Journal of Chromatography, 583 (1992) 35–44
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6552

Gas chromatographic–mass spectrometric method for the determination of selenium in biological samples

V. Ducros and A. Favier

Laboratoire de Biochimie C, Hôpital A. Michallon, B.P. 217, 38043 Grenoble Cedex 9 (France)

(First received February 14th, 1992; revised manuscript received August 24th, 1992)

ABSTRACT

The determination of selenium by capillary gas chromatography–mass spectrometry (GC–MS), using an enriched stable isotope ^{76}Se as internal standard, is described. Reference values for selenium in human biological fluids (serum, red blood cells and urine) are reported. With the advent of new compact capillary GC–MS (benchtop) instruments, this method will be very simple and accurate for routine analysis.

INTRODUCTION

A wide range of methods [1] for the determination of selenium in biological matrices are available, more than for any other trace element. Among these techniques, three groups of methods are commonly used: fluorimetry, atomic absorption spectrometry and gas chromatography (GC). Fluorimetry is the oldest and most popular type, widely used by veterinarians. Although this method is inexpensive to set up, one major drawback is the laborious and delicate sample preparation required to avoid interfering fluorescent impurities, which limit this technique for very low concentration samples (picogram range) [2,3]. GC methods have a similar problem, in that prolonged sample digestion, extraction and purification steps are needed. However, with electron-capture detection, GC is very sensitive (absolute detection limit *ca.* 1–5 pg) [4,5]. Atomic absorption spectrometric (AAS) methods are of three types: flame AAS, hydride-generation AAS and graphite-furnace AAS. Flame AAS cannot be used for biological samples because the detection limit is high. Hydride-generation AAS could be used with a wide variety of matrices (blood, urine, food, etc.) but needs very careful sample digestion (conversion of all selenium into selenite) to obtain precise results [6,7]. Graphite-furnace AAS with Zeeman background correction is suitable for rapid determination of selenium in routine samples, but less precise than isotope dilution mass spectrometry (IDMS) [8], and some matrix interferences have been reported if acid pretreatment of samples is necessary [9]. The main advantage of the IDMS technique is that it does not require quantitative or reproducible recovery once spike equilibration occurs. This method was first described by Reamer and Veillon [10], who used GC–MS with a packed column. An adaptation to capillary column GC–MS available in-house has been developed, which is valuable for different kinds of biological sample, and is easy to use. A capillary column increases the efficiency of the chromatographic separation and the speed of the analysis, and decreases the

Correspondence to: Véronique Ducros, Laboratoire de Biochimie C, Hôpital A. Michallon, B.P. 217, 38043 Grenoble Cedex 9, France.

0378-4347/92/$05.00 © 1992 Elsevier Science Publishers B.V. All rights reserved

"bleeding" of the column, which is a particularly important point in GC–MS. To make the method very precise, an internal standard (an enriched isotope of the element) is used, so we also established an IDMS method with enriched ^{76}Se as the internal standard. Moreover, this technique can be extended to a double-isotope method to perform *in vivo* studies of selenium metabolism [11,12].

EXPERIMENTAL

Instrumentation

A DN200 gas chromatograph coupled to a Nermag R10-10C quadrupole mass spectrometer (Delsi Nermag Instruments, Argenteuil, France) was used. Electron impact (EI) ionization was used, and mass fragmentography was performed to enhance the sensitivity of the detection.

The glass needle injector was set at 250°C with a purge flow-rate of 20 ml/min. The WCOT CPSil 5CB capillary column (Chrompack, Middelburg, Netherlands) (9 m, 0.32 mm I.D., film thickness 1 μm) was connected directly to the ion source. The carrier gas was helium at a flow-rate of 4.5 ml/min. The temperature was programmed from 120 to 250°C at 5°C/min. For MS, the electron energy was 70 eV, the filament intensity 200 μA, the electronic gain 10^7 V/A and the integration set-time 400 ms for ion 225 (^{76}Se) and 400 ms for ion 229 (^{80}Se).

Materials and reagents

Borosilicate glass tubes (160 × 18 mm) with PTFE caps (Verre-Labo Mula, Corbas, France) were used and specially cleaned. The cleaning procedure consisted of water washing, soaking for 2 h in an ultrasonic bath of 1.6 *M* EDTA, ten rinses in deionized water, then soaking for 2 h in an ultrasonic bath of deionized water and, finally, oven-drying at 60°C. All reagents were of ultrapure grade: HNO_3 65% suprapur (Merck, Darmstadt, Germany), $HClO_4$ 70% suprapur (Merck), HCl 30% normatom (Prolabo, Paris, France), ammonium hydroxide 25% suprapur (Merck) and deionized water.

Standards were prepared from a solution of SeO_2 (Titrisol, Merck) at 1 g/l and further diluted to contain 0, 20, 50, 100, 150 and 200 ng/ml selenium.

The derivatizing reagent was 98% 4-nitro-O-phenylenediamine, (NPD) (Janssen Chimica, Beerse, Belgium). A 4 g/l solution in 0.1 *M* HCl was used, purified by cyclohexane extraction (three times). This solution was kept at 4°C in darkness.

The reduction mixture was a solution containing 20 m*M* EDTA, 10 mg/l bromocresol purple and 7 *M* ammonium hydroxide.

The standard reference materials were freeze-dried bovine liver SRM 1577a, purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA) and lyophilized human reference urine Seronorm (batch 108) and serum Seronorm (batch 105) (Nycomed Pharma, Oslo, Norway). Seronorm urine or serum was prepared according to the company's directions.

Isotope dilution analysis

The basis of the IDMS method is to measure the concentration of an element by the change in its isotopic composition caused by the addition of an enriched isotope (spike) to the sample. The concept of isotope dilution has the important advantage that quantative recovery of the element is not required once spike equilibration occurs. The enriched ^{76}Se isotope is an ideal internal standard because it is chemically identical with natural selenium and would be affected to the same extent by any interferences. For example, losses during sample processing are the same for natural selenium and spiked ^{76}Se because they are indistinguishable chemically, so the determination involving the relative amounts (ratio $^{76}Se/^{80}Se$) is unaffected. A 20-mg amount of ^{76}Se (atomic abundance 98.5%) in elemental form was obtained from Commissariat à l'Energie Atomique (CEA, Saclay, France) and dissolved in concentrated nitric acid. The solution was made up to 100 ml with 0.1 *M* HCl and further diluted in deionized water to produce a 100 ng/ml spike solution. The concentration of this spike solution was accurately established by reverse isotope dilution using natural selenium as the internal standard, as described below.

Sample collection

Venous blood samples were drawn in 5-ml trace metal-free heparinized (heparin, sodium salt, Prolabo) polystyrene tubes (CML, Nemours, France). After centrifugation for 10 min at 1600 *g*, plasma was removed, kept in a polystyrene tube and frozen at −20°C until analysis. Red blood cells were washed three times with isotonic NaCl and then frozen. Urine samples were collected over 24 h in a polystyrene vial without additives. An aliquot was stored at −20°C until analysis.

Sample digestion

Biological fluids. The sample (0.5 ml of serum, 1 ml of urine or 0.5 ml of red blood cells) was spiked with 0.5 ml of ^{76}Se (100 ng/ml) and digested with 2 ml of HNO_3–$HClO_4$ (1:1, v/v). The tubes were kept at room temperature for 1–2 h, then heated at 150°C (30 min) and then at 180°C until white fumes of $HClO_4$ appeared. Heating was continued overnight or longer, until the digests were clear of $HClO_4$ fumes. This procedure is an adaptation of Lalonde's technique [13].

Reference materials. Sample volumes of Seronorm urine or serum were the same as for corresponding biological fluids. Weighed samples (0.02 g) of NIST bovine liver 1577a were spiked with 0.05 ml of the internal standard solution (100 ng/ml) and further digested as described previously.

Chelate formation

After the sample tubes had been cooled, 200 μl of 6 *M* HCl were added. This step was repeated five times to eliminate completely fumes of nitrogen dioxide. Between each addition, tubes were heated for 5 min at 150°C to convert selenium to the tetravalent state. The pH was adjusted by the addition of 2 ml of the reduction mixture. Tubes were heated at 150°C until a yellow colour appeared; if necessary, a few drops of 6 *M* HCl were added. After cooling, 5 ml of 0.1 *M* HCl were added. Tubes were left overnight in darkness at room temperature. Then 0.5 ml of derivatizing agent were added, and the tubes were put in a water-bath (40°C) for 30 min. After cooling, 5 ml of chloroform were added to extract the piazselenol complex (Se–NPD). After shaking for 5 min, this organic phase was removed, put in another tube and evaporated under nitrogen to dryness.

Just before injection into the GC–MS system, the dried residue was dissolved in a few microlitres of chloroform.

Gas chromatography–mass spectrometry

Selenite (Se^{4+}) reacted with 4-nitro-O-phenylenediamine in acid medium to give a stable piazselenol (Se–NPD) (Fig. 1). EI ionization was used to produce an ion cluster for the molecular ion Se–$NPD^{+\cdot}$, corresponding to the six selenium isotopes. EI spectra of natural Se–NDP and enriched ^{76}Se–NPD are shown in Fig. 2. Data were acquired by selected-ion monitoring (SIM) of the two major ions of each Se–NPD chelate (*m/z* 225, 229) and by integrating the corresponding ion current (Fig. 3).

RESULTS AND DISCUSSION

Digestion duration

Wet digestion with HNO_3 and $HClO_4$ for selenium analysis is commonly performed until the appearance of $HClO_4$ fumes. The final heating is at *ca.* 200°C (the $HClO_4$ boiling point) and lasts from 3.5 to 12 h (overnight) [13,14]. We tested four different periods of digestion (6, 12, 24 and 48 h). We found that, under our conditions of a final digestion temperature of 180°C and an acid mixture volume of 1 ml, to obtain a clear chromatogram of Se–NPD, 6 h of heating was insufficient. No difference was found between the

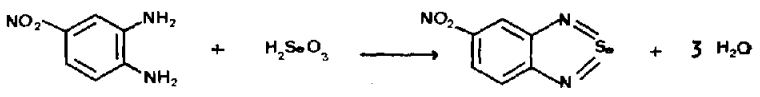

Fig. 1. Formation of nitropiazselenol (Se–NPD) from selenite and 4-nitro-O-phenylenediamine.

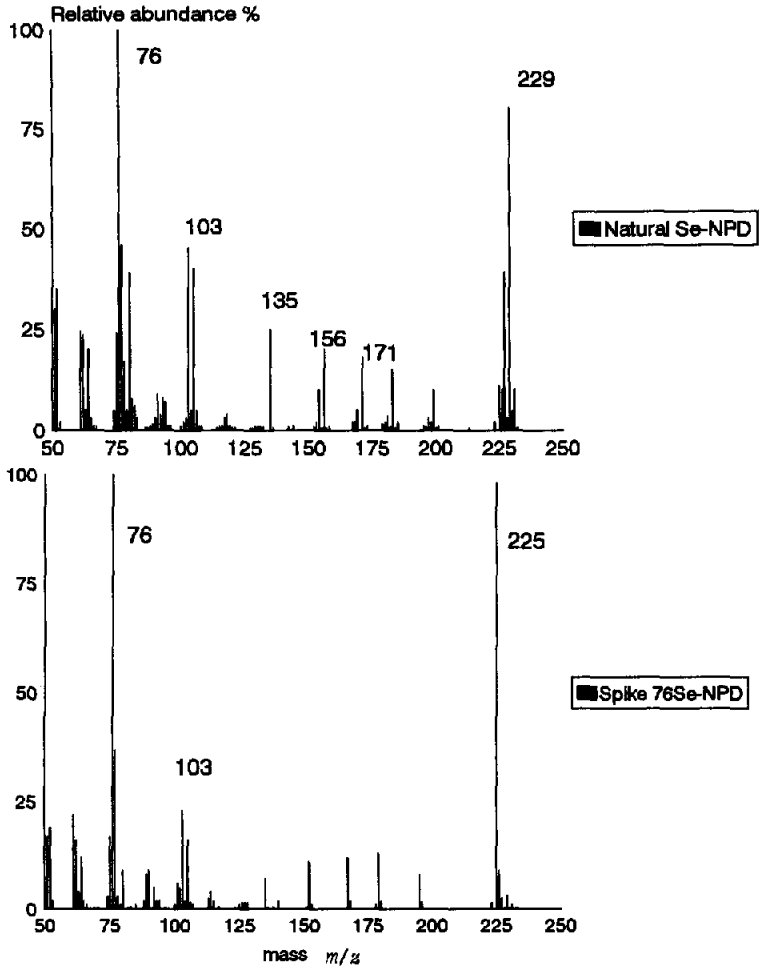

Fig. 2. EI mass spectra of natural Se–NPD and of enriched ^{76}Se–NPD.

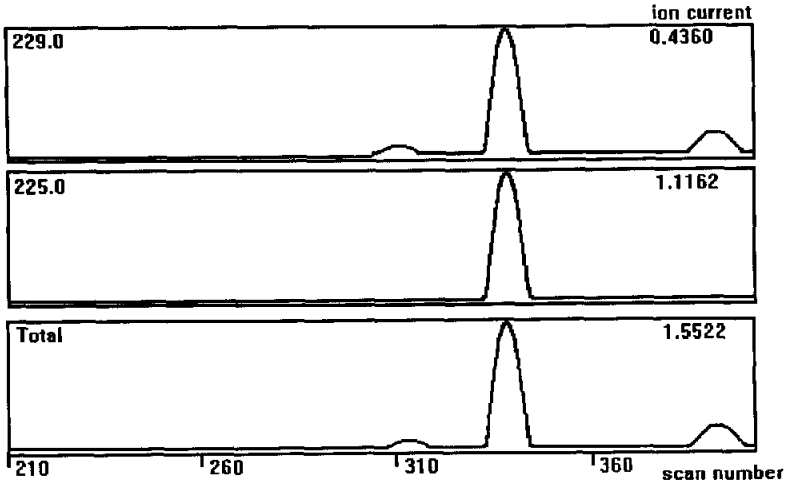

Fig. 3. Reconstructed chromatogram of m/z 225 and 229 of a 78 ng/ml serum sample.

TABLE I

INFLUENCE OF DIGESTION DURATION (12–48 h) ON RECOVERY OF SELENIUM ADDED TO HUMAN SERUM

Selenium added (ng/ml)	Selenium measured (ng/ml)			Recovery (%)		
	12 h	24 h	48 h	12 h	24 h	48 h
0.0	58	64	58	–	–	–
20.0	79	81	73	101.3	96.4	93.6
50.0	110	115	107	101.8	100.8	99.1
100.00	164	164	162	103.8	100.0	102.5

other periods for selenium determinations in a serum pool (Table I), so we decided to perform the digestion overnight.

Analytical performance

Graph calibration and linear zone. The isotopic compositions of Se–NPD in the natural standard and in the spike were determined experimentally by preparing each Se–NPD chelate and analysing them in the selected ion monitoring (SIM) mode (Table II). From standard solutions, a calibration curve was established from 0 to 200 ng/ml natural selenium. The internal standard concentration (100 ng/ml) was chosen so that the isotopic ratio 229/225 should be between 0 and 1.

As natural selenium possesses the two isotopes (^{80}Se and ^{76}Se) with a non-negligible abundance of the isotope 76, a non-linear curve is obtained. Only when this abundance is small in comparison with the amount of enriched ^{76}Se added it can be neglected. So between 0 and 100 ng/ml, the graph is linear. Above this limit, a correction (using the Pickup and McPherson equation) [15] must be applied to take isotopic contributions into account (Fig. 4). Using the same symbols, the Pickup and McPherson equation can be written as

$$x = [E/F\, y\, (R\mathrm{QI} - \mathrm{Qk})]/(\mathrm{Pk} - R\mathrm{PI})$$

where QI, Qk, PI and Pk represent the relative abundance at m/z 225 and 229 for the ^{76}Se spike and natural selenium, respectively, as shown in Fig. 4. The molecular mass ratio E/F is determined experimentally (equal to 1.013). From this value and the data in Table I, this equation can be expressed as

$$x = [y\, (91.48R - 0.48)]/(48.41 - 9.01R)$$

where x is the total amount (nanograms) of selenium in the original sample, y is the amount (nanograms) of ^{76}Se spike added, and R is the observed ratio. This equation is also used to establish the ^{76}Se concentration by reverse isotope dilution, as previously mentioned. Accurate known amounts of natural selenium (x) are spiked with known volumes of ^{76}Se solution, R is measured and then the equation is solved for ^{76}Se.

Detection limit. The detection limit in IDMS is usually defined as three times the standard deviation of the analytical blank [16,17]. Ten blanks (0.5 ml of deionized water plus 0.5 ml of ^{76}Se (100 ng/ml) were analysed in the same experiment as a set of standard solutions (0–100 ng/ml). The mean and standard deviation (S.D.) of the blank signal were calculated. The S.D. was converted into concentration by dividing this value

TABLE II

ISOTOPIC ABUNDANCE OF Se–NPD DERIVED FROM NATURAL SELENIUM AND SPIKED ^{76}Se

Selenium isotope	Se–NPD ion (m/z)	Isotope abundance in Se–NPD (%)	
		With ^{76}Se spike	With natural selenium
74	223	1.19	1.73
76	225	90.31	9.01
77	226	7.10	8.44
78	227	0.88	23.88
80	229	0.47	48.41
82	231	0.05	8.64

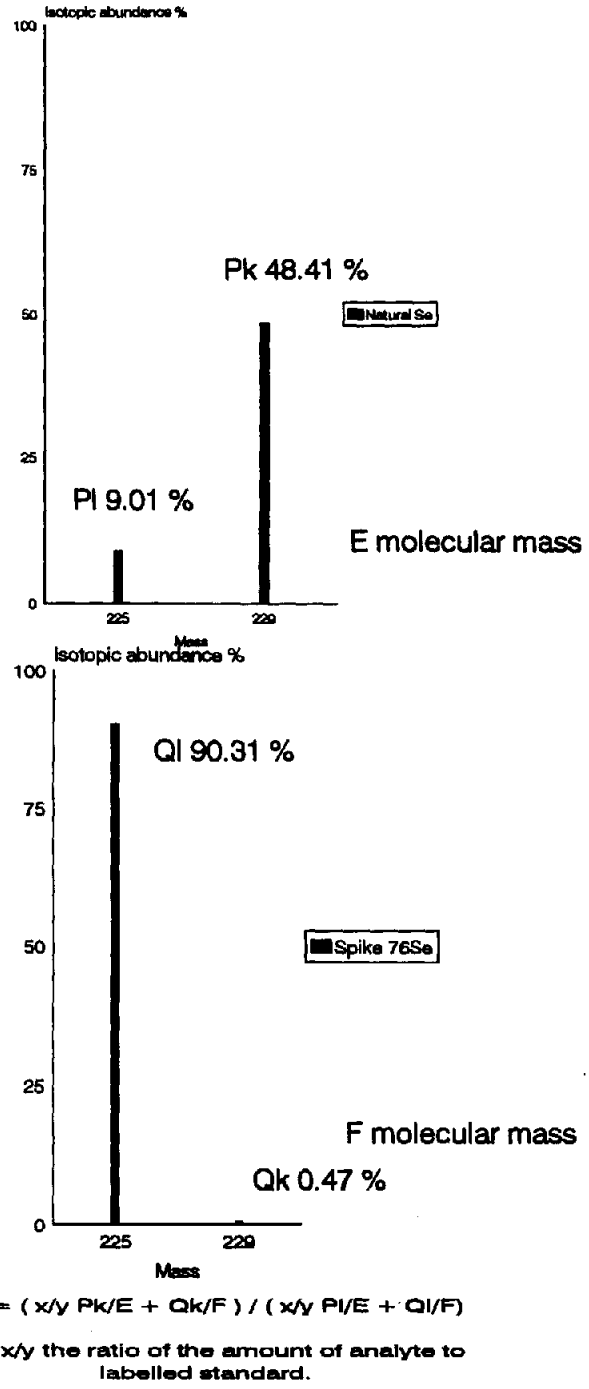

Fig. 4. Calculations using the Pickup and McPherson equation [15].

by the initial slope of the calibration curve according to the Valtec protocol [18]. A detection limit of 2.8 ng/ml was obtained, corresponding to a minimum detectable amount of 200 pg.

Our detection limit is comparable with those in the literature: Al-Attar and Nickless [5] found a 2.1 ng/ml detection limit for selenium determination by GC with electron-capture detection, and

TABLE III

WITHIN-ASSAY PRECISION

Sample	Concentration (mean ± S.D.) (ng/ml)	n	R.S.D. (%)
20 ng/ml aqueous standard	22.1 ± 1.4	10	6.3
50 ng/ml aqueous standard	49.3 ± 2.7	10	5.5
100 ng/ml aqueous standard	94.9 ± 3.3	9	3.5
Human serum[a]	69.6 ± 2.7	17	3.9
Seronorm urine Nycomed (batch 108)	41.5 ± 2.0	10	4.8

[a] A serum pool from Grenoble's blood transfusion centre was used.

Swanson *et al.* [16] reported a 6-pmol detection limit for selenium analysis by GC–MS. This type of detection limit in pure solution allows comparison with the other methods of selenium determinations but is not useful for routine analysis. A preferable detection limit is obtained by analysing a real sample. For example, ten samples of Seronorm urine (batch 108) were analysed the same day. The selenium concentration was 41.5 ± 2.0 ng/ml, which indicated a practical detection limit (three times S.D.) for biological samples of 6 ng/ml.

Precision. The within-assay (repeatability) and between-assay (reproducibility) precisions were evaluated for different aqueous standards of 20, 50 and 100 ng/ml, and for different biological fluids (serum and urine). The repeatability was evaluated from the data obtained by replicate injections on a particular day. The reproducibility was calculated from the mean values obtained on different days. The data are shown in Tables III and IV.

These results are in agreement with those previously reported [10,12,19].

Accuracy. The accuracy of the method was assessed by performing a recovery test and by assaying a certified standard reference material. The recovery of known amounts of standard solutions (20, 50 and 100 ng/ml) added to human serum was evaluated. For each addition of a standard, four samples were prepared and analysed. The recovery varied from 101.8 to 104.5% (Table V).

The selenium content of a reference material (NIST bovine liver 1577a) was determined. Ten samples of 0.02 g of bovine liver 1577a were analysed and showed a mean ± S.D. value at 0.69 ± 0.04 μg/g (certified value = 0.71 ± 0.07 μg/g).

TABLE IV

BETWEEN-ASSAY PRECISION

Sample	Concentration (mean ± S.D.) (ng/ml)	n	R.S.D. (%)
20 ng/ml aqueous standard	17.4 ± 2.9	11	16.6
50 ng/ml aqueous standard	50.9 ± 2.4	11	4.7
100 ng/ml aqueous standard	97.4 ± 5.0	11	5.1
Seronorm serum Nycomed (batch 105)	73.0 ± 4.7	21	6.4
Seronorm urine Nycomed (batch 108)	42.2 ± 2.2	3	5.2
NIST bovine liver 1577a (μg/g)	0.68 ± 0.05	4	7.3

TABLE V

RECOVERY OF SELENIUM ADDED TO HUMAN SERUM (n = 4)

Added (ng/ml)	Measured (ng/ml)	Recovery (%)
0.0	62	–
20.0	85	103.6
50.0	117	104.5
100.0	165	101.8

Thus our value agreed with the assigned value within the uncertainty of the certification.

Reference values in human biological fluids

Serum. These measurements were taken in thirty healthy subjects working in the laboratory and living at Grenoble. The results show a mean value of 66 ng/ml with an S.D. of 13 ng/ml. Our values are a little lower than those in the literature, unless only French population values are considered (Table VI).

Urine. Nine samples of 24-h urine obtained from laboratory staff were analysed. A mean (± S.D.) value of 23.4 ± 10.2 μg per 24 h (or 15.7 ± 5.6 ng/ml) was determined. Most reported values are in the range 20–50 ng/ml.

Red blood cells. Ten samples of erythrocytes were taken from the same staff and analysed. A mean (± S.D.) value of 105 ± 15 ng/ml was established from this small group. Values in red blood cells of children were also determined [29]. For a control group (34 children aged 7 months to 19 years), a value of 76 ± 13 ng/ml was determined, and for another control group (22 children aged 4–19 years) a very similar value was obtained (73 ± 12 ng/ml). These values are a little lower than those in the literature, but again geographical criteria may be involved (Table VII).

CONCLUSION

Selenium determination by isotope dilution GC–MS seems a convenient technique, being extremely versatile for the analysis of total selenium in various matrices. This method is a valuable tool for selenium measurements, given the availability of benchtop instruments of moderate cost. Using this technique, we perform *in vivo* studies of selenium metabolism [11].

TABLE VI

COMPARISON OF OUR RESULTS WITH LITERATURE VALUES FOR SELENIUM IN SERUM OR PLASMA

n	Sex[a]	Concentration (mean ± S.D.) (ng/ml)	Age range (years)	Matrix[b]	Country	Ref.
99	42M, 57F	80 ± 14	–	S	Germany	20
163	110M, 53F	97 ± 12	19–58	P	Belgium	21
206	106M, 100F	104 ± 14	11–60	P	Georgia	22
36	36F	79 ± 11	26–50	S	Yougoslavia	23
50	15M, 35F	80 ± 30	20–65	S	France	24
75	29M, 46F	95 ± 4[c]	<65	P	England	25
145	69M, 76F	84 ± 12[c]	20–79	P	Belgium	26
125	75M, 50F	72 ± 11[c]	20–59	S or P	France	27
100	50M, 50F	90 ± 18[c]	20–60	S	Switzerland	28
30	7M, 23F	66 ± 13	25–60	P	France	Our results

[a] M = male; F = female.

[b] P = plasma; S = serum.

[c] Results given in μmol/l are expressed here in ng/ml.

TABLE VII

COMPARISON OF OUR RESULTS WITH LITERATURE VALUES FOR SELENIUM IN RED BLOOD CELLS FROM ADULTS AND CHILDREN

n	Sex[a]	Concentration (mean ± S.D.) (ng/ml)	Age range (years)	Country	Ref.
Adults					
160	108M, 52F	161 ± 31	19–58	Belgium	21
206	106M, 100F	158 ± 29	11–60	Georgia	22
50	15M, 35F	105 ± 22	20–65	France	24
75	29M, 46F	126 ± 5[b]	<65	England	25
145	69M, 76F	131 ± 29[b]	20–79	Belgium	26
10	3M, 7F	105 ± 15	24–55	France	Our results
Children					
19	11M, 8F	80.6 ± 22 (ng/g)	0–6	Sweden	30
45	25M, 20F	84.4 ± 15.1 (ng/g)	7–17	Sweden	30
21	–	100 ± 30	0.25–2	Poland	31
34	15M, 19F	76 ± 13	0.6–19	France	Our results
22	11M, 11F	73 ± 12	4–19	France	Our results

[a] M = male; F = female.

[b] Results given in μmol/l are expressed here in ng/ml.

ACKNOWLEDGEMENTS

We thank Nicole Belin, Valérie Mathevet and Daniel Ruffieux for their expert technical skill.

REFERENCES

1 G. Tölg, in P. Brätter and P. Schramel (Editors), *Proceedings of the Third International Workshop, Neuherberg, April 1984*, Walter de Gruyter, Berlin, New York, 1984, p. 95.
2 R. C. Ewan, C. A. Bauman and A. L. Pope, *J. Agric. Food Chem.*, 16 (1968) 212.
3 Analytical Methods Committee, *Analyst*, 104 (1979) 778.
4 T. P. McCarthy, B. Brodie, J. A. Milner and R. F. Bevill, *J. Chromatogr.*, 225 (1981) 9.
5 A. F. Al-Attar and G. Nickless, *Analyst*, 115 (1990) 1441.
6 B. Welz, M. Melcher and G. Schlemmer, *Fresenius Z. Anal. Chem.*, 316 (1983) 271.
7 B. Welz and M. Melcher, *Anal. Chim. Acta*, 165 (1984) 131.
8 N. Slavin and D. C. Manning, *Prog. Anal. At. Spectrosc.*, 5 (1982) 243.
9 S. A. Lewis, N. S. Hardison and C. Veillon, *Anal. Chem.*, 58 (1986) 1272.
10 D. C. Reamer and C. A. Veillon, *Anal. Chem.*, 53 (1981) 2166.
11 V. Ducros, A. Favier and M. Guigues, *J. Trace Elem. Electrolytes Health Dis.*, 5 (1991) 145.
12 D. C. Reamer and C. A. Veillon, *J. Nutr.*, 113 (1983) 786.
13 L. Lalonde, Y. Jean, K. D. Roberts, A. Chapdelaine and G. Bleau, *Clin. Chem.*, 28 (1982) 172.
14 T. S. Koh and T. H. Benson, *J. Assoc. Off. Anal. Chem.*, 66 (1983) 918.
15 J. F. Pickup and K. McPherson, *Anal. Chem.*, 48 (1976) 1885.
16 C. A. Swanson, B. H. Patterson, O. A. Levander, C. Veillon, P. R. Taylor, K. Helzlsouer, P. A. McAdam and L. A. Zech, *Am. J. Clin. Nutr.*, 54 (1991) 917.
17 K. G. Heumann, in F. Adams, R. Gijvels and R. Van Grieken (Editors), *Inorganic Mass Spectometry*, Wiley, Chichester, 1988, Ch. 7, p. 301.
18 M. Baud, R. Cohen, G. Dumont, M. Mercier, C. Naudin and A. Vassault, *ISB*, 15 (1989) 157.
19 A. R. Mangels, P. B. Moser-Veillon, K. Y. Patterson and C. Veillon, *Am. J. Clin. Nutr.*, 52 (1990) 621.
20 O. Oster and W. Prellwitz, *Clin. Chim. Acta*, 124 (1982) 277.
21 M. Verlinden, M. Van Sprundel, J. C. Van Der Auwera and W. J. Eylenbosch, *Biol. Trace Elem. Res.*, 5 (1983) 91.
22 P. A. McAdam, D. K. Smith, T. T. Kushe, R. J. Teague, D. S. Feldman, C. Hames and E. B. Feldman, *Nutr. Res.*, Suppl. 1 (1985) S-144.
23 D. Beker, H. Krsnjavi and Z. Petrinec, *Trace Elem. Med.*, 8 (1991) 128.

24 M. Simonoff, C. Conri, B. Fleury, B. Berdeu, P. Moretto, G. Ducloux and Y. Llabador, *Trace Elem. Med.*, 5 (1988) 64.
25 D. Campbell, V. W. Bunker, A. J. Thomas and B. E. Clayton, *Br. J. Nutr.*, 62 (1989) 221.
26 J. Nève, F. Vertongen, A. Peretz and Y. A. Carpentier, *Ann. Biol. Clin.*, 47 (1989) 138.
27 F. Dubois, A. Teby, F. Belleville, P. Nabet and P. Paysant, *Ann. Biol. Clin.*, 48 (1990) 28.
28 R. Forrer, K. Gautschi and H. Lutz, *J. Trace Elem. Electrolytes Health Dis.*, 5 (1991) 101.
29 B. C. Wilke, *Thesis*, University of Nancy I, Nancy, 1989.
30 G. Anneren, M. Gebre-Medhin, K. H. Gustavson and L. O. Plantin, *Acta Paediatr. Scand.*, 74 (1985) 508.
31 B. A. Zachara, W. Wasowicz, J. Gromadzinska, M. Sklodowska and B. Cabalska, *Biomed. Biochim. Acta*, 46 (1987) S209.