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IMPROVED METHOD FOR SELENIUM DETERMINATION IN BIOLOGICAL SAMPLES BY GAS CHROMATOGRAPHY

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SUMMARY

A gas chromatographic assay employing electron-capture detection for the determination of selenium in biological samples is reported. A calibration curve of 4-nitro-*o*-phenylenediamine derivative of selenium as a function of peak area was linear from 5—1000 pg. The limit of detection for the electron-capture detector was approximately 0.5 pg. Recoveries of selenium added to various biological materials ranged from 95—105%. This procedure reduces the number of transfers thereby reducing errors associated with losses or contamination. One advantage of the present method is that interfering compounds occurring in previously employed chromatographic methods are eliminated. This procedure can be used for routine microanalysis of selenium. Samples containing less than 2 ng selenium in 200 μl of biological fluid can be routinely analyzed using this method.

INTRODUCTION

The essentiality of selenium for higher animals, including man, has been clearly established [1—3]. A dietary deficiency or excess of this element has been associated with numerous disorders in mammals [2—6]. Furthermore, recent epidemiological data have suggested that selenium may also have a role in the etiology of cancer. Schrauzer et al. [7, 8] have shown an inverse relationship between selenium consumption and cancer mortality both nationally and internationally. These observations plus increasing experimental data suggest that selenium may have a preventative [9—11] and therapeutic [12—15] role in the etiology of cancer.

Selenium is often considered one of the most toxic elements in nature. Clearly excessive intakes of selenium lead to pronounced toxic symptoms [16—20]. Because of the biological and toxicological implications of selenium consumption, there is need for an analytic method that can be applied to various biological tissues.

Recently, electron-capture gas chromatography (GC) has evolved as a sensitive and reliable method for selenium analysis. Various *o*-diamines derivatives have been used to form piazselenoles which after extraction are analyzed by GC. Some of the *o*-diamines that have been used include 2,3-diaminonaphthalene, and the 4-chloro, 4,5-dichloro, 4-nitro and 3,5-dibromo derivatives of 1,2-diaminobenzene [21—29]. The various reagents employed for fluorometric and electron-capture analysis of selenium were reviewed by Shimoishi [26].

A variety of instruments and conditions have been used for selenium analyses. Most methods of selenium analysis require prolonged sample digestion, extensive extraction and purification steps. This paper describes a GC method for selenium analysis applicable to numerous biological samples. This method minimizes the errors associated with sample transfers, reduces the laboratory glassware needed, and eliminates interferences which have been reported in previous procedures.

MATERIALS AND METHODS

Apparatus

A Hewlett-Packard Model 5830 gas chromatograph equipped with a pulsed current ^{63}Ni electron-capture detector was used for selenium analysis. Instrument operating conditions were as follows: temperatures, detector, 350°C; injector, 225°C; column oven, 200°C. The carrier gas was argon—methane (95:5) at a flow-rate of 30 ml/min. A 1.8 m × xx mm I.D. silanized glass column packed with 10% OV-225 on 120—140 mesh Gas-Chrom Q purchased from Applied Science Labs. (State College, PA, U.S.A.) was used for separation of the *o*-diamine derivative of selenium.

Reagents

All chemicals used were analytical reagent grade. Metallic selenium from J.D. Mackay (New York, NY, U.S.A.) and selenium dioxide were used for preparation of standard selenium solutions and the purified 5-nitropiazselenol reference material, respectively. Glass distilled toluene from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) was used for all extractions. A reference standard of 5-nitropiazselenol prepared as described by Poole et al. [28] was used in the initial calibration.

Formation of 5-nitropiazselenol

The complex reagent 4-nitro-*o*-phenylenediamine (4NPD) from Aldrich (Milwaukee, WI, U.S.A.) was converted to the chloride salt by dissolving in hot 2 *M* hydrochloric acid. The solution was then filtered and chilled in an ice bath. The resulting crystals were collected by suction filtration, rinsed with concentrated hydrochloric acid and stored frozen in a sealed glass container for subsequent use. The complexing solution was prepared by dissolving the chloride salt of 4NPD in 1 *N* hydrochloric acid (0.5%, w/v). The complexing solution was extracted with toluene prior to use. The complexing solution was found to be stable for at least two weeks when kept in a dark bottle and refrigerated.

Biological materials

Heparinized blood samples were collected from humans, cows, pigs, and sheep by venipuncture. Blood samples were obtained from experimental mice by heart puncture. Feed samples were collected from local grown sources. The selenium content of liver homogenates obtained from mice receiving supplemental selenium in their drinking water was also determined. Mice received water supplemented with 0, 1, 3, or 5 ppm selenium as sodium selenite. Liver homogenate (10%) was prepared in Krebs Ringers phosphate buffer, pH 7.4 before analysis.

Recoveries of selenium in various biological samples were also determined by the addition of known quantities of selenium to the sample before digestion. A comparison was made between the present GC method and the fluorometric method of Spallholz et al. [30]. Fluorometric measurements were made with the excitation and emission wavelengths set at 363 and 525 nm, respectively, using an Aminco-Bowman spectrophotofluorometer.

Sample preparation

Biological fluids. Approximately 5—200 μl of blood, plasma or tissue homogenate were added to 7-ml glass stoppered weighing vials equipped with ground glass stoppers. Following the addition of 1.5 ml of concentrated nitric acid containing 30 g $Mg(NO_3)_2$ per 100 ml acid (w/v) (50°C), the vials were placed in a heating block for 60 min at 105°C, followed by 30 min at 115°C. The vials were then removed from the heating block and placed on a hot plate turned to the highest setting, 450°C. All vials were removed from the hot plate when fumes of nitrogen oxide ceased. Nitric acid is used to oxidize the lower oxidation states of selenium to selenium(IV). The digestion was completed by placing the vials in a muffle furnace at 500°C for 60 min. The conversion of selenium(VI) into selenium (IV) is achieved by the addition of hydrochloric acid. After cooling, 1.5 ml of concentrated hydrochlorid acid was added to the vials. The vials were capped and placed in a heating block at 90°C for 15 min and then allowed to cool to room temperature. A 2.5-ml solution containing 1% hydroxylamine sulfate, from Alfa Division, Ventron Corp. (Danvers, MA, U.S.A.), 1% EDTA, 15% urea (w/v) in water was added to each vial, mixed and allowed to stand for 10 min. A 100-μl aliquot of the complexing solution was added and mixed. The vials were then placed in a heating block at 45°C for 30 min. After addition of 1 ml of toluene, the vials were mixed for 5 sec. A portion of the toluene layer was removed and placed in a screw cap tube for subsequent GC analysis.

Feeds. To ensure adequate sampling feed samples had to be prepared in the following manner. Samples (1.0 g or less) ground to pass a 20-mesh screen were added to beakers containing 4 g magnesium nitrate and 10 ml concentrated nitric acid. These samples were digested at a solution temperature of 100°C for 60—90 min. The digest was then heated at the highest hot plate temperature until fumes of nitrogen oxide ceased. The beakers were then placed in a muffle furnace of 500°C for 60 min. After cooling samples were removed from the furnace and 10 ml of concentrated hydrochloric acid added. The samples were then placed on a hot plate and the solution temperature maintained at 90°C for 15 min. An aliquot of the hydrochloric acid was

removed and placed in a weighing vial. The procedure was then the same as for fluids with the additions of the hydroxylamine sulfate, EDTA and urea solution.

RESULTS AND DISCUSSION

The most common procedures used for selenium digestion for GC analysis employ wet digestion with nitric acid or combinations of nitric acid with sulfuric and perchloric acids. These procedures generally require longer digestion times and require greater technical care to prevent selenium loss due to charring than the present method. Although some procedures do employ a shorter digestion period, greater time was required for sample clean-up [29]. Solvent partitioning or liquid column chromatography or combinations have often been used to remove interfering components present with such digestion procedures.

The present digestion procedure was a modification of the original procedure developed by Holkak [31] and later modified by Poole et al. [28]. This procedure was found to result in chromatograms with minimal interferences compared to assays using wet digestion with nitric, sulfuric, or perchloric acids in combination with sample clean-up.

Initial investigations of selenium digestion in the presence of magnesium

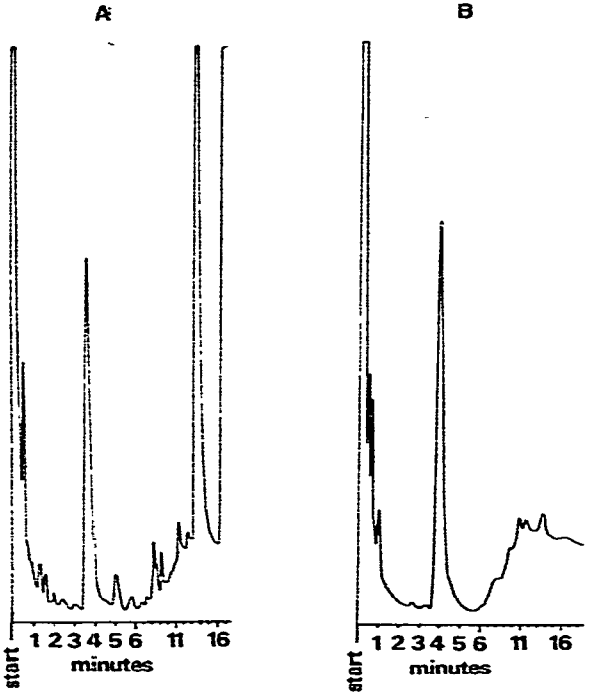

Fig. 1. Selenium analysis of a bovine blood sample containing 12 ng per 200 µl (A) without or (B) with hydroxylamine and EDTA addition before formation of the *o*-diamine complex. Note two interfering peaks occurring at 14 and 17 min during temperature program analysis in Fig. 2B.

nitrate, nitric acid and urea revealed that two interfering peaks occurred in the chromatograms. Fig. 1 shows these interfering peaks occurring at 14 and 17 min post injection when a temperature program was run. These peaks occurred after approximately 25—30 min without temperature programming. These interferences could reduce the number of repetitive samples that can be analyzed. The addition of hydroxylamine sulfate and EDTA in combination with urea was found to eliminate the two interfering peaks occurring during the analysis of selenium in standards and biological materials. The elimination of these peaks allows for repetitive analysis with minimal interferences. Typical chromatograms of a human blood and bovine plasma with the present procedure are shown in Fig. 2.

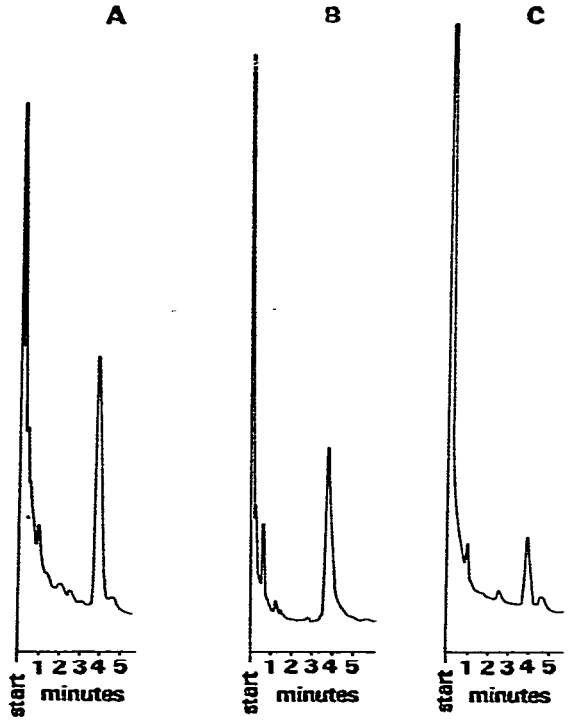

Fig. 2. GC analysis of selenium in (A) human blood (29.0 ng per 200 μl analyzed); (B) 20 ng selenium standard; and (C) bovine plasma (6.5 ng per 200 μl analyzed). Selenium peak occurs at 4 min.

The selenium content of biological materials can be readily detected by this GC procedure. A calibration curve of peak area versus selenium content in the range of 5—1000 pg per injection is linear. The correlation coefficient of selenium content against peak area was in the range of 0.999. The precision of repeated analyses of 4 μl of 30 ng/ml selenium was within 0.2%. The precision of repeated analyses of human blood selenium concentrations was similar. The present procedure can detect as little as 2 ng selenium in 200 μl of fluid. To obtain this sensitivity digestion blanks must be maintained at a level below 0.5 ppb. Hydrochloric acid was found to contribute most to

the content of selenium reagent blanks. Furthermore, large variations in the selenium content of reagent grade hydrochloric acid were observed. Acid specifically prepared for trace metal analysis was found to minimize the selenium background.

Initial calculations were based upon standard curves obtained by analysis of varying quantities of selenium carried completely through the analytical procedures. Recoveries of 5-nitropiazselenol were essentially quantitative as previously reported by Cappon and Smith [29]. Consequently, calculations were based on the standard selenium complex. Elemental analysis of 5-nitropiazselenol revealed 31.6% C, 1.3% H, 18.4% N, 14.0, and 34.7% Se. The theoretical content of C, H, N, and Se are 31.8, 1.2, 18.4, 14.7 and 33.9% respectively. The synthesized 5-nitropiazselenol had a maximum UV absorption at 349 nm and resulted in a single peak when analyzed by GC with flame ionization detection. The GC—mass spectrometric spectra gave two molecular ions corresponding to the two selenium isotopes.

The present procedure also eliminates the excessive use of glassware required in other methods. In addition to the time saved in glassware preparation, this procedure also reduces the errors associated with the losses during the transfer of the test solutions and errors resulting from contamination.

Excellent agreement of selenium content of reference material, as well as fluids and tissues from animals and humans occurred between this procedure and the fluorometric method of Spallholz et al. [30] (Table I). The determination of selenium by fluorescence with 2,3-diaminonaphthalene

TABLE I

DETERMINATION OF SELENIUM CONCENTRATIONS IN NORMAL ADULT BLOOD AND RAT LIVER BY GC AND FLUOROMETRIC ANALYSIS

	Method	
	GC*	Fluorometric**
Orchard leaf standard*** (ng/g)	84.8 ± 4.0	88.6 ± 2.7
Human blood (ng/ml)		
Mean	147.3	162.8
S.E.M.§	6.0	11.4
Range	106—175	106—233
Number of determinations	12	12
Rat liver§§ (μg/g)		
0	0.91 ± 0.1	1.11 ± 0.2
1	2.61 ± 0.4	2.28 ± 0.6
3	2.92 ± 0.8	2.51 ± 0.5

*See Materials and Methods for procedure.

**See ref. 25.

***National Bureau of Standards, Standard Reference Material 1571. Selenium content given as 80 ± 10 ng/g.

§Standard error of the mean.

§§Rats were given supplemental selenium in the drinking water at 0, 1, or 3 ppm as Na_2SeO_3 for 1 week before analysis. Means ± S.E.M. are for four mice per treatment. No significant differences in selenium content by the two analytical methods were detected.

has been well evaluated in biological materials. However, the fluorometric technique suffers from interference of co-extractants originating from the samples plus a low precision at low concentrations. When the selenium content is at trace concentrations or the supply of sample material is limited as may occur in blood samples obtained from infants and small experimental animals, the present method will be of considerable value.

Analysis of various biological samples has been performed using the described method (Table II). In all cases the reproducibility of the analysis

TABLE II

SELENIUM CONTENT OF BIOLOGICAL SAMPLES BY GC ANALYSIS

A. Physiological fluids*			B. Plant materials**	
	Whole blood (ng/ml)	Plasma (ng/ml)	Feed products	ng/g
Porcine	219 ± 11	192 ± 12	Corn	104 ± 1
Bovine	48 ± 3	28 ± 2	Hay Δ1	29 ± 1
Ovine	166 ± 13	57 ± 2	Hay Δ2	66 ± 2
			Silage	24 ± 0
			Grain mixtures	96 ± 3

*Values are means ± S.E.M. for 10 samples per species examined.
**Values are means ± S.E.M. for duplicate determinations of the same sample.

was within 3%. Table III lists typical recoveries of selenium that have been observed. Recovery of added selenium was always found to be in the range of 95—105%. This recovery was observed in the analysis of various samples including grasses, grains and animal tissues or fluids (Table III).

TABLE III

RECOVERY OF SELENIUM IN BIOLOGICAL SAMPLES

Values are means ± S.E.M. for duplicate determinations of two samples.

	Initial concentration (ppb)	Selenium addition	
		50 ppb	100 ppb
Corn	27 ± 1	78 ± 2	124 ± 3
Bovine liver	192 ± 8	248 ± 5	296 ± 4
		10 ppb	40 ppb
Bovine blood	48 ± 3	57 ± 2	90 ± 4

Experience with this GC method for over two years indicates that it is a suitable method for routine determination of selenium in terms of labor and precision. One individual can analyze without difficulty at least 60 samples, including GC and calculations in approximately 12 h.

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