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CRITERIA FOR REFERENCE VALUE ASSESSMENT OF ELEMENTS IN HUMAN TISSUES

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Summary. - *The availability of updated and reliable reference values for elements in biological tissues and fluids plays a key role in the correct planning and performance of clinical, toxicological and environmental studies. The basic principles for a proper analytical approach in order to obtain such data are outlined. This in turn should significantly reduce the wide range of figures reported for most elements as "normal" intervals in the scientific literature. Some applications of these guidelines are illustrated in detail in the case of human lungs, liver and kidneys for which tentative reference values are assessed for a number of minor and trace elements, namely, Al, Ba, Cd, Cr, Cu, Li, Mg, Mn, Pb, Sr, V and Zn.*

Riassunto (Criteri per la produzione dei valori di riferimento per gli elementi nei tessuti umani). - *La disponibilità di valori di riferimento aggiornati ed attendibili per elementi in tessuti e fluidi biologici è di fondamentale importanza per la progettazione e l'esecuzione accurata di indagini cliniche, tossicologiche ed ambientali. Per quanto concerne l'ottenimento di tali dati, si espongono i principi di base che devono ispirare una corretta strategia analitica. Ciò a sua volta favorisce una considerevole riduzione degli intervalli dei valori designati come "normali", ma eccessivamente ampi per buona parte degli elementi stando a quanto riportato nella letteratura scientifica. Alcune applicazioni di tali criteri vengono illustrate in dettaglio nel caso di polmoni, fegato e reni umani, per i quali vengono indicati in via preliminare i valori di riferimento di elementi sia a bassa concentrazione che in traccia e precisamente Al, Ba, Cd, Cr, Cu, Li, Mg, Mn, Pb, Sr, V e Zn.*

Introduction

A proper and self-sufficient assessment of the risk related to the human exposure to heavy metals and other elements is necessarily bound to the availability of reliable information regarding their concentration levels in a wide variety of biological matrices. In spite of the large number of studies carried out so far in recent years in this field, there are still many gaps in our knowledge that need to be filled. The literature is also full of discrepancies which require to be urgently clarified. These problems can to a large extent be due to the unavailability of reliable and updated reference values for many toxicologically, clinically and environmentally relevant elements in well defined population groups. Fortunately enough, general awareness is growing on the key role played by such figures from several points of view, among which one should enumerate the identification of priorities deserving immediate action, the subsequent correct planning of original studies, their actual performance with optimization of the cost-to-benefit ratio and exploitation of the investigation outcomes for enhancing protection of human health and eventually promoting regulatory initiatives. This is all the more understandable if the attention is focused on the fact that numerous biochemical functions need the participation of selected elements to perform properly. Any deviation from the optimal concentrations can cause serious if not lethal consequences. On the other hand, nonessential elements can build up in the organism due to two main factors, i.e. environmental exposure and food consumption. Their presence can be tolerated until the respective threshold values are reached, beyond which toxic effects inevitably

occur. In both instances it is mandatory to arrive at reference data at the same time reliable and representative to correctly interpret the corresponding mechanism. In turn this type of achievement demands the development of accurate and efficient protocols for sample collection and treatment before analyses are carried out as well as the systematic use of measurement procedures capable of providing accurate and precise values [1-5].

These remarks may even seem too plain were it not for the fact that they have been often overlooked or ignored. This is all the more serious given the extremely low concentrations at which many elements of sanitary and ecotoxic importance are present in biological samples. It is paradoxical that most problems are caused precisely by the actual paramount ability of today's analytical tools which in recent years have become available to virtually all laboratories, whereas this process has not been paralleled by an analogous efficacy to control contamination phenomena and to optimize accuracy. In other terms, such analytical shortcomings have greatly affected - and still affect - the achievement of reference values for elements, as can be judged by the information available in the literature. The purpose of this paper is to outline the basic aspects which should be incorporated in studies of this type and to illustrate their applications in an overall research program coordinated by our group at the Istituto Superiore di Sanità (Italian National Institute of Health). The definition of "reference values" has been internationally agreed upon and detailed in specialistic publications [6-9].

Methods and instrumental approaches

Preanalytical procedures

One of the primary sources of error in the analytical sequence is the inappropriate collection of samples, be it from the point of view of correct representativeness of the system being tested as well as of the possible contamination or loss of analytes in this stage. Its erroneous performance makes all subsequent steps useless or even dangerous when such information is used for clinical diagnosis. There are countless examples of such malpractice, ranging from the use of metal needles for liver biopsies to the dissection of organs and bones with conventional scalpels, knives and scissors. Very high and unpredictable amounts of Co, Cr, Cu, Mn, Ni and Zn can thus be released to the specimens and spoil their information content. Therefore, it is absolutely necessary to rely on tools made of non-contaminating high-purity materials like quartz, titanium and synthetic resins or, alternatively, on devices coated with highly inert and strongly adherent compounds like tin. Their choice should be made on a case-by-case basis, considering in the first place which elements are sought for and in which matrix, so that no undesirable interactions take place. These tenets have been summarized and codified in a WHO-IAEA recommendation [10].

Regarding storage and transport (if any), these may lead to serious alterations of the samples. Within this framework, adding of preservatives such as heparin and formalin is definitely to be excluded. Freezing down to - 20 °C is certainly safer, even though this operation does not warrant against the risk of movement of fluids from organ to organ or within the same organ, with subsequent changes in analyte concentration. In a much more subtle way, the contact between sample and container can jeopardize the original content of the former through two competing processes, namely adsorption onto and release from the container walls. The pretreatment itself - subsampling included - of the biological materials is subjected to the obvious, unintentional addition of contaminants from chemical reagents, digestion devices and atmospheric dust as well as to losses caused by inaccurately fixed temperature conditions or chemical reactions forming volatile substances.

With this general scenario in mind, a detailed protocol has been developed enabling the determination of trace elements in biological specimens meeting the requirements of reliability and reproducibility, mandatory for reference value assessment. It prescribes that tissues are to be excised within 24 h from death in a dust-free environment under strictly controlled conditions. Non-contaminating TiN-coated surgical tools should be employed to subsample the organ after it has been removed from the body. Each specimen has to be taken in duplicate, each aliquot being immediately placed into a chemically inert polyethylene vial and quickly frozen at - 80 °C. Two parallel, entirely equivalent series of samples are thus obtained and then subjected to quantification by means of two completely different analytical approaches - one more accessible to most laboratories, the other more suited as a reference method - to guarantee the validity of the final concentration data achieved. This procedure has the advantage that one of the two sets of samples can be stored for checks and analyses at a later stage of the study by new methodologies which can develop in the years to come. Such a specimen bank will obviously be possible only once the analytical methodology has been validated. For each subject, information regarding their habits must be obtained so as to include in the study the persons who turn out to be non-smokers, not occupationally exposed and not affected by ascertained pathologies, at least with respect to the organs from which tissues are to be excised. An example of the form that can be used for this purpose is given in Table 1. The difficulty of this step cannot be overstressed as often the health operators only have recent information on the subject, while relatives of this last are usually reluctant to provide details of past events (or they even ignore them). A thorough description of how these principles were incorporated to a study planning of elements in lungs can be found elsewhere [11, 12].

Table 1. - *Form employed to collect informative data on deceased individuals to decide their eligibility for inclusion in the study*

DATA FROM

Surname and name: _____ Sex: M__ F__
Date of birth: _____ Date of death: _____
Cause of death and other autopsy evidences: _____
_____
Residence*: _____
Activity*: _____
Other relevant data: _____

* *record information covering the last 10 years*

Analytical methodologies

Tasks as the one considered in this paper demand a flexible strategy based on an inherently multielemental analytical technique featuring a wide dynamic range, a large throughput and a limited dependence on matrix composition. Both inductively-coupled plasma atomic emission spectrometry (ICP-AES) and nuclear methods such as neutron activation analysis (NAA) comply with these requirements, although to different extents and from partly opposite standpoints. In fact, while the former is much more prone to routine applications and requires destruction of the sample components, the latter generally preserves the original structure of the specimen, needs very large facilities - the nuclear reactor *in primis* - and can be considered an absolute method. This is only one example of the possible expedient combination of assay procedures meant to accurately establish reference values for elements. At the same time this will allow the ability of a relative technique to be ascertained after validation through an absolute one for systematic, long-range continuation of analogous initiatives. These concepts will be practically illustrated in the next sections devoted to a joint pilot study launched by the Istituto Superiore di Sanità, the Joint Research Centre of Ispra and the Institute of Pathological Anatomy of the University of Perugia.

Ongoing activities

Over the last three years a research programme has been carried out to assess the reference values for Al, Ba, Cd, Cr, Cu, Li, Mg, Mn, Pb, Sr, V and Zn in human lungs, liver and kidneys. These organs were chosen because they are - in this order - the target of atmospheric pollution, the site of metabolic transformation and accumulation, and the port of final excretion. Both urban and rural population subgroups are included in the study, as significant differences are expected between the two overall environmental conditions. The ultimate goal of this project is to reach

Table 2. - *Procedure for the dry-ashing destruction of biological tissues*

A) Transfer, under strictly controlled conditions, of the samples into decontaminated quartz containers with determination of the sample wet weight

B) Sample drying and measurement of the dry weight

C) Slow charring on a heating plate after addition of the smallest amount possible of concentrated HNO_3 (Merck Suprapur)

D) Calcination at 400 ± 10 °C in muffle furnaces, the inner surface of which is entirely coated with either laminar or fibrous quartz

E) Obtainment of completely white ashes at the end of the thermal treatment (if this is not achieved at the completion of the first cycle, a few drops of HNO_3 are added and the procedure is repeated as described above)

F) Dissolution of the white ashes with 1 ml concentrated HNO_3 at 40 °C

G) Transfer of the final solutions into 25 ml flasks and dilution with doubly-distilled water up to the marked volume

ranges of reference values for the concentration of a number of elements as a function of geographical site, sex and age for healthy subjects. All the above considerations were applied in the most rigorous way. In particular, in the case of lungs it was necessary to fix 13 different sampling points to guarantee their representativeness over the entire pulmonary mass, namely the tracheal bifurcation (I), the bronchi (II, III), the sub-pleural tissues (IV, V) the different lobes (VI-XI) and the lymph nodes (XII, XIII). This need was less keenly felt for the other two types of tissues. Albeit the work is still in progress and at least two further years are deemed necessary to complete it, data obtained so far already allow for preliminary and meaningful conclusions to be drawn that deserve prompt diffusion. Digestion of samples, when unavoidable, was achieved by dry ashing in wholly quartz-lined muffle furnaces. In alternative, microwave (MW) dissolution was adopted because of its treatment efficiency and much shorter time required to destroy the matrix. Some doubts still remain about this procedure's reproducibility and analyte recovery in the long-run. For the time being, preference is given to the more conventional approach until the role of various parameters influencing the MW operation is completely clarified. The main steps of the dry-ashing method are set forth in Table 2. The mental attitude behind the selection of proper instrumental methodologies has been detailed above. Here it would be enough to simply mention that analyses are made by both ICP-AES and NAA, the first being occasionally replaced by atomic absorption spectrometry with graphite furnace (GFAAS) whenever the necessary detection power cannot be afforded by ICP-AES. Tables 3-5 summarize the experimental conditions adopted in all three instances.

Table 3. - *Instrumental specifications and operative parameters for ICP-AES*

Instrumentation	
Spectrometer	Jobin-Yvon 32-38 VHR
RF Generator	DURR-JY 3848 frequency 56 MHz nominal power output 2.2 kW
Torch	INSA, demountable, coil with 6 turns
Monochromator	HR 1000 M (Czerny-Turner mounting) with holographic plane grating (3600 grooves/mm) focal length 1 m linear dispersion (first order) 0.27 nm/mm theoretical resolution 504,000 spectral range 170-450 nm
Polychromator	HR 1000 M (Paschen-Runge mounting) with holographic concave grating (3600 grooves/mm) 31 channels focal length 0.5 m linear dispersion (first order) 0.55 nm/mm spectral range 170-410 nm
Computer	Apple II, version Pascal 2-5
Printer	MI 80
Analytical conditions	
Argon flows	plasma 18 l/min coating 0.3 l/min aerosol 0.45 l/min
Slit widths	40 µm (entrance and exit for monochromator) 50 µm (entrance and exit for polychromator)
Spectral lines (nm)	Al(I) 396.2, B(I) 249.8, Ba(III) 455.4, Cd(I) 228.8, Cr(II) 205.6, Cu(I) 324.7, Mg(II) 279.6, Mn(II) 257.4, Sr(II) 407.8, V(II) 292.4, Zn(I) 213.9

Table 4. - *Instrumental specifications and operative parameters for GFAAS determinations*

Instrumentation	
Spectrometer	Perkin-Elmer 5100 with Zeeman corrector
Monochromator	Czerny-Turner mounting with holographic plane grating (2880 grooves/mm in the UV region and 1440 grooves/mm in the visible region) focal length 408 mm linear dispersion (first order) 0.65 nm/mm (UV) 1.30 nm/mm (visible) spectral range 170-900 nm
Furnace assembly	Perkin-Elmer HGA 600 with furnace autosampler AS-60
Computer	Perkin-Elmer 7300 Professional
Printer	Epson FX-85
Analytical conditions	
Thermal program	drying 110 °C ashing 850 °C for Pb and 900 °C for Li atomization 1800 °C for Pb and 2600 °C for Li
Slit widths	0.7 nm for Pb and 1.4 nm for Li
Spectral lines	283.4 nm for Pb and 670.8 nm for Li

Table 5. - *Instrumental specifications and operative parameters for NAA determinations*

Instrumentation	
Reactors	1) Triga Mark 2 with a thermal neutron flux of 5×10^{12} neutrons/cm^2 s 2) HFR with a thermal neutron flux of 2×10^{14} neutrons/cm^2 s
Detector	high resolution Ge (Li) gamma-counter
Analytical conditions	
Counting	1) 60 and 380 s after irradiation, samples counted for 100 s (^{27}Mg, ^{66}Cu, ^{52}V and ^{28}Al) 2) 2 h after irradiation, samples counted for 500 s (^{56}Mn) 3) after radiochemical separation on Chelex 100 (^{115m}In(Cd), ^{51}Cr and ^{65}Zn)

Results

The results obtained thus far for two population subgroups (inhabitants of the urban area of Rome and people living in the industrial area of Terni) are shown in Tables 6-9. From these results it can be concluded that in all cases the range spanned by element concentrations is well within one and the same order of magnitude. It is worth stressing that the figures reported for lungs are the average of those obtained with ICP-AES (or GFAAS) and NAA, both series of values overlapping to a significant extent [12]. The suitability of the former technnique being thus ascertained, the use of NAA was, in the assay of other organs, restricted to a few instances in order to verify the regular performance of the analyses. The constant use of reference materials like the NBS SRM no. 1577a Bovine Liver permitted a continuous checking of the determination reliability. It is also interesting to note that for most elements there are no striking differences in lungs of subjects from urban and highly industrialized areas. In spite of this general behavior, the reasons for which still require a convincing explanation, there are a few examples that are markedly probative of the contrary. This holds true e.g. for the elements Cd, Cr, and Pb, probably because of their higher concentrations in the polluted atmosphere of an industrial site. Nothing further can momentarily be inferred due to the still incomplete set of data.

Table 6. - *Figures of merit for element concentrations in lungs. Source of subjects: Rome (number of subjects: 12)*

Element	Mean (μg/g) *(b)*	Median (μg/g) *(b)*	Range *(a)* (μg/g) *(b)*
Al	10.52	8.88	4.10 - 35.8
Ba	0.140	0.155	0.056 - 0.211
Cd	0.080	0.065	0.023 - 0.255
Cr	0.11	0.10	0.06 - 0.17
Cu	1.48	1.30	0.78 - 5.10
Li	0.03	0.03	0.01 - 0.05
Mg	84.5	83.3	60.5 - 99.6
Mn	0.130	0.128	0.059 - 0.206
Pb	0.17	0.19	0.11 - 0.23
Sr	0.130	0.119	0.072 - 0.236
V	0.03	0.028	0.010 - 0.071
Zn	9.86	10.14	8.42 - 15.4

(a) for a greater number of samples, the criterion of the 5th-95th percentile should be preferred
(b) wet tissue

Table 7. - *Figures of merit for element concentrations in lungs. Source of subjects: Terni (number of subjects: 25)*

Element	Mean (μg/g) *(b)*	Median (μg/g) *(b)*	Range *(a)* (μg/g) *(b)*
Al	8.68	5.51	1.16 - 38.34
Ba	0.171	0.115	0.049 - 0.542
Cd	0.215	0.112	0.006 - 0.832
Cr	1.08	0.21	0.05 - 10.90
Cu	1.71	1.17	0.52 - 14.18
Li	0.06	0.05	0.03 - 0.30
Mg	60.19	61.27	15.80 - 100.7
Mn	0.227	0.057	0.015 - 1.783
Ni	0.44	0.18	0.05 - 3.79
Pb	0.33	0.27	0.09 - 1.30
Sr	0.086	0.075	0.025 - 0.292
V	*(c)*	*(c)*	*(c)*
Zn	9.95	8.78	4.79 - 22.01

(a) for a greater number of samples, the criterion of the 5th-95th percentiles should be preferred
(b) wet tissue
(c) values below or very close to the detection limit (0.010 μg/g)

Table 8. - *Figures of merit for element concentrations in liver. Source of subjects: Terni (number of subjects: 25)*

Element	Mean (μg/g) *(b)*	Median (μg/g) *(b)*	Range *(a)* (μg/g) *(b)*
Al	1.74	1.43	0.82 - 4.74
Ba	0.108	0.075	0.051 - 0.486
Cd	0.832	0.790	0.126 - 2.036
Cr	0.09	0.08	0.05 - 0.21
Cu	4.94	4.37	2.34 - 17.78
Li	0.04	0.02	0.01 - 0.30
Mg	113.0	114.9	24.0 - 147.2
Mn	0.775	0.744	0.057 - 1.437
Ni	0.16	0.10	0.03 - 0.55
Pb	0.54	0.48	0.14 - 1.68
Sr	0.035	0.027	0.009 - 0.259
V	*(c)*	*(c)*	*(c)*
Zn	59.10	53.54	29.7 - 119.1

(a) for a greater number of samples, the criterion of the 5th-95th percentiles should be preferred
(b) wet tissue
(c) values below or very close to the detection limit (0.010 μg/g)

Table 9. - *Figures of merit for element concentrations in kidneys. Source of subjects: Terni (number of subjects: 25)*

Element	Mean (μg/g) *(b)*	Median (μg/g) *(b)*	Range *(a)* (μg/g) *(b)*
Al	1.06	0.94	0.41 - 2.82
Ba	0.101	0.071	0.032 - 0.270
Cd	9.257	5.887	2.45 - 23.50
Cr	0.07	0.06	0.04 - 0.11
Cu	1.75	1.68	0.87 - 2.80
Li	0.03	0.03	0.01 - 0.06
Mg	90.85	91.69	66.52 - 111.84
Mn	0.438	0.403	0.174 - 0.804
Ni	0.10	0.09	0.01 - 0.37
Pb	0.27	0.22	0.09 - 1.48
Sr	0.081	0.078	0.026 - 0.189
V	*(c)*	*(c)*	*(c)*
Zn	25.86	27.70	9.68 - 49.72

(a) for a greater number of samples, the criterion of the 5th-95th percentiles should be preferred
(b) wet tissue
(c) values below or very close to the detection limit (0.010 μg/g)

Conclusions

The validity of the assumptions reported herein regarding the precautions to be taken when launching studies focused on reference values is substantiated by the outoctome of studies carried out so far. The considerable improvements obtained in reducing the amplitude of the range normally reported for reference values proves the correctness of the overall approach, this being is strongly encouraging. The possible identification of "marker" elements, whose concentration variations in given organs may be paralleled by that of other analytes, is also conceivable, thus shedding new light on the mechanisms of accumulation and detoxification. Finally, it should be acknowledged that such a rigorous attitude in planning and performing studies on the assessment of reference values for elements is of great importance to minimize useless duplication of efforts and optimize comparability of data produced by different research groups. This fact alone would constitute a noticeable step forward for a sound and efficient action in the safeguard of human health.

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