the intestinal phase ($\mu\text{g g}^{-1}$), Safety guidelines based on an average adult body weight was 70 Kg.

^b Based on provisional tolerable weekly intake (PTWI) set by the Food and Agricultural Organization/World Health Organization Joint Expert Committee on Food Additives (FAO/WHO).³²

^c California Proposition 65.³³

^d American National Standards Institute/National Sanitation Foundation.³⁴

^e United States Environmental Protection Agency reference doses (EPA RfDs).³⁵

^f Agency for Toxic Substances and Disease Registry (ATSDR).³⁶