Mass Spectrometric Discrimination of Squalene
Monohydroperoxide Isomers

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Abstract: Squalene (SQ), a main component of human sebum, is readily photooxidized by exposure to sunlight, producing six squalene monohydroperoxide (SQ-OOH) isomers. Despite its known connection to various skin conditions, few studies have sought to analyze SQ-OOH at the isomeric level. In this study, we aimed to develop a method to discriminate each SQ-OOH isomer with the use of tandem mass spectrometry (MS/MS). The six standard SQ-OOH isomers were prepared by photooxidizing SQ in the presence of rose bengal, a photosensitizer, and isolated by semipreparative high-performance liquid chromatography (HPLC). To purify each isomer, 2-methoxypropene, which reversibly reacts with the hydroperoxide group of SQ-OOH, was utilized. Product ion scanning was then performed on the standard SQ-OOH isomers in the absence and presence of the sodium ion. In the absence of the sodium ion, the fragmentation patterns produced by atmospheric pressure chemical ionization were similar between the isomers, whereas in the presence of the sodium ion by electrospray ionization, unique fragmentation patterns were achieved. Based on these fragment ions, HPLC-MS/MS multiple reaction monitoring analysis was conducted on a mixture of the standard SQ-OOH isomers. We achieved discrimination of SQ-OOH isomers with high selectivity and detected SQ-OOH isomers at nanogram levels. These results may improve our understanding of the effect of SQ-OOH on skin conditions as well as the mechanism behind SQ peroxidation.

Key words: mass spectrometry, squalene, squalene monohydroperoxide isomers

1 INTRODUCTION

Squalene, a 30-carbon isoprenoid (SQ; Fig. 1A), is a major component of human sebum, present on the outermost surface of human skin¹. SQ on skin is believed to act as a protective barrier to the skin surface², however the compound is vulnerable to environmental stressors such as solar ultraviolet (UV) radiation³,⁴.

We have previously established methods for the sensitive and selective detection of various lipid hydroperoxides⁵–⁶, and have discovered that SQ monohydroperoxide (SQ-OOH; Fig. 1A) is produced in human skin after exposure to sunlight or UV radiation³,⁴. There have been many reports on the accumulation of SQ-OOH on human skin⁷–¹⁰; we reported that the concentration of SQ-OOH on human forehead skin before and after 3 h of sunlight exposure was about 1,000 μg/g and 2,800 μg/g skin lipids respectively⁹. These studies⁷–¹⁰ suggest that SQ on human skin is the principle target of lipid photooxidation. Additionally, it has been reported that the accumulation of SQ-OOH on skin may lead to comedogenesis, the development of inflammatory acne, and other skin conditions such as hyperpigmentation and the formation of wrinkles¹¹–¹⁴.

Despite the significance of skin SQ-OOH and its relationship to various skin conditions, few studies have aimed to analyze it at the isomeric level. SQ-OOH consists of six isomers (Fig. 1A), but to the best of our knowledge, the relative abundance of each isomer in skin is not verified, and it is still unclear whether each isomer has a different effect on skin conditions. This may be due to the lack of a suitable analytical method to distinguish each SQ-OOH isomer, even with the use of tandem mass spectrometry (MS/MS)⁹. In our previous experiments of other lipid hy-
droperoxides (e.g., phosphatidylcholine hydroperoxide; PCOOH), unique fragment ions (hydroperoxy group-derived fragments) were achieved using MS/MS in the presence of the sodium ion $^{15}$ $^{17}$. Therefore, in this study, we evaluated the hypothesis that this technique will allow for better discrimination of SQ-OOH isomers.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

SQ, rose bengal, and 2-methoxypropene (MxP) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Pyridinium $p$-toluenesulfonate (PPTS) and sodium acetate were obtained from Sigma (St. Louis, MO). All other reagents were of the highest grade available.

2.2 Preparation of the six standard SQ-OOH isomers

The six standard SQ-OOH isomers (Fig. 1A) were synthesized from SQ as follows (Fig. 1B): 5 g of SQ was dissolved in 55 mL of ethanol in a round flask, to which rose bengal (0.01 mg/mL), a photosensitizer, was added. The round flask was rotated under an 18 W LED light (CN-304; Guangdong Nanguang Photo & Video Systems Co.; Guangdong, China) for 4 h at 4°C. The lamp was held 5 cm vertically above the round flask and the illumination intensity was approximately 50,000 lux. The resultant solution was loaded onto an ethanol-equilibrated SepPak Plus QMA cartridge (Waters; Milford, MA) and eluted with 5 mL of ethanol. Rose bengal was retained on the cartridge, while the eluate containing SQ-OOH was collected. The eluate was evaporated and dissolved in 20 mL of hexane.

A portion of the solution (4 mL) was evaporated, dissolved in methanol (1 mL), and subjected to high-performance liquid chromatography (HPLC)-UV (210 nm) to monitor the yield of SQ-OOH. An ODS column (COSMOSIL 5C18-MS-II, 4.6 $\times$ 250 mm; Nacalai Tesque; Kyoto, Japan) was used with methanol as the mobile phase at a flow rate of 1 mL/min and a column temperature of 40°C. To separate and isolate the six SQ-OOH isomers, the remaining sample in hexane was subjected to semipreparative HPLC-UV with two silica columns connected (Silica SG120, 10 $\times$ 250 mm; Shiseido; Tokyo, Japan) and a mobile phase of hexane/isopropanol (100:0.3) at 20 mL/min. Each isomer was subjected once more to semipreparative HPLC-UV under the same conditions.

For further purification, each isomer’s hydroperoxide group was protected by a reaction with MxP as described previously $^{18}$ $^{19}$. In brief, SQ-OOH isomers were individually weighed and dissolved in acetonitrile (0.01 mg/mL). MxP (1/5 vol. eq. of sample solution) and 10 mM PPTS in acetonitrile (1/10 vol. eq.) were added to the solutions and reacted at 4°C for 5 min. Water (1 vol. eq.) and methanol (1/10 vol. eq.) were added to stop the reaction. Each solution was loaded on to a SepPak Vac C18 column (Waters), washed with methanol/water (1:1), and eluted with isopropanol. These solutions were evaporated, dissolved in methanol, and subjected to semipreparative HPLC-UV to isolate the SQ-OO-MxP fraction. An ODS column (Inertsil ODS-3, 20 $\times$ 250 mm; GL Sciences Inc.; Tokyo, Japan) was eluted with methanol at 20 mL/min. The fractions were evaporated and dissolved in methanol (0.5 mg/mL), to which 10 mM...
PPTS in methanol (1/10 vol. eq.) was added. The solutions were kept at 4°C for 2 days, and solid phase extraction was performed using a SepPak Vac C18 column under the conditions described above. Semipreparative HPLC-UV with two silica columns connected was performed on the samples, using the above conditions. This procedure was repeated multiple times according to the purity of the isomers.

The purity of each isomer was examined by HPLC-UV using a silica column (Inertsil SIL 100A 5 μm, 4.6 × 250 mm; GL Sciences Inc.) with hexane/isopropanol (100:0.3) at 1.0 mL/min. Each isomer was also subjected to NMR; 1H and 13C NMR spectra were recorded on a Varian (Palo Alto, CA) Unity 600 spectrometer (600 MHz for 1H NMR and 150 MHz for 13C NMR). The standard SQ-OOH isomers were dissolved in chloroform (20 mg/mL) and stored at −30°C until analysis.

### 2.3 MS/MS and HPLC-MS/MS analysis of standard SQ-OOH isomers

A portion (5 μL) of each standard SQ-OOH isomer solution was evaporated and dissolved in 10 mL of either methanol or methanol containing 0.1 mM sodium acetate. To evaluate whether each SQ-OOH isomer produced characteristic fragment ions, product ion scanning was performed on each sample by direct infusion (0.6 mL/h) of the sample solution (10 μg/mL) into the microTOF-Q II mass spectrometer (Bruker Daltonik; Bremen, Germany) MS/MS system. The MS/MS conditions used are shown in Table 1.

Using the fragments observed, multiple reaction monitoring (MRM) of each SQ-OOH isomer was performed with an HPLC-MS/MS system. A Shimadzu liquid chromatography

| Table 1 | Analytical conditions used for Q1, MS/MS and HPLC-MS/MS analysis. |
|---------|---------------------------------------------------------------|
| Condition | Source | Ion polarity | Mass range (m/z) | End plate offset (V) | Capillary (V) | Nebulizer (bar) | Dry gas (L/min) | Dry temp (°C) | Funnel 1 RF (Vpp) | Funnel 2 RF (Vpp) | isCID energy (eV) | Hexapole RF (Vpp) | Ion energy (eV) | Low mass (m/z) | Collision energy (eV) | Collision RF (Vpp) | Transfer time (μs) | Pre pulse storage (μs) |
| 1       | APCI   | Positive     | 50-2500          | 500                | 4500             | 1.6       | 8.0           | 200.0          | 300.0          | 300.0           | 0.0            | 400.0           | 2.0           | 80.0          | 15.0             | 700.0         | 95.0        | 8.5            |
| 2       | APCI   | Positive     | 50-700           | 500                | 4500             | 1.6       | 8.0           | 200.0          | 300.0          | 300.0           | 0.0            | 400.0           | 2.0           | 100.0         | 8.0              | 100.0         | 45.0       | 4.5            |
| 3       | ESI    | Positive     | 50-700           | 500                | 4500             | 0.4       | 4.0           | 180.0          | 300.0          | 400.0           | 0.0            | 400.0           | 6.0           | 100.0         | 12.0             | 400.0         | 45.0      | 10.0           |
| 4       | ESI    | Positive     | 50-700           | 500                | 4500             | 0.4       | 4.0           | 180.0          | 300.0          | 400.0           | 0.0            | 400.0           | 6.0           | 100.0         | 12.0             | 400.0         | 45.0      | 10.0           |
| 5       | ESI    | Positive     | 50-700           | 500                | 4500             | 0.4       | 4.0           | 180.0          | 300.0          | 400.0           | 0.0            | 400.0           | 6.0           | 100.0         | 12.0             | 400.0         | 45.0      | 10.0           |

Conditions 1 and 3 were used for Q1 analysis, whereas conditions 2 and 4 were used for MS/MS analysis of the standard SQ-OOH isomers. Condition 5 was used for HPLC-MS/MS analysis of the mixture of six standard SQ-OOH isomers. The MRM transitions used for HPLC-MS/MS analysis were: 2-OOH-SQ, m/z 465.37/433.34; 3-OOH-SQ, m/z 465.37/395.33; 6-OOH-SQ, m/z 465.37/365.28; 7-OOH-SQ, m/z 465.37/161.10; 10-OOH-SQ, m/z 465.37/297.22; 11-OOH-SQ, m/z 465.37/229.16.
phy system (Shimadzu; Kyoto, Japan), consisting of a vacuum degasser, a quaternary pump, and an autosampler, was connected to a micrOTOF-Q II mass spectrometer. A mixture of six standard SQ-OOH isomers was prepared (10 μg/mL each isomer in methanol) and analyzed using a silica column (Inertsil SIL 100A 5 μm, 4.6 × 250 mm) with hexane/isopropanol (100:0.3) at 1.0 mL/min. Methanol/isopropanol (1:1) containing 0.1 mM sodium acetate was mixed post-column at 0.4 mL/min to enhance ionization. The eluate mixture was split into two streams, where one stream was discarded (0.6 mL/min) and the other entered the MS/MS system (0.8 mL/min). Each SQ-OOH isomer was detected using MRM under the conditions described in Table 1.

3 RESULTS AND DISCUSSION

3.1 Preparation of standard SQ-OOH isomers for MS/MS analysis

The scheme shown in Fig. 1B was used to prepare pure standard SQ-OOH isomers, which are essential in the development of analytical methods to discriminate SQ-OOH isomers. Rose bengal, a singlet oxygen emitter, was chosen as a photosensitizer for the reaction. The UV-chromatogram with an ODS column displayed two peaks, with one corresponding to unoxidized SQ and the other to a mixture of SQ-OOH isomers, confirming that the product of the photooxidation reaction was SQ-OOH (Fig. 2A). Then by performing semipreparative HPLC-UV using silica columns, the six SQ-OOH isomers were separated, generating six peaks (Fig. 2B). The peaks were isolated, and then purified by a reaction with MxP18,19. The standard SQ-OOH isomers were finally achieved using semipreparative HPLC-UV with silica columns. Using NMR, each peak was identified as corresponding to each of the following isomers: peak 1, 11-OOH-SQ; peak 2, 7-OOH-SQ; peak 3, 10-OOH-SQ; peak 4, 6-OOH-SQ; peak 5, 3-OOH-SQ; and peak 6, 2-OOH-SQ (data not shown). Interestingly, no peroxidation was detected at the C4, C8, or C12 positions of SQ. The NMR spectra and HPLC-UV chromatograms with a silica column (Fig. 2C) confirmed that 2-OOH-SQ, 3-OOH-SQ, 7-OOH-SQ, and 11-OOH-SQ were pure (HPLC purity >95%) and did not contain SQ or other compounds. 6-OOH-SQ and 10-OOH-SQ had somewhat lower purities due to the considerable difficulty of their separation. The standard SQ-OOH isomers were used as references in later experiments. It is noteworthy that the application of MxP, involving the protection of the hydroperoxy group as a perketal, was utilized for the first time in the purification of SQ-OOH isomers (Fig. 1B). The resultant SQ-OO-MxP solutions were isolated by semipreparative HPLC-UV using an ODS column, and the SQ-OOH isomers were subsequently regenerated from the perketal. The MxP reaction was effective in providing standard SQ-OOH isomers with high purity.

3.2 MS/MS analysis of standard SQ-OOH isomers in the absence and presence of the sodium ion

In the case of PC-OOH (e.g. 1-palmitoyl-2-hydroperoxy-octadecadienoyl-sn-glycero-3-phosphocholine (16:0/HpODE PC, m/z 790)), HPLC-MS/MS MRM analysis has generally been performed using its product ion (phospho-
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choline, m/z 184)\textsuperscript{20,21}, but the MRM 790/184 could not distinguish the hydroperoxide positional isomers (e.g. 1-palmitoyl-2-(9-hydroperoxy-octadecadienoyl)-sn-glycero-3-phosphocholine (16:0/9-HpODE PC) and 1-palmitoyl-2-(13-hydroperoxy-octadecadienoyl)-sn-glycero-3-phosphocholine (16:0/13-HpODE PC)). As mentioned above, we found that PC-OOH (16:0/HpODE PC (m/z 812)) generates unique hydroperoxy group-derived fragments (i.e., m/z 388 for 16:0/9-HpODE PC and m/z 541 for 16:0/13-HpODE PC) in the presence of the sodium ion, and therefore achieved discrimination of 16:0/9-HpODE PC (MRM 812/388) and 16:0/13-HpODE PC (MRM 812/541) in our previous experiments\textsuperscript{15,22}. Hence, in this study, we evaluated the hypothesis that the use of the sodium ion

![Fig. 3](image-url) Mass spectra (Q1) and product ion mass spectra (MS/MS) of SQ-OOH isomers in the absence (A) and presence of the sodium ion (positive mode) (B). Standard SQ-OOH isomers were dissolved in either methanol or methanol containing 0.1 mM sodium acetate. The sample solutions (10 μg/mL) were infused directly into a microTOF-Q II mass spectrometer at a flow rate of 0.6 mL/h. Detailed analytical conditions are described under EXPERIMENTAL PROCEDURES.
will allow for better discrimination of SQ-OOH isomers. Accordingly, direct infusion of the six SQ-OOH standards into the MS/MS system was performed in both the absence and presence of the sodium ion to investigate their fragmentation patterns. Unless specifically stated otherwise, spectral data were obtained under the optimized conditions. Each spectrum (Fig. 3) is representative of at least a triplicate analysis.

We first analyzed protonated SQ-OOH isomers in the absence of the sodium ion under atmospheric pressure chemical ionization (APCI). Consistent with previous studies, collision-induced dissociation produced many fragments with similar patterns (Fig. 3A), making it difficult to discriminate the SQ-OOH isomers. We thereby analyzed the SQ-OOH isomers in the presence of the sodium ion under electrospray ionization (ESI), which yielded different fragment ions compared to those produced under APCI (Fig. 3B). For example, 2-OOH-SQ formed a characteristic fragment ion at \( m/z \) 433.34 \((\text{M} + \text{Na} - \text{CH}_2\text{O})^+\) corresponding to a 32.03 Da loss from the sodiated molecular ion \((m/z \text{ 465.37[M+Na]}^+)\). The fragment ion was presumably derived from a cleavage of the C1-C2 bond associated with an elimination of an -OH group; this follows the fragmentation patterns observed during the analysis of other lipid hydroperoxides in the presence of sodium. The characteristic fragmentation patterns may be attributed to the ability of alkali metals to promote ionization of neutral loss molecules, and were commonly seen among all SQ-OOH isomers except 3-OOH-SQ, of which we predicted two types of fragmentation patterns (Fig. 3B). The chemical structures of all ions described in Fig. 3B were predicted using the exact mass data from the ACD/MS-ID Workbook (ACD labs; Ontario, Canada). Under the aforementioned conditions, we were able to analyze SQ-OOH isomers at nanogram levels in the presence of the sodium ion. In later experiments, it was evaluated whether this technique will allow for higher selectivity and sensitivity in HPLC-MS/MS MRM analysis of SQ-OOH isomers.

### 3.3 HPLC-MS/MS MRM analysis of a mixture of six standard SQ-OOH isomers

We achieved discrimination of SQ-OOH isomers in a mixture of standards using HPLC-MS/MS with a silica column and MRM based on fragments observed during product ion scanning on each isomer in the presence of the sodium ion (Fig. 4). In the MRM chromatograms, each SQ-OOH isomer was detected at the following retention times: 8.7 min (11-OOH-SQ), 9.5 min (7-OOH-SQ), 11.4 min (10-OOH-SQ), 11.7 min (6-OOH-SQ), 13.9 min (3-OOH-SQ), 16.4 min (2-OOH-SQ). These results are representative of at least a triplicate analysis. To the best of our knowledge, this is the first complete discrimination of SQ-OOH isomers using HPLC-MS/MS MRM analysis. In addition to demonstrating high selectivity, the developed method also allows for the detection of SQ-OOH isomers at nanogram levels. This method may therefore be applied in the quantification of each SQ-OOH isomer on human skin and the evaluation of each isomer’s effect on skin conditions. We are currently assessing the effect of sunlight exposure on the accumulation of each SQ-OOH isomer on human skin utilizing the developed HPLC-MS/MS MRM method. The results may improve our understanding of the effect of SQ-OOH on skin conditions as well as the mechanism behind SQ peroxidation. This will allow for the development of SQ peroxidation inhibitors, providing a therapeutic approach to skin disorders.

### 4 Conclusion

We prepared six standard SQ-OOH isomers on which we performed MS/MS analysis in the presence of the sodium ion. It was revealed that in the presence of the sodium ion, more distinct fragmentation patterns were observed compared to those seen in the absence of the sodium ion. Based on these findings, higher selectivity and sensitivity in HPLC-MS/MS MRM analysis were achieved; this may assist not only in the quantification of each isomer in skin,
but also in the unravelment of each isomer’s effect on human skin conditions.

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