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A highly sensitive LC–ESI-MS/MS method for the quantification of cholesterol ozonolysis products secosterol-A and secosterol-B after derivatization with 2-hydrazino-1-methylpyridine

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ABSTRACT

Cholesterol ozonolysis products, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (secosterol-A) and its aldolization product 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde (secosterol-B) have been found in atherosclerosis plaques and the brain tissues of Alzheimer's disease patients, implicating them in the pathogenesis of cardiovascular and neurodegenerative diseases. We have recently reported that when cholesterol is oxidized with an ozone-like oxidant generated by activated mouse neutrophils, secosterol-A is generated which is then converted to secosterol-B by an aldol reaction. To investigate further pathophysiological roles of secosterols, we have developed a highly sensitive method to detect secosterol-A and -B as derivatives with 2-hydrazino-1-methylpyridine (HMP) by LC–ESI-MS/MS. The limits of detection for the HMP derivatives of secosterol-A and secosterol-B were 0.05 and 0.01 fmol, respectively, which were approximately 400 and 2000 times better than those for underivatized secosterol-A and -B. We also developed a highly reproducible and accurate method to extract, purify and derivatize secosterol in small volumes of biological specimens. Using this method, we determined the levels of secosterol-A and -B as 1.4 ± 0.7 and 4.3 ± 0.8 nM, respectively, in the plasma of normal C57BL/6 mice, and in the range of 10.4 ± 16.3 to 40.7 ± 20.1 pmol/g and 110.9 ± 10.6 to 161.5 ± 56.3 pmol/g, respectively, in the brain, liver and lung tissues.

1. Introduction

3β-Hydroxy-5-oxo-5,6-secocholestan-6-al (secosterol-A) and its aldolization product 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde (secosterol-B) are major cholesterol oxidation products formed by cholesterol ozonolysis [1–3]. Recent studies showed that elevated levels of secosterol-A and -B are detected in human atherosclerotic plaques [4] and brain tissues of neurodegenerative diseases, such as Alzheimer's disease, Lewy body dementia, and α-synuclein overexpressed human neuroblastoma SH-SY5Y cells [5,6]. Secosterols possess strong cytotoxic effects toward various culture cells [7,8] and have the ability to covalently modify proteins, such as the amyloid-β peptide and α-synuclein to accelerate amyloidogenesis and fibrilization [6,9,10]. On the basis of these findings, secosterols have been implicated in the pathogenesis of cardiovascular and neurodegenerative diseases.

Wentworth et al. reported that secosterol-A was formed only when cholesterol was reacted with ozone but not with other reactive oxygen species such as singlet oxygen, superoxide, and hydroxyl radical [11]. Our recent study also showed that secosterol-A but not secosterol-B was formed in serum-free culture of activated mouse neutrophils, and its yield was increased significantly in the presence of IgG where it appeared to catalyze the formation of the ozone-like oxidant. Furthermore, secosterol-A in culture was found to be rapidly converted to secosterol-B and other secosterol metabolites in the presence of fetal bovine serum. Moreover, elevated levels of secosterol-A and secosterol-B were detected in plasma samples of wild-type (WT) mice but not myeloperoxidase (MPO)-deficient mice after i.p. injection of lipopolysaccharide (LPS). Secosterol-A and -B formations in inflamed tissues seem to be mainly mediated by MPO-dependent cholesterol ozonolysis [12]. On the other hand, it has been reported that secosterol-B was formed not only by aldolization of secosterol-A but also via Hock cleavage of cholesterol 5α-hydroperoxide, which is formed by the reaction of cholesterol with singlet oxygen [13,14]. Thus there are at least two pathways for secosterol-B formation, one ozone-dependent and the other independent. Our previous *in vitro*

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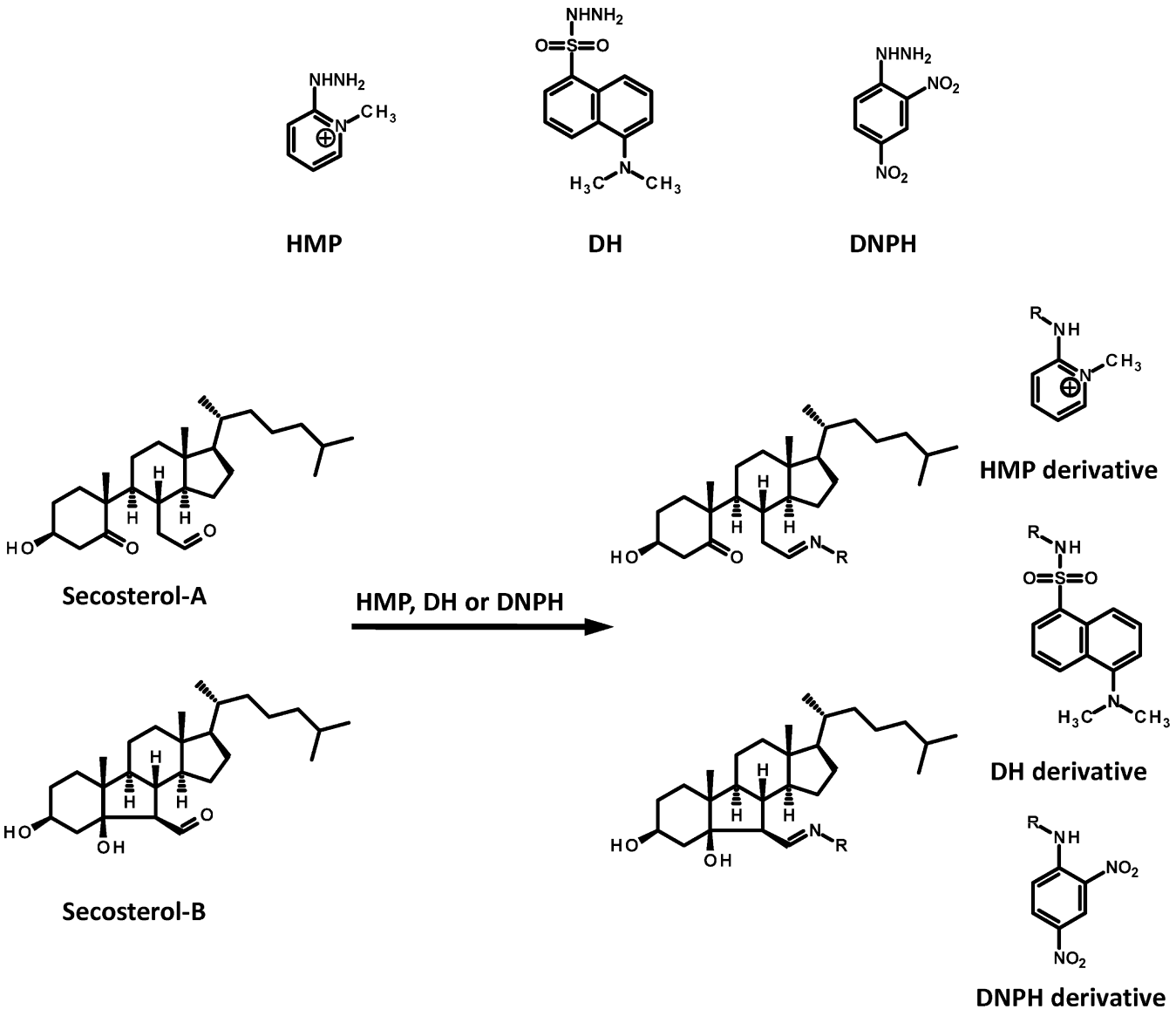

Fig. 1. Derivatization reagents and derivatization procedures of secosterol-A and -B.

experiments also showed that secosterol-B was easily formed by the reaction of cholesterol with a $^1O_2$-releasing compound, 1-methylnaphthalene-4-endoperoxide in phosphate buffer [15]. Moreover the constitutive level of secosterol-B in plasma was lower and less frequently detected in MPO-deficient mice than in WT-mice, whereas plasma levels of secosterol-A was almost below the detection limit in MPO-deficient mice. Therefore these results suggest that secosterol-B may be formed *in vivo* by both the ozone-dependent and independent pathways, whereas secosterol-A may only be formed through an ozone dependent pathway [12].

To quantify the physiological and pathological levels *in vivo* of secosterol-A and -B, they were analyzed by HPLC with UV detection or MS spectrometry after their conversion into dinitrophenylhydrazine (DNPH) derivatives [4]. Recently, Mansano et al. developed a highly sensitive method for the detection of secosterol-A and secosterol-B using HPLC and fluorescent detection after derivatization with 1-pyrenebutyric hydrazine (PBH) [16]. However, these methods lack specificity and/or sensitivity needed for the detection of secosterols in biological specimens from patients with various inflammation related diseases and for the analysis of molecular mechanisms of secosterol generation *in vivo*. We recently reported an analytical method for the quantification of secosterols after derivatization with the fluorescent compound dansyl hydrazine (DH) and detection by fluorescent HPLC and LC–ESI-MS/MS [15]. Using this method, we revealed the basal and increased levels of secosterol-A and -B in mice plasma after the i.p. injection of LPS as an acute inflammation stimulus [12]. However the method was insufficient to determine the levels of secosterols in some tissues [12].

In this study, we have explored a reagent, 2-hydrazino-1-methylpyridine (HMP), to derivatize secosterols because HMP has a 1-methylpyridyl group to act as a permanently charged moiety and a hydrazine group to act as a reactive site (Fig. 1). HMP derivatives provide only their molecular cations, $[M]^+$, and can be detected 70–1600-fold more sensitively than underivatized steroids [17]. We validated the method to quantify secosterols in various biological specimens, and used it to determine the levels of secosterols in the plasma, brain, liver and lung tissues of C57/BL6 mice.

Table 1
LODs of secosterols and their DNPH, DH and HMP derivatives.

Compound (mw)	t_R (min)	Transition (precursor ion, *m/z*/product ion, *m/z*)	LOD (fmol)	Increasing sensitivity
Secosterol-A (intact) (418.4)	25.0	436.4 $[M+NH_4]^+$/383.4 $[M-2H_2O+H]^+$	20	1
Secosterol-A-DNPH (598.4)	27.3	599.4 $[M+H]^+$/581.4 $[M-H_2O+H]^+$	10	2
Secosterol-A-DH (665.4)	26.3	666.4 $[M+H]^+$/236.1 [DH−hydrazine+H]$^+$	1	20
Secosterol-A-HMP (524.4)	20.2	524.0 $[M]^+$/109.1 [*N*-methylpyridine+NH_2]$^+$	0.05	400
Secosterol-B (intact) (418.4)	27.0	419.4 $[M+H]^+$/383.4 $[M-2H_2O+H]^+$	20	1
Secosterol-B-DNPH (598.4)	29.4	599.4 $[M+H]^+$/581.4 $[M-H_2O+H]^+$	10	2
Secosterol-B-DH (665.4)	27.8	666.4 $[M+H]^+$/236.1 [DH−hydrazine+H]$^+$	1	20
Secosterol-B-HMP (524.4)	21.7	524.0 $[M]^+$/109.1 [*N*-methylpyridine+NH_2]$^+$	0.01	2000

2. Materials and methods

2.1. Materials

3,4-^{13}C-Cholesterol, dansyl hydrazine (DH) and *p*-toluenesulfonic acid were purchased from Cambridge Isotope, Invitrogen (Carlsbad, CA) and Sigma (St. Louis, MO, USA), respectively. All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan). Secosterol-A, -B and 3,4-^{13}C-secosterol-A, -B were synthesized according to Wentworth [4]. The purity of the products was verified by TLC and ^{1}H-NMR. The secosterol stock solutions (10 mM) were prepared in ethanol and stored at −20 °C until use. HMP was synthesized according to the method of Higashi et al. [18]. Bond Elute Si cartridge (500 mg adsorbent; Varian, Inc., Palo Alto, CA, USA) were successively washed with 3 ml each of ethyl acetate and *n*-hexane prior to use.

2.2. Pretreatment of animal tissues

The plasma samples (20 μl) were added to 200 μl chloroform–methanol (2:1, v/v) containing 3,4-^{13}C-secosterol-A and -B (20 pmol each). After vigorously mixing for 1 min, supernatant was obtained by centrifugation at 3000 rpm for 10 min. The precipitate was further extracted with 200 μl chloroform–methanol (2:1, v/v) and centrifuged. The combined supernatant was dried and redissolved in 800 μl *n*-hexane, and the sample was passed through a Bond Elute Si cartridge. After washing with 3 ml *n*-hexane and 5 ml ethyl acetate–*n*-hexane (1:4, v/v), the secosterols were eluted with 5 ml ethyl acetate–acetonitrile (1:1, v/v). After evaporation of the solvent, the residue was subjected to derivatization with HMP as described below. The brain, liver or lung tissues (∼0.2 g) were homogenized in 1 ml chloroform–methanol (2:1, v/v) using a Multi-beads Shocker® (Yasui Kikai, Osaka, Japan). The homogenate was centrifuged at 3000 rpm for 10 min and the supernatant was obtained. The precipitate was further extracted with 5 ml chloroform–methanol (2:1, v/v) and centrifuged. The supernatants were combined, added to 3,4-^{13}C-secosterol-A and -B (20 pmol each) and washed with 2 ml water twice. The supernatant was dried and evaporated, and the residue was redissolved in 800 μl *n*-hexane then purified in the same way as the plasma sample. After evaporation of the solvent, the residue was subjected to derivatization with HMP as described below.

2.3. HMP derivatization

The sample was dissolved in 1 ml of acetonitrile, to which 0.2 mg HMP and 0.1 mg *p*-toluenesulfonic acid were added and incubated for 30 min at room temperature. After evaporation of the solvent, the derivatized product was dissolved in 1 ml acetonitrile, 10 μl of which was subjected to LC–MS/MS.

2.4. DNPH and DH derivatization

The sample was dissolved in 1 ml acetonitrile, to which 0.5 mg DNPH or DH and 0.1 mg *p*-toluenesulfonic acid were added and incubated for 4 h at room temperature in dark. After removal of the solvents, the derivatized product was dissolved in 1 ml acetonitrile, 10 μl of which was subjected to LC–MS/MS.

2.5. LC–MS/MS

LC–MS/MS was performed on an Agilent 1200 series HPLC system using a TSK-GEL ODS-100 V column (3 μm, 150 mm × 2.0 mm, TOSOH, Tokyo, Japan) and an Agilent G6410B triple quadrupole tandem mass spectrometer with an electrospray ionization device running in positive ionization mode. The detector conditions were as follows: capillary voltage at 4000 V, source temperature at 300 °C, drying gas flow at 7 l/min, nebulizer gas at 20 psi, and fragmentor at 200 V. The collision activated dissociation gas was set at medium using nitrogen as the collision gas. The detection of intact and derivatized secosterols was performed using the selected reaction monitoring (SRM) mode. The ion transitions monitored for HMP derivatives of secosterol-A and -B were *m/z* 524.0/109.1, 506.1, and 3,4-^{13}C-secosterol-A and -B were *m/z* 526.0/109.1, 508.1. Solvent A was 0.1% formic acid in water and solvent B was acetonitrile containing 0.1% formic acid. The HMP derivatives were eluted from the column using a linear gradient of 70% solvent A and 30% solvent B to 100% solvent B in 20 min and with 100% solvent B for an additional 20 min. The flow rate was 0.2 ml/min.

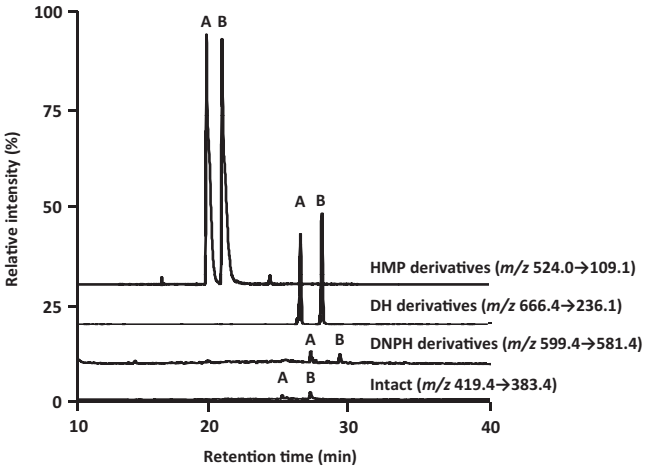

Fig. 2. LC–MS/MS analyses of DNPH, DH and HMP derivatives of secosterol. Ten nanomoles of secosterols were derivatized and then dissolved in the acetonitrile (1 ml). These derivatives (10 μl) were analyzed using the SRM mode. SRM chromatogram of intact secosterol-A (t_R 25.2 min); secosterol-B (t_R 27.2 min); secosterol-A-DNPH (t_R 27.3 min); secosterol-B-DNPH (t_R 29.4 min); secosterol-A-DH (t_R 26.3 min); secosterol-B-DH (t_R 27.9 min); secosterol-A-HMP (t_R 20.2 min); secosterol-B-HMP (t_R 21.7 min).

Table 2
Summary of calibration curves of secosterols.

		Slope[a] (R.S.D.)	Intercept[a]	Correlation coefficient (r^2)
Plasma	SecoA	0.0406 ± 0.00041 (1.0%)	0.0398 ± 0.0237	0.9994[b]
	SecoB	0.0326 ± 0.00047 (1.5%)	0.0239 ± 0.0037	0.9995[b]
Brain	SecoA	0.057 ± 0.00068 (1.2%)	0.0037 ± 0.0025	0.9948[c]
	SecoB	0.0632 ± 0.00038 (0.6%)	0.0887 ± 0.0066	0.9977[c]
Liver	SecoA	0.0498 ± 0.00117 (2.4%)	0.034 ± 0.0109	0.9995[c]
	SecoB	0.0642 ± 0.00023 (0.4%)	0.0482 ± 0.0161	0.9966[c]
Lung	SecoA	0.0476 ± 0.0005 (1.0%)	0.0296 ± 0.0036	0.9995[c]
	SecoB	0.0646 ± 0.0005 (0.7%)	0.052 ± 0.0164	0.9982[c]

[a] Mean ± S.D., $n = 5$.
[b] Measurable range, 1–50 pmol/ml.
[c] Measurable range, 1–50 pmol/g tissues.

Table 3
Precision in determination of secosterols in mice tissues.

Validation	Plasma A		Brain A		Liver A		Lung A	
	SecoA	SecoB	SecoA	SecoB	SecoA	SecoB	SecoA	SecoB
Intra-assay								
Mean ± S.D.	0.65 ± 0.03[a]	3.57 ± 0.22[a]	20.66 ± 0.99[b]	86.40 ± 1.80[b]	44.55 ± 2.13[b]	122.68 ± 3.97[b]	29.06 ± 1.03[b]	89.06 ± 6.6[b]
R.S.D. (%)	5.4	6.1	4.8	2.1	4.8	3.2	3.6	7.4
Inter-assay								
Mean ± S.D.	0.65 ± 0.05[a]	3.58 ± 0.02[a]	20.32 ± 0.72[b]	86.59 ± 1.77[b]	44.80 ± 3.05[b]	121.35 ± 2.62[b]	29.12 ± 1.32[b]	88.92 ± 2.31[b]
R.S.D. (%)	7.7	0.6	3.5	2.0	6.8	2.2	4.5	2.6
Validation	Plasma B		Brain B		Liver B		Lung B	
	SecoA	SecoB	SecoA	SecoB	SecoA	SecoB	SecoA	SecoB
Intra-assay								
Mean ± S.D.	0.90 ± 0.07[a]	4.28 ± 0.08[a]	4.86 ± 0.31[b]	98.42 ± 3.26[b]	12.20 ± 0.93[b]	246.71 ± 7.78[b]	28.45 ± 1.03[b]	76.64 ± 3.65[b]
R.S.D. (%)	8.2	1.8	6.6	3.3	7.6	3.2	3.6	4.8
Inter-assay								
Mean ± S.D.	0.88 ± 0.36[a]	4.25 ± 0.15[a]	4.84 ± 0.14[b]	98.67 ± 1.11[b]	11.28 ± 0.74[b]	247.80 ± 5.64[b]	28.48 ± 0.25[b]	77.46 ± 6.26[b]
R.S.D. (%)	4.1	3.6	2.9	1.1	6.6	2.3	0.9	8.1

[a] pmol/ml, $n = 5$.
[b] pmol/g tissues, $n = 5$.

2.6. Calibration curves for secosterol-A and -B

Calibration and validation studies were carried out by the addition of secosterol-A and -B to the plasma, brain, liver, and lung extracts obtained from untreated mice. The blank extract of brain, liver, lung (0.2 ml) and plasma (20 μl) were spiked with secosterol-A and -B [1, 2, 5, 10, 20 or 50 pmol each] and 3,4-^{13}C-secosterol-A and -B (20 pmol), which were then purified with the cartridge, derivatized and subjected to LC–MS/MS. The calibration curves were constructed by plotting the peak area ratios [(spiked secosterol-A – endogenous secosterol-A)/3,4-^{13}C-secosterol-A or (spiked secosterol-B – endogenous secosterol-B)/3,4-^{13}C-secosterol-B] against the concentration of secosterol-A and -B.

2.7. Assay precision

The intra-assay precision was assessed by determining two individual mice tissues samples at different concentration levels ($n = 5$ for each sample) on 1 day. The inter-assay precision was assessed by determining these samples over 5 days. The precision was determined as the relative standard deviation (R.S.D., %).

2.8. Analytical recovery (assay accuracy)

The extracts of brain, liver, lung and plasma were prepared by homogenizing the samples in chloroform–methanol as described in Section 2.2. Acetonitrile (2 μl; unspiked sample) or secosterol-A and -B dissolved in acetonitrile (0.4 or 1 pmol each in 2 μl; spiked sample) were added to the extracts. The spiked concentrations of secosterols were 0, 20, 50 pmol/ml, respectively ($n = 3$). After the addition of ISs (20 pmol each), each of the resulting samples was purified with the cartridge, derivatized and analyzed by LC–MS/MS. The analytical recoveries of secosterols were defined as secosterol-A or -B spiked sample concentration/(secosterol-A or -B unspiked sample concentration + spiked concentration) × 100 (%).

3. Results and discussion

3.1. Secosterols derivatization with HMP and their LODs

All compounds were analyzed by the ESI-MS in the positive-ion mode (Table 1). A precursor ion for underivatized secosterol-A observed as an ammonium adduct ion $[M+NH_4]^+$ (m/z 436.4) and the protonated molecule ($[M+H]^+$, m/z 419.4) provided two major product ions (m/z 383.4 and 365.4). After optimization, the precursor to product ion of m/z 436.4/383.4 was selected for secosterol-A quantitation by SRM analyses. In the case of secosterol-B, the protonated molecule ($[M+H]^+$, m/z 419.4) was observed as the base peak ion. The optimized precursor to product ion was m/z 419.4/383.4 for secosterol-B quantitation by SRM analyses. For HMP derivatives of secosterols, molecular cations $[M]^+$ (m/z 524.4) provided one major product ion (m/z 109.1). The optimized precursor to product ion was m/z 524.4/109.1 for HMP derivatives of secosterols quantitation by SRM analyses. For DNPH derivatives of secosterols, the protonated ion $[M+H]^+$ of secosterol-A-DNPH and secosterol-B-DNPH (m/z 599.4) provided one major product ion (m/z 581.4). The optimized precursor to product ion was m/z 599.4/581.4 for DNPH derivatives of secosterols quantitation by SRM analyses. For DH derivatives of

Table 4
Analytical recoveries (assay accuracy) in determination of secosterols in mice tissues.

Added[a]	Plasma A				Plasma B			
	SecoA		SecoB		SecoA		SecoB	
	Measured[a]	Recovery (%)	Measured[a]	Recovery (%)	Measured[a]	Recovery (%)	Measured[a]	Recovery (%)
0	0.65 ± 0.03	–	3.57 ± 0.22	–	0.90 ± 0.07	–	4.28 ± 0.08	–
20	20.08 ± 0.67	97.1	23.59 ± 0.47	100.1	20.60 ± 0.10	98.5	25.18 ± 0.93	104.5
50	49.32 ± 2.54	97.3	53.83 ± 1.70	100.5	49.03 ± 0.45	96.3	54.77 ± 2.47	100.9
Added[a]	Brain A				Brain B			
	SecoA		SecoB		SecoA		SecoB	
	Measured[b]	Recovery (%)	Measured[b]	Recovery (%)	Measured[b]	Recovery (%)	Measured[b]	Recovery (%)
0	20.66 ± 0.99	–	86.40 ± 1.80	–	4.86 ± 0.31	–	98.42 ± 3.26	–
20	40.83 ± 0.78	100.8	106.58 ± 0.62	100.9	25.28 ± 2.38	102.1	118.54 ± 1.90	100.6
50	71.41 ± 3.14	101.5	138.12 ± 1.58	103.4	54.90 ± 2.39	100.1	147.07 ± 4.29	97.3
Added[a]	Liver A				Liver B			
	SecoA		SecoB		SecoA		SecoB	
	Measured[b]	Recovery (%)	Measured[b]	Recovery (%)	Measured[b]	Recovery (%)	Measured[b]	Recovery (%)
0	44.55 ± 2.13	–	122.68 ± 3.97	–	12.20 ± 0.93	–	246.71 ± 7.78	–
20	62.90 ± 7.24	91.8	143.68 ± 2.94	105.0	33.10 ± 0.97	104.5	266.78 ± 11.34	101.3
50	98.54 ± 7.26	108.0	177.39 ± 9.30	109.4	60.81 ± 2.61	97.2	297.68 ± 6.09	101.9
Added[a]	Lung A				Lung B			
	SecoA		SecoB		SecoA		SecoB	
	Measured[b]	Recovery (%)	Measured[b]	Recovery (%)	Measured[b]	Recovery (%)	Measured[b]	Recovery (%)
0	29.06 ± 1.03	–	89.06 ± 6.56	–	28.45 ± 1.03	–	76.64 ± 3.65	–
20	48.78 ± 0.78	98.6	109.09 ± 0.79	100.1	48.73 ± 1.37	101.4	96.43 ± 17.24	99.0
50	80.20 ± 2.26	102.3	138.17 ± 3.71	98.2	79.37 ± 1.54	101.8	126.39 ± 9.25	99.5

[a] pmol/ml, $n = 3$.
[b] pmol/g tissues, $n = 3$.

secosterols, the protonated ion $[M+H]^+$ of secosterol-A-DH and secosterol-B-DH (m/z 666.4) provided two major product ions (m/z 236.1 and 170.1). The optimized precursor to product ion was m/z 666.4/236.1 for DH derivatives of secosterols quantitation by SRM analyses.

Fig. 2 shows the chromatogram for HMP derivatives of secosterols and IS. Peaks eluting at 20.2 and 21.7 min, corresponding to HMP derivatives of secosterol-A and -B, respectively, were detected. Although when we derivatized the reaction mixture under the presence of trifluoroacetic acid, or higher amounts of *p*-toluenesulfonic acid (up to 50 mg), or at higher temperatures (up to 60 °C), and for longer incubation time (up to 2 h), ~20% of secosterol-A was converted to secosterol-B, no conversions of secosterol-A to secosterol-B or secosterol-B to secosterol-A during the sample derivatization were observed under our experimental conditions. Typical chromatograms of underivatized secosterols and derivatized secosterols analyzed by optimized SRM conditions are also shown in Fig. 2. The limits of detection (LOD, S/N > 3) are summarized in Table 1. The LODs for various derivatives were as follows: 20 fmol for underivatized secosterol-A and -B, 10 fmol for secosterol-A-DNPH and secosterol-B-DNPH, 1 fmol for secosterol-A-DH and secosterol-B-DH, 0.05 fmol for secosterol-A-HMP, and 0.01 fmol for secosterol-B-HMP. Secosterols derivatized especially by DH and HMP increased the sensitivity of their detection. These increased sensitivities of DH derivatives could be attributed to the dimethylamino group in DH which is amenable to protonation under acidic conditions [19]. On the other hand, because HMP has a 1-methylpyrizyl group as a permanently charged moiety, the ionization efficiencies of HMP derivatized secosterols appear to be high and their product ion at m/z 109 by collision-induced dissociation (CID) derived from the 1-methylpyrizinoamino moiety results in their increased sensitivity for ESI-MS/MS analysis. The detection responses of the HMP-derivatives were increased by 400–2000-fold over the underivatized secosterols and the LODs were at the attomole range. Although the LODs for DNPH and DH derivatives of secosterol-A and -B were much better than those of the underivatized secosterols, they were inferior to the HMP derivatives. This sensitivity enhancement significantly contributed to reducing sample volume, which is important in developing a method applicable in the clinical fields.

3.2. Validation of the proposed method

In order to construct the calibration curves for quantitation of HMP-secosterols in biological samples, tissue extracts of plasma, brain, liver or lung from C57/BL6 mice were examined as a complex matrix (Table 2). The regression lines obtained from the combination of eight calibration curves were ($y = 0.0406x + 0.0398$, $y = 0.057x - 0.0037$, $y = 0.0498x + 0.034$ and $y = 0.0476x + 0.0296$, respectively) with a correlation coefficient (r^2) of (0.9994, 0.9948, 0.9995 and 0.9995, respectively) within the range of 1–50 pmol/ml or 1–50 pmol/g tissue for secosterol-A and ($y = 0.0326x + 0.0239$, $y = 0.0632x + 0.0887$, $y = 0.0642x + 0.047$ and $y = 0.0646x + 0.052$, respectively) with an r^2 of (0.9995, 0.9977, 0.9966 and 0.9982, respectively) within the range of 1–50 pmol/ml or 1–50 pmol/g tissue for secosterol-B. The determination at each concentration was repeated five times. A good calibration curve was obtained for several tissues. The R.S.D. of slope ($n = 5$) for secosterol-A were 1.0, 1.2, 2.4 and 1.0%, respectively, and for secosterol-B were 1.5, 0.6, 0.4 and 0.7%, respectively. These results demonstrate that the individual differences in the animal sample matrix do not affect the detection responses of the derivatized secosterols.

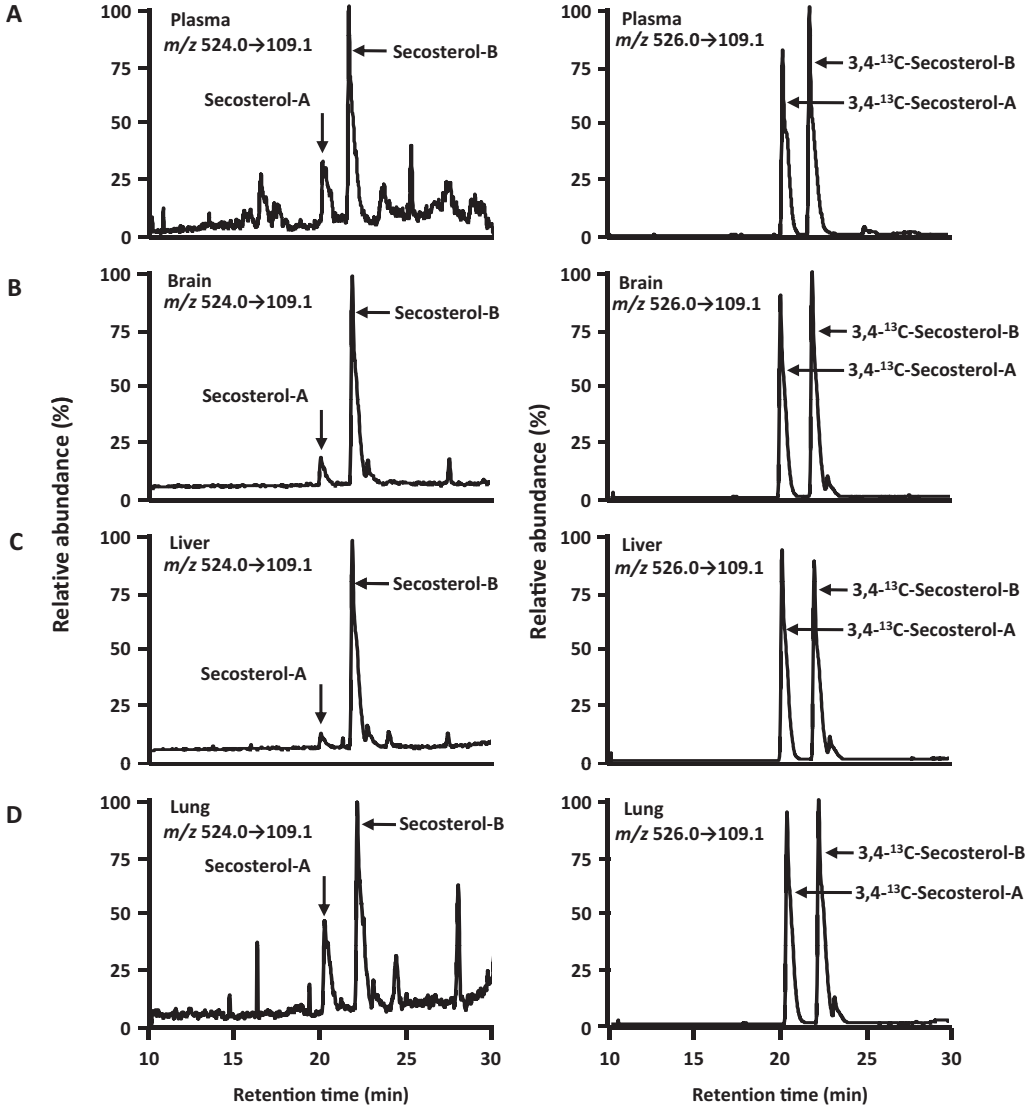

Fig. 3. LC–MS/MS analyses of HMP derivatives of secosterols and IS in mice plasma (A), brain (B), liver (C) and lung (D). The SRM transitions at *m/z* 524.0/109.1 and 52.0/109.1 were monitored for HMP derivatives of secosterol-A, -B and IS (3,4-^{13}C-secosterol-A, -B).

The assay precision was examined using samples of plasma, brain, liver and lung from C57/BL6 mice which contained different concentrations of secosterol-A, -B. R.S.D. were <9% for intraday and interday precision. The assay accuracy was evaluated as the analytical recovery (Table 3). As shown in Table 4, satisfactory recovery rates ranging from 91.8 to 109.4% were obtained. These data indicate that the present method is highly reproducible and accurate. In addition, the good linearity, sensitivity, recovery and

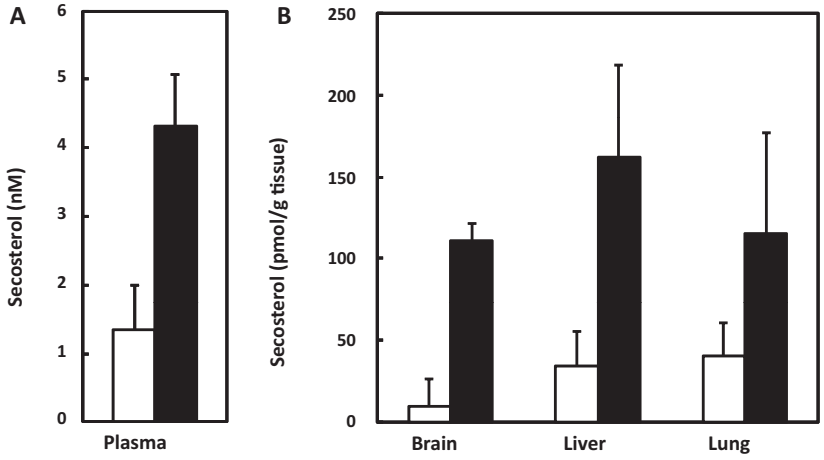

Fig. 4. Secosterols levels in several mice tissues. Value are mean ± S.D. (n = 3). Open and closed bars represent levels of secosterol-A and -B, respectively.

precision demonstrated that the present method is applicable for animal tissues.

3.3. Analysis of secosterol-A and -B in animal samples

The developed method was applied to the analysis of biological samples. We determined the levels of secosterols in the plasma, brain, liver and lung collected from C57/BL6 mice. Fig. 3 shows typical chromatograms obtained for the analyses of secosterols in mice samples. The levels of secosterol-A detected in mice samples were 1.4 ± 0.7 nM (plasma), 10.4 ± 16.3 pmol/g tissue (brain), 34.1 ± 21.6 pmol/g tissue (liver) and 40.7 ± 20.1 pmol/g tissue (lung). Similarly the levels of secosterol-B were 4.3 ± 0.8 nM (plasma), 110.9 ± 10.6 pmol/g tissue (brain), 161.5 ± 56.3 pmol/g tissue (liver) and 115.8 ± 61.3 pmol/g tissue (lung) (Fig. 4). It has been suggested that the ratio of secosterol-A to -B reflects their formation mechanism, which is either ozone-dependent or independent [11,15]. However, their ratios in each tissue were varied which could be due to their individual redox states.

Previous studies demonstrated that secosterols are constitutively present in several tissues, and the levels of secosterols are influenced by genetic and pathological factors. For example, Wentworth et al. reported that levels of secosterol-B were 70–1690 nM in the plasma from atherosclerotic patients, which were much higher than the levels in healthy subjects (less than the detection limit of ~1–10 nM detected by LC–MS after DNPH derivatization) [4]. We also demonstrated that constitutive levels of plasma secosterol-A and -B were significantly lower in MPO-deficient mice than in WT-mice [12]. Secosterol levels in WT-mice plasma, but not in MPO-deficient ones, were significantly elevated by the treatment with an acute inflammatory stimulus [12]. Moreover, Bieschke et al. reported that levels of secosterol-B were increased in the brain cortex tissue of patients with Alzheimer's disease (0.12 pmol/mg) and dementia with Lewy bodies, (0.21 pmol/mg), compared to that of normal subjects (0.09 pmol/mg) [5]. Also, secosterols in rat brain have been quantified at ~100 pg/mg (~0.24 pmol/mg) of secosterol-A and ~300 pg/mg (~0.72 pmol/mg) of secosterol-B using LC–MS/MS [20].

4. Conclusion

In conclusion, we developed a highly sensitive and specific LC–MS/MS method for the simultaneous determination of secosterol-A and -B in biological samples such as plasma, brain, liver and lung after converting them to HMP-derivatives. The derivatization is simple to handle, and the process does not interfere with the analyses. HMP-derivatized secosterols can be easily detected by positive ESI-MS/MS. This derivatization method combined with LC–ESI-MS/MS should prove useful for quantitating the levels of secosterols as biomarkers for early diagnosis of several oxidative stress related diseases.

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