



Identification of phase II metabolites of thiol-conjugated [6]-shogaol in mouse urine using high-performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Ginger is frequently consumed as a spice and has numerous medicinal properties. Extensive research has characterized the anti-inflammatory, antioxidant, and antitumor activities of ginger. Previously, we reported the mercapturic acid pathway as a major metabolic route of [6]-shogaol in mice and the thiol conjugates of [6]-shogaol existed in the glucuronidated and sulfated forms in mouse urine. However, their structures are still unknown. In the present study, we further investigated the phase II metabolism of thiol-conjugated [6]-shogaol in mouse urine, in which we identified sixteen phase II metabolites of thiol-conjugated [6]-shogaol: 5-cysteiny-[6]-shogaol glucuronide (**9**), 5-N-acetylcysteinyl-[6]-shogaol glucuronide (**10**), 5-cysteinylglycyl-[6]-shogaol glucuronide (**11**), 5-methylthio-[6]-shogaol glucuronide (**12**), 5-cysteiny-M6 glucuronide (**13** and **14**), 5-cysteiny-M6 sulfate (**15** and **16**), 5-N-acetylcysteinyl-M6 glucuronide (**17** and **18**), 5-cysteinylglycyl-M6 glucuronide (**19** and **20**), 5-cysteinylglycyl-M6 sulfate (**21** and **22**), and 5-methylthio-M6 glucuronide (**23** and **24**) using liquid chromatography/electrospray ionization tandem mass spectrometry. The structures of these metabolites were confirmed by analyzing their MSⁿ ($n = 1-4$) spectra as well as comparing with the tandem mass spectra of authentic standards. To the best of our knowledge, this is the first report involving identification of phase II urinary metabolites of [6]-shogaol in mice.

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1. Introduction

Ginger (*Zingiber officinale* Rosc.), a tropical and subtropical cultivated plant, has been widely used as a spice, dietary supplement, and traditional medicine for centuries [1]. In recent times, there has been much research conducted to test the validity of the medicinal claims made about ginger, including anti-inflammatory, anti-microbial, anti-allergic or control of nausea and vomiting activities [2]. [6]-Shogaol is one of the predominant pungent constituents in dried ginger [3–6]. A number of *in vitro* and *in vivo* studies have found that [6]-shogaol possesses many medical and biological effects including antiemetic, vasodilating, antibacterial, antifouling, antitussive, and anti-hepatotoxic properties [7–12]. Recently, [6]-shogaol has drawn more attention than [6]-gingerol, another major component in ginger, due to its higher potency. Pan et al. showed that [6]-shogaol suppressed lipopolysaccharide-induced up-expression of iNOS and COX-2 in murine macrophages by inhibiting the activation of NFκB

[13]. Dugasani et al. found that [6]-shogaol exhibited more potent antioxidant and anti-inflammatory properties than [6]-, [8]-, and [10]-gingerols [14]. More recently, along with our collaborators, we have demonstrated that [6]-, [8]-, and [10]-shogaols exhibited much higher anti-proliferative potency than [6]-, [8]-, and [10]-gingerols against human lung cancer cells (H-1299) [5], and [6]-shogaol was more effective than [6]-gingerol in inhibiting 12-O-tetradecanoylphorbol 13-acetate-induced tumor promotion in mice [6].

Due to the prospective health benefits of [6]-shogaol, characterization, identification and structure elucidation of its metabolites is an important step for thorough understanding of its biological activities. Recently, we have identified mercapturic acid pathway as one of the major metabolic routes of [6]-shogaol and found that the thiol-conjugated metabolites of [6]-shogaol existed mostly in the glucuronidated and sulfated form in mouse urine [15]. In the present study, we analyzed the phase II metabolites of thiol-conjugated [6]-shogaol in mouse urine using high-performance liquid chromatography tandem mass spectrometry. The structures of the major metabolites were identified by analyzing their MS²–MS⁴ spectra as well as comparing with the tandem mass spectra of authentic standards.

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2. Experimental

2.1. Chemicals and reagents

[6]-Shogaol was purified from ginger extract in our laboratory [5]. 1-(4'-Hydroxy-3'-methoxyphenyl)-4-decen-3-ol (M6), 5-methylthio-6S, and 5-methylthio-M6 were purified from fecal samples collected from [6]-shogaol treated mice [15]. HPLC-grade solvents and other reagents were obtained from VWR International (South Plainfield, NJ). LC-MS grade solvents and other reagents were obtained from Thermo Fisher Scientific (Pittsburgh, PA). Sulfatase from *Aerobacter aerogenes* and β -glucuronidase from *Helix aspersa* were obtained from Sigma (St. Louis, MN).

2.2. Treatment of mice and urine collection

Experiments with mice were carried out according to a protocol described previously [15]. In brief, female C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and allowed to acclimate for at least 1 week prior to the start of the experiment. The mice were housed 5 per cage and maintained in air-conditioned quarters with a room temperature of $20 \pm 2^\circ\text{C}$, relative humidity of $50 \pm 10\%$, and an alternating 12-h light/dark cycle. Mice were fed Purina Rodent Chow #5001 (Research Diets) and water, and were allowed to eat and drink *ad libitum*. [6]-Shogaol in dimethyl sulfoxide (DMSO) was administered to mice by oral gavage (200 mg/kg). Urine samples were collected in metabolism cages (5 mice per cage) 24 h after administration of vehicle (control group, $n = 5$) or [6]-shogaol (treated group, $n = 5$). These samples were stored at -80°C before analysis.

2.3. Urine sample preparation

The urine samples (50 μL from each group, control group and [6]-shogaol-treated group) were added to 1200 μL methanol to precipitate proteins. After centrifugation at $17 \times 1000\text{rpm}$ for 5 min, the supernatants were transferred into vials for LC/MS analysis. Enzymatic deconjugation was performed as described previously with slight modification [16]. In brief, duplicate samples were prepared in the presence of β -glucuronidase (250 U) and sulfatase (3 U) for 24 h at 37°C and then extracted twice with ethyl acetate. The ethyl acetate fraction was dried under vacuum, and the solid was resuspended in 250 mL of 80% aqueous methanol with 0.1% acetic acid for further LC/MS analysis.

2.4. LC/ESI-MS method

LC/MS analysis was carried out with a Thermo-Finnigan Spectra System which consisted of an Accela high-speed MS pump, an Accela refrigerated autosampler, and an LTQ Velos ion trap mass detector (Thermo Electron, San Jose, CA) incorporated with heated electrospray ionization (H-ESI) interfaces. A Gemini C18 column (50 mm \times 2.0 mm i.d., 3 μm ; Phenomenex, Torrance, CA, USA) was used for separation at a flow rate of 0.2 mL/min. The column was eluted with 100% solvent A (5% aqueous methanol with 0.2% acetic acid) for 3 min, followed by linear increases in B (95% aqueous methanol with 0.2% acetic acid) to 40% from 3 to 15 min, to 85% from 15 to 45 min, to 100% from 45 to 50 min, and then with 100% B from 50 to 55 min. The column was then re-equilibrated with 100% A for 5 min. The LC eluent was introduced into the ESI interface. The positive ion polarity mode was set for the H-ESI source with the voltage on the H-ESI interface maintained at approximately 4.5 kV. Nitrogen gas was used as the sheath gas and auxiliary gas. Optimized source parameters, including ESI capillary temperature (300°C), capillary voltage (50 V), ion

spray voltage (3.6 kV), sheath gas flow rate (30 units), auxiliary gas flow rate (5 units), and tube lens (120 V), were tuned using authentic 5-N-acetylcysteinyl-[6]-shogaol. The negative ion polarity mode was set for the H-ESI source with the voltage on the H-ESI interface maintained at approximately 4.5 kV. Nitrogen gas was used as the sheath gas and auxiliary gas. Optimized source parameters, including ESI capillary temperature (300°C), capillary voltage (50 V), ion spray voltage (3.6 kV), sheath gas flow rate (30 units), auxiliary gas flow rate (5 units), and tube lens (120 V), were tuned using authentic 5-N-acetylcysteinyl-5-shogaol. The collision-induced dissociation (CID) for both positive and negative was conducted with an isolation width of 2 Da and normalized collision energy of 35 eV for MS^2 , MS^3 and MS^4 . Default automated gain control target ion values were used for $\text{MS}-\text{MS}^4$ analyses. The mass range was measured from 50 to 1000 m/z . Data acquisition was performed with Xcalibur 2.0 version (Thermo Electron, San Jose, CA).

3. Results and discussion

3.1. Strategies for data analysis

Previously, we have reported that the mercapturic acid pathway is one of the major metabolic routes of [6]-shogaol and have identified seven thiol-conjugated metabolites of [6]-shogaol and its ketone-reduced metabolite 1-(4'-hydroxy-3'-methoxyphenyl)-4-decen-3-ol (M6) (Fig. 1). It was determined that the thiol-conjugated metabolites of [6]-shogaol were undetectable without incubation with glucuronidase and sulfatase in mouse urine and plasma indicating that these thiol-conjugated metabolites exist in the glucuronidated and/or sulfated forms [15]. The current study further investigated the phase II metabolites of these thiol-conjugated compounds in mouse urine. In this study, we used selected-ion monitoring (SIM) mode to search all the potential phase II metabolites of the thiol-conjugated metabolites of [6]-shogaol from urine samples collected from [6]-shogaol treated mice. The possible glucuronidated metabolites included 5-cysteinyl-[6]-shogaol glucuronide (5-Cys-6S-Glu, m/z 573), 5-N-acetylcysteinyl-[6]-shogaol glucuronide (5-NAC-6S-Glu, m/z 615), 5-cysteinylglycyl-[6]-shogaol glucuronide (5-Cys-Gly-6S-Glu, m/z 630), 5-glutathione-[6]-shogaol glucuronide (5-GSH-6S-Glu, m/z 759), 5-methylthio-[6]-shogaol glucuronide (5-Methylthio-6S-Glu, m/z 500), 5-cysteinyl-1-(4'-hydroxy-3'-methoxyphenyl)-4-decan-3-ol glucuronide (5-Cys-M6-Glu, m/z 575), 5-N-acetylcysteinyl-1-(4'-hydroxy-3'-methoxyphenyl)-4-decan-3-ol glucuronide (5-NAC-M6-Glu, m/z 617), 5-cysteinylglycyl-1-(4'-hydroxy-3'-methoxyphenyl)-4-decan-3-ol glucuronide (5-Cys-Gly-M6-Glu, m/z 632), 5-glutathione-1-(4'-hydroxy-3'-methoxyphenyl)-4-decan-3-ol glucuronide (5-GSH-M6-Glu, m/z 761), and 5-methylthio-1-(4'-hydroxy-3'-methoxyphenyl)-4-decan-3-ol glucuronide (5-methylthio-M6-Glu, m/z 502). The possible sulfated metabolites included 5-cysteinyl-[6]-shogaol sulfate (5-Cys-6S-Sul, m/z 477), 5-N-acetylcysteinyl-[6]-shogaol sulfate (5-NAC-6S-Sul, m/z 519), 5-cysteinylglycyl-[6]-shogaol sulfate (5-Cys-Gly-6S-Sul, m/z 534), 5-glutathione-[6]-shogaol sulfate (5-GSH-6S-Sul, m/z 663), 5-methylthio-[6]-shogaol sulfate (5-Methylthio-6S-Sul, m/z 404), 5-cysteinyl-1-(4'-hydroxy-3'-methoxyphenyl)-4-decan-3-ol sulfate (5-Cys-M6-Sul, m/z 479), 5-N-acetylcysteinyl-1-(4'-hydroxy-3'-methoxyphenyl)-4-decan-3-ol sulfate (5-NAC-M6-Sul, m/z 521), 5-cysteinylglycyl-1-(4'-hydroxy-3'-methoxyphenyl)-4-decan-3-ol sulfate (5-Cys-Gly-M6-Sul, m/z 536), 5-glutathione-1-(4'-hydroxy-3'-methoxyphenyl)-4-decan-3-ol sulfate (5-GSH-M6-Sul, m/z 665), and 5-methylthio-1-(4'-hydroxy-3'-methoxyphenyl)-4-decan-3-ol sulfate (5-methylthio-M6-Sul, m/z 406).

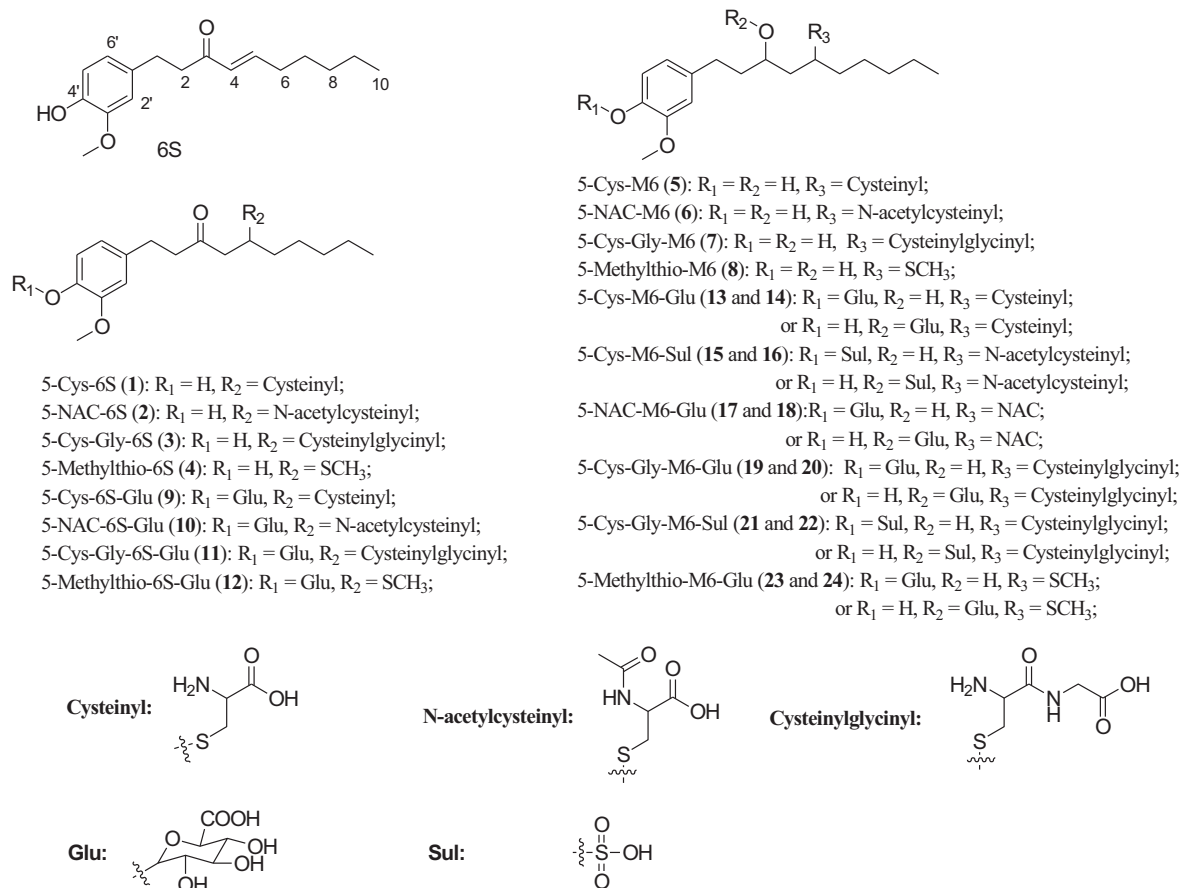


Fig. 1. Structures of [6]-shogaol, thiol-conjugated [6]-shogaol and their phase II metabolites, and thiol-conjugated M6 and their phase II metabolites.

We analyzed the urine samples collected from both control mice and mice treated with 200 mg/kg [6]-shogaol through oral gavage using both positive and negative ESI-MS (Fig. 2). Among all the possible metabolites, we identified 5-Cys-6S-Glu (9), 5-NAC-6S-Glu (10), 5-Cys-Gly-6S-Glu (11), 5-Methylthio-6S-Glu (12), 5-Cys-M6-Glu (13 and 14), 5-NAC-M6-Glu (15 and 16), 5-Cys-Gly-M6-Glu (17 and 18), 5-Cys-M6-Sul (19 and 20), 5-Cys-Gly-M6-Sul (21 and 22), and 5-methylthio-M6-Glu (23 and 24) as the phase II metabolites of thiol-conjugated [6]-shogaol in mouse urine (Fig. 1 and Table 1).

3.2. Identification of the glucuronidated metabolites of thiol-conjugated [6]-shogaol

In the extracted ion chromatogram of m/z 574 $[M+H]^+$ (5-cysteinylyl-[6]-shogaol glucuronide under positive mode), one new peak (peak 9 at 25.77 min) was observed from urine samples collected from [6]-shogaol treated mice (Figs. 2(A) and 3(A)). This peak showed tandem mass spectrum with m/z 398 (molecular ion of 5-cysteinylyl-[6]-shogaol under positive mode) and 277 (molecular ion of [6]-shogaol under positive mode) as the major product ions, indicating that 9 was the potential mono-glucuronidated metabolite of 5-cysteinylyl-[6]-shogaol. The tandem mass spectrum of product ion 398 (MS^3 : m/z 398/574) of 9 (Fig. 3(A)) was almost identical to the MS^2 spectrum of 5-cysteinylyl-[6]-shogaol (MS^2 : m/z 398) [15], suggesting that 9 was the mono-glucuronidated metabolite of 5-cysteinylyl-[6]-shogaol. The MS^4 spectrum of the product ion m/z 277 (MS^4 : 277/398/574) of 9 (Fig. 3(A)) showed a major product ion at m/z 137 which was almost identical to the MS^3 spectrum of 5-cysteinylyl-[6]-shogaol (MS^3 : 277/398) [15], confirming our definition of 9. The presence of the glucuronidated

metabolite of 5-cysteinylyl-[6]-shogaol was also verified by the observation that peak 9 disappeared and a new peak (peak 1 at 23.10 min) appeared in the extracted ion chromatogram of urine samples treated with β -glucuronidase and sulfatase (Fig. 2(A) and (B)). Peak 1 had almost the same retention time

Table 1
Major phase II metabolites of thiol-conjugated [6]-shogaol in mouse urine.

Compound/metabolite name	Peak #	Retention time (min)
5-Cys-6S	1	23.10
5-NAC-6S	2	33.50
5-Cys-Gly-6S	3	19.52
5-Methylthio-6S	4	35.34
5-Cys-M6	5	25.48
5-NAC-M6	6	35.39
5-Cys-Gly-M6	7	21.30
5-Methylthio-M6	8	35.73
5-Cys-6S-Glu	9	25.77
5-NAC-6S-Glu	10	35.89
5-Cys-Gly-6S-Glu	11	22.05
5-Methylthio-6S-Glu	12	40.42
5-Cys-M6-Glu	13	24.48
	14	28.29
5-Cys-M6-Sul	15	36.41
	16	39.30
5-NAC-M6-Glu	17	33.90
	18	36.52
5-Cys-Gly-M6-Glu	19	20.91
	20	23.98
5-Cys-Gly-M6-Sul	21	27.09
	22	30.57
5-Methylthio-M6-Glu	23	36.79
	24	40.60

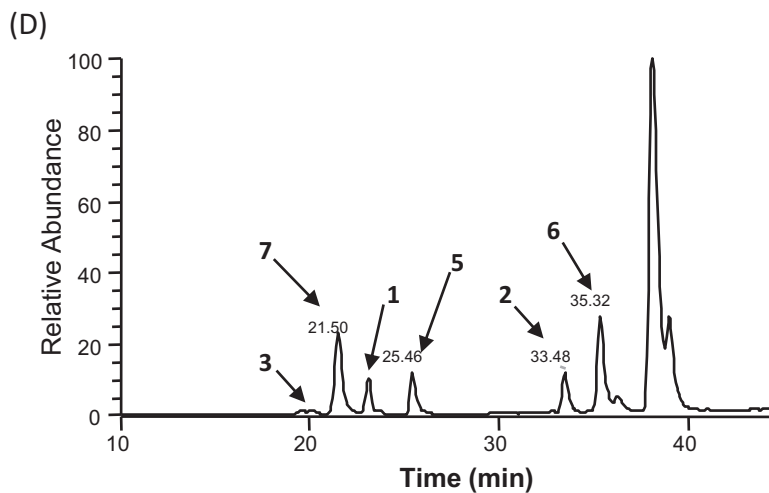
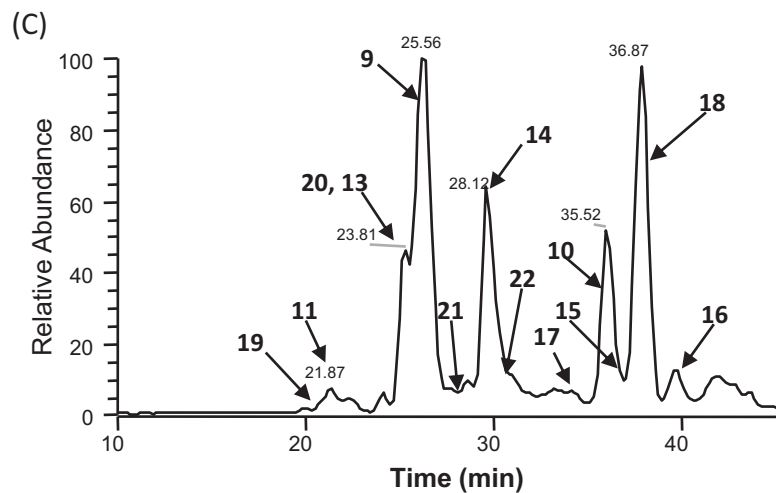
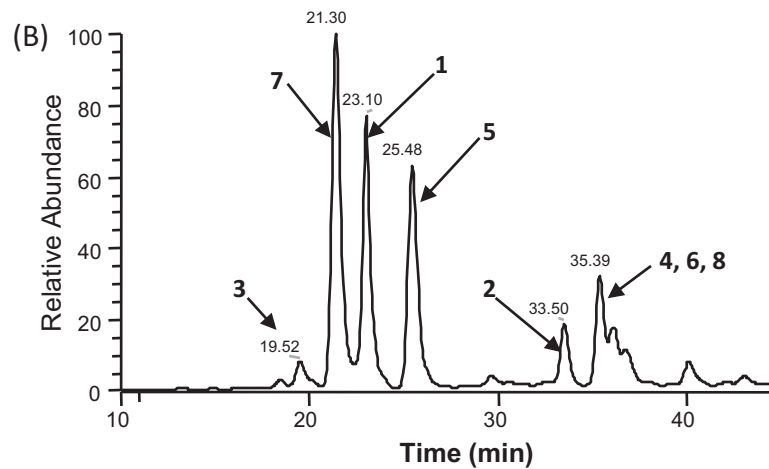
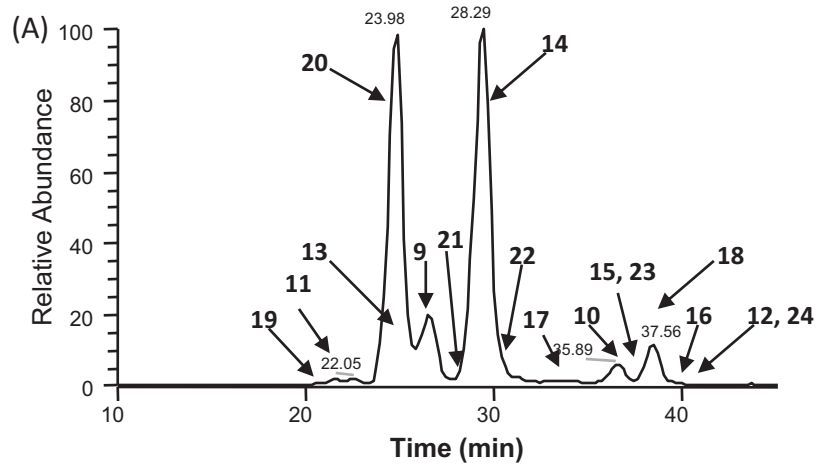


Fig. 2. Extracted ion chromatograms of urine samples collected from [6]-shogaol-treated mice obtained by positive ESI-MS interface: (A) before and (B) after enzymatic hydrolysis; and by negative ESI-MS interface: (C) before and (D) after enzymatic hydrolysis.

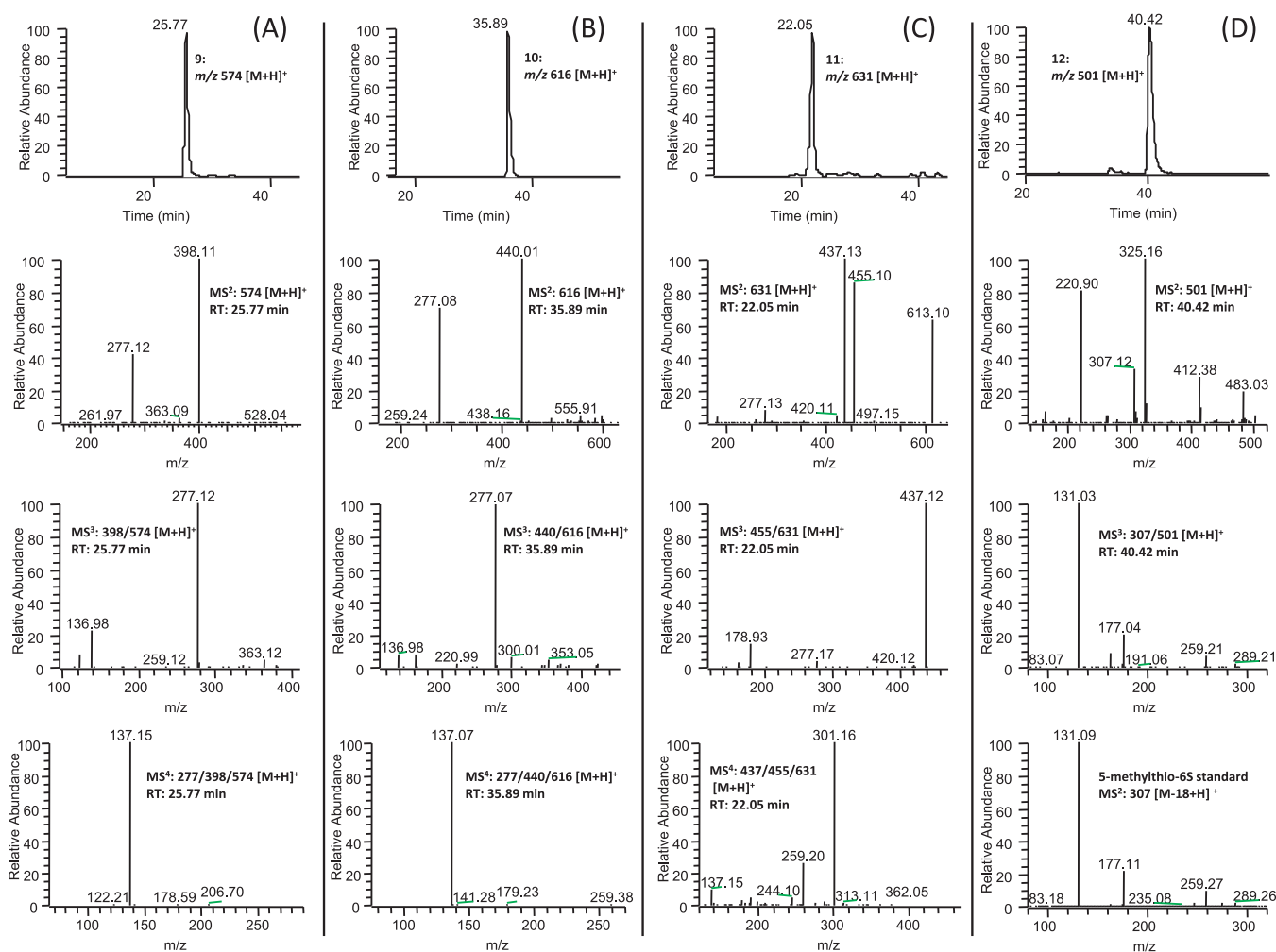


Fig. 3. LC-MS², MS³ and MS⁴ (positive) spectra of (A) 5-Cys-6S-Glu (**9**), (B) 5-NAC-6S-Glu (**10**), and (C) 5-Cys-Gly-6S-Glu (**11**); and (D) LC-MS² and MS³ (positive) spectra of 5-methylthio-6S-Glu (**12**) and MS² spectra of authentic 5-methylthio-6S (**4**).

and tandem mass spectrum as those of the authentic 5-cysteinyll-6]-shogaol (data not shown), suggesting that peak **9** was the mono-glucuronidated metabolite of 5-cysteinyll-6]-shogaol. Since 5-cysteinyll-6]-shogaol has only one hydroxyl group, we then identified peak **9** as 4'-glucuronide-5-cysteinyll-6]-shogaol (Fig. 1).

One new peak was observed (peak **10** at 35.89 min) in the extracted ion chromatogram of m/z 616 [M+H]⁺ (molecular ion of mono-glucuronidated 5-N-acetylcysteinyll-6]-shogaol under positive mode) in the urine samples collected from [6]-shogaol treated mice (Figs. 2(A) and 3(B)). This peak showed 176 mass units higher than that of 5-N-acetylcysteinyll-6]-shogaol indicating it was the mono-glucuronidated 5-N-acetylcysteinyll-6]-shogaol. Similar to 5-cysteinyll-6]-shogaol glucuronide, **10** showed tandem mass spectrum with m/z 440 (molecular ion of 5-N-acetylcysteinyll-6]-shogaol under positive mode) and 277 (molecular ion of [6]-shogaol under positive mode) as the major product ions and the tandem mass of m/z 440 (MS³: m/z 440/616) (Fig. 3(B)) was almost identical to the MS² spectrum (MS²: m/z 440) of 5-N-acetylcysteinyll-6]-shogaol [15]. Besides, the presence of peak **10** was also confirmed by the disappearance of peak **10** and the appearance of a new peak (peak **2** at 33.50 min) in the extracted ion chromatogram of urine samples treated with β -glucuronidase and sulfatase (Fig. 2(B)). Peak **2** had almost the same retention time and tandem mass spectrum as those of the authentic 5-N-acetylcysteinyll-6]-shogaol (data not shown), indicating that peak **10** was the mono-glucuronidated metabolite of

5-N-acetylcysteinyll-6]-shogaol. There is only one hydroxyl group in 5-N-acetylcysteinyll-6]-shogaol, we then identified peak **10** as 4'-glucuronide-5-N-acetylcysteinyll-6]-shogaol (Fig. 1).

Additionally, in the SIM mode for 5-cysteinyllglycinyll-6]-shogaol glucuronide (m/z 631 [M+H]⁺ under ESI positive mode), one major peak (peak **11** at 22.05 min) was observed from mouse urine treated with [6]-shogaol (Figs. 2(A) and 3(C)). This peak showed 176 mass units higher than that of 5-cysteinyllglycinyll-6]-shogaol, indicating it was the mono-glucuronidated 5-cysteinyllglycinyll-6]-shogaol. Peak **11** showed tandem mass spectrum with m/z 613 (molecular ion of dehydrated **11** under positive mode), 455 (molecular ion of 5-cysteinyllglycinyll-6]-shogaol under positive mode), and 437 (molecular ion of dehydrated 5-cysteinyllglycinyll-6]-shogaol under positive mode) as the major product ions. After treatment with β -glucuronidase and sulfatase, peak **11** disappeared and one new peak (peak **3** at 19.52 min) was shown in the extracted ion chromatogram of urine samples collected from [6]-shogaol treated mice (Fig. 2(A) and (B)). Peak **3** had a molecular weight of 454 as determined by the mass ion at m/z 455 [M+H]⁺, which gave 178 mass units higher than that of [6]-shogaol, indicating that **3** was possible the cysteinyllglycine conjugated metabolite of [6]-shogaol. The MS² spectrum (MS²: m/z 455) of **3** showed a dominant fragment ion at m/z 437 (Fig. 4(B)), which was the loss of H₂O from **3**, and the tandem mass spectrum of this product ion [MS³: m/z 437/455] gave a major product ion at m/z 301, which was the loss of m/z 137 (the major product ion of [6]-shogaol) from the

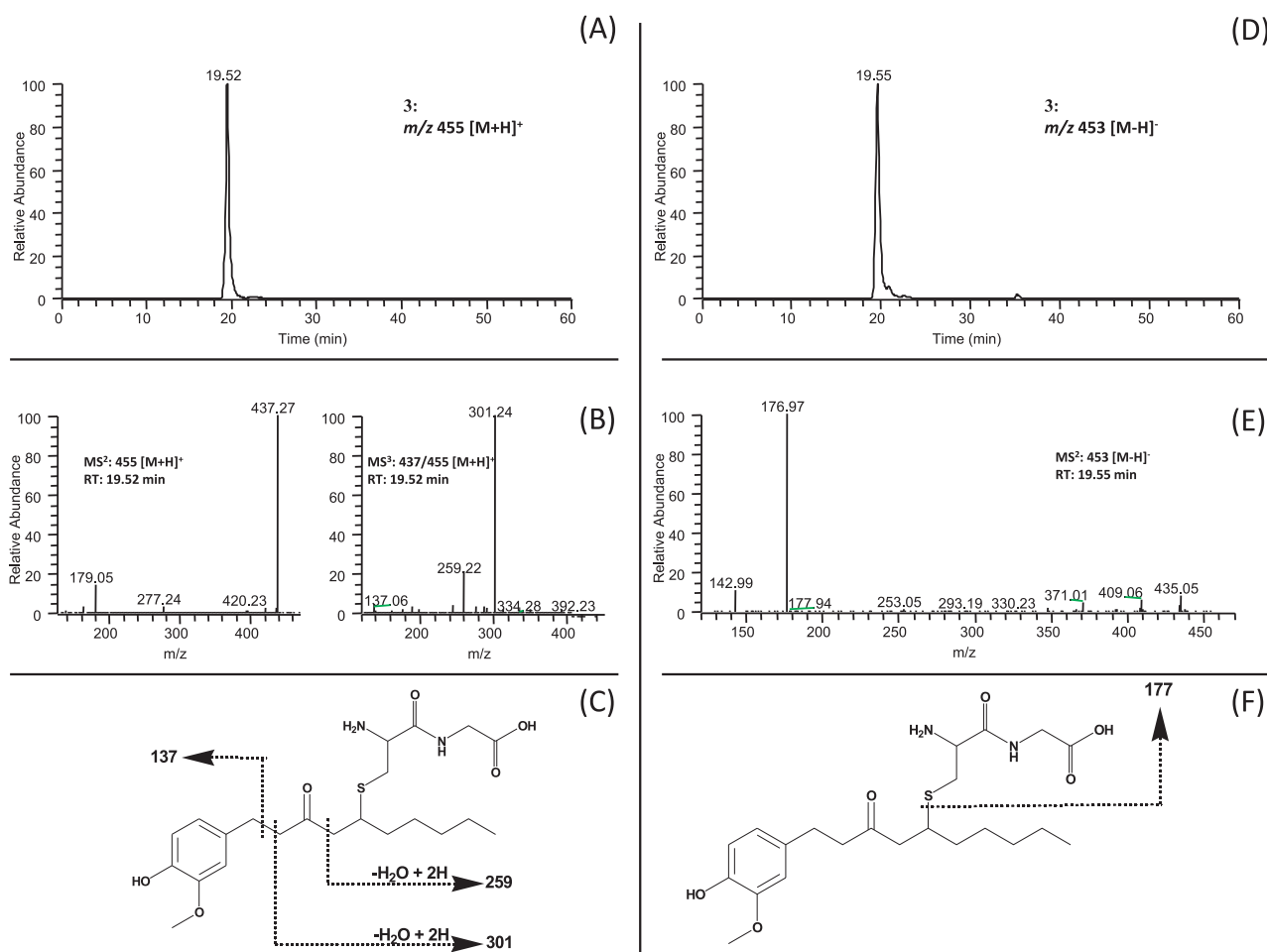


Fig. 4. LC-MS² and MS³ spectra of 5-Cys-Gly-6S (**3**) and its potential fragmentation pathways in both positive (A–C) and negative (D–F) ESI detection.

molecular ion at m/z 437 (Fig. 4(B) and (C)). In addition, under negative ESI-MS detection, peak **3** showed a major product ion at m/z 177, which was the deprotonated cysteineglycine (Fig. 4(E) and (F)). All of these spectral features identified **3** as 5-cysteinyglyciny-[6]-shogaol (Fig. 1). The tandem mass of the product ion m/z 455 of **11** (MS³: m/z 455/631) (Fig. 3(C)) was almost identical to the MS² spectrum of peak **3** (m/z 455) (Fig. 4(B)), indicating that **11** was 5-cysteinyglyciny-[6]-shogaol glucuronide. Since there is only one hydroxyl group in 5-cysteinyglyciny-[6]-shogaol, **11** was thus confirmed as 4'-glucuronide-5-cysteinyglyciny-[6]-shogaol (Fig. 1).

Furthermore, in the extracted chromatogram of m/z 501 [M+H]⁺ (molecular ion of mono-glucuronidated 5-methylthio-[6]-shogaol under positive mode), one new peak (peak **12** at 40.42 min) was observed from urine samples collected from [6]-shogaol treated mice (Figs. 2(A) and 3(D)). Peak **12** showed 176 mass units higher than that of 5-methylthio-[6]-shogaol, indicating that it was the mono-glucuronidated 5-methylthio-[6]-shogaol. Its MS² spectrum showed product ions of m/z 325 (−176 Da, neutral loss of one glucuronide moiety) and m/z 307 (−194 Da, neutral loss of one glucuronide moiety and one water moiety). The MS³ spectrum of the product ion m/z 307 of **12** was almost identical to the MS² spectrum of m/z 307 [M-18+H]⁺ of the authentic 5-methylthio-[6]-shogaol (Fig. 3(D)), indicating **12** was the mono-glucuronidated metabolite of 5-methylthio-[6]-shogaol. The presence of the glucuronide metabolite of 5-methylthio-[6]-shogaol was also confirmed by the observation that peak **12** disappeared and one new peak (peak **4**) was shown in the extracted ion chromatogram of the urine

samples treated with β-glucuronidase and sulfatase (Fig. 2(A) and (B)). Peak **4** and authentic 5-methylthio-[6]-shogaol had almost identical retention time and MS/MS spectrum (data not shown), further suggesting that peak **12** was the mono-glucuronidated 5-methylthio-[6]-shogaol. Therefore, peak **12** was identified as 4'-glucuronide-5-methylthio-[6]-shogaol (Fig. 1).

3.3. Identification of glucuronidated and sulfated metabolites of 5-cysteiny-M6

We observed two new peaks (peaks **13** and **14** at 24.48 and 28.29 min, respectively) in the extracted ion chromatogram obtained from positive ESI-MS detection with the molecular ion m/z 576 [M+H]⁺ (400+176), indicating that these two peaks were the glucuronidated metabolites of 5-cysteiny-M6 (Molecular weight: m/z 399). Both **13** and **14** showed tandem mass spectra (Fig. 5(B) and (C)) with m/z 400 (molecular ion of 5-cysteiny-M6 under positive mode) and 261 (dehydrated molecular ion of M6 under positive mode) as the major product ions, indicating that the two compounds were feasible mono-glucuronidated metabolites of 5-cysteiny-M6. In order to corroborate this proposition, we compared the tandem mass spectra of m/z 400 (MS³: m/z 400/476) of these two peaks (Fig. 5(B) and (C)) with the MS² spectrum of authentic 5-cysteiny-M6 (MS²: m/z 400) [15]. Our results clearly indicated that the tandem mass spectra of this product ion of both peaks were almost identical to the MS² spectrum of 5-cysteiny-M6, suggesting that these two compounds were the mono-glucuronidated metabolites of 5-cysteiny-M6. In

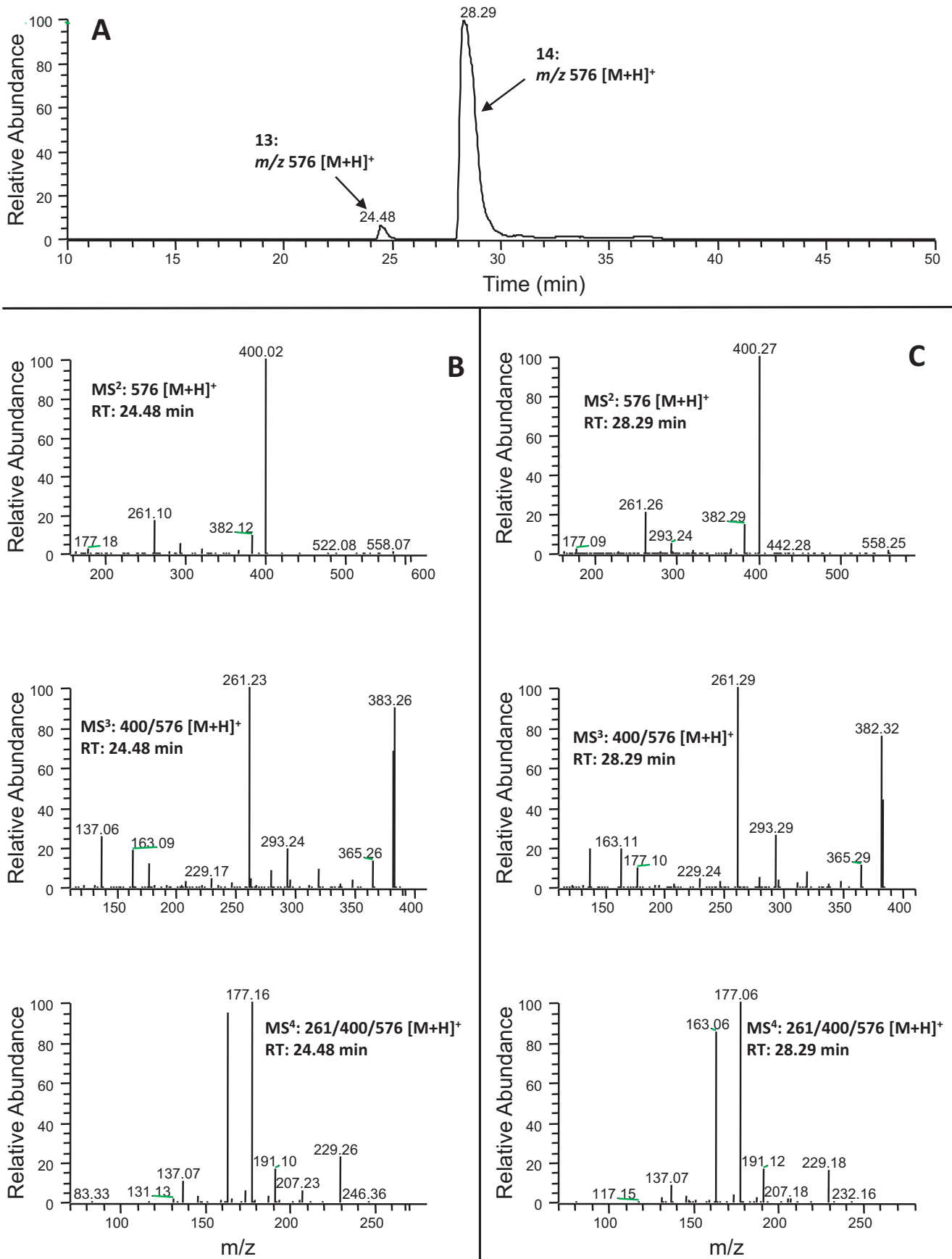


Fig. 5. (A) Extracted ion chromatogram and (B and C) ESI- MS^2 , MS^3 and MS^4 (positive ion) spectra of 5-Cys-M6-Glu (**13** and **14**).

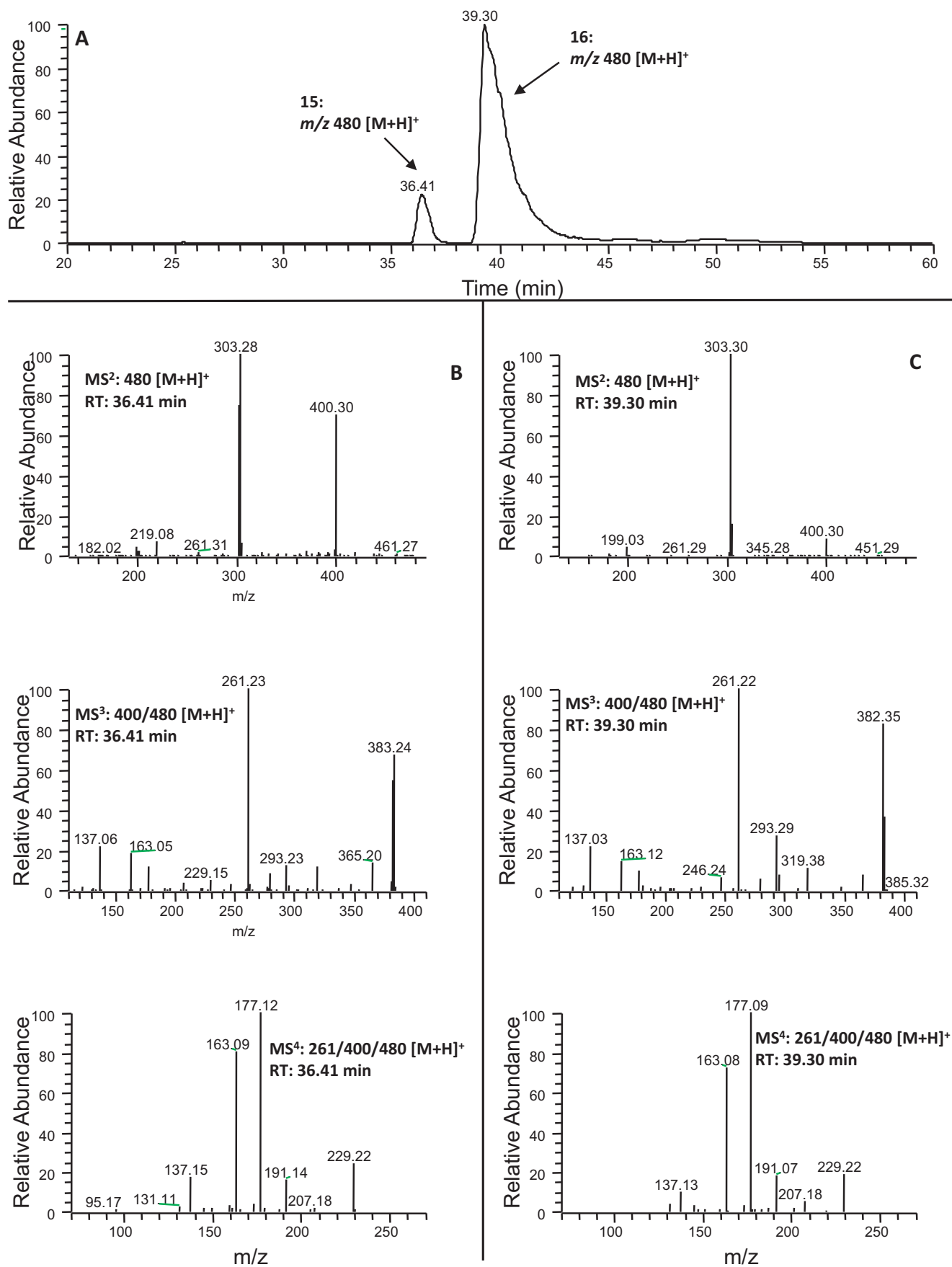


Fig. 6. (A) Extracted ion chromatogram and (B and C) ESI-MS², MS³ and MS⁴ (positive ion) spectra of 5-Cys-M6-Sul (**15** and **16**).

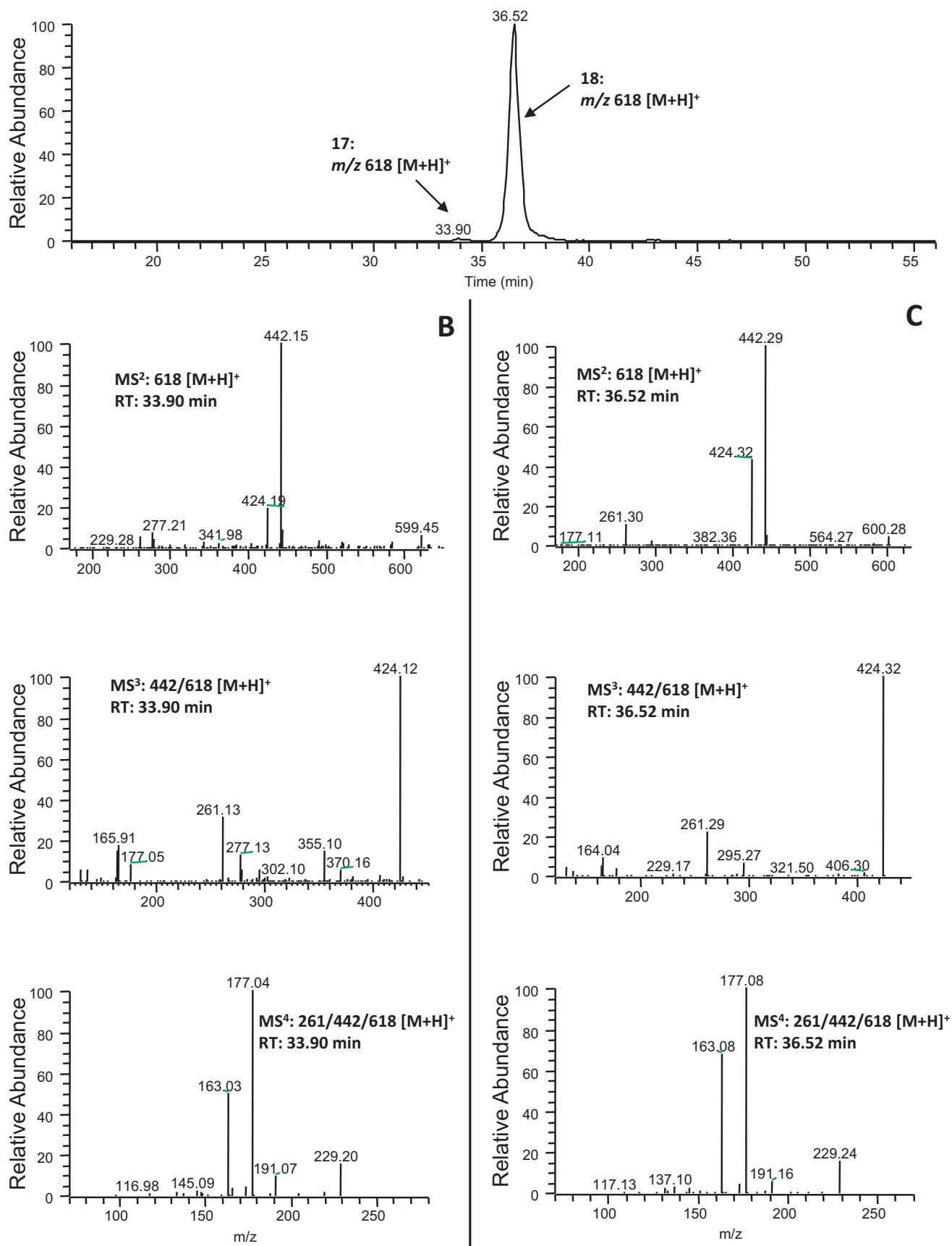


Fig. 7. (A) Extracted ion chromatogram and (B and C) ESI-MS², MS³ and MS⁴ (positive ion) spectra of 5-NAC-M6-Glu (**17** and **18**).

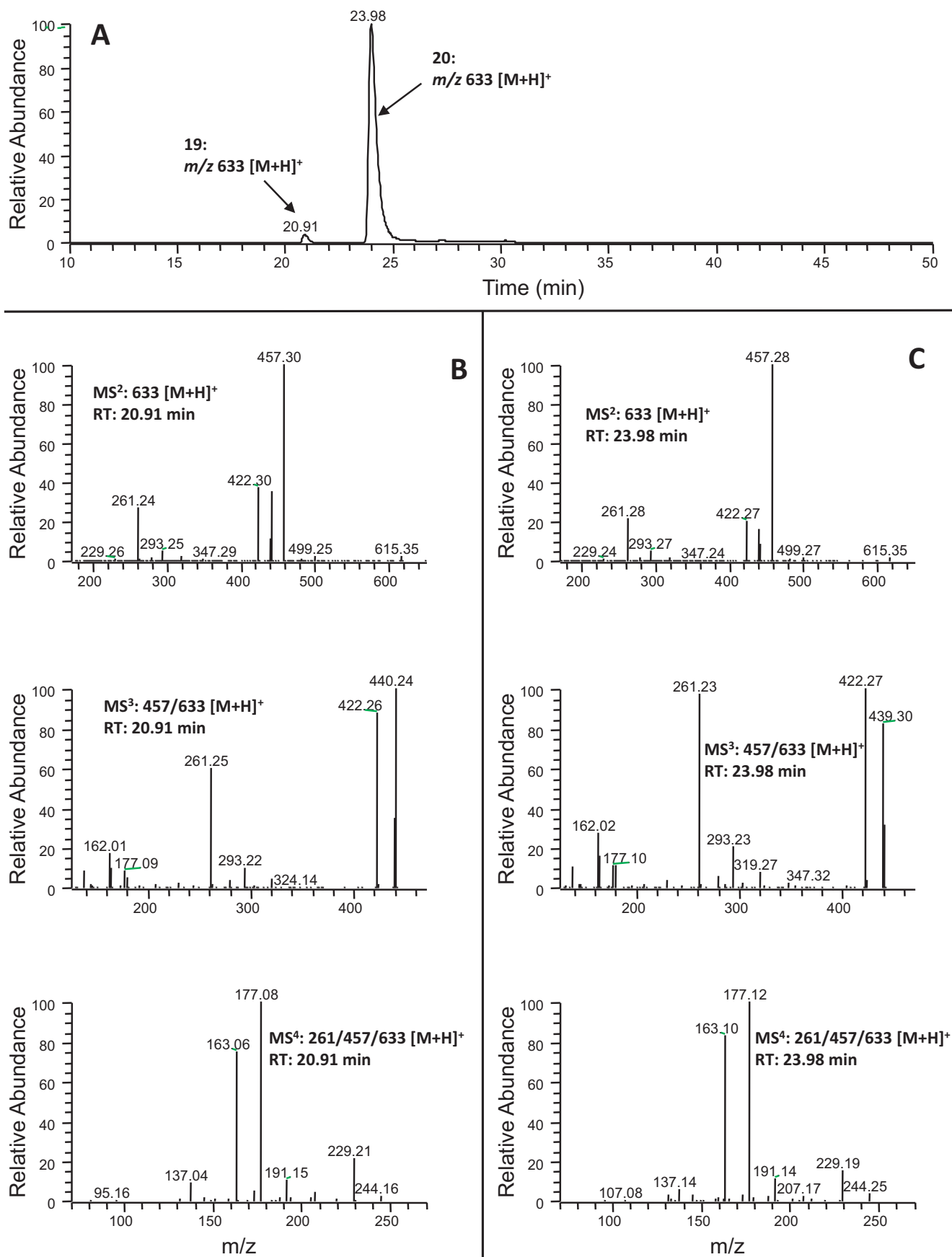


Fig. 8. (A) Extracted ion chromatogram and (B and C) ESI- MS^2 , MS^3 and MS^4 (positive ion) spectra of 5-Cys-Gly-Glu (**19** and **20**).

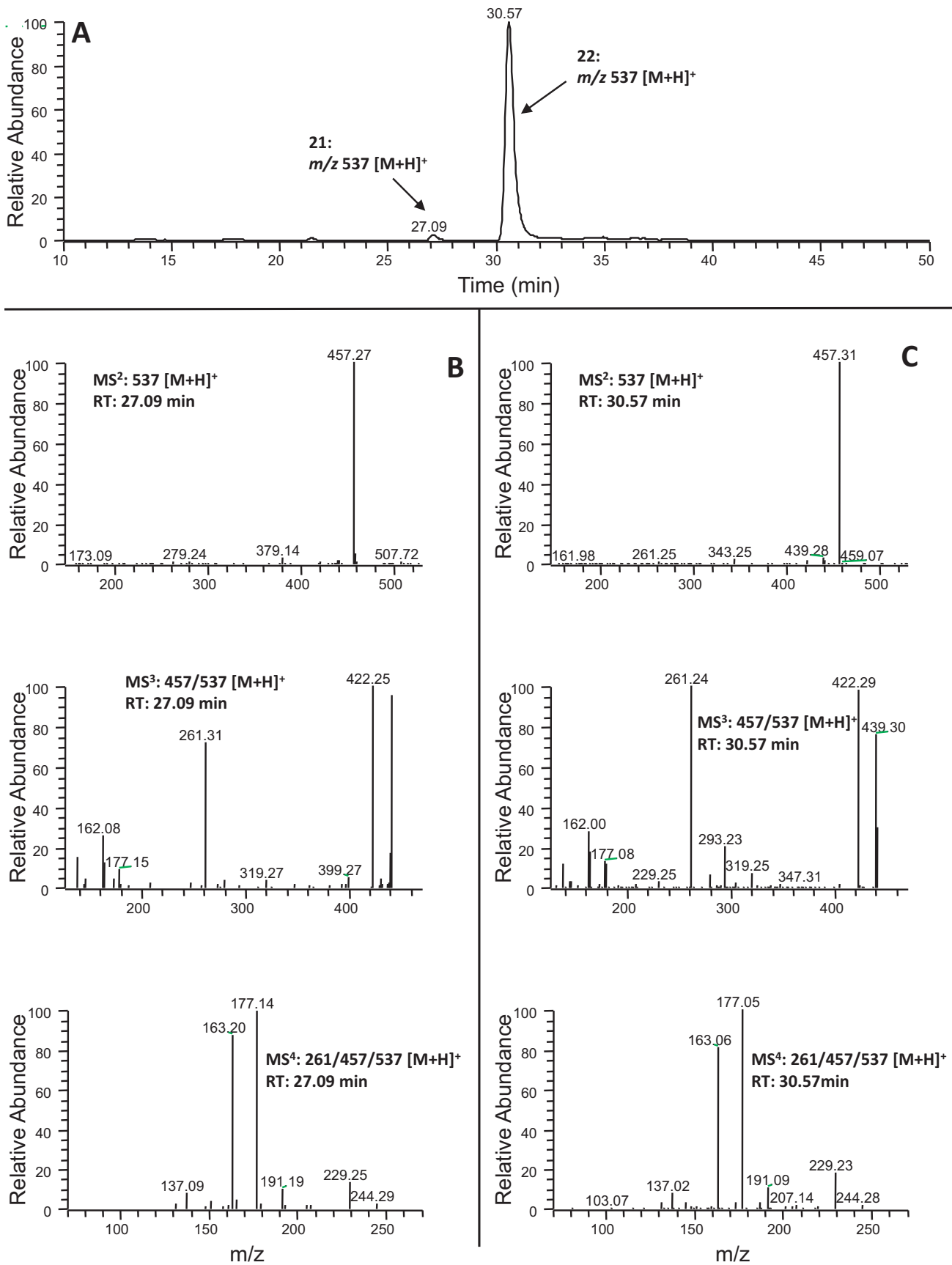


Fig. 9. (A) Extracted ion chromatogram and (B and C) ESI-MS², MS³ and MS⁴ (positive ion) spectra of 5-Cys-Gly-Sul (**21** and **22**).

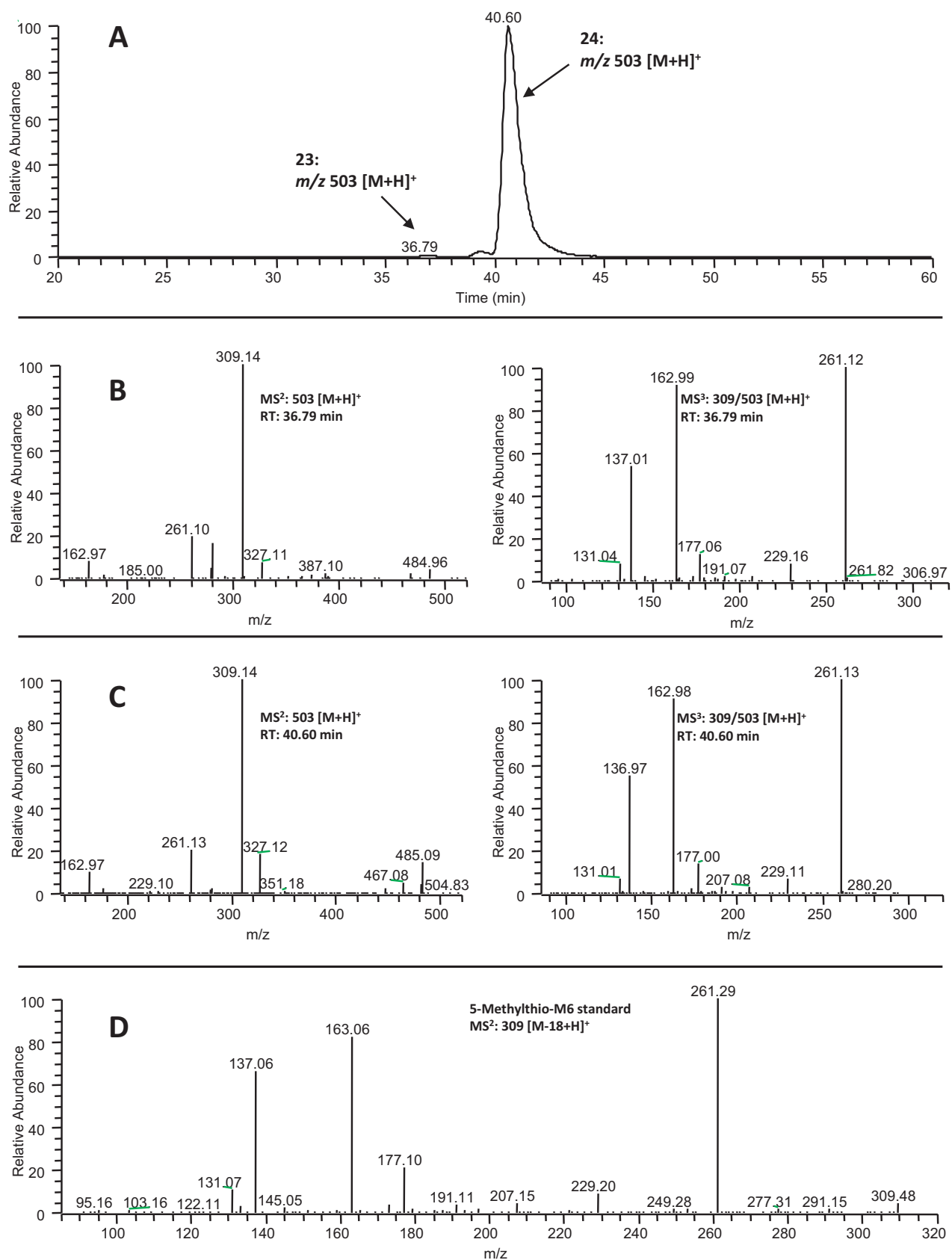


Fig. 10. (A) Extracted ion chromatogram and (B and C) ESI-MS² and MS³ (positive ion) spectra of 5-methylthio-M6-Glu (**23** and **24**), and (D) MS² spectrum of authentic 5-methylthio-M6 (**8**).

addition, the MS⁴ spectrum of the product ion m/z 261 (MS⁴: m/z 261/400/576) of **13** and **14** (Fig. 5(B) and (C)) demonstrated almost identical readings to the MS³ spectrum of 5-cysteiny-M6 (MS³: m/z 261/400) [15], which also confirmed our conclusion. The presence of the glucuronidated metabolites of 5-cysteiny-M6 was also verified by the observation that peaks **13** and **14** disappeared and a new peak (peak **5** at 25.48 min) appeared in the extracted ion chromatogram of the urine samples after treatment with β -glucuronidase and sulfatase (Fig. 2(B)). All of these features suggested that peaks **13** and **14** were 5-cysteiny-M6 glucuronide. Since the active sites of 5-cysteiny-M6 for glucuronidation have not been identified and metabolites **13** and **14** had identical tandem mass spectra, we could not determine the exact structures of the two mono-glucuronidated 5-cysteiny-M6 (Fig. 1). It has been reported that the hydroxyl group at the benzene ring of [6]-gingerol, an analog of [6]-shogaol, is more active for glucuronidation than the hydroxyl group on the side chain [17]. Therefore, we tentatively identified the major peak (**14**, RT: 28.29 min) as 4'-glucuronide-5-cysteiny-M6 and the minor peak (**13**, 24.48 min) as 3-glucuronide-5-cysteiny-M6. However, this deduction needs to be further confirmed by corresponding authentic standards.

Similarly, in the extracted ion chromatogram of m/z 480 [M+H]⁺ (molecular ion of mono-sulfated 5-cysteiny-M6 under positive mode), two new peaks (peaks **15** and **16** at 36.41 and 39.30 min, respectively) were observed from urine samples collected from [6]-shogaol treated mice (Fig. 6(A)). The molecular weight of these two peaks showed 80 mass units higher than that of 5-cysteiny-M6 and both peaks had product ion m/z 400 [M-80+H]⁺ in their tandem mass spectra, suggesting that they were potential mono-sulfated metabolites of 5-cysteiny-M6. The tandem mass spectra of the product ion m/z 400 (MS³: m/z 400/480) of those two compounds (Fig. 6(B) and (C)) and the MS² spectrum of 5-cysteiny-M6 [15] showed the same fragment ion mass spectra, indicating that both of them were the mono-sulfated 5-cysteiny-M6. Similar to the mono-glucuronidated metabolites of 5-cysteiny-M6, we tentatively identified the major peak (**16**, RT: 39.30 min) as 4'-sulfate-5-cysteiny-M6 and the minor peak (**15**, 36.41 min) as 3-sulfate-5-cysteiny-M6.

3.4. Identification of glucuronidated metabolite of 5-N-acetylcysteiny-M6

In the extracted ion chromatogram of m/z 442 [M+H]⁺ taken from the MS² data set obtained from m/z 618 [M+H]⁺ (molecular ion of mono-sulfated 5-N-acetylcysteiny-M6 under positive mode), two new peaks (peaks **17** and **18** at 33.90 and 36.52 min, respectively) were observed from the urine samples collected from [6]-shogaol treated mice. This suggested that they were likely the mono-glucuronidated metabolites of 5-N-acetylcysteiny-M6. The tandem mass spectra of the product ion m/z 442 (MS³: m/z 442/618) of these two peaks (Fig. 7(B) and (C)) were almost identical to the MS² spectrum of 5-N-acetylcysteiny [15], confirming that they were the mono-glucuronidated metabolites of 5-N-acetylcysteiny-M6. Similar to the mono-glucuronidated metabolites of 5-cysteiny-M6, we tentatively identified the major peak (**18**, RT: 36.52 min) as 4'-glucuronide-5-N-acetylcysteiny-M6 and the minor peak (**17**, 33.90 min) as 3-glucuronide-5-N-acetylcysteiny-M6.

3.5. Identification of glucuronidated and sulfated metabolites of 5-cysteinyglyciny-M6

In the SIM mode for mono-glucuronidated 5-cysteinyglyciny-M6 (m/z 633 [M+H]⁺ under ESI positive mode), two new

peaks (peaks **19** and **20** at 20.91 and 23.98 min, respectively) were detected in the urine samples collected from [6]-shogaol treated mice (Figs. 2(A) and 8(A)). Both **19** and **20** showed similar tandem mass spectra with m/z 457 (molecular ion of 5-cysteinyglyciny-M6 under positive mode) as the major product ion, indicating that they were potential mono-glucuronidated metabolites of 5-cysteinyglyciny-M6. In order to further confirm this deduction, we compared the MS³ (MS³: m/z 457/633) and MS⁴ (MS⁴: m/z 301/457/633) spectra of **19** and **20** (Fig. 8(B) and (C)) with the MS² (MS²: m/z 457) and MS³ (MS³: m/z 301/457) spectra of 5-cysteinyglyciny-M6 [15], respectively. Our results clearly indicated that **19** and **20** were mono-glucuronidated 5-cysteinyglyciny-M6. Similar to the mono-glucuronidated metabolites of 5-cysteiny-M6, we tentatively identified the major peak (**20**, RT: 23.98 min) as 4'-glucuronide-5-cysteinyglyciny-M6 and the minor peak (**19**, 20.91 min) as 3-glucuronide-5-cysteinyglyciny-M6.

Similarly, two new peaks (peaks **21** and **22** at 27.09 and 30.57 min, respectively) were observed at m/z 537 (mono-sulfated 5-cysteinyglyciny-M6 under positive mode), which were 80 mass units higher than that of 5-cysteinyglyciny-M6, indicating that they were the mono-sulfate conjugated 5-cysteinyglyciny-M6. We then compared the tandem mass spectrum of the major product ion m/z 457 (MS³: m/z 457/537) (Fig. 9(B) and (C)) of these two peaks with that of 5-cysteinyglyciny-M6 (MS²: m/z 457) [15]. Our results indicated that they had almost identical mass fragments. Hence, we established these metabolites as mono-sulfated 5-cysteinyglyciny-M6 (Fig. 1). Similar to the mono-glucuronidated metabolites of 5-cysteiny-M6, we tentatively identified the major peak (**22**, RT: 30.57 min) as 4'-sulfate-5-cysteinyglyciny-M6 and the minor peak (**21**, 27.09 min) as 3-sulfate-5-cysteinyglyciny-M6.

3.6. Identification of glucuronidated metabolites of 5-methylthio-M6

In the extracted ion chromatogram of m/z 503 [M+H]⁺ (molecular ion of mono-glucuronidated 5-methylthio-M6 under positive mode), two new peaks (peaks **23** and **24** at 36.79 and 40.60 min, respectively) were observed in the urine samples collected from [6]-shogaol treated mice (Figs. 2(A) and 10(A)). These two peaks showed 176 mass units higher than that of 5-methylthio-M6, indicating that they were 5-methylthio-M6 glucuronide. Both **23** and **24** showed the same product ion m/z 309 (Fig. 10(B) and (C)) and the tandem mass of this product ion was almost identical to the tandem mass of authentic 5-methylthio-M6 (Fig. 10(D)). Similar to the mono-glucuronidated metabolites of 5-cysteiny-M6, we tentatively identified the major peak (**24**, RT: 40.60 min) as 4'-glucuronide-5-methylthio-M6 and the minor peak (**23**, 36.79 min) as 3-glucuronide-5-methylthio-M6.

4. Conclusions

In this study, using LC/ESI-MSⁿ analysis we successfully identified sixteen phase II metabolites of thiol-conjugated [6]-shogaol from mouse urine collected 24 h after administration of 200 mg/kg [6]-shogaol through oral gavage (Fig. 2 and Table 1). To the best of our knowledge, this is the first study to establish the phase II metabolites of thiol-conjugated [6]-shogaol in mouse urine using multi-stage tandem mass spectrometry. Our results indicated that all the thiol conjugated metabolites of [6]-shogaol are the prime substrates for glucuronidation, and some of them are also the substrates for sulfation. Besides the thiol-conjugated metabolites of [6]-shogaol, our previous study has also identified several non-thiol conjugated metabolites of [6]-shogaol (M6-M11) [15]. These

non-thiol conjugated metabolites of [6]-shogaol exist in phase II conjugated form since they are barely detectable in mouse urine samples before treatment with glucuronidase and sulfatase (data not shown). However, these phase II metabolites are poorly ionized under ESI detection; we could not get publishable LC/MS spectra of these metabolites. New method is needed to analyze these phase II metabolites.

Our results suggested that the metabolites of [6]-shogaol are in the phase II conjugated form in mice. Glucuronidation and sulfation are typically considered as the means of inactivating biologically active compounds and facilitating excretion. However, examples of biologically active metabolites have also been reported. The most prominent example is that morphine-6-glucuronide has a more potent analgesic action than morphine [18]. Whether the thiol conjugates and their glucuronidated and sulfated metabolites are bioactive remains to be determined. The results would help us assess the relative contribution of [6]-shogaol metabolites to the disease preventive effects of [6]-shogaol. Results from this work are important for understanding the metabolism of [6]-shogaol and related analogs in humans and to provide useful information that may act as a reference in clinical pharmacology. Knowledge of the metabolism of [6]-shogaol may also help in comprehending the mechanism of action and therapeutic effects of [6]-shogaol, as well as ginger extract.

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