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Simultaneous determination of selenomethionine enantiomers in biological fluids by stable isotope dilution gas chromatography–mass spectrometry☆

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ABSTRACT

A method for the stereoselective determination of D- and L-enantiomers of selenomethionine in mouse plasma was developed using gas chromatography–mass spectrometry with selected-ion monitoring (GC–MS-SIM). DL-[$^{2}H_{3}$,^{82}Se]selenomethionine was used as analytical internal standard to account for losses associated with the extraction, derivatization and chromatography. Selenomethionine enantiomers in mouse plasma were purified by cation-exchange chromatography using BondElut SCX cartridge and derivatized with HCl in methanol to form methyl ester followed by subsequent *N*-acylation with optically active (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride to form diastereomeric amide. Quantification was performed by SIM of the molecular-related ions of the diastereomers on the chemical ionization mode. The intra- and inter-day precision for D- and L-selenomethionine spiked to mouse plasma gave good reproducibility with relative standard deviation of 3% and 3% for D-selenomethionine and 6% and 3% for L-selenomethionine, respectively. The estimated amounts were in good agreement with the actual amounts spiked, the intra- and inter-day relative error being 5% and 2% for D-selenomethionine and 2% and 1% for L-selenomethionine, respectively. The present method is sensitive enough to determine pharmacokinetics of selenomethionine enantiomers.

1. Introduction

Cisplatin is an inorganic platinum compound with a broad-spectrum antineoplastic activity against human tumors [1–8]. Both clinical and experimental studies have reported nephrotoxicity of cisplatin, which restricted its optimal usefulness in cancer chemotherapy [9,10]. Since the prevention of nephrotoxicity of cisplatin is considered to be of clinically great importance, several protective measures have been employed in the chemotherapy of cancer.

Selenium, which has been recognized as an essential element, protects against nephrotoxicity of cisplatin [11–14]. Among the various forms of selenium, selenomethionine is commonly used as a selenium source for human dietary supplements. Recently, pretreatment with L-selenomethionine resulted to reduce the nephrotoxicity of cisplatin without reducing the antitumor activity [15]. McAdam and Levander [16] showed little difference in the nutritional bioavailability between D- and L-selenomethionine in rats and suggested that D-selenomethionine might be converted into the L-enantiomer. L-Selenomethionine is known to be metabolized to L-selenohomocysteine via adenosyl-L-selenomethionine and adenosyl-L-selenohomocysteine as intermediates. Condensation of L-selenohomocysteine with L-serine gives L-selenocystathionine, which catabolizes to L-selenocysteine and 2-oxobutanoic acid. However, little information is available on the metabolic fate of D-selenomethionine, especially conversion of D-selenomethionine into the L-enantiomer. We have initiated studies to characterize the pharmacokinetic behavior of selenomethionine enantiomers.

Several methods have been reported for separation of selenomethionine enantiomers using GC [17,18] and HPLC [19–21] in combination with detection by inductively coupled plasma mass spectrometry (ICP-MS). In these methods, separation has been carried out directly with a chiral stationary phase column or indirectly with an achiral column following derivatization with a chiral reagent to form the diastereomer. However, determination of selenomethionine by ICP-MS is often difficult due to isobaric interferences from argon dimers on most selenium isotopes [22].

GC–MS has also been used as a powerful technique for characterizing amino acids in various samples [23,24], due to its inherent

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capability for structure identification. Selected ion monitoring (SIM) method and a stable isotope labeled analog as an internal standard have been extremely important for isotope dilution GC–MS. However, this method has rarely been applied to determine selenomethionine due to the complex molecular ion-cluster spectrum derived from selenium isotopes and the need to calculate relative isotope abundance of molecular ions. To overcome the complicated quantification procedure, a new stable isotope labeled selenomethionine is required for use as an analytical internal standard.

The present paper describes the procedure for the simultaneous determination of D- and L-selenomethionine by GC–MS-SIM. This study involves synthesis of DL-selenomethionine double-labeled with selenium-82 (^{82}Se) and deuteriums, chiral GC separation of DL-selenomethionine by diastereomeric method and quantification by stable isotope dilution method.

2. Experimental

2.1. Materials

DL-Selenomethionine was purchased from Wako Pure Chemicals (Osaka, Japan). (*RS*)-2-amino-4-bromobutyric acid hydrobromide was purchased from Aldrich (Milwaukee, WI, USA). [$^2H_3$]methyl iodide (>99.5 at.% ^{2}H) was purchased from ISOTEC (Tokyo, Japan). ^{82}Se metal powder (>99.72% enriched) was purchased from Eurisotop (Gif-Sur-Yvette, France). (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (MTPA-Cl) and 10% HCl/methanol were purchased from Tokyo Kasei (Tokyo, Japan). A strong cation-exchange solid-phase extraction column BondElut SCX (H^+ form, size 1 ml/100 mg) was purchased from Varian (Harbor City, CA, USA). Chloroform stabilized with amylene was purchased from Cica-Merck (Tokyo, Japan). All other chemicals and solvents were of analytical-reagent grade and were used without further purification.

2.2. Synthesis

2.2.1. (RS)-[$^2H_3$,^{82}Se]2-amino-4-methylselenylbutanoic acid (DL-[$^2H_3$,^{82}Se]selenomethionine)

[$^2H_3$]methyl iodide (10 g, 69.0 mmol) was added with stirring to lithium (1.1 g, 158 mmol) in dry diethyl ether (50 ml) under nitrogen atmosphere at a rate adequate to maintain gentle reflux of diethyl ether. The concentration of [$^2H_3$]methyllithium was estimated by hydrolysis of an aliquot (0.2 ml) and titration with 0.1 M HCl and was found to 1.1 mol/l. To a solution of [^{82}Se]selenium (metal powder, 80 mg, 0.98 mmol) in dry THF (30 ml) was added 6 ml of [$^2H_3$]methyllithium solution, and the resulting solution was stirred at room temperature until all [^{82}Se]selenium was dissolved. A solution of (*RS*)-2-amino-4-bromobutanoic acid hydrobromide (398 mg, 1.5 mmol) in dry ethanol (10 ml) was gradually added and stirred for 1 h. After evaporating the solvent under reduced pressure, the residue was dissolved in 1 M HCl (50 ml) and washed with diethyl ether (30 ml × 2). The aqueous layer was neutralized with 1 M NaOH. The solution was applied to a strong cation-exchange Dowex 50 W X8 (50–100 mesh) column (130 × 20 mm I.D., H^+ form), washed with water (200 ml) and eluted with 1 M ammonia (300 ml). After evaporating the solvent under reduced pressure, the crude product was obtained as a colorless solid, which was recrystallized with water-ethanol to yield DL-[$^2H_3$,^{82}Se]selenomethionine (67 mg, 34%) as colorless crystalline solid. m.p. 227 °C (dec.). ^{1}H NMR (400 MHz, ^2H_2O) δ 2.16–2.30 (2H, m, 4-H), 2.65 (2H, t, J = 7.7 Hz, 3-H), 3.87 (1H, dd, J = 5.4, 7.0 Hz, 2-H). ^{13}C-NMR (100 MHz, ^2H_2O) δ: 20.0 (4-C), 31.9 (3-C), 55.7 (2-C), 175.1 (1-C). Anal. Calcd for $C_5H_8{}^2H_3NO_2{}^{82}Se$: C, 29.71; H(^{2}H), 5.49; N, 6.93; O, 15.84; ^{82}Se, 40.58. Found: C, 29.67; H, 5.66; N, 6.83.

2.2.2. N-(2-Methoxy-2-trifluoromethyl-phenylacetyl)-DL-selenomethionine methyl ester (MTPA-DL-selenomethionine-OMe)

A solution of DL-selenomethionine (0.51 g, 2.57 mmol) in 10% HCl/methanol (20 ml) was heated at 60 °C for 2 h. The solvent was evaporated under reduced pressure. To the residue were added 10 ml of 2% triethylamine in chloroform and 1.0 g of MTPA-Cl. The resulting solution was stirred at room temperature for 2 h. After evaporating the solvent under reduced pressure, the residue was dissolved in ethyl acetate (15 ml) and washed with water (10 ml × 2) and dried over Na_2SO_4. The solvent was evaporated to yield MTPA-DL-selenomethionine-OMe (1.0 g, 53%) as gummy oil. Anal. Calcd. for $C_{16}H_{20}F_3NO_4Se$: C, 45.08; H, 4.73; F, 13.37; N, 3.29; O, 15.01; Se, 18.52. Found: C, 45.25; H, 4.75; N, 3.31.

2.3. Stock solutions

Stock solutions of DL-selenomethionine (37.0 nmol/ml) were prepared in methanol. The stock solutions were further diluted serially with methanol to prepare the standard solutions for calibration curve. Quality control (QC) standard solutions of DL-selenomethionine were prepared in the same manner as the calibration standard solutions and the respective concentration of each enantiomer were 1.7, 12.5 and 20.8 nmol/ml. The standard solution of DL-[$^2H_3$,^{82}Se]selenomethionine as internal standard was prepared at 50.6 nmol/ml in methanol. Storage of these solutions at 4 °C did not result in any detectable decomposition for at least six months.

2.4. Sample preparation

DL-[$^2H_3$,^{82}Se]selenomethionine (5.06 nmol in 100 μl) was added to 50 μl-aliquot of mouse plasma as an analytical internal standard. The plasma sample was deproteinized and extracted with ethanol (1 ml) on a vortex mixer for 0.5 min. After centrifugation at 1000 × *g* for 10 min, the supernatant was evaporated with a gentle stream of nitrogen at 40 °C. The residue was dissolved in 1 ml of 40 mM HCl and then applied to an activated BondElut SCX cartridge. The cartridge was pre-washed and activated with 3 ml of methanol, 3 ml of a mixture of methanol and 0.1 M HCl (1:1, v/v) and 3 ml of 0.1 M HCl. The cartridge was washed with 1 ml of water and 1 ml of methanol, and selenomethionine species were then eluted with 0.5 ml of 10% HCl in methanol. The eluate was heated at 60 °C for 1 h. After removal of the solvent under a stream of nitrogen, the residue was reconstituted in 100 μl of 2% triethylamine in chloroform and 100 μl of 2% MTPA-Cl in chloroform, shaken for 0.5 min on a vortex mixer and left at room temperature for 1 h. After washing the reaction mixture with water (1 ml × 2), the solvent was evaporated at room temperature under a stream of nitrogen. The residue was dissolved in 20 μl of ethyl acetate and a 1–2 μl of the solution was subject to GC–MS-SIM.

2.5. GC–MS-SIM

GC–MS-SIM analyses were performed on a Shimadzu (Kyoto, Japan) QP2010 quadrupole gas chromatography–mass spectrometer equipped with a data processing system. A methylsilicone bonded-phase fused-silica capillary column SPB-1 (15 m × 0.25 mm I.D.) with a 0.25 μm film thickness (Supelco, Bellefonte, PA, USA) was connected directly into the ion source. Helium was used as the carrier gas at a column head pressure of 100 kPa and total column flow rate was maintained at 1.0 ml/min. A split–splitless injection system Shimadzu SPL-G9 was operated in the splitless mode with a septum purge flow-rate of 3 ml/min after 2 min. The initial column temperature was set at 120 °C. After the sample injection, it was maintained for 2 min, increased at

25 °C/min to 270 °C and hold at 270 °C for 1 min. The temperature of the injector was 280 °C. The mass spectrometer was operated in chemical ionization mode with isobutane as the reactant gas at an electron energy of 70 eV and an emission current of 60 μA. The ion source temperature was 280 °C. SIM was performed on the molecular related ions at *m/z* 428 and 433 for the MTPA-OMe derivatives of selenomethionine and [$^2H_3$,^{82}Se]selenomethionine, respectively.

2.6. Calibration curves and quantification

To 50 μl portions of mouse plasma were added each of standard solutions containing known amounts of DL-selenomethionine (0.02, 0.04, 0.19, 0.37, 1.84, 3.70 and 5.56 nmol as racemate). DL-[$^2H_3$,^{82}Se]selenomethionine (5.06 nmol as racemate) was added as an internal standard. The samples were extracted, derivatized and analyzed as described above.

The peak area values were determined at *m/z* 428 for MTPA-OMe derivatives of D- and L-selenomethionine and at *m/z* 433 for those of D- and L-[$^2H_3$,^{82}Se]selenomethionine, and the peak area ratios (D-selenomethionine/D-[$^2H_3$,^{82}Se]selenomethionine and L-selenomethionine/L-[$^2H_3$,^{82}Se]selenomethionine) were calculated. The calibration curves were obtained by an unweighted least-squares linear fitting of the peak area ratios versus the amounts added on each sample. D- and L-selenomethionine concentrations in plasma were calculated by comparing the peak area ratios obtained from the unknown samples with those obtained from the standard mixtures.

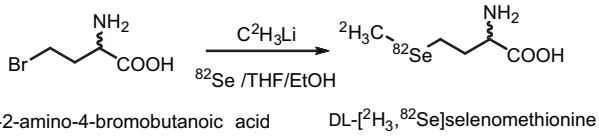
2.7. Accuracy and precision

QC samples were prepared by spiking the QC solution for DL-selenomethionine into 50 μl-aliquot of mouse plasma. Final concentrations of selenomethionine were 3.3, 24.9 and 41.6 nmol/ml for each enantiomer. After preparation of the sample for GC–MS-SIM as described above, the peak area ratios were determined.

3. Results and discussion

3.1. Stable isotope labeled selenomethionine

The use of GC–MS-SIM and stable isotopically labeled compounds as diluents has found broad application in pharmacokinetic studies. In this technique, stable isotopically labeled analogues have served as the ideal internal standard to correct for losses of a substance at all steps of sample handling, extraction, derivatization and chromatographic procedures. We have first synthesized selenomethionine labeled at predesigned positions for use as an internal standard. Selenium has six naturally occurring isotopes [^{74}Se (0.89%), ^{76}Se (9.37%), ^{77}Se (7.63%), ^{78}Se (23.77%), ^{80}Se (49.61%) and ^{82}Se (8.73%)], which give rise to a cluster of isotope peaks in mass spectrometry. To avoid the interference of the isotope clusters, we have chosen to introduce three deuteriums and ^{82}Se into the Se-methyl group of selenomethionine.



(RS)-2-amino-4-bromobutanoic acid DL-[$^2H_3$,^{82}Se]selenomethionine

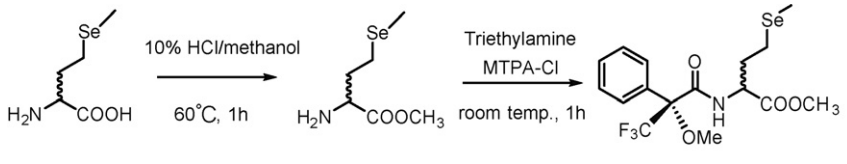
Fig. 1. Synthesis of DL-[$^2H_3$,^{82}Se]selenomethionine.

DL-[$^2H_3$,^{82}Se]selenomethionine was synthesized by condensation of commercially available (*RS*)-2-amino-4-bromobutanoic acid with lithium [$^2H_3$, ^{82}Se]methaneselenolate, which was prepared from ^{82}Se metal and [$^2H_3$]methyl iodide (Fig. 1). ^{1}H NMR data for DL-[$^2H_3$, ^{82}Se]selenomethionine were identical to the corresponding non-labeled DL-selenomethionine, except for the absence of signals of Se-methyl group. The DL-[$^2H_3$,^{82}Se]selenomethionine was used as an analytical internal standard.

3.2. GC–MS-SIM

The simultaneous determination of D- and L-enantiomers of selenomethionine by GC–MS requires the GC separation of the compounds. Since selenomethionine is not volatile and does not permit direct analysis on GC, it must be converted into suitable derivatives. Méndez et al. [17] have shown the direct resolution of DL-selenomethionine by a chiral stationary Chirasil-L-Val (*N*-propionyl-L-valine-tert-butylamide) column after derivatization to *N*-trifluoroacetyl isopropyl ester. In the indirect diastereomer method, DL-selenomethionine is converted to *N*-ethoxycarbonyl (+)-3-methyl-2-butyl ester derivative followed by separation on a conventional achiral column [25]. We have previously used MTPA-OMe derivative for separation of the leucine enantiomers [23]. One of the major advantages for using MTPA-Cl as a derivatizing reagent is that the reagent, lacking α-hydrogen, is highly resistant to racemization. The reaction scheme for the derivatization of selenomethionine was illustrated in Fig. 2. DL-Selenomethionine was converted into the methyl ester by HCl in methanol followed by subsequent chiral *N*-acylation with MTPA-Cl, and the diastereomeric MTPA-OMe derivative was subjected to GC–MS. The MTPA-OMe derivatives of D- and L-enantiomer underwent baseline separation (Rs = 1.52) on GC within 6.5 min and eluted in this order (Fig. 3).

In isotope dilution GC–MS, the presence of non-labeled molecule in the internal standard and naturally occurring isotope in the analyte arises the interference between the measured peak area of the analyte and that of its isotopic variant [26]. Fig. 4 shows the chemical ionization mass spectra for MTPA-OMe derivatives of non-labeled DL-selenomethionine and DL-[$^2H_3$,^{82}Se]selenomethionine. The molecular related ion $[M+H]^+$ clusters for the derivatives of non-labeled D- and L-selenomethionine appeared in the range of *m/z* 422–431, which corresponded to the isotopic isomer with natural abundances of C, H, O, N and Se isotopes. The relative intensities of the ion clusters were close to the theoretical values (Table 1). The ion clusters derived from selenium isotopomers was disappeared on the mass spectrum for the MTPA-OMe derivatives of D- and L-[$^2H_3$,^{82}Se]selenomethionine. The mass peak at *m/z* 433 of the



MTPA-DL-selenomethionine-OMe

Fig. 2. Derivatization of selenomethionine.

Table 1
Isotopic distributions for the *m*/*z* ions in the CI mass spectrum of the MTPA-OMe derivatives of selenomethionine and [$^2H_3$,^{82}Se]selenomethionine.

m/*z*	Corresponding Se isotope	Relative intensity (%)					
		MTPA-selenomethionine-OMe			MTPA-[$^2H_3$,^{82}Se]selenomethionine-OMe		
		Calculated	Found		Calculated	Found	
			D	L		D	L
422	74	1.8	1.2	0.1	0.0	0.0	0.0
423		0.3	1.5	0.1	0.0	0.0	0.0
424	76	18.7	20.2	17.7	0.0	0.0	0.0
425	77	18.6	23.3	20.6	0.0	0.0	0.0
426	78	50.6	51.7	48.7	0.0	0.0	0.0
427		9.0	18.3	16.0	0.0	0.0	0.0
428	80	100.0	100.0	100.0	0.1	0.1	0.1
429		18.0	19.7	17.0	0.0	0.0	0.1
430	82	19.7	19.4	16.7	0.0	0.1	0.1
431		3.4	2.8	0.8	0.2	0.8	1.1
432		0.4	0.0	0.0	0.0	6.7	6.5
433	82(+$^2H_3$)	0.0	0.0	0.0	100.0	100.0	100.0
434		0.0	0.0	0.0	18.0	17.5	17.8
435		0.0	0.0	0.0	2.4	2.3	2.4
436		0.0	0.0	0.0	0.2	0.2	0.2
437		0.0	0.0	0.0	0.0	0.0	0.0

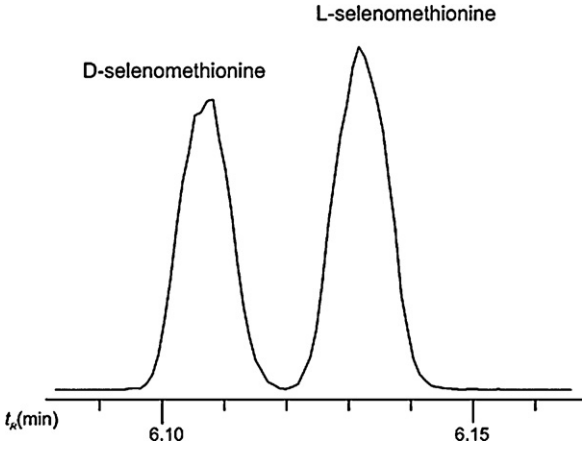


Fig. 3. Total ion chromatogram for MTPA-OMe derivatives of D- and L-selenomethionine. The retention times of the MTPA-OMe derivative of D- and L-methionine are 6.11 min and 6.13 min, respectively.

MTPA-OMe derivatives of D- and L-[$^2H_3$,^{82}Se]selenomethionine did not overlap with the *m*/*z* peaks known from that of corresponding non-labeled D- and L-selenomethionine. This result indicates that no correction for mutual contribution between non-labeled selenomethionine and [$^2H_3$,^{82}Se]selenomethionine is necessary. Because the respective MTPA-OMe derivatives produce strong molecular related ions $[M+H]^+$ at *m*/*z* 428 and 433, we have chosen the ions as the selected-ion monitoring ions.

To determine the limit of detection, known amounts of MTPA-OMe derivative of DL-selenomethionine, which was prepared as a reference, was analyzed by GC–MS-SIM. When a signal-to-noise (S/N) ratio of at least 3.0 was used as a criterion for a significant response, the limit of detection of the present GC–MS-SIM method was found to 300 fmol per injection for each D- and L-selenomethionine (Fig. 5).

3.3. Method validation

Cation-exchange column has been used as a simple method for extracting amino acids from biological fluids prior to GC–MS analysis. Aqueous ammonia was usually used as a solvent for elution of amino acids. However, evaporation of the eluent solvent was labo-

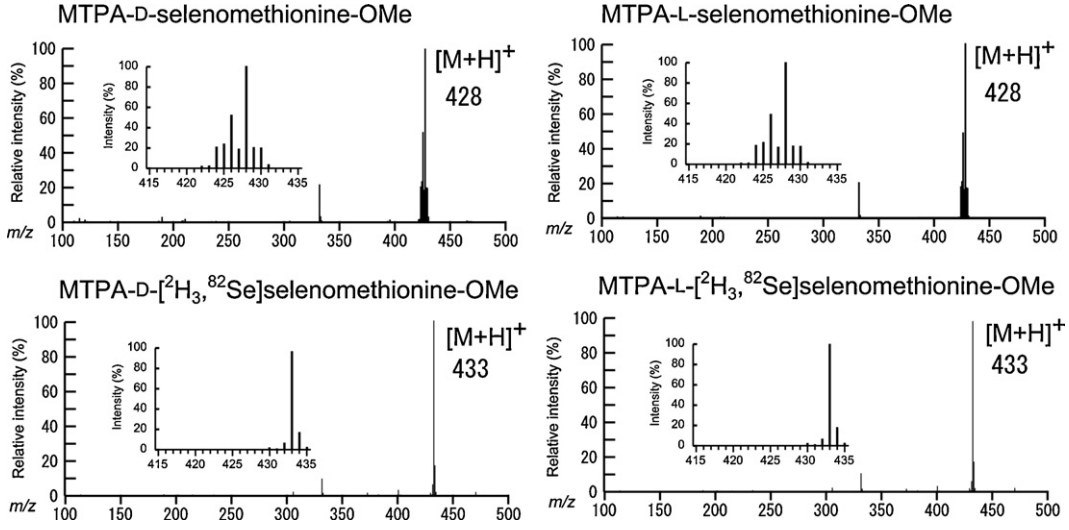


Fig. 4. Chemical-ionization mass spectra of MTPA-OMe derivatives of selenomethionine and [$^2H_3$,^{82}Se]selenomethionine enantiomers.

Table 2
Intra- and inter-day accuracy and precision for D- and L-selenomethionine spiked to mouse plasma.

Spiked (nmol)	Expected (nmol/ml)	Intra-day ($n=4$)			Inter-day ($n=3$)		
		Found (nmol/ml)	R.S.D. (%)	R.E. (%)	Found (nmol/ml)	R.S.D. (%)	R.E. (%)
D-Selenomethionine							
0.17	3.33	3.50 ± 0.10	2.79	+5.03	3.36 ± 0.07	1.98	+0.95
1.25	24.97	24.46 ± 0.59	2.43	−2.04	24.47 ± 0.69	2.83	−2.00
2.08	41.62	42.04 ± 0.61	1.45	+1.01	41.59 ± 0.29	0.70	−0.07
L-Selenomethionine							
0.17	3.33	3.35 ± 0.19	5.54	+0.70	3.32 ± 0.02	0.55	−0.33
1.25	24.97	24.49 ± 0.64	2.63	−1.94	24.76 ± 0.57	2.29	−0.88
2.08	41.62	42.25 ± 0.84	1.99	+1.51	41.91 ± 0.56	1.34	+0.69

rious and time-consuming. In the present study, we have used 10% HCl in methanol as the eluent solvent from BondElut SCX column and the eluate was directly heated to yield methyl ester. The recovery of DL-selenomethionine was found to be high yield (72 ± 4%, $n=3$).

The lower limit of quantification (LOQ) for the present method was determined by spiking 50 μl aliquots mouse plasma with DL-selenomethionine. Endogenous D- and L-selenomethionine were not detected in mouse plasma, which was consistent with the previous study in human plasma [27]. When a relative standard deviation of 10% or greater was a criteria for an LOQ, the values for D- and L-selenomethionine were around 0.3 nmol/ml plasma. The relative intensity of the monitoring ion species (m/z 428) against the total molecular related ions in the range of 422–431 of MTPA-OMe derivative of D- and L-selenomethionine was 41.5%. Therefore, the relative low sensitivity might have resulted in the ion clusters.

Calibration curves for D- and L-selenomethionine were constructed by the addition of known amounts of DL-selenomethionine in the range of 0.02–5.6 nmol to 50 μl each of mouse plasma. When the peak-area ratio (y) were plotted against the amounts added (x), good correlations were found, $y=2.6272x+0.0237$ ($r=0.9995$) for D-selenomethionine, $y=2.5560x-0.0177$ ($r=0.9993$) for L-selenomethionine.

The accuracy and precision of the method were determined by spiking QC solutions of DL-selenomethionine to 50 μl aliquots of mouse plasma. The final concentrations of each enantiomer were 3.3, 24.9 and 41.6 nmol/ml. These concentrations were chosen because the expected maximum plasma concentration of selenomethionine was around 40 nmol/ml after a bolus intravenous administration of D- or L-selenomethionine at a dose of 0.5 mg/kg (=2.5 μmol/kg), which corresponded to 5% of the minimal lethal dose [28]. The estimated amounts were in good agreement with the actual amounts spiked, the intra- and inter-day relative error (R.E.) being 5% and 2% for D-selenomethionine and 2% and 1% for L-selenomethionine, respectively (Table 2). The intra- and inter-day precisions with relative standard deviation (R.S.D.) of the assay were less than 3% and 3% for D-selenomethionine and those were less than 6% and 3% for L-selenomethionine, respectively. The results demonstrated an excellent reproducibility.

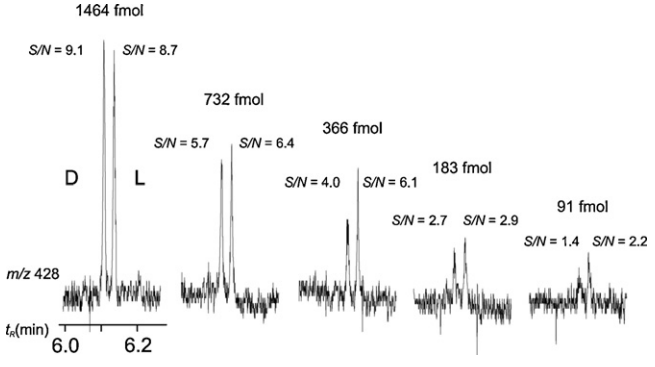


Fig. 5. Sensitivity of MTPA-OMe derivative of DL-selenomethionine by selected-ion monitoring.

4. Conclusions

Since DL-[$^2H_3$,^{82}Se]selenomethionine synthesized possessed sufficiently high isotopic purity, the mutual contribution between DL-[$^2H_3$, ^{82}Se]selenomethionine and the non-labeled selenomethionine were negligibly small. By using the DL-[$^2H_3$,^{82}Se]selenomethionine as internal standard, the present method provides a sensitive and reliable technique for the simultaneous determination of D- and L-selenomethionine in mouse plasma. The method can be applied pharmacokinetic and metabolic studies of selenomethionine enantiomer with a particular interest in evaluating the extent of conversion of D-selenomethionine into the L-enantiomer *in vivo*.

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