The Science of the Total Environment, 95 (1990) 89–105
Elsevier

TRACE ELEMENT REFERENCE VALUES IN TISSUES FROM INHABITANTS OF THE EUROPEAN COMMUNITY I. A STUDY OF 46 ELEMENTS IN URINE, BLOOD AND SERUM OF ITALIAN SUBJECTS

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(Received September 29th, 1989; accepted November 1st, 1989)

ABSTRACT

Neutron activation analysis–electrothermal atomic absorption spectroscopy (ETA-AAS) and inductively coupled plasma atomic emission spectrometry (ICP-AES) have been used for the determination of 46 elements in urine, 35 in blood and 26 in serum of unexposed Italian subjects living in the same region (Lombardy). The results allowed the proposal of reference values for various elements determined in more than 350 healthy subjects, these being Ag, Al, As, Be, Bi, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Se, Tl, V, Zn, in urine; Ag, As, Bi, Cd, Cr, Co, Cu, Hg, Pb, Se, Tl, Zn in blood; and Ag, Al, Be, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Se, Tl, V, Zn in serum (or plasma). For all other elements indicative values are suggested. In addition to the mean value and the "reference range", a "range of uncertainty" and an upper limit above which metabolic abnormalities could be expected have also been defined on the basis of simple statistical considerations.

INTRODUCTION

The definition of health criteria for trace elements imposes the assessment of the risk to humans arising from trace element exposure. In assessing this risk, knowledge of the current levels of trace elements in humans and the definition of reference values (baseline data) in such tissues are of paramount importance [1]. There is no doubt that progress in this field has been made during the last few years, which has been facilitated by gaining more and more awareness of the analytical problems related to ultratrace element analysis [2] and by the availability of a new generation of biological Reference Materials [3]. However, most of the data reported in the literature on trace elements in

0048-9697/90/$03.50

human tissues are of limited value for the assessment of their toxicological impact. This is not only due to individual biological variations, but also to errors caused by analytical inconsistencies [4], mostly as a consequence of insufficient attention being paid to the pre-analytical factors, such as sampling, sample handling and storage. This has led to data of too poor analytical quality to provide an adequate scientific basis for formulating baseline concentrations, representing a serious obstacle for practical problems for several reasons. First, because of the confusion and waste of time caused by unreliable and erroneous data. Second, because important decisions can depend on the accuracy of such data, such as in clinical diagnoses or in the implementation of governmental regulations pertaining to the maximum acceptable levels of trace elements for the general population or professionally exposed workers. In addition, the absence of adequate reference values for elements in human tissues makes it difficult to establish relationships between element concentrations and toxic effects in the general population and professionally exposed workers, as well as pathological states in biomedical research into trace element-related diseases [5–7].

The objectives of this study were: (i) to determine trace element concentrations in the urine, whole blood and serum (or plasma) of unexposed healthy subjects living in three provinces of north Italy; (ii) to determine reliable reference values for the concentration of 18 elements in urine, 12 elements in whole blood and 15 elements in serum; (iii) to give an indication of approximate concentrations of other elements in these biological fluids.

The work was carried out in three laboratories by means of atomic absorption spectroscopy (AAS) and neutron activation analysis (NAA) in the context of a programme aimed at the establishment of criteria to assess the clinical and toxicological significance of environmental and occupational overexposure to trace elements [8].

MATERIALS AND METHODS

Examined populations

The populations sampled consisted of subjects living in the provinces of Brescia, Pavia and Varese of the Lombardy region, north Italy.

Subjects considered were selected as being representative of five sub-groups resident in urban, suburban, rural, and low and high hill areas. A questionnaire supplied detailed information on age, sex, area of residence, occupation, smoking habits, body weight, alimentary habits, socio-economic and ethnic factors, as well as on the elemental composition of the drinking water from the municipal supply and mineral water used. For subjects living near industrialized zones, information about the production processes and products of these industries was also obtained.

In order to identify possible physiological anomalies or pathological cases, the selected subjects were submitted to clinical examinations, including hema-

tological tests, hemocrome, VES, azotemia, glycemia, hepatic tests, lipid and urine analysis. Subjects showing clinical values outside the normal range were excluded from the study. In addition, the following groups of subjects were excluded: smokers consuming more than 10 cigarettes per day; subjects under particular physiological stress such as pregnancy or sports training; subjects with obesity and hypertension; subjects at high risk or affected by chronic diseases such as alcoholism, cardiovascular disorder, mental diseases and cancer; subjects with a history of occupational exposure to metal compounds; and subjects consuming drugs or contraceptives.

All sample collections were made at various hospitals, or medical services, located in the three provinces considered.

Table 1 shows the number of subjects examined and the analytical techniques used for the determination of the elemental composition of the biological fluids.

Reagents and laboratory ware

Ultrapure HNO_3 and HCl were obtained by a sub-boiling procedure or were supplied by BDH (U.K.). All other reagents were Aristar grade. Working standards for AAS analysis were prepared from the appropriate AAS standard [$1\,g\,l^{-1}$, Spectrosil solutions, BDH (U.K.)]. Bidistilled water was prepared from deionized water obtained from a Milli-Q Millipore system.

The standards for AAS were international Standard Reference Materials from the Community Bureau of Reference, Brussels (CRM 194, Blood), the National Bureau of Standards, Gaithersburg, MD (U.S.A.) (SRM 2670, Toxic Metals in Freeze-dried Urine, and SRM 8419, Bovine Serum), and Nycomed, Oslo (Norway) (Blood, Urine and Serum).

The standards for NAA were of two types. The first were multielement international Standard Reference Materials Orchard Leaves and Pine Needles (SRM 1571 and 1575) supplied by the National Bureau of Standards, Washington. The second were synthetic standards prepared from Spectrosil solutions [$1\,g\,l^{-1}$, BDH (U.K.)] used for AAS by dissolving ultrapure salts of various elements (Johnson and Matthey, U.K.) in bidistilled water or ultrapure dilute acids.

Teflon cannula for blood collection were supplied by Terumo Co., Tokyo; commercial syringes with stainless steel needles by Terumo Europa, N.V., Leuven (Belgium); pipettes for serum preparation by Eppendhorf, Hamburg (F.R.G.).

Collection and storage containers for AAS analysis of whole blood were made of polypropylene, polystyrene and polycarbonate (LP Italiana, Milan). Suprasil quartz was supplied by Passoni or Italquartz, Milan. For the preparation of serum, whole blood was collected in heparinized containers (LP Italiana, Milan), with the exception of that for Al determination [vacuotainer vials, Becton-Dickinson Co., Rutheford, NY (U.S.A.)]; in the case of Be, as well as Pb and Se in plasma, Suprasil quartz containers were used.

TABLE 1

Italian populations and analytical techniques used for the elemental analysis of urine, blood and serum

Province	Subjects			Analytical technique	Elements determined		
	Male	Female	Age (years)		Urine	Blood	Serum
Brescia	260	270	41 ± 15	Flame AAS, AAS Zeeman	Al, As, Be, Co, Cr, Cu, Mn, Ni, Tl, V, Zn	Cd, Pb	Al, Zn
Pavia	290	300	39 ± 16	Flame AAS, ETA–AAS Zeeman, ICP–AES	Ag, Al, As, B, Ba, Be, Bi, Cd, Co, Cr, Cu, Gd, Hg, Mn, Ni, Pb, Pd, Pt, Sb, Se, Si, Te, Ti, Tl, V, Zn, Zr	Ag, As, Ba, Bi, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Se, Tl, Zn	Ag, Al, Au, Be, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Se, Tl, V, Zn
Varese	85	32	46 ± 13	NAA (instrumental and/or radiochemical)	Ag, As, Au, Ba, Cd, Ce, Co, Cr, Cs, Cu, Eu, Fe, Ga, Hf, Hg, In, Ir, La, Lu, Nd, Rb, Sb, Sc, Se, Sm, Ta, Th, Tl, U, W, Zn, Yb	Ag, As, Au, Cd, Ce, Co, Cs, Cu, Eu, Ga, Hf, Hg, Ir, La, Lu, Nd, Rb, Sb, Sc, Se, Sm, Ta, Th, U, V, W, Zn, Yb	Ag, Au, Co, Cr, Cs, Cu, Eu, Hg, La, Lu, Rb, Sb, Sc, Se, Ta, Th, W, Zn

Urine collection and storage containers were 1 l unused polyethylene bottles (Kartell, Binasco, Milan). Blood samples for NAA were collected directly into 10 ml polyethylene vials (Kartell, Binasco, Milan).

Sample collection and handling

Whole blood

Twenty milliliters of whole blood for AAS elemental analysis were collected using a Teflon cannula (determination of Co, Cr, Mn, Pb, V and Zn) or a commercial syringe with a stainless steel needle (all other elements) into heparinized vials. Immediately after sampling, blood samples were subdivided into aliquots of from 0.5 to 1 ml and stored at 5 °C for periods ranging from 5 to 10 days before analysis.

All blood samples for NAA were collected using a Teflon cannula without addition of anticoagulants. The vials were stored at 5 °C and freeze-dried in the same collection containers before neutron irradiation.

Serum (or plasma)

Serum samples for AAS and NAA analysis were prepared by centrifugation of the blood (collected without addition of anticoagulants) at 2500 rpm and separation from the erythrocytes by pipetting the supernatant into unused vials (see *Reagents and laboratory ware*) previously washed as described for whole blood collection. The samples were stored at −20 °C, with the exception of those for the analysis of serum Hg (5 °C). The time lapse before elemental determinations were carried out ranged from 10 days (Hg) to 60 days (serum Al, Au, Be, Cu and Mn). Lead, Se and Zn were determined in plasma instead of in serum after addition of heparin during blood collection.

Urine

Containers for 24 h urine collection were subjected to different decontamination procedures according to the elements to be analyzed: 1–3% HNO_3 and bidistilled water (Be, Bi, Cd, Co, Cr, Cu, Mn, Hg, Ni, Pd, Sb, Se, Tl, Zn); aqua regia and bidistilled water (Ag and Pd); 5% HCl (As); Na_2EDTA solution (5 mmol l^{-1}) and bidistilled water (Al).

Urine samples were stored at −2 °C for 20–60 days before analysis, with the exception of the samples for the analysis of Hg (5 °C for 3 days). In the case of analysis for Be, Co, Cu, Ni, Sb or Zn, 1 ml 1% HNO_3 or 1 ml 1% HCl was added immediately after urine collection.

Atomic absorption analysis (AAS)

Atomic absorption measurements were made using the following instruments: (i) Perkin-Elmer atomic absorption spectrometers (Models 5000 and 3030) equipped with graphite furnace HGA (Models 500 and 600) and

TABLE 2

Trace elements in urine of healthy Italians. **Bold type**, reference values; light type, informative values

Element	Number of subjects	Elemental concentration ($\mu g\,l^{-1}$)				
		Mean ± σmt	Experimental range ($X_L - X_M$)	Reference values (range)	Range of uncertainty (undefined indicative values)	Upper limit (X_M) for metabolic anomalies
Ag	472	**0.46** ± 0.12	(0.06 – 2.5)	**0.04 – 0.88**	> 0.88 – 2.5	**>2.5**
Al	766	**10.9** ± 1.06	(1 – 31)	**2.3 – 19.5**	> 19.5 – 31	**>31**
As	540	**16.7** ± 1.9	(1 – 64.5)	**2.3 – 31.1**	> 31.1 – 64.5	**> 64.5**
Au[a]	43	0.07 ± 0.068	(0.003 – 0.85)	0.001 – 0.6	> 0.6 – 0.85	> 0.85
B	119	1890 ± 126	(470 – 7800)	490 – 3290	> 3290 – 7800	> 7800
Ba	35	2.7 ± 0.5	(0.25 – 10.1)	0.25 – 5.7	> 5.7 – 10.1	> 10.1
Be	579	**0.4** ± 0.09	(< 0.02 – 0.82)	**0.04 – 0.76**	> 0.76 – 0.82	**>0.82**
Bi	368	**1.2** ± 0.02	(0.2 – 2.55)	**0.8 – 1.6**	> 1.6 – 2.55	**>2.6**
Cd	392	**0.86** ± 0.06	(0.15 – 2)	**0.38 – 1.34**	> 1.34 – 2	**>2**
Ce	23	3.1 ± 1.95	(0.15 – 20)	0.1 – 12.1	> 12.1 – 20	> 20
Co	468	**0.57** ± 0.1	(0.12 – 2)	**0.18 – 0.96**	> 0.96 – 2	**>2**
Cr	879	**0.61** ± 0.11	(0.04 – 5.1)	**0.04 – 1.5**	> 1.5 – 5.1	**>5.1**
Cs	70	8.1 ± 1.5	(1.1 – 22)	0.1 – 17.5	> 17.5 – 22	> 22
Cu	507	**23** ± 6.9	(4.2 – 75)	**4.2 – 50**	> 50 – 75	**>75**
Eu	13	0.11 ± 0.08	(0.003 – 0.4)	0.003 – 0.36	> 0.36 – 0.4	> 0.4
Ga	10	< 0.5				> 0.5
Gd	26	< 1				> 1
Hf	16	0.49 ± 0.22	(0.01 – 1.4)	0.01 – 1.31	> 1.31 – 1.4	> 1.4
Hg	380	**3.5** ± 0.2	(0.3 – 16.5)	**0.1 – 6.9**	> 6.9 – 16.5	**>16.5**
In	42	< 0.15				> 0.15
Ir	17	0.018 ± 0.009	(0.0007 – 0.07)	0.0005 – 0.054	> 0.054 – 0.07	> 0.07
La	28	0.73 ± 0.55	(0.015 – 6)	0.015 – 3.6	> 3.6 – 6	> 6
Lu	16	0.05 ± 0.04	(0.001 – 0.3)	0.001 – 0.22	> 0.22 – 0.3	> 0.3
Mn	777	**1.02** ± 0.05	(0.1 – 3)	**0.12 – 1.9**	> 1.9 – 3	**>3**
Nd	15	3.84 ± 1.9	(0.2 – 12)	0.2 – 10.6	> 10.6 – 12	> 12
Ni	878	**0.9** ± 0.11	(0.1 – 3.9)	**0.06 – 1.74**	> 1.74 – 3.9	**>3.9**

Pb	456	**17** ± 0.46	(4 – 39)	**12 – 27**	> 27 – 39	**>39**
Pd	136	< 0.15				> 0.15
Pt	25	< 1				> 1
Rb	87	2190 ± 203	(240 – 4450)	284 – 4096	> 4096 – 4450	> 4450
Sb	360	**0.79** ± 0.07	(0.1 – 3.6)	**0.19 – 1.1**	> 1.1 – 3.6	**>3.6**
Sc	28	0.038 ± 0.018	(0.0003 – 0.16)	0.0003 – 0.13	> 0.13 – 0.16	> 0.16
Se	484	**22.1** ± 2.4	(2.1 – 68)	**2.1 – 30.9**	> 30.9 – 68	**>68**
Si	92	7500 ± 470	(2000 – 14500)	2900 – 12100	> 12100 – 14500	> 14500
Sm	19	0.055 ± 0.038	(0.0015 – 0.25)	0.001 – 0.21	> 0.21 – 0.25	> 0.25
Ta	16	0.16 ± 0.12	(0.02 – 0.9)	0.01 – 0.6	> 0.6 – 0.9	> 0.9
Th	25	0.085 ± 0.04	(0.01 – 0.7)	0.01 – 0.28	> 0.28 – 0.7	> 0.7
Te	20	< 1				> 1
Ti	18	2.1 ± 0.19	(0.6 – 3.7)	1.3 – 2.9	> 2.9 – 3.7	> 3.7
Tl	496	**0.42** ± 0.09	(0.06 – 0.82)	**0.07 – 0.7**	> 0.7 – 0.82	**>0.82**
U	14	< 0.1				> 0.1
V	382	**0.8** ± 0.08	(0.05 – 1.44)	**0.2 – 1**	> 1.0 – 1.44	**>1.44**
W	11	0.32 ± 0.19	(0.07 – 0.9)	0.05 – 0.85	> 0.85 – 0.9	> 0.9
Yb	6	0.028 ± 0.02	(0.0015 – 0.09)	0.005 – 0.086	> 0.086 – 0.09	> 0.09
Zn	683	**456** ± 58	(302 – 1300)	**266 – 846**	> 846 – 1300	**>1300**
Zr	30	< 2				< 2

[a] 124 subjects from Pavia analyzed by ETA–AAS gave results below the detection limit ($0.08\,\mu g\,l^{-1}$).

TABLE 3

Trace elements in blood of healthy Italians. **Bold type**, reference values; light type, informative values

Element	Number of subjects	Elemental concentration ($\mu g\,l^{-1}$)				
		Mean ± σmt	Experimental range ($X_L - X_M$)	Reference values (range)	Range of uncertainty (undefined indicative values)	Upper limit (X_M) for metabolic anomalies
Ag	437	**0.37** ± 0.07	(0.05 – 0.78)	**0.13 – 0.61**	> 0.61 – 0.78	**> 0.78**
As	470	**7.9** ± 1.75	(0.4 – 70.5)	**0.4 – 11.9**	> 11.9 – 70.5	**> 70.5**
Au[a]	35	0.045 ± 0.0007	(0.002 – 0.06)	0.006 – 0.049	> 0.049 – 0.06	> 0.06
Ba	25	1.2 ± 0.26	(0.47 – 2.9)	0.47 – 2.4	> 2.4 – 2.9	> 2.9
Bi	358	**0.49** ± 0.023	(0.12 – 0.89)	**0.12 – 0.8**	> 0.8 – 0.9	**> 0.9**
Cd	900	**0.6** ± 0.3	(0.1 – 5.5)	**0.1 – 1.7**	> 1.7 – 5.5	**> 5.5**
Ce	12	3.1 ± 2.15	(0.7 – 10)	0.5 – 9	> 9 – 10	> 10
Co	441	**0.39** ± 0.13	(0.1 – 4.2)	**0.01 – 0.91**	> 0.91 – 4.2	**> 4.2**
Cr	519	**0.23** ± 0.01	(0.09 – 0.75)	**0.01 – 0.45**	> 0.45 – 0.75	**> 0.75**
Cs	62	3 ± 0.52	(0.5 – 8.5)	0.5 – 7.0	> 7 – 8.5	> 8.5
Cu	475	**1225** ± 64.3	(535 – 1940)	**807 – 1643**	> 1643 – 1940	**> 1940**
Eu	15	0.21 ± 0.08	(0.005 – 0.62)	0.005 – 0.5	> 0.5 – 0.62	> 0.62
Ga	5	0.26 ± 0.16	(0.1 – 0.38)	0.1 – 0.52		> 0.52
Hf	29	0.21 ± 0.064	(0.012 – 0.6)	0.012 – 0.53	> 0.53 – 0.6	> 0.6
Hg	368	**5.3** ± 0.95	(0.5 – 17.3)	**1.7 – 9.9**	> 9.9 – 17.3	**> 17.3**
Ir	22	0.0074 ± 0.004	(0.0002 – 0.035)	0.0002 – 0.02	> 0.02 – 0.035	> 0.035
La	21	1.42 ± 0.71	(0.13 – 4.75)	0.1 – 4.4	> 4.4 – 4.75	> 4.75
Lu	42	0.2 ± 0.09	(0.00025 – 0.8)	0.0002 – 0.5	> 0.5 – 0.8	> 0.8
Mn	88	8.8 ± 0.2	(5 – 12.4)	7.1 – 10.5	> 10.5 – 12.4	> 12.4
Nd	13	1.39 ± 0.82	(0.075 – 3.75)	0.075 – 3.1	> 3.1 – 3.75	> 3.75
Ni	36	2.3 ± 0.16	(0.6 – 3.8)	1.3 – 3.3	> 3.3 – 3.8	> 3.8
Pb	959	**157.7** ± 9.9	(30 – 390)	**39.7 – 275.7**	> 275.7 – 390	**> 390**
Rb	67	2805 ± 408	(900 – 6800)	900 – 4145	> 4145 – 6800	> 6800
Sb	27	2.16 ± 0.45	(0.03 – 5)	0.03 – 3.5	> 3.5 – 5	> 5
Sc	40	0.061 ± 0.015	(0.002 – 0.18)	0.002 – 0.12	> 0.12 – 0.18	> 0.18
Se	455	**107.5** ± 6.4	(40 – 180)	**76 – 140**	> 140 – 180	**> 180**

Sm	10	0.26 ± 0.14	(0.03 – 0.5)	0.03 – 0.45	> 0.45 – 0.5	> 0.5
Ta	20	0.23 ± 0.09	(0.04 – 0.7)	0.04 – 0.6	< 0.6 – 0.7	> 0.7
Th	17	0.21 ± 0.1	(0.03 – 0.73)	0.03 – 0.61	> 0.61 – 0.73	> 0.73
Tl	418	**0.39** ± 0.05	(0.1 – 1.1)	**0.15 – 0.63**	> 0.63 – 1.1	> 1.1
U	17	< 0.1				> 0.1
V	65	0.35 ± 0.11	(0.09 – 1.1)	0.09 – 0.75	> 0.75 – 1.1	> 1.1
W	10	0.39 ± 0.15	(0.05 – 0.75)	0.05 – 0.7	> 0.7 – 0.75	> 0.75
Yb	7	0.15 ± 0.083	(0.05 – 0.3)	0.05 – 0.3		
Zn	502	**6340** ± 210	(3500 – 8800)	**4076 – 7594**	> 7594 – 8800	**> 8800**

[a] 118 subjects from Pavia analyzed by ETA–AAS gave results below the detection limit (0.08 $\mu g\, l^{-1}$).

TABLE 4

Trace elements in serum (or plasma) of healthy Italians. **Bold type**, reference values; light type, informative values

Element	Number of subjects	Elemental concentration ($\mu g\,l^{-1}$)				
		Mean ± σmt	Experimental range ($X_L - X_M$)	Reference values (range)	Range of uncertainty (undefined indicative values)	Upper limit (X_M) for metabolic anomalies
Ag	394	**0.18** ± 0.04	(0.06 – 0.46)	**0.06 – 0.3**	> 0.3 – 0.46	**>0.46**
Al	916	**6** ± 0.36	(1 – 10.9)	**0.3 – 7.5**	> 7.5 – 10.9	**>10.9**
Au	22	0.012 ± 0.003	(0.001 – 0.09)	0.002 – 0.08	> 0.08 – 0.09	> 0.09
Be	398	**0.15** ± 0.006	(< 0.08 – 0.36)	**0.03 – 0.27**	> 0.27 – 0.36	**>0.36**
Cd	360	**0.2** ± 0.008	(0.09 – 0.66)	**0.04 – 0.36**	> 0.36 – 0.66	**>0.66**
Co	405	**0.21** ± 0.008	(0.08 – 0.52)	**0.08 – 0.4**	> 0.4 – 0.52	**>0.52**
Cr	530	**0.17** ± 0.01	(0.04 – 0.6)	**0.04 – 0.41**	> 0.41 – 0.6	**>0.6**
Cs	25	1.5 ± 0.12	(0.11 – 6.8)	0.11 – 5.2	> 5.2 – 6.8	> 6.8
Cu	901	**985** ± 36	(600 – 1760)	**601 – 1373**	> 1373 – 1700	**>1760**
Hg	349	**2.1** ± 0.082	(0.39 – 4.8)	**0.6 – 3.8**	> 3.8 – 4.8	**>4.8**
Ir	8	0.005 ± 0.001	(0.0002 – 0.04)	0.0002 – 0.018	> 0.018 – 0.04	> 0.04
La	12	< 1				> 1
Lu	14	< 0.05				> 0.05
Mn	414	**0.6** ± 0.014	(0.3 – 1.35)	**0.3 – 0.9**	> 0.9 – 1.35	**>1.35**
Ni	385	**1.2** ± 0.079	(0.24 – 3.7)	**0.24 – 2.8**	> 2.8 – 3.7	**>3.7**
Pb[a]	228	**0.3** ± 0.023	(0.25 – 0.54)	**0.1 – 0.5**	> 0.5 – 0.54	**>0.54**
Rb	57	230 ± 48	(78 – 511)	78 – 317	> 317 – 511	> 511
Sb	22	0.5 ± 0.1	(0.01 – 3.1)	0.01 – 1.7	> 1.7 – 3.1	> 3.1
Sc	24	0.043 ± 0.013	(0.003 – 0.1)	0.003 – 0.09	> 0.09 – 0.1	> 0.1
Se[a]	441	**81** ± 1.12	(33 – 121)	**56 – 105**	> 105 – 121	**>121**
Ta	16	< 0.1				> 0.1
Th	13	< 0.1				> 0.1
Tl	360	**0.18** ± 0.009	(< 0.05 – 0.4)	**0.02 – 0.34**	> 0.34 – 0.4	**>0.4**
V	415	**0.62** ± 0.03	(0.07 – 1.8)	**0.07 – 1.1**	> 1.1 – 1.8	**>1.8**
W	10	0.045 ± 0.01	(0.004 – 0.5)	0.004 – 0.35	> 0.36 – 0.5	> 0.5
Zn[a]	682	**922** ± 68	(540 – 1510)	**587 – 1215**	> 1215 – 1510	**>1510**

[a] Plasma.

furnace autosamplers AS (Models 40 and 60). The first instrument was converted to an ICP–AES system using a Perkin-Elmer Data System 10 for data treatment. The second instrument was equipped with a Zeeman-effect background correction system involving a Zeeman burner head assembly with a line frequency-modulated AC magnetic field applied to the atomic vapour in the graphite tube, perpendicular to the optical path; (ii) Perkin-Elmer atomic absorption spectrometer Model 603 with an air–acetylene flame. The instrument was equipped with the MHS 20 analysis system to detect hydride-forming elements such as As, Hg and Se; (iii) Varian atomic absorption spectrometer Model 40 equipped with a deuterium background correction system connected to an IBM-PC for data treatment; (iv) Model 400 Zeeman connected to an IBM-PC.

The methods used for AAS analysis of the elements in whole blood, serum and urine involved a direct extraction procedure or STPF (stabilized temperature platform furnace) [9] for samples ranging from 10 μl to 2 ml. The coefficients of variation (CV) within and between series were also determined to assess the precision. The lowest CVs observed were for Zn (4.7–2.3%) followed by Pb, Cu, Cd and As, while the highest values were for Cr (67.3–28.7%) followed by Hg, Pt and Ni. Between series the highest CVs were for Cr (67.8–34.6%) followed by Hg, Pt, Co and Ni; the lowest ($< 10\%$) were for Zn, Cu and Pb, depending on the analytical technique as well as the concentration of the element in the matrix.

Neutron activation analysis (NAA)

Multielement analysis, using neutron activation, was carried out on urine and blood by instrumental (INAA) and/or radiochemical (RNAA) procedures, as previously described [10]. Briefly, dried samples in sealed polyethylene or quartz vials were irradiated for 10 h in the Triga Mark II reactor, University of Pavia (thermal neutron flux of the order of 10^{13} neutrons $cm^{-2}s^{-1}$), or for 40 h in the HFR reactor at the JRC, Petten (The Netherlands) (2×10^{14} neutrons $cm^{-2}s^{-1}$). The irradiated samples were then counted by computer-based, gamma-ray spectroscopy using a Ge(Li) detector and/or submitted to radiochemical separation involving: mineralization in a Teflon bomb, chromatography on a set of ion-exchangers or ion-exchange resins, including TDO (tin dioxide, C. Erba, Milan), Dowex 1-X8 resin (Bio Rad) and CuS (copper sulphide, C. Erba), and counting of the fractions by gamma-ray spectroscopy. Thallium was determined after isolation of the induced ^{204}Tl by another radiochemical separation scheme [11], and vanadium by a special procedure involving its preseparation prior to neutron activation [12].

Statistical treatment

The data have been arranged in Tables 2–4 as follows:
(i) mean value (x) $\pm$ σmt where σm is $\sigma/n^{1/2}$ (σ is the standard deviation), n

is the number of observations and t is the Fischer coefficient for $n - 1$ ($P = 0.05$). The experimentally observed range (X_L–X_M) is also shown. When the same element was determined in two or three population samples, the mean value was calculated according to the equation:

$$\bar{X} = n\bar{X}_1 + m\bar{Y} + l\bar{Z}/\, n + m + l$$

where X_1, Y and Z are the mean value of each set; n, m and l are the corresponding number of subjects analyzed. In this case, σmt was calculated according to the equation:

$$\left\{\frac{[n\,(\bar{X}_1^2 + (\sigma mt)_1^2)] + m\,[\bar{Y}^2 + (\sigma mt)_2^2] + l\,[\bar{Z}^2 + (\sigma mt)^2\,] - \bar{X}(\sigma mt)^2}{n + m + l}\right\}^{1/2}$$

where $(\sigma mt)_{1,2,3}$ are related to each sample population;

(ii) the range "reference values" defined as $(\bar{X} - 2\sigma)$–$(\bar{X} + 2\sigma)$. If $(\bar{X} - 2\sigma) < X_L$, then the range was taken from X_L to $\bar{X} + 2\sigma$;

(iii) the "uncertainty range" defined from $\bar{X} + 2\sigma$ to X_M (highest value observed experimentally);

(iv) the threshold limits defined as values $> X_M$.

The ranges defined by (ii) and (iii) were obtained by considering the data set for each element as a normal distribution when the number of subjects was $\geqslant 30$. When this number was equal to 30, the "Student t" distribution was considered [13].

RESULTS

Tables 2–4 give the elemental composition of the urine, blood and serum of the subjects analyzed. These tables also report the "reference range", the "range of uncertainty" (undefined informative values) and the "upper threshold limit" above which the levels may possibly be related to metabolic alterations.

In urine (Table 2), Si, Rb and B were the only elements whose mean value was at the milligrams per litre level, followed by Zn (456 $\mu g\,l^{-1}$). Concentrations between 10 and 30 $\mu g\,l^{-1}$ were observed for Al, As, Cu, Pb and Se; and between 1 and 10 $\mu g\,l^{-1}$ for Ba, Bi, Ce, Cs, Hg, Mn, Nd and Ti. All other elements (Ag, Au, Be, Cd, Co, Cr, Eu, Hf, Ir, La, Lu, Ni, Sb, Sc, Sm, Ta, Th, Tl, V, W, Yb) were at levels of $< \mu g\,l^{-1}$ with the lowest value for Ir (0.018 $\mu g\,l^{-1}$). Limits of sensitivity are reported for Ga, Gd, In, Pd, Pt, Te, U and Zr.

The mean values of the elements in blood (Table 3) at the milligrams per litre level were Zn, Rb and Cu (6.34, 2.8 and 1.225 $mg\,l^{-1}$, respectively), followed by Pb and Se at concentrations $> 100\,\mu g\,l^{-1}$. All other elements were in the ranges 1–10 $\mu g\,l^{-1}$ (As, Ba, Ce, Cr, Cs, Hg, La, Mn, Nd, Ni, Sb); 0.01–1 $\mu g\,l^{-1}$ (Ag, Bi, Cd, Co, Cr, Eu, Ga, Hf, Lu, Sm, Ta, Th, V, W, Yb) or $< 0.1\,\mu g\,l^{-1}$ (Au, Ir, Sc, U), with the lowest value for Ir (7.4 $ng\,l^{-1}$).

Among the elements determined in serum, only for Zn and Cu were the mean concentrations nearly at the milligrams per litre level (922 and 985 $\mu g\,l^{-1}$),

followed by Rb ($230\,\mu g\,l^{-1}$). Selenium was at a level of $> 50\,\mu g\,l^{-1}$ ($81\,\mu g\,l^{-1}$). Concentrations of the order of a few micrograms per litre were observed for Al, Cs, Hg, Ni, Pb and V, while all other elements (Ag, Au, Be, Cd, Co, Cr, Ir, Lu, Mn, Sb, Sc, Tl, W) were at concentrations of $< 1\,\mu g\,l^{-1}$, with Ir being the lowest ($5\,ng\,l^{-1}$). The limit of sensitivity is reported for La, Ta and Th.

DISCUSSION

This work is the result of studies by three laboratories, operating independently, using analytical techniques such as flame-AAS, ETA-AAS or ICP-AES and NAA to determine metal concentrations in tissues of three population groups living in the same region of Italy. Although we realize that some elements were determined by one technique only, or in subjects from one province, and that discrepancies due to analytical differences cannot be completely excluded, there are reasons for believing that the work is of good analytical quality and of practical interest.

AAS and NAA are two analytical techniques that are recognised as being sufficiently accurate and reliable for establishing baseline data for elements in biological specimens [14]. A recent intercomparison exercise among our laboratories on the determination of As, Co, Mn, Tl and V in human urine leads us to believe the results to be satisfactory for practical purposes, with differences among the mean values not exceeding 20% (results not reported here). In addition, great effort has been made by the three laboratories to operate in agreement with good practices as stressed at the 1987 Economic Summit for a common strategy to establish reliable and internationally acceptable analytical data [15]. We have taken into account aspects of the control of pre-analytical factors, perhaps the most critical point in ultratrace analysis of elements in biological specimens, to establish reference values. Cornelis and Versieck [16,17] have critically examined the modifications of reported levels of elements in biological specimens in the last 20 years. They showed differences in the data of the various authors which cannot be explained simply by the evolution of the analytical techniques. Many data were certainly affected by contamination during sampling, sample handling and storage of the specimens analyzed.

In our study, sampling, sample handling and storage of the samples tested were carried out under rigorous standardized protocols. In addition, the availability of systems for background correction in AAS, in combination with STPF, has made possible the direct analysis of many elements. In this case, sample pre-treatment was not necessary, which greatly reduced the risk of contamination of the samples prior to instrumental analysis. Furthermore, this risk, in practice, is absent in NAA [18].

Two other interesting aspects of this work merit further comment. First, the criteria used for selection of the "reference population group" included anamnesis and clinical examinations to exclude as much as possible physiological anomalies or pathological states. Most of the reported studies in this field

have been based on groups of subjects that only partly corresponded to those of a "reference population". Second, the number of subjects considered; in the literature, most of the data reported are often based on sample numbers generally too small to establish reliable guidelines for reference values. In our study, many elements were determined in a number of subjects which can be considered sufficiently high to suggest guidelines for "normal" concentrations. In particular, 18 elements (Ag, Al, As, Be, Bi, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Se, Tl, V and Zn) were determined in the urine of a number of subjects, ranging from 360 (Sb) to 879 (Cr) (Table 2); 12 elements (Ag, As, Bi, Cd, Co, Cr, Cu, Hg, Pb, Se, Tl and Zn) were analyzed in blood, the number of subjects ranging from 358 (Bi) to 959 (Pb) (Table 3); and 15 elements were determined in serum with the number of subjects ranging from 228 (Pb) to 901 (Cu) (Table 4). For all other elements the reported values must be taken as indicative.

Interestingly, the only elements found at milligrams per litre levels in the blood were Zn, Cu and Rb, and in the urine Si, Rb and B. The presence of Rb at the same level as Zn and Cu in the blood, mainly associated with the red blood cells as shown in Table 4 and by in vitro experiments using ^{86}Rb radiotracer [19], is of particular interest and tends to support the reported essential role of this element in man [20]. Iridium had the lowest concentrations in urine, blood and serum (18, 7.4 and 5 $ng\,l^{-1}$, respectively).

One of the problems in establishing "reference values" is the question of how much the experimentally determined range of values for each element is representative of "normal values", and how one can define an upper limit above which metabolic alterations or pathological states can be expected. This is a difficult question, as this limit may be influenced by various factors, for example interrelationships among the elements and analytical inconsistencies due to sensitivity problems and inaccuracy. In fact, such a limit may well cover a range of values and may vary under different circumstances. In an effort to overcome this problem we have considered:

(i) a "reference range" or "normal" values calculated using basic statistical distributions, i.e. "normal" or "Student t", depending on the number of subjects analyzed;

(ii) a "range of uncertainty" beyond the "reference range" that defines concentrations which at present can be considered informative values, undefined in relation to the reference range. In the future, particular attention could be paid to this "range of uncertainty" so that these levels and the factors affecting them may be accurately determined;

(iii) an upper limit of concentration above which one would expect the presence of abnormal metabolic manifestations.

We stress that, in presenting the data and in an attempt to set the limit of concentration of an element beyond which toxicity may occur, the following conventions, arbitrarily chosen, were adopted:

(i) let the data be a normal population (statistically, i.e. normal distribution);

(ii) let a limit of 2σ be chosen to define the reference range.

TABLE 5

Comparison between Iyengar and Woittiez's reference values (median) for urine, blood and serum and the mean values of the present work

Element	Elemental concentration ($\mu g\,l^{-1}$)					
	Urine		Blood		Serum	
	Iyengar	Present work	Iyengar	Present work	Iyengar	Present work
As	20	16.7	5	7.9	3.5	
Cd	0.8	0.86	1	0.6	~0.1[b]	0.2
Cr	0.4	0.61	2.8[b]	0.23	0.19	0.17
Co		0.57	20[b]	0.39	0.29	0.21
Cu	38	23	960	1225	1100	987
Pb	11	17	123	157.7	< 1[b]	0.3[c]
Mn	0.6	1.2	13.6	8.8[b]	0.63	0.6
Hg	4.3	3.5	9.5[b]	5.3	(2.2–5.8)	2.1
Ni	2.5	0.9		2.3[b]	(2.6–7.5)	1.2
Se	40	22.1	105	107.5	96	81[c]
Zn	449	456	6400	6340	930	922[c]

[a] From ref. 21.
[b] Indicative values.
[c] Plasma.

In the absence of other valid criteria these assumptions appear to be a useful attempt to organize the many data as a guideline for defining current limits for clinical purposes as well as for establishing priorities for future research in this field.

Recently, baseline concentrations for 15 elements in human urine, whole blood and serum clinical specimens have been suggested by Iyengar and Woittez [21]. In blood, the median (or mean) values for As, Cd, Cu, Pb, Se and Zn are within our suggested "reference ranges" (Table 5). However, Iyengar's upper limit (X_M) for As falls in our "range of uncertainty", and those for Cd, Hg, Ni and Se exceed our reported threshold limits of toxicity for these elements. Only for Mn is Iyengar's lower limit (X_L) within our "reference range". Iyengar's median values for As, Cd, Cr, Cu Mn and Pb in urine fall within our "reference range", while the values for Ni and Se are within our "range of uncertainty". This can be considered satisfactory for general reference values for an unexposed population. In addition, considering that Iyengar's reference values were obtained by critical evaluation of data from different parts of the world, including analysis of the quality of the data, we conclude that the trace element content of blood, urine and serum of the general population reported here are not so different from those reported in the literature, which confirms the great importance of the influence of pre-analytical factors. We are probably at the limit of actual experimental possibilities in which variations due to pre-analytical factors become negligible in comparison with those of a

biological nature. In this context, it is interesting to compare the present data with element concentrations in the blood of subjects from the United Kingdom [22] as published by Hamilton et al. more than 15 years ago; these authors paid particular attention to possible influences of pre-analytical factors and the risks of contamination prior to analysis. Among the reported data for 14 elements, satisfactory agreement with our results is observed for As, Cs, Cu, Hg, Pb, Rb, Sb, Tl and Zn. Higher values were reported only for Ag, Cr, Mn and Sc. This confirms the view that old results are acceptable if the determinations are carried out on a sufficiently high number of subjects to reduce statistical variation and if adequate contamination control procedures are also adopted.

The data reported here conclude the first phase of the study: the global presentation of the data. On the basis of the information acquired, a more detailed study to establish the influences on the reference ranges of different factors such as sex and age will be undertaken, as well as a discussion of the clinical significance of the levels found. In addition, sophisticated computer-based statistical tests for normality, such as Lilliefors test [23], are to be applied to determine whether or not the distributions of the trace elements agree with some specific distribution function [24].

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