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Synthesis of novel selenium-containing choline and acetylcholine analogues and their quantitation using a pyrolysis–gas chromatography–mass spectrometry assay

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ABSTRACT

Methods for the synthesis and quantitation of the novel choline analogues, selenonium choline and acetylselenonium choline, are described. An assay procedure utilizing pyrolysis–gas chromatography–mass spectrometry (Py–GC–MS) with cold trapping was developed with deuterated d_4-selenonium choline and d_4-acetylselenonium choline as internal standards. The selenonium compounds were ion-pair extracted from tissue with dipicrylamine, washed with 2-butanone, and pyrolyzed prior to GC–MS analysis. The compounds were monitored using selected ion monitoring at *m/z* 122 and *m/z* 125 for the non-deuterated and deuterated compounds, respectively. The assay had a sensitivity of 20 pmol of compound taken through the assay and was linear through 20 nmol.

INTRODUCTION

The role of choline in cholinergic neurotransmission has been studied using a number of analogues of choline and acetylcholine. Those compounds studied include alkyl side chain derivatives [1] (homocholine and β-methylcholine), and quaternary nitrogen alkyl derivatives [2] (monoethylcholine, diethylcholine, triethylcholine, and pyrolidiniumcholine). A third group of choline analogues has been synthesized utilizing isosteric quaternary nitrogen replacements with a positive cationic feature. Examples include phosphocholine and arsenocholine [3], stibocholine [4], sulfocholine [5], and silicocholine and carbocholine [6]. A number of these choline analogues appear to qualify as cholinergic false transmitters [2–9], and can prevent acetylcholinesterase inhibitor toxicity [10].

Data indicate that some choline analogues exist naturally in living organisms including propionylcholine [11,12], arsenocholine [13–15], and arsenobetaine [16]. Selenium, like arsenic, is abundant in the earth's mantle [17] and is found in various food products [18]. Several selenoamino acids have been reported incorporated into proteins including selenocystine, selenocysteine, and selenomethionine [19]. Although selenobetaine does not occur naturally, it is extensively metabolized in male rats [16].

Because of the structural similarity of selenonium choline [$(CH_3)_2Se^+CH_2CH_2OH$, selenocholine] to choline, and the possibility that selenonium choline might occur in nature as well as act as a cholinergic false transmitter, it was of interest to undertake its synthesis and to develop a method for its quantitation.

Quantitation of choline or its analogues by gas

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chromatography (GC) or GC–mass spectrometric (MS) methods is problematic because of their cationic and non-volatile properties and therefore must be demethylated using either chemical or pyrolytic procedures. Pyrolysis (Py)–GC–MS has proven useful in the identification and quantitation of many substances which are difficult to chromatograph such as paints, polymers, matrix trapped plasticizers, and bacteria [20], as well as choline and acetylcholine [21,22].

This work, therefore, describes the synthesis of the novel choline analogues selenonium choline and acetylselenonium choline and their quantitation using a Py–GC–MS assay. Although the name selenocholine appears in the literature [23], selenium was utilized in that study as an oxygen replacement for the alcohol functionality (*i.e.*, a selenol) of choline, rather than for the quaternary nitrogen function.

METHODS

Spectroscopic studies

NMR spectra were recorded as C^2HCl_3 solutions, unless otherwise noted, on either a Bruker AM-300 (7.05 T) or a Bruker AM-500 (11.70 T) spectrometer. Resonance frequencies on the AM-300 are ^1H (300.133 MHz), ^{13}C (75.427 MHz), and ^{77}Se (57.19 MHz). On the AM-500, resonance frequencies are ^1H (500.13 MHz), ^{13}C (125.767 MHz), and ^{77}Se (95.314 MHz). Chemical shifts for ^{13}C [referenced with respect to internal C^2HCl_3 (δ 77.0 ppm)] and ^1H are reported in parts per million (ppm) relative to tetramethylsilane. The ^{77}Se chemical shifts are reported in ppm relative to a 60% C^2HCl_3 solution of dimethyl selenide [24]. All spectra were acquired at ambient probe temperature in 5-mm tubes. Typically, for ^{77}Se spectra 100–1000 scans were acquired using a pulse angle of 35° (90° pulse = 15.4 ms on the AM-500) and a recycle time of 2.2 s. A sweep width of 50 000 Hz and 64K data points resulted in a digitization of 1.7 Hz.

Infrared spectra were recorded on a Perkin-Elmer Fourier transform spectrometer as C^2HCl_3 solutions or neat unless otherwise noted. Mass spectra (including exact masses) were obtained on a VG 705Q mass spectrometer.

Reagents and chemicals

Elemental selenium was obtained from Alfa Products as a gray powder (100 mesh) and used without further purification. Bromoethanol was obtained from Aldrich, distilled, and stored over 4-Å molecular sieves before use. Iodomethane and methyllithium were obtained from Aldrich, and used without further purification. [1-$^2H_2$][2-$^2H_2$]-bromoethanol was obtained from Cambridge Isotopes, and used without further purification. The concentration of methyllithium reagents in commercial solutions was determined by titration of diphenylacetic acid to the yellow end point [25]. Tetrahydrofuran (THF) was distilled from sodium–benzophenone prior to use.

*Preparation of 2-(methylselenenyl)ethanol (**1**).* Elemental selenium (6.50 g; 82.3 mmol) and 100 ml of dry THF were added to a flame-dried 250-ml 3-neck flask, which was purged with nitrogen and fitted with a septum, a gas inlet, a ground glass stopper and a magnetic stir bar. Under nitrogen, methyl lithium was added dropwise at 0°C to the stirred Se–THF suspension. As the addition reached the end-point (1.0 equivalent), the dark brown suspension lightened to give a white suspension (if a more dilute solution was employed the resulting lithium methyl selenolate was a clear yellow solution). Stirring was continued for an additional 5 min, and the temperature was subsequently lowered to −78°C. 2-Chloroethanol (5.51 ml; 82.3 mmol) was then added dropwise and stirring was continued for 1 h at −78°C. The mixture was then allowed to warm to ambient temperature and stirring was continued for an additional hour. The reaction mixture was then filtered through a pad of SiO_2 (EM Science; 230–400 mesh) and all volatiles were removed *in vacuo*. The crude material was then subjected to flash column chromatography (SiO_2, 230–400 mesh) using a diethyl ether–hexane (30:70) mixture. Purified yield, 57%. Infrared (in cm^{-1}): 3670 (m), 3616 (s), 3475 (s), 2933 (s), 2976 (s), 1604 (m), 1468 (s), 1430 (s), 1392 (s), 1380 (s), 1341 (s), 1290 (s), 1255 (s), 1197 (s), 1181 (s), 1047 (s). NMR (in ppm): ^1H, δ 1.77 {s, 3H [^1H–^{77}Se satellites, J (^1H–^{77}Se) = 10.5 Hz]}, 2.46 (t, J = 6.7 Hz, 2H), 3.53 (m, 2H), 3.64 (t, J = 5.7 Hz, 1H (O–**H**)]; ^{13}C, 61.0, 28.1 [J(^{13}C–^{77}Se) = 61.6 Hz], 3.96 [J(^{13}–^{77}Se) = 62.3 Hz]: ^{77}Se, δ 34.

*Preparation of 2-(dimethylselenonium)ethanol iodide, selenonium choline (**2**).* Compound **1** (6.54 g; 47.1 mmol) was placed in a 100-ml single-neck round bottom flask fitted with a septum and containing a magnetic stir bar. The flask was chilled to −78°C and iodomethane was added dropwise. The mixture was slowly warmed to ambient temperature and stirring was continued for about 8 h. The resulting oil was washed three times with 50-ml portions of diethyl ether. All volatile materials were then removed *in vacuo* to yield 11.56 g (87%) of a viscous yellow oil. Infrared (in cm^{-1}): 3355 (s), 3011 (s), 2920 (m), 2876 (m), 1620 (w), 1417 (m), 1295 (m), 1270 (m), 1209 (w), 1142 (m), 1063 (s), 1010 (m), 984 (m). NMR (in ppm): ^1H [in dimethyl sulfoxide (DMSO-d_6)], δ 2.79 (s, 6H), 3.50 (m, 2H), 3.80 (dd, J = 5.1 Hz, 5.3 Hz, 2H), 5.24 (t, J = 4.7 Hz, 1H; ^{13}C (in DMSO-d_6), δ 57.0, 44.1, 21.5 [$J(^{13}C–^{77}Se)$ = 53 Hz]; ^{77}Se (in DMSO-d_6), δ 285 ppm. Elemental analysis calculated for $C_4H_{11}OSe^+$: C, 17.10; H, 3.95; Se, 28.10. Found: C, 17.20; H, 3.99; Se, 28.47.

*Preparation of 2-(methylselenenyl)-[1-$^2H_2$]-[2-$^2H_2$]ethanol (**3**).* Compound **3** was prepared using a procedure identical to that used to prepare **1** except that [1-$^2H_2$][2-$^2H_2$]bromoethanol was employed. The purified yield was 67%. NMR: ^1H (DMSO-d_6), 81.90 (s, 3H), 2.50 (s, 1H, OH).

*Preparation of 2-(dimethylselenonium)-[1-$^2H_2$]-[2-$^2H_2$]ethanol iodide (**4**).* Compound **4** was prepared from **3** using a procedure identical to that used to prepare **2**. The purified yield was 70%. NMR: ^1H(DMSO-d_6), δ 2.73 (s, 6H), 3.40 (s, 1H, OH).

Demethylation experiment

Compound **2** (100 mg) was placed in a single-neck 100-ml round bottom flask equipped with a high vacuum stopcock. The flask was attached to a high vacuum system, evacuated, and subjected to dynamic vacuum for 18 h. Volatile materials that evolved from the solid over this period were collected in a trap at −196°C. These materials were then placed in an NMR tube, dissolved in C^2HCl_3 and a ^1H spectrum obtained. The major compound was shown to be CH_3I (δ 2.15) by comparison to a ^1H NMR spectrum of a known sample of CH_3I in C^2HCl_3 (δ 2.13).

Quantitation of selenonium choline and acetylselenonium choline

Synthesis of the acetyl esters of d_0- and d_4-selenonium choline was required for the construction of a standard curve used to quantitate selenonium choline and acetylselenonium choline. Amounts of 5 μmol each of d_0- or d_4-selenonium choline dissolved in acetonitrile were added to 10-ml assay tubes and evaporated to dryness with nitrogen. Further, the tubes were desiccated under vacuum for 5 min and the residue dissolved in 2 ml of 12.5 m*M* silver *p*-toluenesulfonate–acetonitrile solution. An aliquot of 100 μl of doubly distilled acetylchloride was added, the solution vortexed briefly, and left at room temperature for 30 min. The tube was then evaporated to dryness under nitrogen and desiccated under vacuum for 5 min. The dried compound was reconstituted in 1 ml of sodium acetate buffer (0.05 *M*, pH 4) and used without further purification.

Standard curves for selenonium choline and acetylselenonium choline were prepared using 2.5 nmol of d_4-selenonium choline and d_4-acetylselenonium choline as internal standards along with various amounts of unlabelled selenonium choline and acetylselenonium choline. Acetonitrile–1 *M* formic acid (85:15, v/v) (2 ml) was then added and the supernatant transferred to a 10-ml screw-capped conical centrifuge tube. Diethylether was added, vortexed, and centrifuged for 2 min. The organic phase was then aspirated and residual ether and acetonitrile evaporated under nitrogen at 70°C for 5 min. The selenonium compounds were ion-pair extracted by adding 2 ml of 2 m*M* dipicrylamine in dichloromethane (DCM) and 0.5 ml of tris(hydroxymethyl)-methylaminopropanesulfonic acid (TAPS) buffer (pH 9.2). The mixture was immediately vortexed for 2 min and centrifuged. The upper aqueous layer was aspirated and the DCM layer was transferred to another tube, evaporated under a stream of nitrogen, and desiccated under vacuum for 5 min. Silver *p*-toluenesulfonate (5 m*M*) in acetonitrile (0.5 ml) was added plus 50 μl of doubly distilled propionyl chloride. The solution was then vortexed, and left at room temperature for 10 min. The tube was then evaporated to dryness under nitrogen and desiccated under vacuum for 5 min. The residue was dissolved in 250 μl of sodium acetate buffer (0.05 *M*, pH 4) and washed twice with 500 μl of 2-butanone.

The aqueous layer was then evaporated to dryness with nitrogen at 50°C and desiccated under vacuum for 5 min. The sample was then dissolved in an acetonitrile–water mixture (90:10, v/v), vortexted, and briefly kept on ice until analyzed by Py–GC–MS. For the assay of tissue, the procedure was the same as described above except that the tissue was homogenized in formic acid–acetonitrile with an internal standard using a glass Elvehjem apparatus and PTFE pestle. After centrifugation at 26 000 *g* for 20 min, the supernatant was decanted and processed as above.

The prepared samples were analyzed by Py–GC–MS using a Hewlett-Packard gas chromatograph (5890)–mass spectrometer (5970) and a Stabilwax column (Restek, 30 m × 0.25 mm I.D., 0.5-μm phase). Helium was used as the carrier gas at a head pressure of 5 p.s.i. and a linear flow velocity of 25 cm/s. Samples were pyrolyzed (Pyroprobe 122, Chemical Data Systems) using a quartz tube, for 10 s at 325°C. The pyrolysis interface temperature was 150°C, and the ramp setting was off. The pyrolysis products were trapped on the column by a CO_2 cold trapping attachment (see Fig. 1). Liquid CO_2 was supplied to the cold trapping area of the column from a CO_2 syphon cylinder controlled with a hand-operated valve. The 1/8 in. copper supply line terminated in a brass "T" fitting which served to deflect the CO_2 to maximize the cooled area and to support a 10-cm stainless-steel tube column jacket. The column jacket was necessary to prevent column breakage from CO_2 pressure surges.

After pyrolysis of the sample and cold trapping for 3 min, the oven door was closed and the oven heating ramp initiated. The initial oven temperature was 30°C, and was increased at 30°C/min to 200°C and held for 2.5 min. To prevent late eluting pyrolysis products from contaminating the next run, the oven temperature was further increased by 30°C/min to 225°C and held for 5.0 min. GC was operated in the splitless mode and the injector purge initiated at 3 min. The mass spectrometer was operated at 68 eV ionization energy, a filament current of 220 μA, an ion source temperature of 200°C and a vacuum of $5 \cdot 10^{-5}$ torr. Selected ion monitoring was chosen for sample analysis using *m/z* 122 ($[CH_3\text{-}Se\text{-}CH{=}CH_2]^+$) and *m/z* 125 ($[CH_3\text{-}Se\text{-}C^2H{=}C^2H_2]^+$) which corresponded to the base peaks for the d_0- and d_4-compounds, respectively (see Fig. 2). Quantitation was based on the d_0/d_4 ratios of the corrected areas and referenced to a standard curve. Areas were corrected and samples

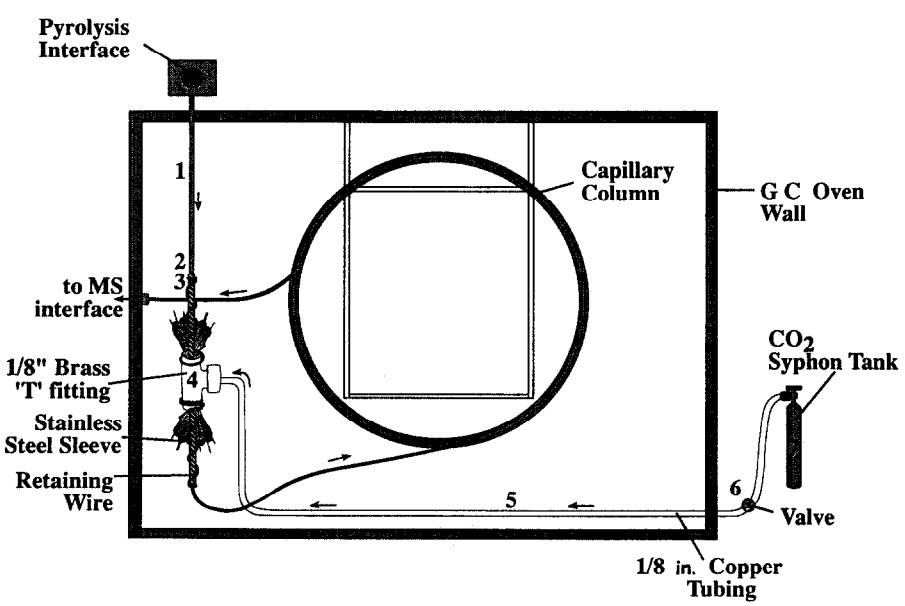

Fig. 1. Diagram of the CO_2 cold trapping apparatus. Key: 1 = capillary column, 2 = stainless-steel sleeve, 3 = retaining wire, 4 = 1/8 in. brass "T" fitting, 5 = 1/8 in. copper tubing, 6 = hand-operated control valve, 7 = CO_2 syphon tank.

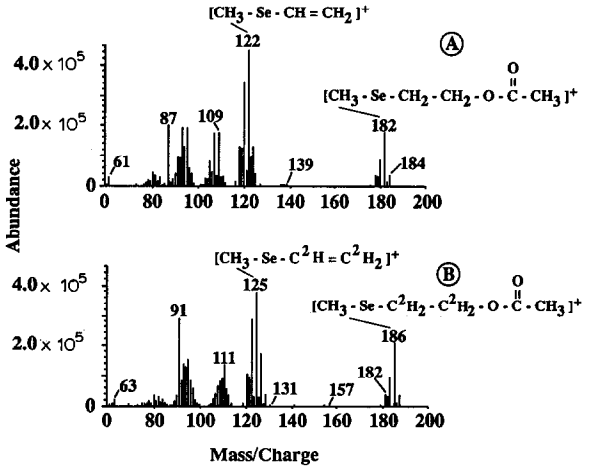

Fig. 2. Mass spectra of (A) d_0- and (B) d_4-acetylselenonium choline. The selenonium compounds were subjected to the complete assay protocol and analyzed by Py–GC–MS. Data were obtained using the HP5890/5970 GC–MS system operated in the "scan" mode.

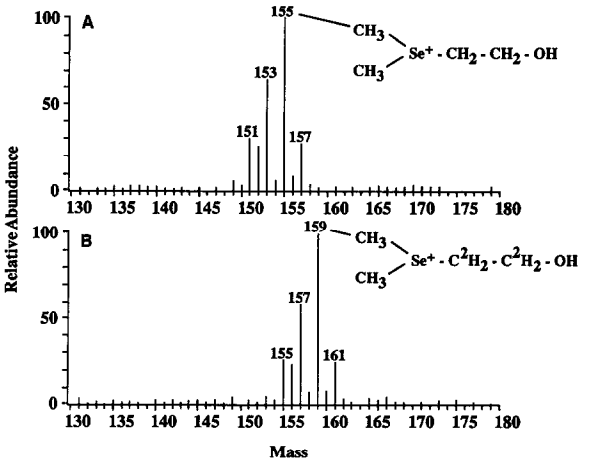

Fig. 3. Fast atom bombardment (FAB) spectra (positive ion) of (A) d_0- and (B) d_4-selenonium choline. Spectra were obtained following a standard static procedure using a VG 705Q mass spectrometer and data system.

automatically quantitated through the use of a matrix-based Pascal program written by the authors for the Hewlett-Packard system.

RESULTS AND DISCUSSION

The purpose of the present study was to synthesize the novel choline analogue selenonium choline and to develop a method to quantitate this analogue and its acetyl ester in biological tissue. A thorough spectroscopic characterization of the products synthesized according to the procedures detailed in the Methods section leaves no doubt that selenonium choline and the deuterated (d_4) analogue can be prepared in good yield (49.6% and 49%, respectively) with analytical purity. Proof of synthesis of these analogues is provided in Fig. 3. An exact mass peak using the glycerin m/z = 185 peak as a reference gave a mass of the parent 155 peak of 154.9972 for selenonium choline. The calculated mass for $C_4H_{11}O^{80}Se$ was 154.9975. The most intense peak in the parent ion envelope for d_4-selenonium choline was m/z 159. During the vacuum desiccation of this analogue at room temperature, spontaneous demethylation of selenonium choline iodide was found to occur with evolution of methyl iodide.

The quantitation of selenonium choline and acetylselenonium choline was accomplished using a Py–GC–MS procedure. Briefly, the selenonium compounds were ion-pair extracted from tissue samples, and selenonium choline was converted to the propionyl ester (Fig. 4). The acetyl and propionyl esters were then pyrolyzed and quantitated. It was necessary to propionylate selenonium choline in order to adequately resolve the peak areas representing acetylselenonium choline and selenonium choline. Several washes with 2-butanone were necessary to remove contaminating background due to dipicrylamine (DPA) used in the ion-pairing step. The column adopted for routine use was a Stabilwax column; however, HP20, and HP5 columns were also found to give satisfactory results. The assay procedure appears to be compatible with the column choice since over 300 injections have been made without decrements in performance. Since the selenonium compounds are positively charged, demethylation was necessary to allow volatilization and GC separation. Chemical demethylation was initially tried using the following agents: lithium butylselenolate, hexamethyldisilazane, lithium diisopropylamide mono(tetrahydrofuran), lithium bis-(trimethylsilyl)amide, lithium phenylselenolate and sodium thiophenoxide. Chemical demethylation was not quantitative for any of the reagents tried.

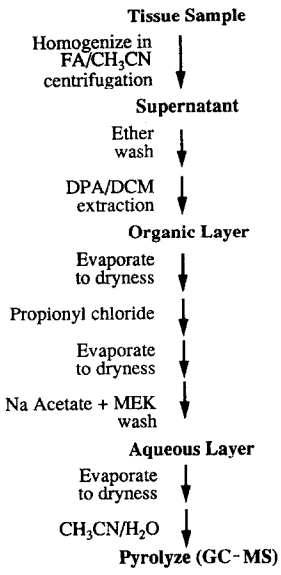

Fig. 4. Flow chart for assay of selenonium choline and acetylselenonium choline. Internal standards (d_4-selenonium choline and d_4-acetylselenonium choline) are added prior to homogenization. An aliquot of 2 μl of final extract is utilized for Py–GC–MS analysis. Abbreviations used are: FA = formic acid; MEK = methyl ethyl ketone = 2-butanone, DPA = dipicrylamine, DCM = dichloromethane.

Although sodium thiophenoxide was the most active, only 10% demethylation was observed. Quantitative demethylation of the selenonium compounds could only be obtained using pyrolysis. A similar problem has been reported with arsenocholine and related compounds [15].

Due to large void volumes in the pyrolysis interface, it was necessary to cold trap the demethylated selenonium compounds on the column prior to initiating the oven temperature program. The authors constructed a simple apparatus (see Fig. 1) at very low cost to achieve the cold trapping effect. A hand valve with low mass was necessary to allow control of CO_2 release and to minimize heat sink effects that would occur with a typical two stage pressure regulator. Also, a key part of the design is the stainless-steel tubing used to encase the column to minimize column breakage due to pressure surges. Although the temperature control of the cold trapped area was variable, little effect was observed on peak retention times and areas.

Fig. 2 illustrates representative mass spectra obtained on the HP5870 mass spectrometer for d_0- and d_4-acetylselenonium choline. Fragmentation patterns for the acetyl and propionyl esters were identical and only the acetyl ester is presented here. The peaks *m/z* 182 and 186 correspond to the molecular ion of the demethylated d_0- and d_4-acetyl esters, respectively. The most abundant peaks in the mass spectra for the acetyl ester were *m/z* 122 for the d_0-variant and *m/z* 125 for the d_4-variant. The *m/z* 122 peak corresponds to the olefinic ion $[CH_3\text{-}Se\text{-}CH=CH_2]^+$ for the d_0-compound and *m/z* 12 corresponds to the ion $[CH_3\text{-}Se\text{-}C^2H=C^2H_2]^+$. Other prominent ions were: *m/z* 109 and 95 for the d_0-compounds and *m/z* 111 and 91 for the d_4-variant. The peaks *m/z* 87 and 91 correspond to the fragments $[CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}CH_3]^+$ and $[C^2H_2\text{-}C^2H_2\text{-}O\text{-}CO\text{-}CH_3]^+$ for the d_0- and d_4-compounds, respectively.

Fig. 5 illustrates the linearity of the standard curve obtained for selenonium choline (as the propionyl ester) and acetylselenonium choline. Excellent linearity with low standard error was observed from 20 pmol (1.6 pmol injected) through 20 nmol for both selenonium compounds. Plotted points

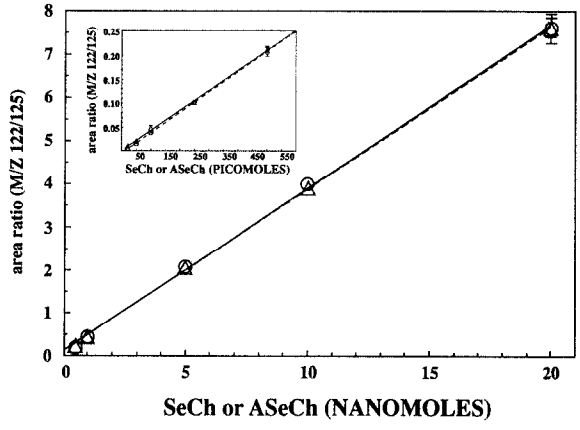

Fig. 5. Standard curves for selenonium choline and acetylselenonium choline. Known quantities of selenonium analogues with 2.5 nmol of internal standard (d_4-selenonium variants) were processed using the described assay protocol and analyzed by Py–GC–MS. Areas of the *m/z* 122 and 125 ions were corrected as described in Methods and the ratio of *m/z* 122/125 plotted *versus* the d_0-selenonium analogue concentration. The large graph demonstrates the linearity of 0.5 to 20 nmol quantities and the inset 20 to 500 pmol quantities of d_0-selenonium analogues. ○ = Acetylselenonium choline (ASeCh); △ = selenonium choline (SeCh).

represent the ratio of the corrected areas of the d_0- and d_4-compounds for each selenonium compound. Peak areas were corrected for isotopic spillover between *m/z* 122 and *m/z* 125 using a matrix calculation procedure similar to that used for the analysis of choline and acetylcholine [26]. The necessity for area corrections can be seen by inspection of a representative matrix table used in the calculations (Table I). The spillover from the *m/z* 122 ion into the *m/z* 125 ion area was usually low (5–8%), whereas the spillover from the *m/z* 125 ion into the *m/z* 122 ion area was higher and ranged from 16 to 17%. For the mixture of both compounds the *m/z* 125 ion area was typically 90–95% of the *m/z* 122 ion area. Generally one set of matrix tubes was analyzed and used as the basis for area corrections for each set of experimental samples (12–16 tubes).

Fig. 6 illustrates a representative selected ion monitoring (SIM) tracing of a 20 pmol sample (1.6 pmol injected) of selenonium and acetylselenonium choline processed in the presence of brain tissue and taken through the assay. Complete resolution and good separation (approximately 0.5 min) of the selenonium analogues were observed. Peak widths at half-height usually ranged from 0.03 to 0.05 min for peaks in this concentration range. The peak for the propionylated ester (selenonium choline) eluted after, and was usually larger than, the acetyl ester peak. The difference in peak areas for the same quantity of each ester is a pattern which is also seen in the GC–MS assay for choline and acetylcholine (data not presented). Table II illustrates the precision of the assay and recovery of the selenonium compounds in the presence of brain tissue. Considering the inherent problems associated with pyrolysis techniques [27], the data obtained show reasonable accuracy with slightly more variation at the lower concentrations of analogue. The presence of mouse brain tissue produced only marginal effects on the accuracy and precision of the method. The assayed values for selenonium choline were generally lower than theoretical in the absence of tissue, and higher than theoretical in the presence of tissue. The assayed values for acetylselenonium choline

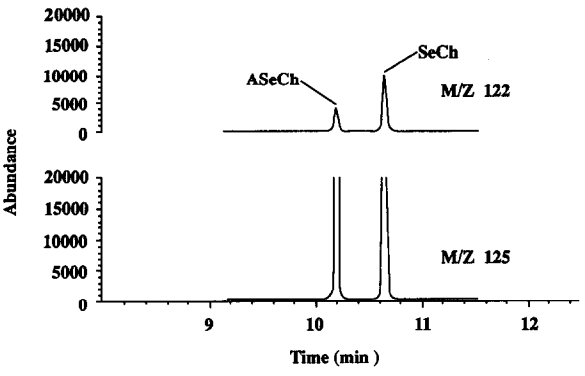

Fig. 6. Representative selected ion chromatogram of acetylselenonium choline and selenonium choline. The selenonium compounds were taken through the Py–GC–MS assay in the presence of 100 mg of mouse brain tissue. The top tracing corresponds to 20 pmol of d_0-analogues and the bottom tracing corresponds to 2.5 nmol of d_4-internal standards.

TABLE I

REPRESENTATIVE NORMALIZED AREA CONTRIBUTIONS BETWEEN MASS 122 AND MASS 125 IONS

Assay tubes containing only d_0-, or d_4-, or a mixture of d_0- + d_4-selenonium choline and acetylselenonium choline were processed according to the assay protocol and subjected to Py–GC–MS analysis. The areas obtained during selected ion monitoring analysis for *m/z* 122 and *m/z* 125 were normalized by dividing each area by the largest value obtained. The normalized areas were subsequently used in matrix calculations to provide corrected area values for the experimental samples.

Acetylselenonium choline			Selenonium choline		
Analogue	*m/z*		Analogue	*m/z*	
	122	125		122	125
d_0	1.0	0.07961	d_0	1.0	0.06562
d_4	0.16692	1.0	d_4	0.16645	1.0
$d_0 + d_4$	1.0	0.93483	$d_0 + d_4$	1.0	0.94651

TABLE II

ASSAY REPRODUCIBILITY IN THE PRESENCE AND ABSENCE OF BRAIN TISSUE

Known quantities of selenonium choline and acetylselenonium choline were homogenized in formic acid–acetonitrile in the presence and absence of 100 mg of mouse brain tissue. The samples were then processed according to the assay protocol and subjected to Py–GC–MS analysis.

Compound	Added (pmol)	Observed			
		Control (pmol)		Brain tissue (pmol)	
		(mean ± S.E.)[a]	*n*	(mean ± S.E.)	*n*
Selenonium choline	20.0	18 ± 1.5	9	25 ± 6	7
	100.0	94 ± 3	5	102 ± 4	6
	1000.0	1000 ± 70	6	990 ± 20	6
Acetylselenonium choline	20.0	27 ± 1	11	23 ± 1	8
	100.0	111 ± 4	6	103 ± 6	6
	1000.0	1040 ± 40	6	1000 ± 10	6

were generally higher than theoretical in the absence and presence of tissue. The majority of the values shown in Table II were well within the standard error of the standard curve points (Fig. 5).

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