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Selenium can be determined quantitatively in biological samples after digestion using nitric acid, orthophosphoric acid, and hydrogen peroxide and the formation of 5-nitropiazselenol. Samples are spiked with enriched ^{82}Se and isotopic ratio of ^{80}Se to ^{82}Se is measured by combined gas chromatography–mass spectrometry using dual ion monitoring. Precise determination at the parts-per-billion level is possible. The accuracy of the method is verified by using standard reference materials.

Selenium was found to be an essential trace amount element for animals by Schwarz (*1*) and was subsequently found to be essential for man (*2*). The role of selenium has been shown to be closely related to that of vitamin E (*3*). Recommended methods for selenium analysis include fluorimetry, X-ray fluorescence, neutron activation analysis, atomic absorption, and gas chromatography. The standard fluorescence method using 2,3-diaminonaphthalene (DAN) has been well evaluated for biological materials and found to be reasonably interference free (*4*, *5*). The sensitivity of atomic absorption for selenium in a flame is poor (*6*), but the sensitivity can be increased with carbon furnace techniques (*7–10*). However, the atomic absorption method of choice has been selenium hydride formation (*4*, *11–13*). The methods of atomization and analysis have included flame (*14*, *15*), quartz tube (*16*, *17*), and atomic fluorescence spectrometry (*18*, *19*). However, there have been some problems associated with the hydride generation method (*4*, *20*). Several authors have used X-ray fluorescence with some success (*21*, *22*). Neutron activation methods are sensitive enough but require time and elaborate equipment (*2*,

23, 24). Cathodic stripping has also been tried with success in some matrices (25).

The procedure adapted here was first reported by Ariyoshi et al. (26) as a spectrophotometric method and then developed into a gas chromatographic method (27–30). It is based on the reaction between selenium(IV) and o-phenylenediamine in acidic solution to form the piazselenol, which can be extracted into organic solvents. By use of a modification of the sample preparation procedure described earlier (31), the thermally stable nitropiazselenol compound is isolated and measured by gas chromatography–mass spectrometry (GC/MS). Stable isotope dilution techniques permit an accurate, sensitive, and precise measurement of selenium levels with no observed losses or matrix effects.

EXPERIMENTAL SECTION

Instrumentation. A Finnigan Model 4000 quadrupole GC/MS equipped with programmable multiple ion monitor (PROMIM) capabilities was used for the selenium determinations. Instrument operating conditions were as follows: injector, 175 °C; column, 160 °C; transfer line, 175 °C; and a carrier gas of helium at a flow rate of 20 mL/min. A 1.2 m × 2 mm i.d. silanized glass column packed with 1% SP2401 on 100/120 mesh Chromosorb 750 (AW/DMCS) was used for the separation. For the radiotracer studies, ^{75}Se in samples was counted in a Searle Analytic 1185 γ spectrometer.

Reagents. All reagents were at least reagent grade, and high-purity deionized water was used throughout. The enriched stable isotope (^{82}Se) was obtained in the black elemental state from Oak Ridge National Laboratory, Oak Ridge, TN. The radioactive ^{75}Se (sodium selenite) was obtained from New England Nuclear, Boston, MA. The derivatizing reagent used was 4-nitro-o-phenylenediamine (NPD). The NPD was converted to the HCl salt and purified by the following procedure: It was dissolved in hot 2 M HCl, which contained some carbon black, filtered, and chilled in an ice bath. The crystals were collected by suction filtration and rinsed with 6 M HCl and stored frozen. The derivatizing solution was prepared by dissolving 1.0% (w/v) NPD in 1 N HCl and extracting with toluene until a 5 μL aliquot analyzed on the GS/MS was free of any nitropiazselenol (Se-N-PD). This generally required four to five toluene extractions. The derivatizing solution was found to be stable for at least 30 days when kept refrigerated in a plastic bottle.

Reference Standards. The enriched stable isotope solution for the isotope dilution method was prepared from a stock solution of ^{82}Se containing 950 ppm Se (96.81% ^{82}Se). The enriched elemental selenium was oxidized with HNO_3 to H_2SeO_3 and diluted to volume with H_2O. The ^{82}Se concentration was verified by GC/MS using selenium standards prepared from Na_2SeO_3, SeO_2, and elemental selenium, using essentially the inverse of the isotope dilution method.

Tracer Procedure. For verification of the reliability of the analytical method, radiotracer ^{75}Se was added to samples and standards prior to digestion or endogenously radiolabeled rat tissue was used to evaluate recoveries and derivatization efficiency.

Analytical Procedure. Samples endogenously or exogenously radiolabeled with ^{75}Se or spiked with ^{82}Se were weighed into Kjeldahl flasks and digested using the procedure previously described (31). After digestion, 2 mL of concentrated HCl was added and the samples were boiled for 5 min to reduce any selenate to selenite. The samples were then diluted with 20 mL of deionized H_2O and extracted twice with 5 mL of toluene to remove all undigested lipids. A 0.5-mL aliquot of the derivatizing solution was added and the solutions were allowed to stand at room temperature (24 °C) for 1 h. The Se-NPD formed was then extracted into 2 mL of toluene by shaking for 15 min on a mechanical shaker. The toluene extract was separated from the aqueous phase and stored in small glass test tubes for subsequent GC/MS analysis when spiked with ^{82}Se or counted in the γ counter when spiked with ^{75}Se.

RESULTS AND DISCUSSION

The sample preparation and derivatizing method was evaluated with the use of radiotracers. Previous work (31) has shown quantitative selenium recoveries when using HNO_3, H_3PO_4, and H_2O_2 to digest biological samples. To be evaluated here is the derivatization of selenium in this digestion matrix. Figure 1 shows that consistent recoveries were achieved for Se-NPD when using ^{75}Se spiked selenium standard or a ^{75}Se endogenously labeled rats whole blood sample when the quantity of H_3PO_4 was varied. The procedure adopted uses 1 mL of H_3PO_4 and Figure 1 indicates that at least 5 mL can be used without any matrix interferences. The 10% selenium loss was due to the incomplete extraction by the single toluene extract. Two 2-mL toluene extracts can remove 97% of the Se-NPD, but it was felt that a single extract was sufficient. Experiments using ^{75}Se tracer to optimize reaction conditions indicate that for a 20-mL sample, using 0.5 mL of a 1% (w/v) solution of NPD, maximum recovery could be achieved after 30 min at room temperature (25 °C). A study of the effect of pH on the recovery (Figure 2) showed a constant recovery at pHs between 0 and 1.5, in agreement with previous results (27, 32). By extraction of the nondigested lipids from the sample with toluene prior to derivatization, the chromatogram was free from any interfering compounds (Figure 3). Peak height and peak area measurements were compared and peak height measurements were found to be as accurate as peak area measurements.

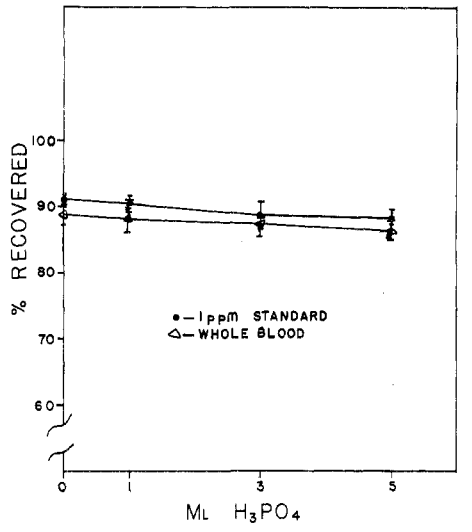

Figure 1. Effect of H_3PO_4 concentration on recovery of 5-nitropiazselenol from standards and whole blood.

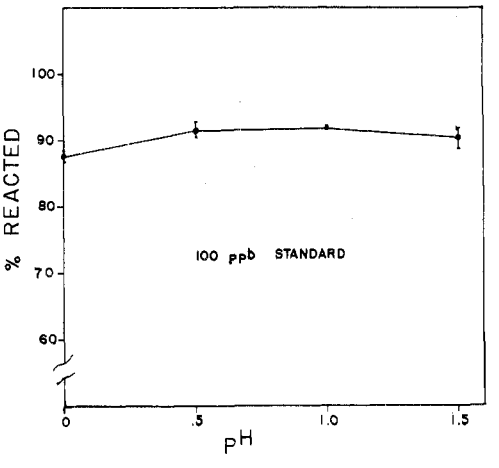

Figure 2. Effect of pH on recovery of 5-nitropiazselenol.

Table I shows the selenium distribution in samples spiked or endogenously labeled with ^{75}Se. Recoveries for a single extract were approximately 90% with less than 2% present in the lipid toluene extract.

For stable isotope dilution analysis, selenium has six stable isotopes (Table II), which can be purchased as enriched

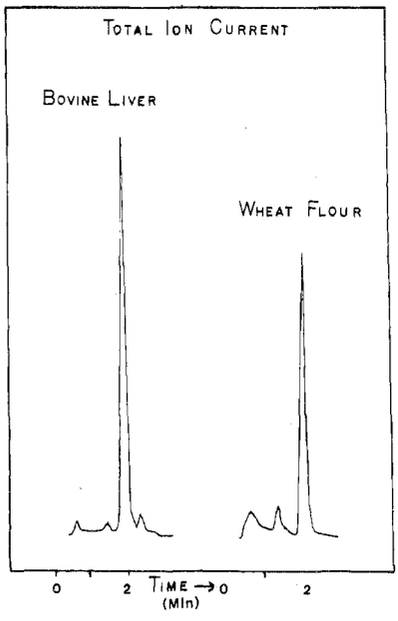

Figure 3. Total ion current chromatogram of 5-nitropiazselenol, NBS bovine liver, and NBS wheat flour.

Table I. Recoveries of ^{75}Se in Biological Materials

sample	no. of samples	% lipid extract	% recovered
Spiked Samples			
soybeans	4	1.1	86.4 ± 2.8
human urine	4	*a*	86.3 ± 0.87
bacon	3	2.0	83.5 ± 5.7
pork	3	1.6	83.9 ± 6.2
cereal	3	0.54	87.8 ± 0.4
pork	3	0.62	86.2 ± 2.1
100 ng std	8	*a*	90.4 ± 2.2
human serum	4	*a*	89.2 ± 4.6
Endogenously Labeled Samples			
rat urine	4	*a*	84.4 ± 1.3
rat urine	3	*a*	78.7 ± 1.7
rat liver (4 h)	5	0.84	93.4 ± 5.4
rat liver (24 h)	7	1.5	93.4 ± 2.8
rat liver (4 h)	3	0.1	93.4 ± 0.7
rat liver (24 h)	3	0.1	92.0 ± 1.1
rat whole blood	5	*a*	93.0 ± 3.0

[a] Samples not extracted.

Table II. Relative Abundance of Se Isotopes in Natural and Enriched Solutions

isotope	atomic %: natural abundance	atomic %: enriched ^{82}Se
74	0.87	0.132
76	9.02	0.192
77	7.58	0.304
78	23.52	0.599
80	49.82	1.960
82	9.19	96.813

preparations. We chose to use an enriched preparation of ^{82}Se (column 2) as the internal standard. The mass spectrum of the Se–NPD compound showed that the parent ion that occurs at 229 amu was the most intense and was therefore used throughout this study. For quantitative analysis, the enriched ^{82}Se was ratioed to ^{80}Se, the most abundant natural isotope. The masses monitored were 229 and 231 amu, corresponding to ^{80}Se–NPD and ^{82}Se–NPD, respectively. Because there are natural isotopes of ^{13}C, ^{15}N, and ^{18}O in the Se–NPD compound, their contribution to the ^{80}Se to ^{82}Se ratio must be calculated to determine if there is any effect on the ratio. The isotopic correction calculation shows that the natural isotopic ratio changed from 5.42 to 5.36, indicating that their isotopic effect was small. This is due to the 2 amu separation of the two isotopes. The observed natural isotopic ratio was 5.36, as calculated. When using ^{82}Se as the internal standard and the isotopic abundances listed in Table II the following relationship is obtained

$$Se_{(n)} = 0.964[Se_{(sp)}]\frac{0.9681R - 0.0196}{0.4982 - 0.0919R} \quad (1)$$

where $Se_{(n)}$ is the total nanograms of selenium present in the original sample. $Se_{(sp)}$ is the nanograms of ^{82}Se spike added and R is the observed ratio of the 229 to 231 amu (^{80}Se–NPD to ^{82}Se–NPD). The correction factor of 0.964 is for the difference in the atomic weights of natural selenium (78.96) and the ^{82}Se spike (81.90) and the isotope correction factor of 1.01 for the contribution from ^{13}C, ^{15}N, and ^{18}O (5.42/5.36) is included in the values multiplied by "R". Frew et al. (*33*) have discussed this relationship in detail. The numerical values of the constants in eq 1 are only valid for the ^{82}Se enriched isotopic spike given in Table II.

With isotope dilution methods, there is an internal standard so that quantitative recovery of the analyte is not required, but care during the various stages of analysis is still needed. As was shown with the ^{75}Se tracer study, the single toluene extraction only recovered approximately 90% of the Se–NPD, but the internal standard stable isotope also reacts the same enabling quantification of the analyte.

To further assess the validity of the method, three standard reference materials were analyzed. The results are given in Table III, and the agreement between the experimentally found and the certified values is excellent. The analytical procedure outlined has been used to determine the concentration of selenium in various biological materials. The results obtained are summarized in Table IV and are in good agreement with values obtained by using the standard perchloric acid digestion and DAN fluorometric method (*34*).

Table III. Analysis of Standard Reference Material for Selenium

sample[a]	no. of samples	certified value, μg/g	exptl value, μg/g
bovine liver SRM (NBS 1577)	4	1.1 ± 0.1	1.07 ± 0.04
wheat flour SRM (NBS 1567)	9	1.1 ± 0.2	0.95 ± 0.04
rice flour SRM (NBS 1568)	3	0.4 ± 0.1	0.39 ± 0.02

[a] 0.475 μg of ^{82}Se added.

Table IV. Selenium Concentration in Biological Materials

sample	selenium concn, ng/g: GC-MS, ng/g	selenium concn, ng/g: fluorometry[a]
egg yolk (normal)	567 ± 3.8 (*5*)	570 ± 5 (*3*)
egg yolk (Se depleted)	112 ± 30 (*5*)	150 ± 50 (*6*)
egg white (Se depleted)	31 ± 7 (*3*)	25 ± 5 (*6*)
chicken feed	359 ± 16 (*6*)	362 ± 25 (*4*)
human whole blood	166 ± 0.2 (*3*)	

[a] DAN method (*34*).

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