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Note

High-performance liquid chromatography of selenium in biological samples

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Selenium is an essential micronutrient that has an important role in health and disease [1–3]. The wide spectrum of physiological and pathological conditions linked to selenium probably results from the diversity of molecular species which contain this element. Glutathione peroxidase, a selenoenzyme, can probably not account for all of the biological effects associated with selenium; other selenoproteins must be sought as there is strong evidence that they exist. Thus, when ^{75}Se was injected into animals, several radioactive proteins were detected in tissues [4,5].

Fluorometry is one of the most popular methods of assay; 61% of the participants in an inter-laboratory study of blood selenium determinations employed the fluorometric measurement of the selenium–diaminonaphthalene (Se–DAN) complex [6]. At very low levels of selenium (picogram range), the fluorometric assay becomes unreliable mainly because of interfering fluorescent contaminants.

In the first high-performance liquid chromatographic (HPLC) method that was described [7], the Se–DAN complex was monitored using UV absorption; the solvent that was needed in order to elute the complex from the column caused complete quenching of the fluorescence. More recently, Shibata et al. [8] used HPLC coupled to a fluorometer of very high sensitivity in order to measure the strongly quenched fluorescence of the Se–DAN complex. Under these conditions, the measurements are possible but this method has not yet been applied to biological samples which are much more difficult to analyse than pure aqueous standards.

In order to allow the identification of selenoproteins in human tissues and biological fluids we developed a highly specific and sensitive HPLC method for

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the assay of selenium. This method has been applied with success to human serum and seminal plasma.

EXPERIMENTAL

Apparatus

We used a Waters Assoc. (Milford, MA, U.S.A.) Model AOLC/GPC-204 HPLC system with a 6000A solvent delivery system, a U6K injector, a data module, a Model 730 recorder and a Model 720 system controller. The fluorescence detector, Model 720, was equipped with filters No. 78154 (peak of excitation at 360 nm) and No. 78233 (emission from 500 nm).

Analyses were performed on a water-deactivated μPorasil column (10 μm, 30 cm×3.9 mm, Waters Assoc.) washed with 100 ml of methanol, 100 ml of water, 10 ml of methanol and finally 30 ml of ethyl acetate. The mobile phase was cyclohexane–ethyl acetate (9:1, v/v) at a flow-rate of 1.0 ml/min. After use, the column was washed with 30 ml of ethyl acetate followed by 30 ml of mobile phase.

Chemicals

Doubly distilled and demineralized water was used. The purest nitric acid (Ultrex, J.T. Baker, Phillipsburg, NJ, U.S.A.) and perchloric acid (BDH Chemicals, Montreal, Canada) were used for digestion. Different purity grades of diaminonaphthalene dihydrochloride were used: 95–98% pure (Sigma, St. Louis, MO, U.S.A.) 97% and 99% pure (Aldrich, Milwaukee, WI, U.S.A.).

Standards were prepared by dissolving known amounts of selenium oxide (Puratronic, Johnson Matthey Chemicals, Royston, U.K.) in 0.1 mol/l hydrochloric acid.

Digestion of samples

Standard solutions of biological samples (40 μl) were transferred to 13×100 mm borosilicate glass tubes. A 100-μl volume of a mixture of nitric acid–perchloric acid (1:1, v/v) was added. The sand bath that was used for the digestion was custom-made from a Model 53015 hot plate (Lindberg, Watertown, WI, U.S.A.) around which a metal border (10 cm high) was soldered directly to the plate. A layer (5 cm) of fine sand was used to cover the hot plate. The tubes were then placed in the sand bath (pre-heated to 140°C) at a depth that did not allow direct contact of the tubes with the hot plate itself. After 1 h at 140°C, the temperature was raised to 180°C (maintained for 2 h) and finally to 190°C (maintained for 30 min). The tubes were then removed from the sand bath and allowed to cool. The temperature of the sand bath was then lowered to 140°C.

Reduction of selenate to selenite

Reduction to selenite was achieved by adding 20 μl of (5 mol/l) hydrochloric acid and the tubes were then heated in the sand bath at 140°C for about 3 min; when fumes of nitrogen dioxide were still detectable, this step was repeated. The tubes were cooled, the pH was adjusted by adding 200 μl of a solution containing

20 mmol/l EDTA, 10 mg/l bromocresol purple and 7 mol/l ammonium hydroxide; the tubes were heated in the sand bath at 140°C.

Each tube was removed as soon as a distinct yellow color developed (this is best observed using a mirror held at angle over the tubes). The tubes were allowed to cool, 500 μl of 0.1 mol/l hydrochloric acid were added, and the volume was completed to 1.0 ml with water. The tubes were left overnight at room temperature.

Formation of the piazselenol complex

The piazselenol complex was formed under subdued light by adding 50 μl of a solution of DAN (4 g/l) in hydrochloric acid (0.1 mol/l). After 30 min of incubation in a water bath at 40°C, the fluorescent complex was extracted with 1.0 ml of cyclohexane.

Fluorescence measurements

Cyclohexane–ethyl acetate (9:1, v/v) was used as the eluent at a flow-rate of 1.0 ml/min, and 200 μl of the extract of cyclohexane were injected into the HPLC system.

RESULTS

Elution conditions

The solvent mixture of cyclohexane–ethyl acetate (9:1, v/v) caused very little quenching (only 13%) of the fluorescence of the piazselenol complex. In addition, this mobile phase can be efficiently used to elute the piazselenol complex from the water-deactivated μPorasil column (Fig. 1) with a relatively short retention time of 6 min.

Chromatograms

The chromatogram of the piazselenol complex differed depending upon the purity of the DAN that was used. The lowest quality grade (95–98%) produced several peaks, the 97% pure DAN yielded two peaks while the highest quality grade (99% pure) produced only one peak. The same results were obtained with selenium from standard solutions as well as with selenium from biological fluids (Figs. 1 and 2). The low value of the blank (Fig. 2D) was substracted from all of the readings.

Linearity

In the range of 0–1000 pg of selenium, the standard curves of the area (10^{-6} units) under the selenium peak versus the amount of selenium injected was $y=7.45x+7.16$ ($r=0.999$) with the 99% pure DAN; for the 97% pure DAN, we obtained $y=10.19x+97.10$ ($r=0.9989$) while the lowest quality grade DAN standard curve was $y=7.66x+42.79$ ($r=0.9987$).

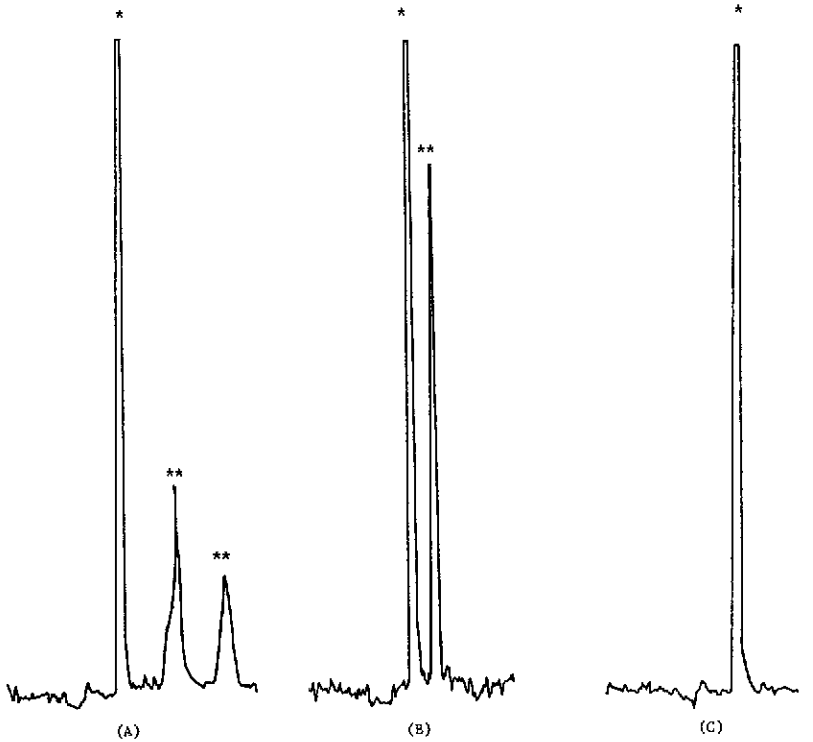

Fig. 1. Chromatograms of a standard solution of selenium obtained with (A) 95–98% pure DAN, (B) 97% pure DAN and (C) 99% pure DAN. * = Se-DAN; * * = non-specific fluorescence.

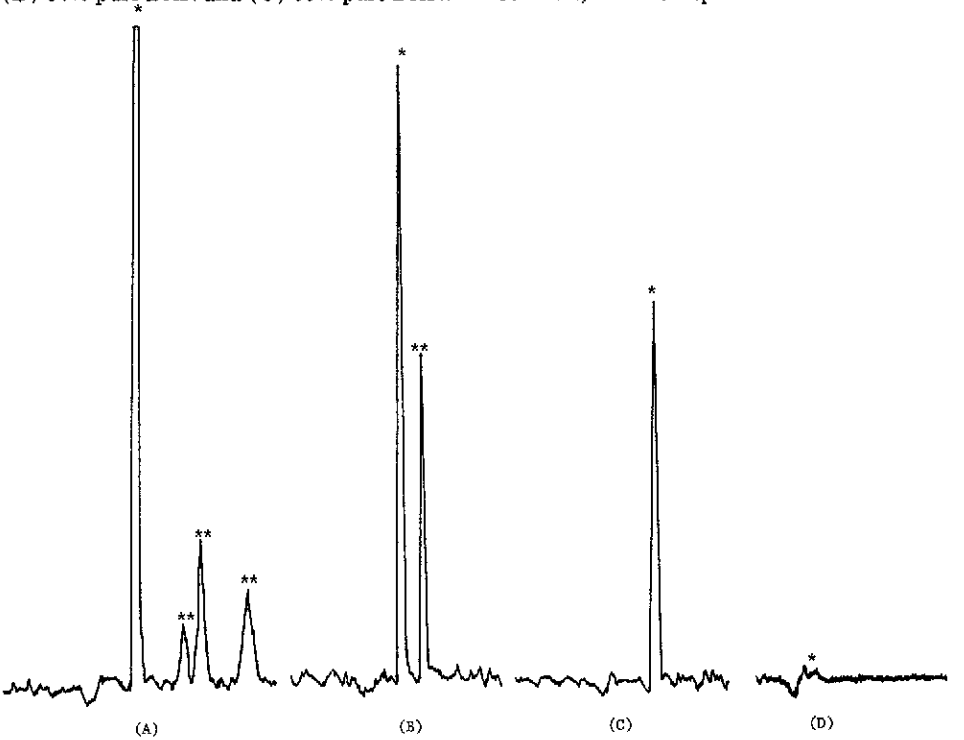

Fig. 2. Chromatograms of biological human fluids and blank obtained using (A) 95–98% pure DAN (serum sample), (B) 97% pure DAN (semen sample), (C) 99% pure DAN (seminal plasma) and (D) blank analysed using 99% pure DAN. * = Se-DAN; * * = non-specific fluorescence.

TABLE I

RECOVERY OF SELENIUM ADDED TO SERUM AND SEMINAL PLASMA ($n=3$)

Serum			Seminal plasma		
Added (ng/ml)	Measured (ng/ml)	Recovery (%)	Added (ng/ml)	Measured (ng/ml)	Recovery (%)
0.0	66.5	–	0.0	21.7	–
39.4	114.0	107.6	11.1	32.5	99.1
74.9	141.1	99.8	23.7	48.5	106.8
88.9	168.5	108.4	45.6	73.1	108.6

Recovery

When known amounts of selenium (from the standard solutions) were added to serum or seminal plasma, the recovery varied between 99.1 and 108.6% (Table I). These experiments were performed using 99% pure DAN.

Reproducibility

Intra-assay reproducibility was evaluated by measuring the selenium concentration in the same sample (seminal plasma and serum). The coefficient of variation was calculated from the mean and standard deviation of the values ($n=9$). We obtained coefficients of variation of 1.7 and 3.8% for seminal plasma and serum, respectively. These experiments were also performed with 99% pure DAN.

Origin of the non-specific fluorescence

Since non-specific fluorescent peaks were present on all chromatograms where the lower quality grade DAN was used (serum, seminal plasma and semen), we incubated solutions of DAN (4 g/l) obtained from different sources in hydrochloric acid (0.1 mol/l) for 30 min at 40°C. Aliquots of 1 ml of these solutions were extracted with 1 ml of cyclohexane and the extracts were analysed by HPLC (Fig. 3). The amount of DAN reagent that was extracted corresponds to 50 times the amount used for reaction in the assay. With the 99% pure DAN, no peak of fluorescence was recorded whereas the 95–98% pure DAN produced several large peaks of non-specific fluorescence.

DISCUSSION

An HPLC method coupled to fluorescence detection of the piazselenol complex proved difficult to develop because the polarity of the solvents that are required to elute the Se–DAN complex from the column caused a strong quenching effect of the fluorescence [7]. Recently, Shibata et al. [8] partly resolved this problem by using a reversed-phase system with acetonitrile as the eluent. Even though acetonitrile causes a 74% decrease in fluorescence of the piazselenol complex (compared to cyclohexane as solvent), the use of a highly sensitive fluorescence detector allowed the measurement of picogram amounts of selenium in water.

In studies on the biochemistry of selenium, a method is required for the mea-

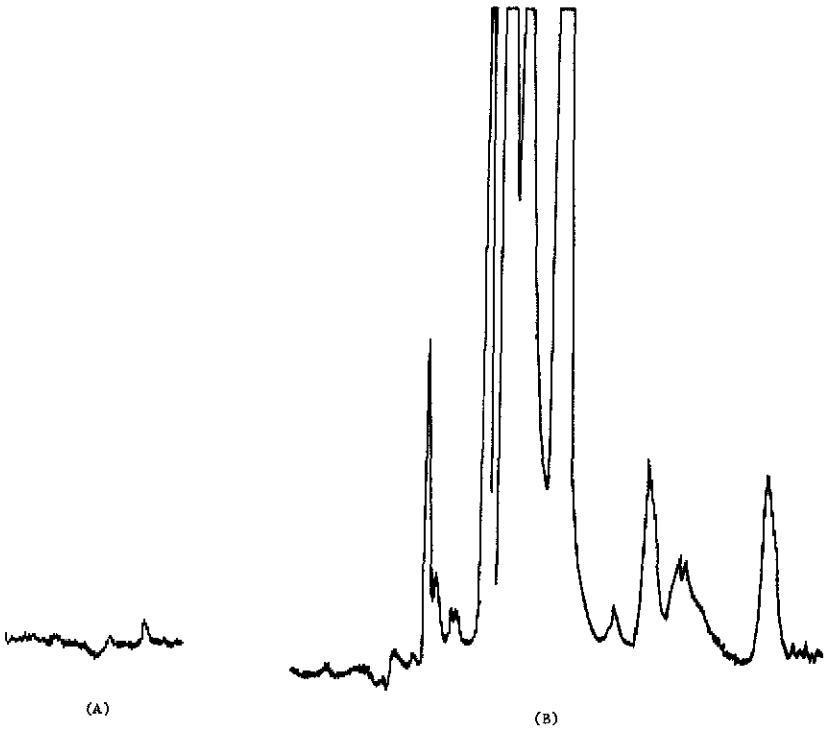

Fig. 3. Chromatograms of extracts of DAN solutions: (A) 99% pure; (B) 95–98% pure.

surement of this element in micro-samples during the purification of selenoproteins (such as fractions from analytical HPLC). We thus developed an HPLC method coupled with fluorescence detection. In preliminary assays, it was observed that a mixture of cyclohexane–ethyl acetate (9:1, v/v) did not produce a strong quenching of the fluorescence of the piazselenol complex (only a 13% decrease) and that the polarity of this mixture was sufficient to elute the complex from a water-deactivated μPorasil column. The treatment of the μPorasil column with water considerably reduces the retention time.

The digestion procedure is an adaptation of a technique previously described [9]. Under these conditions, the mixture of nitric and perchloric acids proved to be efficient in digesting biological fluids. In addition, perchloric acid prevents selenium loss and transforms selenium to selenates and selenites. The duration of the digestion procedure was such that it did not cause any loss of selenium. We used hydrochloric acid to reduce selenium(VI) to selenium(IV); this acid also eliminates any residual nitric acid that would interfere with DAN. Furthermore, this step allows us to obtain a pH of about 2.0, which is optimal for the reaction of selenium with DAN. Dilli and Sutikno [10] reported the different pK_a values for DAN; $pK_{a1} = 0.95$ and $pK_{a2} = 3.99$; since the difference between these values is wide enough the adjustment of the pH is less critical.

When 97% pure DAN and 95–98% pure DAN were used, several fluorescence peaks were observed on the chromatograms. These non-specific peaks originate

from the DAN solutions themselves. Hence, the direct extraction of these solutions followed by their injection into the HPLC system yielded the same non-specific fluorescence peaks. The problem of contaminants in the HPLC assay of selenium can be avoided by a prior purification of low-grade DAN [8]. Alternatively, we found that commercially available 99% pure DAN did not produce any non-specific fluorescence peak and this reagent can be used directly for the assay of selenium.

Our HPLC method is selective and accurate for the determination of selenium in blood and semen. The procedure is highly sensitive (detection limit of 50 pg) and can be adapted to a variety of biological samples. Moreover, it has the advantage of requiring only small aliquots of test material.

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