Journal of Chromatography, 136 (1977) 73–83

CHROM. 9877

DETERMINATION OF SELENIUM IN BIOLOGICAL SAMPLES BY GAS–LIQUID CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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(Received December 10th, 1976)

SUMMARY

Selenium can be determined quantitatively in biological samples after nitric acid–magnesium nitrate digestion and formation of 5-nitropiazselenole, by extraction into toluene for gas–liquid chromatography with electron-capture detection. The method is suitable for the determination of selenium in orchard leaves, bovine liver and human placenta, hair, blood and urine.

INTRODUCTION

Primary sources of selenium in the environment include volcanic emanations, the roasting of metallic ores, the combustion of fossil fuels and natural weathering of selenium-containing minerals. The crustal abundance of selenium is estimated to be 0.05 ppm[1]. Coal and petroleum from the U.S.A. average 3.0 ppm and 0.2 ppm of selenium, respectively[1,2]. Selenium is also present in significant amounts in tobacco (0.3–0.4 ppm)[3]. Plants show a widely varying ability to accumulate selenium and in certain cases are an important source of selenium in higher animals[1,4].

The commercial uses of selenium include the manufacture of coloured glass, ceramics, rubber products and, within controlled limits, as an additive to alloys to improve their machinability and electrical properties. Pure selenium is used in the production of photometers, rectifiers and semiconductors. Organoselenium compounds are used as bactericides, fugicides and herbicides in agriculture[3,5].

Schroeder *et al.*[3], in summarizing the effects of selenium in mammals, concluded that excess of selenium is teratogenic, hepatoxic and neurotoxic, retards growth and causes muscular degeneration and infertility. Selenium has long been recognised as the causal agent of the "blind staggers" or "alkali disease" in lambs, calves and foals; impaired hepatic function in pigs, stunted growth and reduced fertility in sheep and exudative diathesis in poultry[4,6]. Of particular interest to this study are reports of the teratogenic effects of selenium in mammals[3–6], chicks[7,8] and fish[9].

Biochemically, the role of selenium is closely related to that of vitamin E[10], and recently glutathione peroxidase of rat erthythrocytes has been identified as a selenoprotein and enzyme acting to prevent oxidative haemolysis of the cell membrane[11]. Selenium also seems to be important in protecting mammals from the toxic

action of arsenic, cadmium and mercury[5,12–15]. The biological and environmental chemistry of selenium has been reviewed previously[1,3,4,16–19].

Recommended methods of selenium analysis include gravimetry, spectrophotometry, fluorimetry, X-ray fluorescense, neutron-activation analysis and atomic-absorption spectrometry. Gravimetric[20], spectrophotometric[21,22] and X-ray fluorescence[23] techniques are insufficiently sensitive for biological analysis. Neutron-activation analysis has the required sensitivity, but is slow and not available to many laboratories[24,25]. The determination of selenium by fluorescence with 2,3-diaminonaphthalene has been well evaluated for biological work[26–29] but suffers from interference by co-extractants originating from the samples and has poor precision at low levels[3]. The sensitivity of atomic absorption for selenium is limited by flame absorption in the region of the resonance line (at 196 nm) and by scattered light from inorganic ions at this short wavelength[30]. Sensitivity can be increased by the use of carbon furnace techniques[31], although the preferred method is the chemical reduction of selenium to hydrogen selenide, which is then aspirated into an argon–hydrogen–entrained air flame[32–34] or quartz tube[35] or determined by atomic-fluorescence spectrometry[36]. The hydrogen selenide generation technique has sufficient sensitivity for biological work, but recent reports have indicated severe interference in the reduction step by several common cations[37,38]. Cathodic stripping voltammetry is also much affected by cation interference, requiring either removal of selenium from the digest by hydride generation or column chromatography to remove the interfering cations[39,40]. This considerably adds to the time and difficulty of the analysis.

The procedure adopted in this work is based on the reaction between selenium(IV) and *o*-phenylenediamines in acidic solution to form piazselenoles (Fig. 1), which can be extracted into organic solvents. This reaction is well established and forms the basis of the spectrophotometric and fluorescence assay for selenium. By subjecting the organic extract to gas–liquid chromatography (GLC), it was hoped to overcome interference from organic co-extractants, which is the principle limitation of the fluorescence method. Also, by using substituted phenylenediamines containing a group that can act as an electrophore, it was hoped to take advantage of the very high sensitivity and selectivity of the electron-capture detector (ECD). Reports of the GLC of naphthylpiazselenole[41], 5-chloropiazselenole[42,43], 5,6-dichloropiazselenole[44] and 5-nitropiazselenole[45–51] have been made.

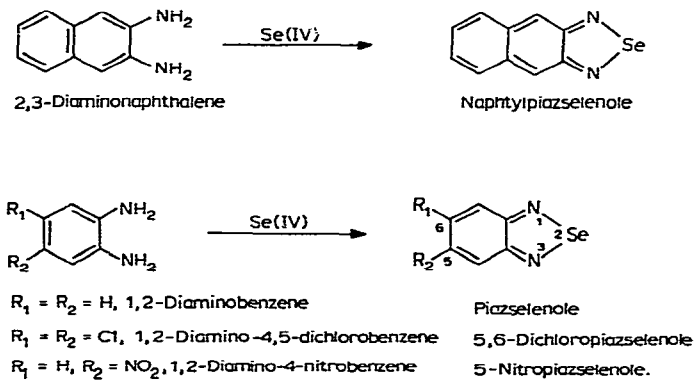

Fig. 1. Structure and nomenclature for piazselenoles.

EXPERIMENTAL

All glass apparatus was washed with water, detergent solution, distilled water and acetone before oven drying at 60°. De-ionised water was distilled in an all-glass apparatus.

Aristar hydrochloric, nitric, sulphuric and perchloric acids, AnalaR magnesium nitrate hexahydrate, urea, toluene and black selenium and reagent-grade selenium dioxide and hydrogen peroxide (30%) were obtained from BDH (Poole, Great Britain). Toluene was used as supplied, without further purification.

Reagent-grade 1,2-diaminobenzene, 1,2-diamino-4-nitrobenzene, 1,2-diamino-4,5-dichlorobenzene and 2,3-diaminonaphthalene dihydrochloride were obtained from Aldrich (Gillingham, Great Britain). The 2,3-diaminonaphthalene dihydrochloride was used without further purification and 1,2-diamino-4,5-dichlorobenzene was purified by the method of Stijve and Cardinale[44]. The 1,2-diaminobenzene was purified as for 1,2-diamino-4-nitrobenzene, detailed below.

The 1,2-diamino-4-nitrobenzene was dissolved in boiling 2 *M* hydrochloric acid and activated charcoal added. The solution was filtered while hot, an equal volume of concentrated hydrochloric acid was added to the filtrate and the solution cooled in an ice–salt bath. The crystals of the dihydrochloride were collected on a glass-fibre filter-paper supported on a glass sinter, washed with concentrated hydrochloric acid and dried by suction.

For the determination of selenium in biological samples, the diamine salts were prepared as a 1% (w/v) solution in 10% (v/v) hydrochloric acid. Freshly prepared solutions were allowed to stand for 1 h at room temperature and then extracted consecutively with toluene until analysis of the toluene extract by GLC–ECD gave a zero blank at the sensitivity setting used for the analysis of samples. The 1,2-diamino-4-nitrobenzene dihydrochloride solution, prepared as above, was stable for at least 2 weeks if stored in a refrigerator.

Whole-blood samples were supplied in sealed containers and stored at 3° until used. Samples that showed signs of precipitation were discarded.

Human hair samples were analyzed without prior treatment.

Urine samples were obtained from male volunteers and freeze-dried prior to analysis.

Fresh human placentae were stored in a deep-freeze until analyzed. Small samples were dissected from the whole and excess of blood was removed with paper tissues prior to weighing. Alternatively, whole placentae were defrosted, rinsed with distilled water to remove excess of blood, homogenized in glass beakers with a stainless-steel Turrax tissue grinder and freeze-dried. The dried material was commuted by hand using a glass mortar and pestle to pass through a 60-mesh sieve. More recently, a micro hammer mill (Glen Creston, London, Great Britain) has been used successfully to grind freeze-dried placentae.

GLC was carried out with a Pye-Unicam GCV instrument using either flame-ionization detectors (FID) or a constant-current ECD. The ECD was operated with a detector current of 7 nA and a detector oven temperature of 350°. Extracts were analyzed on a 1.5 ft. × 0.25 in. column of 7% OV-225 on Supasorb (AW-HMDS, 100–120 mesh) at a column temperature of 210° and a nitrogen flow-rate of 35 ml/min. The retention time of 5-nitropiazselenole was 5.06 min.

Preparation of piazselenole standards

For the synthesis of piazselenole standards, equimolar amounts of the diamine hydrochloride salt and selenium dioxide were mixed together in 0.2 *M* hydrochloric acid and the pH adjusted to the optimal value (piazselenole, 1.5–2.5; naphthylpiazselenole, 2.0; 5,6-dichloropiazselenole, 1.5; 5-nitropiazselenole, 0–2) with dilute ammonia solution. The mixtures were allowed to stand at room temperature for approximately 1 h, the solid piazselenoles filtered off through a glass-fibre filter-paper and the damp solids recrystallized from 95% ethanol–acetone. The purity of the standards was established by thin-layer chromatography, GLC, UV spectroscopy and mass spectrometry (Table I). Melting-point data (measured in air) were imprecise, as the samples showed evidence of decomposition in the region of the melting-point.

Sample digestion

Accurately weighed samples (0.1–1.0 g) were digested with a mixture of 10 ml of concentrated nitric acid and 4.0 g of magnesium nitrate hexahydrate in 100-ml Pyrex glass beakers on a low-temperature hot-plate. A typical digestion time was 2–3 h with an average solution temperature of 80–90°. The solutions were evaporated to dryness and the hot-plate turned to its maximum setting until the production of brown fumes of nitrogen dioxide ceased. The digestion was completed by heating in a muffle furnace at 500° for 30 min. The samples were allowed to cool to room temperature, 8 ml of concentrated hydrochloric acid added, the beakers covered with watch-glasses and heated on a water-bath (100°) for 10–15 min. The acid digest was transferred to a 100-ml stoppered erlenmeyer flask with the aid of 10 ml of 20% (w/v) urea solution. The solutions were allowed to cool to room temperature.

Formation of the selenium complex

To the acid digest solution prepared as above were added 2.0 ml of the 1% (w/v) 1,2-diamino-4-nitrobenzene dihydrochloride solution, and the mixture was allowed to stand for 4 h at room temperature. The solution was transferred to a 50-ml separating funnel with the aid of 5 ml of distilled water and extracted with 5 ml of toluene by shaking for 20 sec and allowing the phases to separate for 10 min. An aliquot of 2–5 μl of the organic phase was injected into the gas chromatograph.

Calibration graph

A selenium standard solution (1000 μg/ml) was prepared by dissolving 0.1 g of black selenium, with heating, in 10 ml of concentrated nitric acid. The solution was allowed to cool and diluted to 100 ml with distilled water. Working solutions were prepared on the day of analysis by dilution of the master standard with 5% (v/v) nitric acid.

A calibration graph was prepared by adding 0.05–2.0 μg of selenium to the nitric acid–magnesium nitrate digestion mixture, which was then treated in an identical manner to the biological samples. The final extraction of the 5-nitropiazselenole was into 5 ml of toluene and 2 μl of the solution were injected into the gas chromatograph. The calibration graph was linear over the range studied.

RESULTS AND DISCUSSION

Selection of selenium reagent

The ECD is a very sensitive and selective detector for those compounds which can capture thermal electrons[52]. As can be seen from Table I, the piazselenole structure is naturally electron capturing, but lacks sufficient sensitivity for the determination of trace levels of selenium found in biological samples. The introduction of a second electrophore, such as the chloro or nitro group, into the molecule, considerably improves the sensitivity, allowing picogram (10^{-12} g) amounts of selenium to be detected. On this basis, the 5,6-dichloropiazselenole and 5-nitropiazselenole were selected for further study. An investigation into the stability of the respective diamine hydrochloride reagents in acidic solution indicated that the 1,2-diamino-4,5-dichlorobenzene reagent was unstable and produced several artefact peaks on the chromatogram. Also, the optimal pH for the formation of the 5,6-dichloropiazselenole differed from that of the acid digest, which meant that some neutralization was required, adding to the analysis time and requiring the use of further reagents with the possibility of increasing the selenium blank. The formation of the 5-nitropiazselenole complex was not influenced by the above problems. Our initial objection to its use centred around the poor peak shape of the complex when subjected to GLC. Peak tailing was observed on non-polar phases such as OV-101, but attempts to use more polar polyester phases were inconvenient owing to interference in the operation of the ECD by column bleeding. Eventually, we found that the 5-nitropiazselenole could be chromatographed as symmetrical peaks on the polar silicone oil OV-225. This phase was compatible with the operation of the ECD as it has very good bleed characteristics after conditioning. Subsequently, we have found the silicone OV-225 phase to be very useful for the GLC of nitro-aromatic compounds in general.

The response of the ECD to various compounds is often markedly temperature dependent[53,54] and the optimal detector temperature for the determination of a particular compound can be ascertained from a plot of ln $AT^{3/2}$ *versus* $1/T$, where A = peak area for a constant mass of piazselenole and T = absolute temperature of the detector oven. Also, plots of this type can provide a valuable insight into the mech-

TABLE I

PHYSICAL PROPERTIES OF PIAZSELENOLES

1.5-ft. column of 7% OV-225, nitrogen flow-rate 35 ml/min. ECD, detector current 10 nA; range, ×8; detector oven, 350°. The least detectable amount is defined as the smallest amount of selenium required to produce a peak which was twice the average base-line noise.

Selenium complex	*UV spectra (toluene)*		*Gas chromatography*		*Least detectable amount of Se (g)*
	$\lambda_{max.}$	$\varepsilon_{max.}$	*Column temperature (°C)*	*Retention time (min)*	
Piazselenole	335	$1.5 \cdot 10^4$	180	1.59	$90.0 \cdot 10^{-12}$
Naphthylpiazselenole	379*	$1.97 \cdot 10^4$	230	4.69	$20.0 \cdot 10^{-12}$
5,6-Dichloropiazselenole	349	$2.1 \cdot 10^4$	220	1.78	$1.0 \cdot 10^{-12}$
5-Nitropiazselenole	349	$1.57 \cdot 10^4$	220	3.94	$1.0 \cdot 10^{-12}$

* Side bands at 360 and 368 nm.

anism of the electron-capture process[53]. For the four piazselenoles studied here (Fig. 2), the nature of the slope indicates that a dissociative type of mechanism is operating. The information gathered in this study is insufficient to propose a molecular basis for the mechanism of electron capture without some knowledge of the products generated. Although the evidence confirms that bond breaking is the determinative process, it is difficult to visualise the possible products produced from a molecule such as piazselenole. A proposal of the mechanism must await the identification of the products by either plasma chromatography[55] or API mass spectrometry[56].

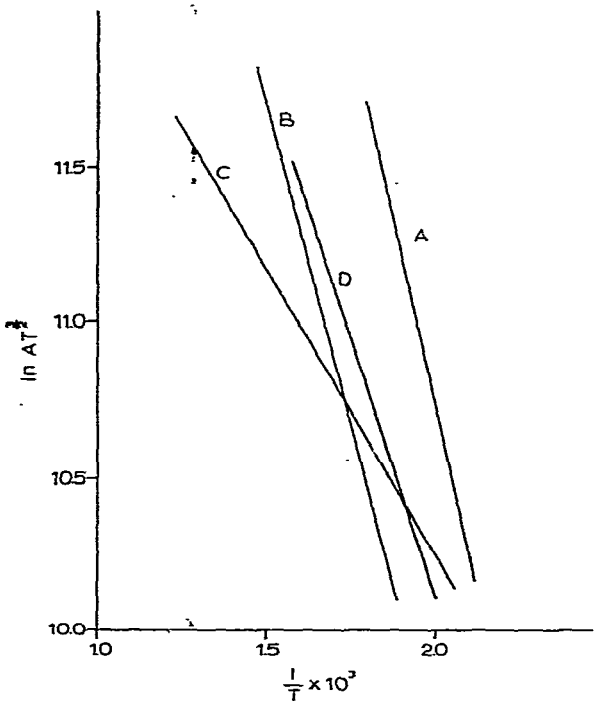

Fig. 2. Plot of ln $AT^{3/2}$ *versus* $1/T$ for piazselenole (A), naphthylpiazselenole (B), 5-nitropiazselenole (C) and 5,6-dichloropiazselenole (D). The negative slope of the lines indicates a dissociative mechanism of electron capture.

For all of the piazselenoles studied here, the maximal detector response was obtained at the highest detector oven temperature. The difference between the detection limits quoted here and those elsewhere[41–50] is probably due partly to a failure to appreciate the importance of detector temperature in influencing detector response.

Selection of digestion technique

Many methods have been described for the digestion of biological samples for selenium analysis and several reviews are available[57–59]. In this study, we have evaluated wet acid digestion techniques for sample dissolution. The advantages of this method are its simplicity, the availability of the necessary reagents in a high de-

gree of purity, suitability for batch processing, high sample capacity and the avoidance of excessive losses of selenium by volatilization. Further points of note are:

(1) The most popular methods involve digestion with nitric acid–perchloric acid–sulphuric acid–hydrogen peroxide mixtures. Recoveries when nitric and sulphuric acids are used together are low unless perchloric acid is also present.

(2) Concentrated hydrochloric acid at reflux should be avoided as selenium can be lost as volatile chloride adducts (*e.g.*, $SeOCl_2$, $SeO_2 \cdot 2HCl$).

(3) Evaporation of acid digests until visible sample charring occurs should be avoided as this leads to a considerable loss of selenium.

(4) Selenium in biological samples exists in several different oxidation states and the use of strong oxidizing acids can give a substantial conversion of selenium(IV) to selenium(VI). For the formation of piazselenoles it is necessary that all the selenium be present in the selenium(IV) oxidation state.

(5) Reduction of selenium(VI) to selenium(IV) is most conveniently carried out by heating in concentrated hydrochloric acid below its boiling point.

With the above framework in mind, several wet acid digestion procedures were evaluated using a laboratory standard reference placental sample*. Shimoishi[50] and Stijve and Cardinale[44] have used concentrated nitric acid to digest biological samples in which the selenium content was determined by GLC of the extracted piazselenole. GLC of the extract produces a chromatogram containing several peaks and interference with the piazselenole peak was found on several GLC columns. Stijve and Cardinale[44] overcame this problem by sample clean-up using Florisil column chromatography, but this considerably adds to the time and difficulty of the assay. An investigation into the origins of the multiple peaks in the chromatogram indicated that many of those in the region of the piazselenole peak were due to the action of nitric acid or its decomposition products on the phenylenediamine reagent. Reduction of the volume of nitric acid in the sample digest to 0.2–0.3 ml, addition of urea solution and prior extraction of the acid digest with toluene before formation of the piazselenole as described by Shimoishi[50] did not completely overcome the problem. A typical result for a placental sample is shown in Fig. 3a. A great deal of care was required in evaporating the nitric acid to a small volume as the sample easily charred with a considerable loss of selenium.

Although nitric acid was an excellent reagent for sample digestion, its presence during the formation of the piazselenole complex should be avoided. A possible solution to this problem is the use of mixed acid media in which a second acid of high boiling point is added to the nitric acid and the temperature of the acid mixture raised until all the nitric acid has distilled off. The use of phosphoric acid for this purpose was unsuccessful, as phosphoric acid was a poor choice for the solubilization of placental samples and charring rapidly followed the removal of nitric acid[51]. Nitric acid–perchloric acid–sulphuric acid–hydrogen peroxide mixtures have been used successfully for the digestion of biological samples, in conjunction with the fluorescence assay of naphthylpiazselenole. We adopted the method described by Ihnat[28] with the addition of an extra step in which urea solution was used instead of EDTA and the resultant digest was extracted with toluene prior to piazselenole formation.

* Refers to pooled samples of freeze-dried human placentae that have been ground to pass a 60-mesh sieve and intimately mixed.

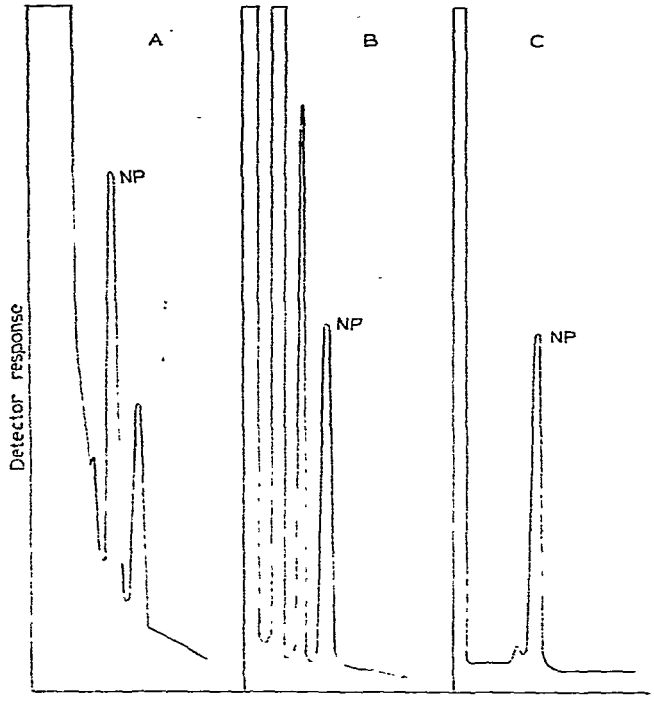

Fig. 3. Determination of selenium as 5-nitropiazselenole (NP) by GLC–ECD in approximately 0.1 g of standard reference placental material after digestion with (a) nitric acid, (b) nitric acid–perchloric acid–sulphuric acid–hydrogen peroxide and (c) nitric acid–magnesium nitrate.

In this procedure, the nitric acid is distilled off in the presence of perchloric acid, which is able to maintain the digest in solution without charring or loss of selenium. GLC analysis of the 5-nitropiazselenole extract produced several peaks on the chromatogram and interference with the 5-nitropiazselenole peak was severe when large samples (>100 mg) were digested. Samples up to 100 mg could be digested successfully (Fig. 3b) but this is a considerable limitation on the technique for general use in trace analysis.

The most convenient method of sample digestion is the nitric acid–magnesium nitrate procedure, described in full under Experimental. The method given here is based on that described by Holak[60] and was modified to take account of the special requirement of the GLC assay. Analysis of the sample digest by GLC after the formation of the 5-nitropiazselenole indicated the presence of two peaks only, an impurity peak (R_t = 3.80 min) and 5-nitropiazselenole (R_t = 5.06 min). An investigation of the reagent blank indicated that the source of the impurity peak was due partly to the reagents used. The intensity of this peak was sufficient to interfere in the quantitative determination of the piazselenole. Fortunately, the impurity peak can be masked by the addition of urea to the acid digest prior to the formation of the 5-nitropiazselenole. In this way a single peak was obtained on the chromatogram free from any interference (Fig. 3c).

In early studies with this digestion technique, some variability in the recovery

of selenium was observed. This was shown to be due to overheating the digest mixture when it had reached a small volume, resulting in a violent expulsion of vapour from the flasks or beakers which could not be contained by the use of a micro Snyder column. This feature is eliminated by carrying out the digestion at a low temperature until all the nitric acid has evaporated. Heating in a muffle furnace completes the digestion and eliminates organic interferents from the chromatogram.

Recovery and precision

To assess if loss of selenium occurred during the digestion procedure and to establish whether the digestion technique was sufficiently vigorous to free all organically bound selenium, three standard reference materials were analyzed. The results are given in Table II, and for the two certified samples the agreement between the experimentally found and the certified values is excellent. Standard additions of selenium (1.0 μg) to either the placental material or the bovine liver standard consistently gave recoveries of 95–105%. The data presented in Table II also indicate that the method has acceptable precision for the analysis of biological samples.

TABLE II

ANALYSIS OF STANDARD REFERENCE MATERIAL FOR SELENIUM

Sample	*Number of samples*	*Weight taken (g)*	*Certified value (μg/g)*	*Experimental value (μg/g)*
Placental reference sample	10	0.2–1.0	—	1.9 ± 0.1
Bovine liver SRM (NBS 1577)	10	0.2–0.5	1.1 ± 0.1	1.1 ± 0.1
Orchard leaves SRM (NBS 1571)	10	0.2–1.0	0.08 ± 0.01	0.078 ± 0.005

Applications

The analytical procedure outlined has been used to determine the concentration of selenium in human placenta, hair, blood and urine. The results obtained are summarized in Table III. The general agreement with literature values is good.

TABLE III

CONCENTRATION OF SELENIUM IN BIOLOGICAL SAMPLES

Sample	*No. of samples*	*Concentration of selenium (μg/g)*	*Range (μg/g)*	*Literature values (μg/g)*	*Reference*
Freeze-dried human placenta*	15	2.0 ± 0.1	1.5 –2.6	1.7 ± 0.6	61
Human placenta (fresh weight)	50	0.34 ± 0.01	0.23 –0.48	0.37 ± 0.09	62
				0.18 ± 0.007	63
Human hair	12	1.47 ± 0.7	0.1 –7.6	0.57 ± 0.038	3
Human blood	20	0.267 ± 0.027	0.11 –0.43	0.32	23
				0.206	64
Human urine**	5	0.005 ± 0.002	0.002–0.011	—	—

* Corrected for moisture content.

** Values expressed as $\mu g \cdot ml^{-1}$.

Of particular interest to this laboratory is the use of human placenta as a monitor of environmental pollution and, because of its unique relationship to the foetus, a monitor of foetal exposure[65]. The data obtained so far indicate excellent agreement between the two studies in the U.S.A.[61,62] and our data obtained for samples from the Birmingham area. The placenta selected for analysis were obtained from women who had a normal birth. The extension of this study to the determination of selenium in placenta obtained from mothers in which a foetal malformation has occurred is considered of importance to see if a correlation exists between foetal exposure to selenium and foetal malformations as a result of environmental insult. A knowledge of selenium concentration in relation to toxic heavy metals in human placentae could provide valuable information on the role of selenium in mammalian tissues. Animal studies have indicated that selenium is important in protecting against the toxic action of cadmium, mercury and arsenic[6,9–12,61].

CONCLUSIONS

The routine determination of trace levels of selenium in biological samples has posed many difficulties. In this report, selenium was determined as 5-nitropiazselenole by GLC with electron-capture detection after sample dissolution with a mixture of nitric acid and magnesium nitrate. The method is simple, suitable for batch processing, free from interference and very sensitive. It has been successfully applied to the determination of selenium in bovine liver, orchard leaves, human placenta, blood, hair and urine.

ACKNOWLEDGEMENTS

Work in this laboratory is supported by the Department of Health and Social Security and by the Commission of the European Communities under the environmental research programme (contract No. 8304). We thank Professor J. H. Edwards of the Infant Care and Development Unit, Queen Elizabeth Medical Centre, Birmingham, for the provision of placental samples, Mr. P. J. Barlow of the Department of Construction and Environmental Health, the University of Aston in Birmingham for hair samples and Dr. H. A. Waldron, Department of Social Medicine, University of Birmingham, for blood samples.

REFERENCES

1 H. W. Lakin, in E. L. Kothny (Editor), *Trace Elements in the Environment*, American Chemical Society, Washington, D.C., 1973, p. 96.
2 Y. Talmi and A. W. Andren, *Anal. Chem.*, 46 (1974) 2122.
3 H. A. Schroeder, D. V. Frost and J. J. Balassa, *J. Chron. Dis.*, 23 (1970) 227.
4 E. J. Underwood, *Trace Elements in Human and Animal Nutrition*, Academic Press, New York, 1962, p. 291.
5 R. E. Holmberg, V. H. Ferm and N. H. Hanover, *Arch. Environ. Health*, 18 (1969) 873.
6 H. A. Schroeder and M. Mitchener, *Arch. Environ. Health*, 23 (1971) 102.
7 K. W. Franke, A. L. Moxon, W. E. Polley and W. C. Tulley, *Anat. Rec.*, 65 (1936) 15.
8 W. J. Birge, O. W. Roberts and J. A. Black, *Bull. Environ. Contam. Toxicol.*, 16 (1976) 314.
9 J. W. Huckabee and N. A. Griffith, *Trans. Amer. Fish. Soc.*, 103 (1974) 822.
10 A. T. Diplock, *Proc. Nutr. Soc.*, 33 (1974) 315.
11 A. Rotruck and M. Pope, *Science*, 179 (1973) 588.
12 D. Jensen, *Proc. Soc. Exp. Biol. Med.*, (1975) 149.

13 G. Ohi, S. Mishigaki, H. Seki, Y. Tamura, T. Maki, K. Konno, S. Ochiai and H. Yamada, *Environ. Res.*, 12 (1976) 49.
14 S. Nishigaki and M. Harada, *Nature (London)*, 258 (1975) 324.
15 S. W. Fowler and G. Benayoun, *Bull. Environ. Contam. Toxicol.*, 16 (1976) 339.
16 E. Browning, *Toxicity of Industrial Metals*, Butterworths, London, 1969, p. 286.
17 O. H. Muth (Editor), *Selenium in Biomedicine*, Avi Publishing Co., Westport, Conn., 1967.
18 L. Fishbein, *The Chromatography of Environmental Hazards*, Elsevier, Amsterdam, 1973, Vol. 2, p. 125.
19 R. A. Zingaro and W. C. Cooper (Editors), *Selenium*, Van Nostrand Reinhold, New York, 1974.
20 C. A. Strace, L. D. Wiersma and P. F. Lott, *Chemist-Analyst*, 55 (1966) 74.
21 H. Ariyoshi, M. Kiniwa and K. Toei, *Talanta*, 5 (1960) 112.
22 M. Tanaka and T. Kawashima, *Talanta*, 12 (1965) 211.
23 K. I. Strausz, J. T. Purdham and O. P. Strausz, *Anal. Chem.*, 47 (1975) 2032.
24 H. J. M. Bowen and P. A. Cawse, *Analyst (London)*, 88 (1963) 721.
25 O. J. Kronborg and E. Steinnes, *Analyst (London)*, 100 (1975) 835.
26 J. H. Watkinson, *Anal. Chem.*, 38 (1966) 92.
27 J. Hall and P. L. Gupta, *Analyst (London)*, 94 (1969) 292.
28 M. Ihnat, *J. Ass. Offic. Anal. Chem.*, 57 (1974) 368.
29 S. Michael and C. L. White, *Anal. Chem.*, 48 (1976) 1484.
30 J. Henn, *Anal. Chem.*, 47 (1975) 428.
31 M. Ihnat, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 911.
32 H. D. Fleming and F. G. Ide, *Anal. Chim. Acta*, 83 (1976) 67.
33 S. Ng and W. McSharry, *J. Ass. Offic. Anal. Chem.*, 58 (1975) 987.
34 K. C. Thompson and D. R. Thomerson, *Analyst (London)*, 99 (1974) 595.
35 R. N. Vijan and G. R. Wood, *Talanta*, 23 (1976) 89.
36 K. C. Thompson, *Analyst (London)*, 100 (1975) 307.
37 A. E. Smith, *Analyst (London)*, 100 (1975) 300.
38 F. D. Pierce and H. R. Brown, *Anal. Chem.*, 48 (1976) 693.
39 R. W. Andrews and D. C. Johnson, *Anal. Chem.*, 48 (1976) 1056.
40 B. L. Dennis, J. Moyers and G. S. Wilson, *Anal. Chem.*, 48 (1976) 1611.
41 J. W. Young and C. D. Christian, *Anal. Chim. Acta*, 65 (1973) 127.
42 S. Nakashima and K. Toei, *Talanta*, 15 (1968) 1475.
43 M. Akiba, Y. Shimoishi and K. Toei, *Analyst (London)*, 100 (1975) 648.
44 T. Stijve and E. Cardinale, *J. Chromatogr.*, 109 (1975) 239.
45 Y. Shimoishi and K. Toei, *Talanta*, 17 (1970) 165.
46 Y. Shimoishi, *Bull. Chem. Soc. Jap.*, 44 (1971) 3370.
47 Y. Shimoishi, *Anal. Chim. Acta*, 64 (1973) 465.
48 Y. Shimoishi, *Bull. Chem. Soc. Jap.*, 47 (1974) 997.
49 Y. Shimoishi, *Bull. Chem. Soc. Jap.*, 48 (1975) 2797.
50 Y. Shimoishi, *Analyst (London)*, 101 (1976) 298.
51 M. Akiba, Y. Shimoishi and K. Toei, *Analyst (London)*, 101 (1976) 644.
52 C. F. Poole, *Chem. Ind. (London)*, (1976) 479.
53 C. F. Poole, *J. Chromatogr.*, 118 (1976) 280; and references therein.
54 C. F. Poole, *Lab. Pract.*, 25 (1976) 309.
55 K. W. Karasek, *Anal. Chem.*, 46 (1974) 710A.
56 M. W. Siegel and M. C. McKeown, *J. Chromatogr.*, 122 (1976) 397.
57 O. E. Olson, I. S. Palmer and E. I. Whithead, *Methods Biochem. Anal.*, 21 (1973) 39.
58 J. H. Watkinson, in O. H. Muth (Editor), *Selenium in Biomedicine*, Avi Publishing Co., Westport, Conn., 1967, p. 97.
59 W. C. Cooper, in R. A. Zingaro and W. C. Cooper (Editors), *Selenium*, Van Nostrand Reinhold, New York, 1974, p. 615.
60 W. Holak, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 650.
61 R. J. Baglan, A. B. Brill, A. Schubert, D. Wilson, K. Larsen, N. Dyer, M. Mansour, W. Schaffiner, L. Hoffman and J. Davies, *Environ. Res.*, 8 (1970) 64.
62 E. B. Dawson, M. P. Menon, R. E. Wainerdi and W. J. McGanity, *J. Nucl. Med.*, 9 (1968) 161.
63 D. Hadjimarkos, C. Bonhurst and J. Mattice, *J. Pediatr.*, 54 (1959) 296.
64 W. H. Allaway, J. Kubota, F. Losee and N. Roth, *Arch. Environ. Health*, 16 (1968) 342.
65 C. F. Poole and D. G. Wibberley, *J. Chromatogr.*, 132 (1977) 511.