Determination of Stigmasterol Hydroperoxides Using High-Performance Liquid Chromatography-Mass Spectrometry with Atmospheric Pressure Chemical Ionization

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Abstract: A new specific method using high-performance liquid chromatography-mass spectrometry (HPLC-MS) for the detection of stigmasterol hydroperoxides was developed. Hydroperoxides of stigmasterol were obtained by photo-oxidation (90 min) in the presence of methylene blue as a sensitizer. The separation was performed using normal-phase chromatographic conditions. The MS detection was carried out with an ion-trap mass spectrometer using atmospheric pressure chemical ionization (APCI) and positive ion mode. Stigmasterol hydroperoxides were seen to produce no protonated molecular ions $[M + H]^+$ but instead fragments representing loss of one or two water molecules $[M - H_2O + H]^+$, $[M - 2H_2O + H]^+$, loss of hydrogen peroxide $[M - H_2O_2 + H]^+$ or loss of hydrogen peroxide and water $[M - H_2O_2 - H_2O + H]^+$. The results showed that positional isomers of hydroperoxides had different fragmentation patterns and relative ion abundances. On the other hand anomeric isomers had more similar fragmentation. As a conclusion the method developed showed to be a useful tool for investigation the oxidation mechanism of sterols.

Keywords: HPLC-MS; APCI; stigmasterol; hydroperoxide

INTRODUCTION

Distinct cholesterol oxidation products have been reported to be mutagenic, cytotoxic and carcinogenic [1]. Due to the structural similarity, plant sterol oxidation products may have similar health implications. Especially in plant sterol-enriched functional foods oxidation susceptibility of plant sterols should thus be known. HPLC has became increasingly popular in the analysis of lipid primary oxidation products, hydroperoxides. The major advantages of HPLC compared to previously commonly used gas chromatography is operation at room temperature. Neither time-consuming sample pretreatment nor derivatization is needed. Moreover, by using MS detection analytes with incomplete separation can be analyzed [2, 3]. The aim of this study was to develop a new sensitive and selective HPLC-MS-method for analysis of plant sterol hydroperoxides. Stigmasterol was used as the model compound.

EXPERIMENTAL

Photo-oxidation. The hydroperoxides of stigmasterol were obtained by photo-oxidation. Stigmasterol (20 mg) was dissolved in 1.95 ml of dichloromethane. The photo-oxidation was sensitized with 50 μl methylene blue (0.5 mg/ml) in dichloromethane. The solution in the flask (45 mm × 25 mm i.d.) was exposed to a 75-watt light source (2000 lux) through a 3-cm layer of water. The water layer filters out the infrared radiation. After photo-oxidation (90 min), dichloromethane was evaporated under a stream of nitrogen and the residual photo-oxidized stigmasterol was dissolved in 4 ml of *n*-heptane-isopropyl alcohol (97:3, v/v). After that the solution was filtered through GHP membrane filters (PALL, Gelman laboratory, USA) to remove the photosensitizer methylene blue.

HPLC-MS analysis. The determination of stigmasterol hydroperoxides was carried out by using HPLC-MS analysis. The separation of the hydroper-

oxides was based on method published earlier [4]. The mobile phase was *n*-heptane-isopropyl alcohol (97:3, v/v) and the separation was performed on a silica Supercosil column (250 mm × 2.1 mm i.d., 5 μm; Supelco, Bellefonte, PA, USA). The flow rate of the mobile phase was 0.6 ml/min and injection volume was 25 μl. The mass spectrometric detection was carried out with an ion-trap mass spectrometer (Esquire-LC, Bruker Daltonik, Bremen, Germany) using APCI positive ion mode. Collision-induced dissociation was produced with helium (99.96% AGA, Finland) at the pressure of 0.6 mPa (87 npsi) in the ion trap. The temperature and flow rate of the dry gas (nitrogen) were set at 300°C and 7.00 l/min, respectively, and the APCI interface temperature was 400°C. The pressure of the nebulizer gas (nitrogen) was 50 psi and voltage of the corona discharge needle was 3.5 kV. Trap drive, skimmer 1 and the capillary exit offset were set at 36.8, 30.0 V and 110 V, respectively. Ethanol (0.5 ml/min) was added throug *T*-valve to the eluent flow to enhance ionization efficiency.

RESULTS AND DISCUSSION

Photo-oxidation of stigmasterol resulted in formation of seven hydroperoxide peaks (Figure 1). The structures of the main hydroperoxides: 6β-hydroperoxide (6β-OOH), 6α- hydroperoxide (6α-OOH), 5α- hydroperoxide (5α-OOH), 7β-hydroperoxide (7β-OOH) and 7α-hydroperoxide (7α-OOH) were identified after their reduction to the corresponding hydroxides by GC-MS [4, 5]. With this HPLC-method the separation of the different positional isomers of stigmasterol hydroperoxides was achieved within 15 min. Only anomeric 6α-OOH and 6β-OOH coeluted. However, with the MS detector we could detect 6α-OOH and 6β-OOH despite their incomplete separation.

Stigmasterol hydroperoxides were seen to produce no protonated molecular ions $[M + H]^+$ but instead fragments representing loss of one or two water molecules $[M - H_2O + H]^+$, $[M - 2H_2O + H]^+$, loss of hydrogen peroxide $[M - H_2O_2 + H]^+$ or hydrogen peroxide and water $[M - H_2O_2 - H_2O + H]^+$ (Table 1). Our findings were similar to those of Kusaka and Ikeda [6], who reported that fatty acid hydroperoxides formed no molecular ion, but instead produce fragments representing loss of water or loss of entire hydrogen peroxide [6]. The results showed that positional isomers of hydroperoxides of stigmasterol had different fragmentation patterns and relative ion abundances. On the other hand the anomeric isomers had a more similar fragmentation behaviour. Compounds eluted at RT 2.9 and RT 7.4 were tentatively identified to

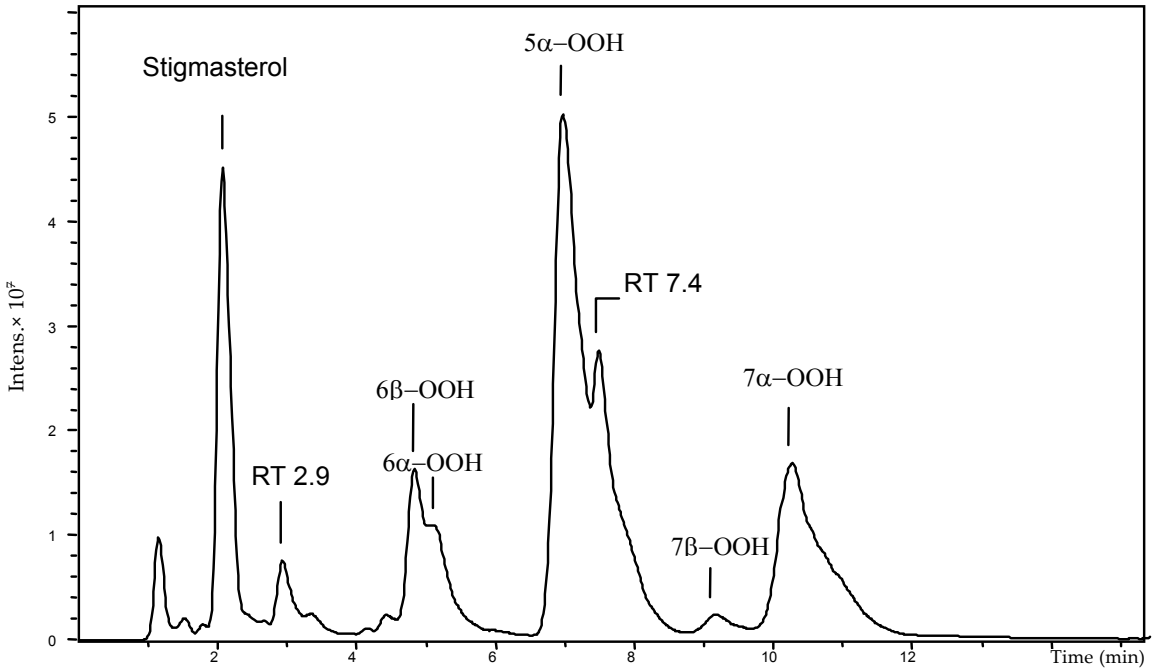

Figure 1. Total ion chromatogram of photo-oxidized stigmasterol. RT 2.9 and RT 7.4 are unidentified hydroperoxides

Table 1. Specific mass spectral ions and relative ion abundances (%) of stigmasterol hydroperoxides (M = molecular ion). A dash indicates that the fragment was not observed

Stigmasterol oxides	Retention time (min)	Specific ions (*m*/*z*)				
		$[M + H]^+$	$[M - H_2O + H]^+$	$[M - 2H_2O + H]^+$	$[M - H_2O_2 + H]^+$	$[M - H_2O_2 - H_2O + H]^+$
Stigmasterol	2.1	413.4 (4)	395.4 (100)			
RT 2.9	2.9	445.4 (–)	427.4 (87)	409.4 (7)	411.4 (100)	393.4 (47)
6β-OOH	4.8	445.4 (–)	427.4 (17)	409.4 (100)	411.4 (27)	393.4 (–)
6α-OOH	5.1	445.4 (–)	427.4 (17)	409.4 (100)	411.4 (53)	393.4 (–)
5α-OOH	6.9	445.4 (–)	427.4 (14)	409.4 (45)	411.4 (20)	393.4 (100)
RT 7.4	7.4	445.4 (–)	427.4 (100)	409.4 (47)	411.4 (13)	393.4 (62)
7β-OOH	9.1	445.4 (–)	427.4 (75)	409.4 (100)	411.4 (24)	393.4 (74)
7α-OOH	10.2	445.4 (–)	427.4 (39)	409.4 (100)	411.4 (23)	393.4 (90)

be hydroperoxides because of their hydroperoxide specific fragmentation behaviour.

Effect of different reagents on the ionization efficiency of the instrument was also investigated. Ethanol, methanol and isopropyl alcohol were added to the eluent (0.5 ml/min) through a *T*-valve and the best sensitivity was observed when using ethanol.

CONCLUSIONS

As a conclusion, a new specific HPLC-MS-method for detection of plant sterol hydroperoxides was developed. The major advantage is the capability of the MS detection to identify analytes even in the case of incomplete separation. With this new method we could detect positional and anomeric hydroperoxides of stigmasterol, without time-consuming sample pretreatment and derivatization. Therefore, the method is a useful tool for investigation of the oxidation mechanism of sterols.

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