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Selenium speciation in human serum of cystic fibrosis patients compared to serum from healthy persons

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Abstract

A formerly developed Se speciation method was applied to human serum. Chomatographic performance was checked regularly by measuring control standards after four sample run, each, to ensure sufficient separation of the species and sensitivity of the method even after a considerable number of serum samples. Detection limits of investigated species were similar to those reported and showed values below 1 μg/L related to Se for each Se compound. Sera of cystic fibrosis (CF) patients were investigated in comparison to sera from healthy volunteers with respect to total selenium and Se species. Generally, CF sera showed lower values of total Se, Selenocystine (SeC), and cationic/neutral Se compounds compared to serum of healthy persons. No significant gender-specific differences were found. Total Se in sera of healthy persons was determined at 102 ± 12 μg/L (n = 12 individuals, mean value from male and female, age 4–38 years), whereas CF patients showed 58 ± 10 μg/L (n = 31 individuals, mean value from male and female, age 3–35 years). Se-cystine showed significant differences between the CF and healthy group with a lowered SeC value in sera of CF patients by −75% (mean ca. 26 μg/L in healthy sera compared to about 6.5 μg/L (mean) in CF sera). A similar situation is seen for neutral/cationic Se compounds, which partly may comprise of Se proteins. The lowered SeC values together with lowered cationic/neutral Se compounds (probably Se enzymes) point to a Se-depleted regulated pathway combined with a reduced capability of protective functions such as protection from peroxides.

Keywords: Cystic fibrosis patients; Human serum; Selenium speciation

1. Introduction

1.1. Role of selenium

Selenium plays an important role in environmental analysis as well as in health studies. Excess Se intake can cause toxic reactions in living organisms [1]. On the other hand, selenium is an essential trace element for humans, which plays a role in prevention of heart diseases and cancer [2,3]. Furthermore, the thyroid metabolism may be impaired, because many de-iodinases are Se proteins [4]. Biological and toxicological effects of Se are strongly dependent on its chemical speciation, resulting in an increasing interest in selenium speciation in various liquid or solid matrices, either in food stuff or food supplementation or in body fluids such as serum and urine [4–6].

1.2. Selenium and cystic fibrosis

Especially for children selenium deficiency has negative effects for growth and the development of the brain. Children often show decreased selenium levels compared to adults. This may become more pronounced when having malabsorptions or diseases like cystic fibrosis (CF) which is caused by an autosomal-recessive gene defect. These persons are suffering from altering of their mucose production, predominantly viscose mucose production, resulting in breath insufficience

Abbreviations: SeM, selenomethionine; GFAAS, graphite furnace atomic absorption spectrometry; SeC, selenocystine; SeU, selenourea; SeE, selenoethionine; ICP-MS, inductively coupled plasma mass spectrometry; SAX, strong anion exchange chromatography; TMAH, tetramethylammonium hydroxide

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and gastro-intestinal disorders like maldigestion [7–11]. The exact alteration of this mechanism, as well as absence or presence of Se metabolites, is unknown up to now [12]. Generally, cystic fibrosis leads to a lack of anti-oxidant agents, mostly Se species and Vitamin E in addition to a parallel increase of free radicals, partly caused by broncho-pulmonal infections [13]. A couple of studies proved the reduced Se status of CF patients [7–11]. The analysis of Se species in serum of such patients thus is of great importance.

1.3. Se-speciation method

Our formerly developed method for speciation of anionic Se species [14] was applied to investigate serum samples from 31 CF patients and 12 healthy persons.

This method employed a Dionex AS 11 column with diluted aqueous NaOH and TMAH as eluents. Eluent concentrations and gradients as well as column temperature were optimized with respect to effective separation and short retention time of Se species in serum. It turned out that some modifications in the chromatographic gradient were necessary. Total Se was determined and mass balances were carried out. The different pattern of Se species related to the two groups (CF, healthy) and related to age of donors will be shown and discussed.

2. Experimental

2.1. Chemicals and reagents

NaOH, HNO_3 (each pro analysis (p.a.)) and HCl (suprapure) were obtained from Merck (Darmstadt, Germany). HNO_3 was subboiling distilled (purification) before use. Tetramethyl ammonium hydroxide (TMAH, 25%, suprapure) was bought from TAMA Chemicals (Kawasaki City, Japan). The respective stock concentrations were achieved by weighing the appropriate amounts into 1000 mL flask and subsequent dissolution in Milli-Q H_2O ($18 M\Omega\,cm^{-1}$ throughout) The Se-standard compounds (selenite, selenate, selenoethionine (SeE), selenourea (SeU), selenocystine (SeC), selenomethionine (SeM)) were purchased from Sigma–Aldrich (Deisenhofen, Germany). Stock solutions of 1 mg Se/L were prepared by dissolving the appropriate amounts of the respective Se species in Milli-Q H_2O, except for SeC (dissolved in 1% HCl) and SeU (dissolved in 0.5% TMAH). The stock solutions were stored in the dark at 4 °C in maximum for 1 week. Working standards were prepared daily by dilution with Milli-Q H_2O. $Argon_{liqu}$ (evaporated at the tank) and Ar/H_2 were bought from Messer (Munich, Germany).

2.2. Samples

Standard mixtures of the Se species were used for generating six-point calibration curves at concentrations between 0.45 and 50 μg/L. They served also as control standards (each 5 μg/L) which were measured after every four serum samples for quality control, to check separation performance and constancy of the methodical response factor. This factor was defined as

$$F = \frac{\text{fixed concentration of standard (5 μg/L)}}{\text{measured concentration of standard (μg/L)}}$$

with decreasing response (measured concentration) F increased. F was used for quantification correction as long as it was below 1.2. If F or the separation became worse the column was purged with 0.5% TMAH until separation or methodical response were acceptable again. Single standards were used for standard addition to clearly identify the species in the samples.

Serum samples were collected from CF patients and healthy persons as described in [15,16]. Few selected sera of healthy persons were analyzed immediately for monitoring unaltered speciation in fresh samples. These sera were analyzed again within 2 days after short-time storage at 4 °C. Aliquots of the samples were stored at −20 °C, too, for several weeks and reanalyzed for comparison, however no significant differences could be found between the results of respective sera after immediate analysis and short-time or long-term storage. This proved that differences in speciation were not caused by storage. Thus, sera of healthy persons were usually analyzed within 2 days (short-time storage at 4 °C) whilst CF sera were stored at −20 °C for several weeks at the hospital and transferred frozen to the laboratory. Before analysis the CF sera were thawed slowly at 4 °C and subsequently analyzed immediately. The samples were collected in nearly equal numbers from male and female persons. Thirty-one CF sera and 12 sera from healthy persons were available. All serum samples were measured in duplicate.

2.3. Selenium determination

Total Se was determined according to [15,16] with graphite furnace atomic absorption spectrometry (model 4100 ZL with Zeeman correction, Perkin-Elmer, Rodgau-Jügesheim, Germany). The value of total Se was taken for comparison with former papers upon Se in CF patients [16] and for calculating Se mass balances (sum of quantified Se peaks/total Se) for quality control. The determined mass balances were around 100% (mean: 104 ± 18%).

For selenium speciation a coupling of strong anion exchange HPLC to ICP-MS was used. The ICP-MS (ELAN 5000, Perkin-Elmer, Sciex, Canada) operated in the graphic mode. It was equipped with a Meinhard nebuliser and a cyclon spray chamber (Perkin-Elmer). Selenium was determined at isotopes 77, 78 and 82. ^{82}Se was taken for peak quantification, because this trace showed lowest noise and baseline values. The measurement of the three isotopes was used to exclude wrong assignment such as possibly occurring interferences were not assigned as Se species. Thus, only peaks detected on each of the three isotopes and showing the natural isotope

Table 1
Gradient of chromatographic elution

Time (min)	Percent eluent B	Comments
0–3	0	–
3–4	10	–
4–7.5	10–45	Flattened gradient compared to [14]
7.5–10	45–100	–
10–13	100	Cleaning step
13–18	0	Re-equilibration to A
18	0	End, ready for next injection

ratios were taken as Se compounds. The HPLC column effluent was introduced directly into the nebulizer.

RF power: 1300 W; nebulizer gas: Ar 0.915 L/min. The dwell time was set to 500 ms. These parameter were optimal for this instrument.

2.4. Chromatographic parameter and instrumentation

The eluent delivery system was a HPLC-gradient pump from Beckman, Munich, Germany, model 127, biocompatible, equipped with a Rheodyne injector, having a 100 μL sample loop.

Column: Dionex AS 11. The column temperature was set to 30 °C using a water jacket linked to a Haake-D8 circulator.

Flow rate: 1.5 mL/min; eluents: A = 0.6 mM NaOH, B = 0.5% TMAH.

The chromatographic gradient is given in Table 1. The gradient was slightly modified compared to former experiments to maintain complete resolution at later retention times. Thus, the increase of eluent B after 4 min was flattened. HPLC injection and start of ICP-MS detection was triggered manually. Retention times of Se compounds, LOD (3σ criterion) and calibration characteristics are given in Table 2. Standard addition procedure was used in addition for peak identification.

The average recovery of Se in sera (sum of quantified Se species/total Se determination) was 104 ± 18%.

3. Results and discussion

3.1. Se total

According to statistical evaluation there was no significant difference in total Se concentration between male and female persons. Values found were 71 ± 16 μg/L (n = 22) for males and 61 ± 18 μg/L (n = 21) for females. This is similar to [16] where no significant difference between genders was found, too. However, significant differences were seen between healthy and CF persons, shown in Fig. 1. In average, healthy persons showed a value of 102 ± 12 μg/L (n = 12 individuals), whereas CF patients showed 58 ± 10 μg/L (n = 31 individuals). Interestingly no significant changes according to age groups were seen (Fig. 1). The values of CF patients varied between ∼50 and 70 μg/L, three of the age groups meeting the average value. The values of healthy persons were varying between 80 and 120 μg/L. Unfortunately, values in the groups from 10 to 20 years were not available. This is due to the lower number of sera from healthy persons. Muntau et al. [16] reported about reduced Se levels in very young persons, however, this was related to the first year of life. As the first group here is 0–5 years this result cannot be seen in this study. In general the total Se values of healthy persons and CF patients were in the expected ranges. Summarizing [17–19] ranges of 32–84 μg/L (0–5 years), 41–74 μg/L (6–10 years), 40–82 μg/L (10–16 years) and 50–120 μg/L (adults, >18 years) are reported.

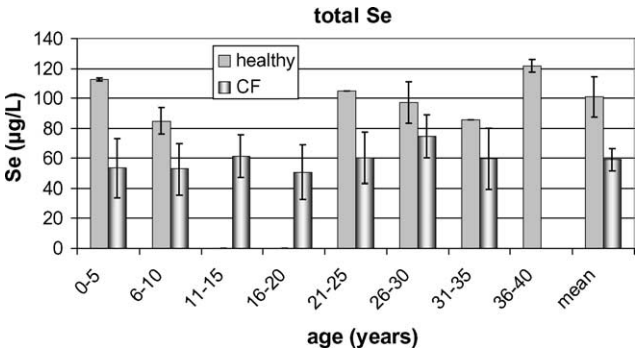

Fig. 1. Total Se concentration in serum is shown of healthy persons and CF patients. Mean values (±S.D.) and values distinguished according to age are given. No healthy volunteers were available at age between 11 and 20 years.

3.2. Se speciation

Chromatograms from Se speciation of both serum types, diluted 1:4 with eluent A, are demonstrated in Fig. 2 in comparison to a 5 μg/L control standard, which was run after a group of four samples. It is shown that separation of the late eluting compounds may get critical especially in samples with high organic load. Thus dilution of samples is advisable to prevent co-elution. Serum samples were measured in duplicate. Standard addition experiments were analyzed

Table 2
Methodical specifications

	Retention time	Limit of detection (μg/L)	Calibration function	r^2
Neutral/cationic	1.27 min ± 2.1%	0.11	$y = 860.41x + 1196$	0.9999
Se–methionine	1.79 min ± 1.6%	0.15	$y = 1091.7x + 1555$	0.9996
Se–ethionine	2.08 min ± 1.8%	0.19	$y = 587.61x + 1150$	0.9999
Se(IV)	7.94 min ± 2.0%	0.16	$y = 816.27x + 1036$	0.9971
Se–cystine	8.25 min ± 1.9%	0.22	$y = 536.27x - 438$	0.9993
Se(VI)	8.45 min ± 1.7%	0.09	$y = 1088.3x + 3069$	0.9991

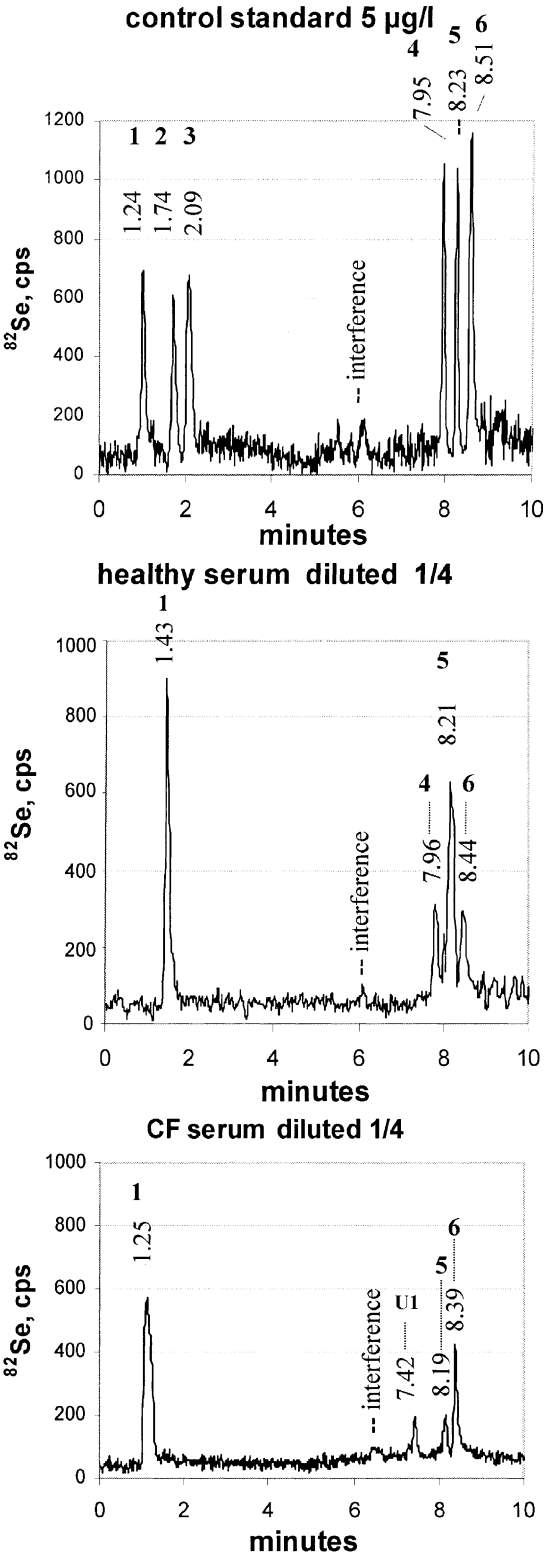

Fig. 2. Chromatograms are shown of sera from CF patients and healthy persons (each diluted 1:4 with eluent A) in comparison to a control standard mixture (5 μg/L). The standard compounds are (in consecutive order of elution): (1) SeU (co-eluting with neutral/cationic Se species); (2) SeM; (3) SeE; (4) Se(IV); (5) SeC; (6) Se(VI); (U1) Se compound not identified by RT or standard addition. Interference on ^{82}Se checked according to isotope ratio using Se isotopes 77, 78, 82.

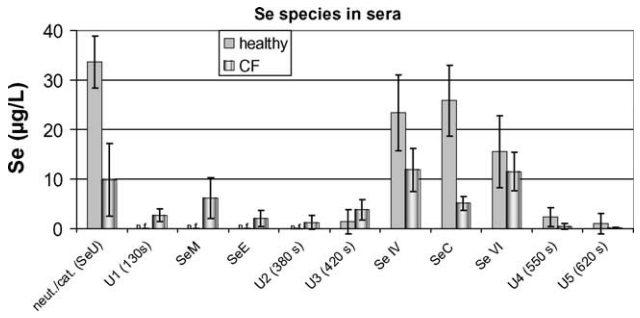

Fig. 3. Mean Se concentration of the Se species in serum are shown from healthy persons and CF patients. Significant differences are seen for neutral/cationic species, and SeC. Borderline significant difference is measured for Se(IV): n.f., not found; U(1–5), Se compounds not identified by RT or standard addition.

additionally to clearly identify peaks or to exclude the presence of respective compounds in serum. The speciation results of both serum types are summarized in Fig. 3.

Generally, again no significant difference between males and females was observed in Se species pattern. Remarkable shifts, however, were observed when comparing the Se species pattern from sera of healthy and CF persons. The predominant differences were as follows. (1) The presence of several low concentrated unknown Se compounds as well as SeM and SeE in most CF sera. These compounds were generally not detected in sera from healthy persons. (2) In each case where (free) SeM was found (21 of 31 CF sera and never in healthy sera) SeC was reduced by 51% (p = 0.0137). (3) The first peak, which is comprising of SeU and neutral/cationic Se compounds, and SeC were significantly higher concentrated in sera of healthy persons. The concentrations of Se(IV) and Se(VI) tended to higher concentrations in healthy sera, too. However, specifically the difference for Se(VI) (17 ± 8 μg/L healthy, 15 ± 4 μg/L, CF sera) is not significant as standard deviations show wide overlap. The increased values especially of the first peak and of SeC are the reason also for the higher total Se concentration in these sera. The concentration profile along age groups shows no specific trend. Variations are fully covered by standard deviations of the single age-group values. The significance at Se(IV) values is borderline as here standard deviations show still some overlap (22 ± 7 μg/L healthy, 15 ± 4 μg/L, CF sera, p < 0.1000). Again, the concentration profile according to age groups shows no specific trend. For the first peak (SeU, neutrals, cationic) and especially SeC the lower values in CF sera, however, are strongly significant. The cationic/neutral/SeU peak may include several Se species. Their concentration shows only small variation in healthy sera around 30 μg/L. In CF sera, however, the Se concentration of this peak increase from 7 μg/L in the 0–5 years group to 25 μg/L in the group 11–15 years. For older CF patients the values drop again to around 7 μg/L (Fig. 4a). For SeC the reduction is 75 % in CF sera compared to healthy ones (p = 0.0002). Looking at SeC along age groups the values in CF sera keep nearly constant (in analogy to total Se) at a low level around 5 μg/L. In

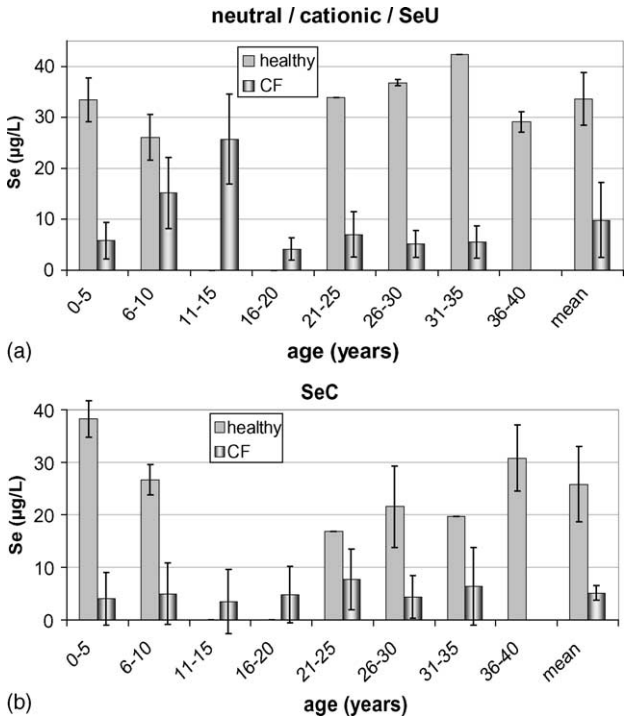

Fig. 4. Age-dependent Se-concentration profile as well as mean values of (a) the neutral/cationic peak from CF patients and healthy persons. No healthy volunteers were available at age between 11 and 20 years; (b) SeC from CF patients and healthy persons. No healthy volunteers were available at age between 11 and 20 years.

healthy sera SeC amounts are considerably higher, starting at 38.3 μg/L in the youngest group, decreasing to 26.7 and 16.9 μg/L in the groups 6–10 and 21–25 years, respectively, followed by an increase again to approximately 25–30 μg/L for older groups.

The fact that SeC is lowered in CF sera is very important. SeC is a central key metabolite in the regulated pathway of selenium. It is required for the synthesis of selenoproteins. Thus a reduced synthesis of SeC is discussed to result in a reduced production of the essential Se enzymes [20]. This lowers the anti-oxidative protection as well as the normal function of many metabolic reactions such as, e.g. control of thyroid metabolism or sufficient protection against several cardiovascular diseases [20].

Fig. 5 shows a simplified overview about the Se metabolic pathway and about the role of, e.g. SeC. The Se metabolites Se(IV) and SeC as well as the cationic/neutral compounds are lowered in CF patients (whilst SeM tends to be increased). The cationic/neutral Se peak in chromatograms may be supposed to contain Se proteins such as selenoprotein P (Sel P) or glutathione peroxidase. This assumption is supported by the fact that both enzymes show concentrations high enough in serum to be detected easily. Sel P is even that Se compound in serum which is carrying the major amount of serum-Se [20]. Preliminary experiments performed together with [21] focused on SEC fractions from serum having high Se amount and serum-GPx activity. Such fractions were analyzed by SAX-ICP-MS (employing the same method as in this study). It turned out that SAX-ICP-MS analysis of these fractions resulted in chromatograms with predominantly the neutral/cationic peak. This might be explained by exclusion of proteins from the chromatography separation matrix [22]. Further, the cationic/neutral Se peak in chromatograms contains the major amount of Se from serum samples and mass balances are determined around 100% (which proves that no major Se compound is missing). Taking these facts together it seems likely that these Se enzymes are monitored within this peak, of which the concentration is significantly reduced in CF sera. Thus important Se compounds from the regulated Se pathway seem to be considerably depleted in CF sera, resulting in a reduced impact concerning the essential functions, such as, e.g. oxidative protection. This is in good accordance to [21], where a reduced glutathion peroxidase activity was determined in CF sera.

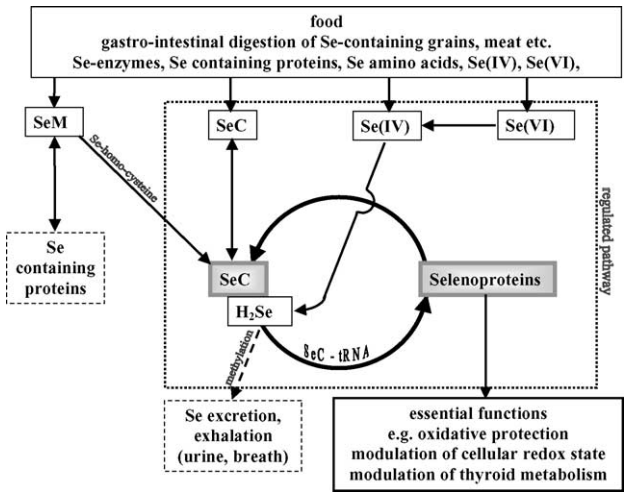

Fig. 5. Simplified scheme of the Se metabolism in humans, based on [23].

Summarizing, the formerly developed Se speciation method was applicable for human serum. Performance checks were done regularly after four samples, each, to ensure sufficient separation of species and sensitivity even after a considerable number of serum samples. CF sera showed lower values of total Se, SeC, and cationic/neutral Se compounds compared to healthy serum. No significant differences were measured between gender. The lowered SeC values together with lowered cationic/neutral Se compounds (probably Se enzymes) point to a Se-depleted regulated pathway combined with a reduced capability of protective functions such as protection from peroxides. Future experiments will focus on further investigations of the neutral/cationic or excluded compounds, e.g. using heparin-column-based separation and ESI-MS detection.

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