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Determination of Reference Ranges for Elements in Human Scalp Hair

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ABSTRACT

Expected values, reference ranges, or reference limits are necessary to enable clinicians to apply analytical chemical data in the delivery of health care. Determination of references ranges is not straightforward in terms of either selecting a reference population or performing statistical analysis. In light of logistical, scientific, and economic obstacles, it is understandable that clinical laboratories often combine approaches in developing health associated reference values. A laboratory may choose to:

1. Validate either reference ranges of other laboratories or published data from clinical research or both, through comparison of patients test data.
2. Base the laboratory's reference values on statistical analysis of results from specimens assayed by the clinical reference laboratory itself.
3. Adopt standards or recommendations of regulatory agencies and governmental bodies.
4. Initiate population studies to validate transferred reference ranges or to determine them anew.

Effects of external contamination and anecdotal information from clinicians may be considered.

The clinical utility of hair analysis is well accepted for some elements. For others, it remains in the realm of clinical investigation. This article elucidates an approach for establishment of reference

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ranges for elements in human scalp hair. Observed levels of analytes from hair specimens from both our laboratory's total patient population and from a physician-defined healthy American population have been evaluated. Examination of levels of elements often associated with toxicity serves to exemplify the process of determining reference ranges in hair. In addition the approach serves as a model for setting reference ranges for analytes in a variety of matrices.

Index Entries: Hair analysis; elements; minerals; reference range; reference limit.

INTRODUCTION

Assessment of health status of an individual often relies on interpretation of laboratory assays of specimens obtained from that individual. Comparison of laboratory data for a given patient with data expected for individuals in good health is an aid to making medical diagnoses, determining appropriate therapeutic management, and monitoring efficacy of treatments. The worth of the laboratory assays depends on construction of reference ranges and an understanding by health care providers of what those reference ranges mean. It is the expected values or reference ranges that permit meaningful comparisons of an individual's values with those of a reference population *(1)*. Although expected values for some analytes in some matrices are familiar to many health professionals and the public alike, e.g., cholesterol or glucose in serum, reference ranges for elements in scalp hair require considered, thoughtful interpretation. Analysis of metals in hair has been performed for almost 150 years. In 1857, Casper determined the presence of arsenic in the hair of a body exhumed after 11 years. Paleopathologists have performed studies providing evidence that elemental hair concentrations reflect biogenic processes *(2)*. In recent years, hair analysis has led to identification and amelioration of industrial or environmental exposures to potentially toxic metals. *(3–5)* Reference ranges for metals in hair are needed for determination of adequacy (in the case of some analytes) and toxicity. The setting of reference ranges for essential elements and elements frequently associated with toxicity will necessarily depend on the types of frequency distributions observed in healthy people and, when known, on the clinical consequences of relatively high or low values.

Sample selection is often governed by convenience and availability of tissues or fluids. Hair is an attractive tissue for analysis, because obtaining a sample is noninvasive and because hair is relatively inert. Therefore, when elemental analysis of hair can be effected quantitatively or even qualitatively, it can be a cost-effective means of screening an individual or population *(6–8)*, monitoring an individual during putative

exposure, or following a patient during therapy. However, even for a screening method, reference ranges must be provided.

The determination of reference ranges is not straightforward in terms of either selecting a reference population or performing statistical analyses. Convenience samples may be skewed. Patients in clinics, hospitals, or laboratory populations may include the relatively well, the very sick, and everything in between. Exogenous variables, including environmental exposures, season of the year, cultural elements of lifestyle, and source of food as well as endogenous variables, such as genetics, gender, age, chronic disease, and medications are confounding *(9)*. It may be inappropriate to transfer published reference values based on one population to a different population. Transfer of reference ranges requires validation. Even subtle procedural differences may yield results that differ from laboratory to laboratory, so that laboratories must establish their own determinants of accuracy, and set and review their own reference ranges *(9)*. Beyond the technical difficulties, assessment of values in a healthy population presumes that one can identify a healthy population. Population studies are expensive, and prospective epidemiological studies are considerably more expensive and, by their nature, take decades. Laboratories are expected to provide reference ranges routinely and cannot wait until a definitive epidemiological investigation is completed. These reference ranges, however, need to be reviewed periodically in light of any new information or simply as a function of the passage of time (which can alter exogenous variables).

Because of the logistical, scientific, and economic obstacles, it is understandable that clinical laboratories often combine approaches in developing health-associated reference values. A laboratory may consider:

1. Validating either reference ranges used by other laboratories or published data from clinical research or both.
2. Results from specimens assayed by the clinical reference laboratory itself.
3. Adoption of standards or recommendations of regulatory agencies and governmental bodies.
4. Initiation of population studies to determine *de novo* or to validate transferred reference ranges.

Whenever possible, correction for or allowance for external contamination is recommended. In addition, communication between a reference laboratory and clinicians permits periodic review of correlations between levels of analytes and clinical signs and symptoms.

In 1996, we undertook a Healthy American Population Study (HAPS) to validate and/or review current reference ranges. The analysis of data is still in progress; however, the project has both provided data required for setting reference ranges and stimulated discussions, which led to adoption of a feasible approach to a difficult problem.

METHODS

Physicians across the US were invited to admit to the epidemiological study five individuals in good health. The study's subjects were thus identified as being "healthy" by physicians. A questionnaire was sent to each potential subject. Individuals were excluded if they had risk factors associated with nonoptimal health, e.g., a history of disease or chronic condition or history of smoking. Those admitted to the study were informed of the HAPS protocol. Data from subjects' blood chemistry profiles and complete blood counts (obtained from an outside laboratory), heights, weights, and urine volumes from 24-h collections were entered into a database. All of our usual analytical tests (elements, amino acids, and so forth) were performed on HAPS specimens (whole blood, packed red blood cells, urine, and hair). Clinicians were provided with our usual instructions regarding specimen collection and handling, so data derived from the HAPS specimens reflect overall procedures, from specimen collection to final analysis. The range, mean, distribution, and other descriptive statistics were determined for each analyte in each matrix.

Hair specimens were prepared for analysis as described elsewhere in this volume *(10)*. Physicians were instructed to collect approx 0.25 g of hair within 1 in. (2.5 cm) of the scalp from five or six areas at the back of the head. In the laboratory, the hair was cut and washed using a modified method developed by the International Atomic Energy Agency (IAEA) *(11)*. Approximately 0.2 g of the specimens was accurately weighed into 50-mL disposable polypropylene centrifuge tubes. Samples were then solubilized in concentrated nitric acid (trace metal grade) and digested using an automated flowthrough microwave oven (SpectroPrep; CEM Corporation, Matthews, NC). The SpectroPrep has been described in detail elsewhere *(12,13)*. All element determinations were performed with an ICP-MS (Elan 5000, Perkin-Elmer, Norwalk, CT) using a flow injection sample uptake system (FIAS 400, Perkin-Elmer).

In addition, specimens were digested using a low-temperature, atmospheric pressure method *(10)*, and the data using both preparative methods were analyzed. All statistical analysis was conducted with SPSS v. 7.5 for Windows (SPSS, Inc, Chicago, IL) on a personal computer.

RESULTS AND DISCUSSION

Design of the HAPS

Completed questionnaires were received from about 500 potential study subjects. Of these, 250 were excluded because of chronic disease(s), a history of smoking, or acute condition. Those included as study subjects, on the basis of the questionnaires, received kits for specimens. Over 150 individuals submitted specimens and the study submittal form. The

resulting reference population was not ideal. There were more women (n = 91) than men (n = 68). The age range of the population was from 3 to 85 yr, but there were very few children (five under 10 yr of age) in the study. Many physicians enrolled several members of one family or household who are likely to have genetic and/or environmental factors in common. Minimal information was collected on dietary habits, exercise, or taking of over-the-counter drugs or supplements.

Analytical Confounders and Limitations

Although it is virtually impossible to control for "biological noise" (genetics, environment, lifestyle, and so forth) *(14)* in a relatively small population, every effort is made to obtain sensitive and accurate analytical data for setting reference ranges as well as for routine clinical assessments. Validity of reference ranges depends on reliability and limitations of the analytical method. Factors that have an impact on reference ranges and interpretations of data include: sampling and analytical sensitivity, accuracy, and precision. Procedures for obtaining specimens, sampling, and sample preparation in our HAPS were the same as those routinely used in the laboratory.

Sampling of hair specimens: Sampling considerations include the sites from which the hair is taken, cutting of the specimen, quantity of hair that is cut, and preparation of the sample for analysis. Hair specimens should be obtained as close to the scalp as possible from multiple sites on the scalp. We request that hair be cut from five or six different areas from the back of the head, not more than 1 in. (2.5 cm) from the scalp. In the laboratory, each hair specimen is further cut to approx 0.125-in. (0.3 cm) pieces and mixed to assure that samples are homogenous and representative. Since the cutting of the hair may introduce contamination, clean, high-quality, stainless-steel scissors are used. Prior to analysis, the hair specimen is washed to remove external contaminants. A number of publications explore different washing procedures and their effectiveness at removing external contamination while minimizing impact on endogenous elements in the hair *(11,15–20)*. Although our washing procedure removes most external contaminants, some hair preparations are known to contain significant amounts of elements that may not be removed in washing. Clinicians are advised to ask patients about hair treatments and other possible exposures, e.g., occupational or recreational exposures (swimming pools, ceramics work). A study is currently in progress that examines specific effects of hair treatment products, including the efficacy of washing procedures in removing the artifactual analytes. Although most shampoos and treatment products do not have an impact on elemental hair analysis, one dandruff shampoo significantly raises hair levels of selenium and one hair dye, known to contain lead acetate, significantly raises lead hair levels. Thus, a little information from a patient will greatly assist in sorting exogenous from

endogenous sources of an element. Detailed findings from the study on hair products will be published elsewhere.

Analytical sensitivity: Analytical sensitivity specifies at what concentration an element can be reproducibly detected. Measured values at or below a detection limit (dl) are not reported. Sensitivity (minimum dls and coefficients of variation for controls at low concentrations) *(21)* affects establishment of the low reference limit. Both sensitivity and specificity (overcoming interferences and noise) affect the diagnostic utility of an assay. The analytical technique used should be able to detect levels below the low reference limit. If the dl were significantly over the warning limit, since results would be reported as "less than the detection limit" for a clinically significant range, the ability to identify individuals at risk for toxicity or deficiency would be severely compromised. There would be limited clinical utility of such a test.

Analytical accuracy: Analytical accuracy refers to the degree to which a measured result matches the actual level of the analyte. Interferences, loss of analyte, and contamination can all affect accuracy. These contributions to bias depend on the procedure used as well as the analytical technique. Differences in accuracy from one laboratory to another make comparison of absolute results difficult. There are extramural programs, such as the Interlaboratory Comparison Program, Le Centre de Toxicologie du Quebec, designed to assess accuracy. Participation in such programs is an aid to identifying analytical biases. These programs send samples to a large number of laboratories for analysis and often evaluate accuracy in terms of the analytical method used. In most cases, our biases are small, and compensation for such biases can be effected. If biases are small and remain constant, a laboratory can report relative (high or low, for example) levels of given elements, even if absolute levels are not determined.

An understanding of bias analysis is exemplified by assessment following a recent change in methodology. First precision and accuracy of the new method were assessed and validated by our usual quality-control procedures. Then the issue of any need for change in reference ranges required attention. Elemental levels from paired samples, i.e., samples analyzed by both methods were analyzed by regression analysis, as is customary (*see* Fig 1).

For every methodological change we introduce, we perform a regression analysis of paired samples for each analyte. If the slope is approx 1 ($0.92 \leq \text{slope} \leq 1.2$), the correlation coefficient, r, is ≥ 0.9 and the intercept is $\leq$ the dl, we do not feel a change in reference values is justified. If these parameters suggest that detailed analysis is in order, paired frequency distributions of a large sample population (from the pool of the total patient population) are required. Comparison of the distributions will clearly indicate marked shifts or trends, and paired t-tests will provide the degree of significance of any differences. An example of one of the linear regression analyses is presented in Fig. 1. It is clear that

there is no justification for altering the reference range for lead in hair based on our criteria.

Analytical precision: Analytical precision is the measure of variation of a result. Precision of analyses affects reference ranges as follows. Very precise numbers permit a tight reference range, whereas less precise results broaden a reference range. For some analytes, clinical relevance requires that the standard deviation for a given level of analyte be a small fraction, say 10–20% of a small reference range. For toxic elements, which generally have one-tailed distributions, the relative standard deviation for a sample (standard deviation of the sample divided by the average measured concentration of that sample) is usually < 20% of the average measured concentration of the sample.

Statistical Distributions Encountered and Their Interpretations

If analytical and biological variation were not enough, semantic confusion can contribute to poor communication. In environmental science, "trace element" simply means a particular element occurs in small amounts. In health sciences, "trace element" generally refers to an element that is a micronutrient and therefore is essential. Nonessential elements that may or may not be present in small amounts can be potentially toxic. The level at which signs or symptoms of toxicity occur may be poorly defined or undefined, and a level suggesting risk of potential toxicity may be even less well documented or justified. Health scientists therefore differentiate between essential and nonessential elements, and usually do not apply the term "trace" to nonessential elements unless the term is carefully restricted to analytical use.

The essential elements, whether they occur in relatively large or small amounts in vivo, may be expected to exhibit a bell-shaped or Gaussian curve in at least some matrices (e.g., blood or tissue from organs) from a healthy human reference population. The nonessential, potentially toxic elements would not necessarily be expected to have such a distribution. Even essential elements can be associated with signs or symptoms of toxicity if they are present at high enough levels, and setting an upper reference limit to preclude even subclinical imbalances or dysfunction is particularly challenging. For example, body manganese content is regulated largely by the level of absorption (2–3%). However, since essentially all manganese is excreted in the bile, liver disease or impaired bile flow can result in excess retention of manganese and consequent degeneration of dopaminergic neurons *(22)*.

It is challenging but important to define expected distributions or levels of metals in hair and to correlate hair levels with circulatory or tissue levels for those analytes. Since healthy people by definition should not be fraught with toxicity, setting an upper limit of safety for a potentially toxic analyte frequently requires inspection of data from a general

population (including some with elevated levels of potentially toxic analytes) with confirmation from values observed in a healthy population, as discussed below.

By definition, a reference range, interval, or value is the interval between and including two numbers: a lower and upper reference limit. In cases for which clear clinical variables can be correlated with levels for an analyte (e.g., serum glucose or urine protein), many laboratories report reference ranges in terms of what is physiologically normal. For many of our analytes and for elements in hair specifically, it is not appropriate to assume *a priori* that some range corresponds to optimal physiological status, and we define ranges judged statistically to be representative of values observed in practically all healthy persons. That is, the reference interval is the range found in a specified percentage of individuals in a reference population.

Frequently, it is presumed that the parameter for which a reference range is being established has a statistically normal distribution for a reference population, and the values within 1 SD of the mean are therefore obtained for 68% of the reference population. Values within 2 and 3 SD of the mean apply to 95 and 99.7% of the reference population, respectively. The corresponding percentiles for a normally distributed variable in a population are shown in Table 1.

For variables with a clear physiological role, whether or not they exhibit a statistically normal distribution, the designation of an expected range—the range in which 68% of the population falls—is taken to be the 16th–84th percentile. The designation of the low and high ranges is set at the 2.5 and 97.5 percentile (95% of the population), respectively. However, in no case is a lower limit set below the limit of detection. Data from the HAPS together with examination of values obtained from other laboratories were sufficient to determine reference ranges for essential as well as nonessential elements.

Some analytes, including elements whose biological significance is unknown or poorly understood, have distributions with a single tail at the high end of the observed range. For these analytes, no low reference limit is assigned, and the high reference limit is set at the value for the 95th percentile of our total patient population (TPP), as opposed to a special, healthy population. Individuals with values above that level may be at risk for adverse health effects. Clinicians often use the level of analyte at the 50th percentile (median) or 68th percentile for more thorough assessment of risk. Our data indicate that there is rarely a large difference between the 95th percentile of the total patient population pool and the HAPS subjects. However, analysis of the large population assures statistical power. This may not result in a conservative designation of the high reference limit, because the TPP includes some individuals who are likely to have a very high level of a toxic element, which is the very analyte being monitored.

Table 1
Relationship Among Percentiles, Standard Deviations, and Percent of a Population with a Variable Exhibiting a Statistically Normal Distribution

Percent of population:						
				99.7%		
			95%			
		68%				
	3 s.d. below the mean	2 s.d. below the mean	1 s.d. below the mean	1 s.d. above the mean	2 s.d above the mean	3 s.d above the mean
Percentile	0.15	2.5	16	84	97.5	99.85

In the case of mercury, it is important to realize that methyl and inorganic mercury come from different sources (fish and dental amalgams, respectively) and have different biochemistries. According to the World Health Organization *(23)*, variation in hair and blood levels of mercury reflects primarily variation in fish consumption. In a large population of men in eastern Finland, the level of mercury in both hair and urine was correlated with fish consumption *(24)*. Hair is an appropriate indicator medium for methyl mercury, since there is good correspondence among hair levels, blood levels, and fish consumption *(25)*. However, suitability of hair as an indicator of exposure to inorganic mercury has been questioned *(26)* and requires further investigation. To set our laboratory's reference range for hair levels of total mercury, we applied our rules for setting percentile-based reference ranges relying on data from the HAPS for confirmation and from our TPP. Unfortunately, the relatively small HAPS population precludes analysis for narrow age ranges. However, data from the small subsets may be used for confirmation of appropriateness of our reference ranges. Gender differences do occur, particularly in lead, but these may be owing to hair treatments or other environmental exposures. Speciation studies are in process that should improve the clinical interpretation of mercury assays.

Transference of Reference Ranges

Extensive population studies are prohibitive in terms of cost and time. Therefore, we rely heavily on published data from other laboratories or research studies. When transferring reference intervals established by another laboratory, we evaluate the appropriateness of adopting those reference intervals. Such studies as the HAPS permit us to assure that reference values are applicable for our sample preparatory and analytical techniques and for our population of patients.

Presentation of Reference Ranges

For variables that exhibit a normal (or approximately normal) distribution *(27)* as well as for variables not normally distributed, the desig-

nation of an expected range is taken to be 68% of the reference population. For physiologically essential analytes, this range covers the 16th–84th percentiles *(1,27,28)*. For these essential analytes exhibiting a fairly normal distribution, the reference range (as opposed to expected range) indicated on a clinical report corresponds to the range observed within 2 SD from the mean. Levels of an analyte between the percentiles 2.5 and 16 or between percentiles 84 and 97.5 are still within the expected range, and signs and symptoms are either absent or expected to be mild. Levels below 2 SD or above 2 SD from the mean (percentiles 2.5 and 97.5, respectively) are more likely to have clinical significance. Clinicians making diagnoses and designing treatment plans based on our test results are expected to interpret the data in terms of a patient's history, medical condition, diet, lifestyle, and environment as well as other laboratory data. For analytes other than the physiologically essential, the designation of a low value makes no sense, and the designation of a high value is problematic. For nonessential elements with a single-tailed distribution, the reference limit is that seen at the 95th percentile. Clear presentation of reference ranges for such analytes is important, and the data should be interpreted with care.

There may be trends observed (e.g., relative levels of elements) in individuals' analyses that suggest risk of some adverse condition. We are in the process of analyzing our data to probe further the correlation of hypothesized trends with clinical findings.

Reference Ranges and Interpretations of Hair Analyses: Challenges and Caveats

The scientific community and groups of health care professionals have some reservations about the clinical utility of elemental analysis of hair specimens both in terms of analytical validity and interpretation of results. A number of worthwhile publications indicate considerable potential value in analysis of hair specimens. For example, exposure to arsenic may be monitored in hair of corpses, even after other tissues have decomposed *(29)*. Elevated levels of arsenic in both urine and hair confirmed arsenic toxicity from pesticide exposure in an individual with peripheral neuropathy and macrocytosis, but without anemia *(30)*. The relationship among lead, cadmium, and mercury levels in children's hair has been correlated with childhood intelligence *(31)*. Hair analysis has been utilized to identify historical as opposed to current exposure to lead *(32)*. Schoolchildren with relatively high levels of lead in their hair had slower reaction times and less flexibility in changing their focus of attention than children with relatively low concentrations of lead in hair *(33)*. Although lead levels in hair correlate with blood levels, copper content of hair was not elevated in patients with Wilson's disease, although there were high copper levels in other tissues *(34)*. History of fish consumption and mercury in hair samples are considered the best indicators of human

exposure to methylmercury *(35)*. Fish consumption is positively correlated with hair mercury levels *(24,36)*. Both high hair and high urinary mercury levels have been found by some researchers *(1,37)*.

Both absolute concentrations and ratios of essential elements in hair have been found to be significantly different in epileptic and nonepileptic subjects *(38)*. Hair levels of zinc, but not copper were considered by Italian investigators to represent one means of assessing nutritional status of groups of individuals *(39)*. Industrial exposure to manganese can be assessed through measurement of blood or hair *(40)*.

It has been found that X-ray technicians were relatively depleted in zinc and copper as a function of occupational exposure to low-dose radiation. Hair levels of zinc, copper, and iron were higher in the technicians than in unexposed individuals, but blood levels of zinc and copper were depleted and iron levels in blood were increased. Using the ratio of zinc:iron as an indicator of nutritional risk, blood analysis gave a stronger indication of nutritional risk, but hair served as an indicator tissue *(41)*.

Compared to interpretation of commonly measured analytes in blood or serum, interpretation of elemental analyses from hair seems primitive. In the early half of the 20th century, there was a gradual awareness that blood levels of lipids are correlated with coronary heart disease (CHD), but only since the 1950s has there been sufficient substantial epidemiological evidence to identify clear relationships between a given blood level of total cholesterol and LDL cholesterol and risk of CHD. It is unlikely that anything approaching large epidemiological studies will be undertaken for elemental hair analysis in the near future. Therefore, even though descriptive statistics may be obtained for element concentrations in hair, setting up percentiles or quartiles in which a given value or range of values for an analyte is related to a probability of risk or predictability of a treatable condition can only be inferred from available data.

In the absence of sufficient data, a conservative clinical policy is prudent. Ultimately, a laboratory can identify values obtained for a given percentile of a population or values consistent with known health effects. In the case of minerals of known biological value, adopting a reference range consistent with what is observed in 95% of a healthy population is consistent with prudence and traditions in health science *(42)*. There is no implication that such a reference range is optimal. After all, a population may have a mean cholesterol value of well over 200 mg/dL, yet no one would consider that optimal.

We are interested in being more proactive than merely determining a level at which elemental presence has clear toxic effects. Ideally one would like to know what level of an element constitutes danger, and therefore requires, or at least justifies, intervention. Our percentile presentation is assistive in indicating probabilities of levels consistent with a healthy population. Of course, the clinician evaluating a patient's sta-

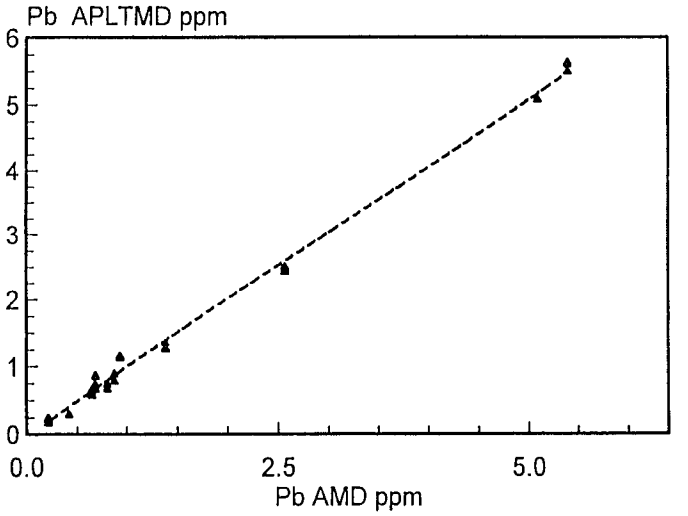

Fig. 1 Linear regression curve of the low-temperature atmospheric pressure method (new method) as a function of the previous method for analysis of lead in hair. R = 0.98854. Lead levels are reported in parts per million. Lead in hair—linear regression. Y = – .0262 + 1.0184*X. Automated microwave digestion method (AMD). – – – Regress; ▲ Plot

tus or considering a plan of treatment must use laboratory data in conjunction with other means of assessment, so that medical judgments are not made purely from objective data out of clinical context.

Some metals, e.g., arsenic, exist in different forms (valence states) with variable toxicity. If an analytical technique measures total arsenic, consideration should be given to contributions from nontoxic forms of the metal. A high arsenic level may be clinically unimportant, if the arsenic is a nontoxic species, but an average level of total arsenic could be consistent with a dangerous exposure if all of the arsenic present were in a toxic form. Methylmercury is far more toxic than elemental mercury. *(43,44)*. If methylmercury toxicity is suspected in an individual, organic mercury, rather than total mercury, may be the preferred analyte. In addition, one matrix may be more suitable for analysis in terms of physiology and biochemistry.

CONCLUSION

The WHO and EPA have indicated benefits of hair testing for heavy metals in some cases. According to the EPA *(45)*, human hair is one of the important biological materials for worldwide environmental monitoring. Human hair is also utilized to monitor trace metals under the aegis of the International Atomic Energy Agency.

There is a need for more standardized methodology in analysis of elements from hair specimens. We have a consistent presentation of reference ranges for analytes in hair as well as other matrices. The approach is valid for one- and two-tailed distributions. The application of percentile-based values as presented is highly recommended in lieu of reference ranges lying within 2 or 3 SD from means for each analyte.

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