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Gas chromatographic-mass spectrometric analyses of cholesterol and its precursors in rat plasma as *tert*-butyldimethylsilyl derivatives

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ABSTRACT

Background: Cholesterol and its metabolic precursors occurring in metabolic pathways are important biochemical indicators in pathological conditions.
Methods: A method for the simultaneous determination of cholesterol and its metabolic precursors, such as lanosterol and 7-dehydrocholesterol, in rat plasma is demonstrated. It involves their extraction after saponification, followed by conversion to *tert*-butyldimethylsilyl (TBDMS) derivatives for analysis by gas chromatography-mass spectrometry (GC-MS) in selected ion monitoring (SIM) mode (GC-SIM-MS).
Results: The characteristic fragment ions of [M-57], *m/z* 443, 483, and 441 permitted the accurate and selective detection of cholesterol and its precursors in rat plasma. The whole procedure of TBDMS derivatization, with subsequent GC-SIM-MS analysis, was linear ($r \geq 0.9994$), reproducible (% relative standard deviation = 2.2 to 7.5), and accurate (% relative error = −5.6 to 7.7), with detection limits of 0.02 to 0.07 ng/ml. Recoveries were measured to be ranged from 89.5 to 95.4%.
Conclusion: The present method was useful for the quantification of cholesterol and its precursors in rat plasma samples of 1 μl.

1. Introduction

Cholesterol has been reported to be a risk factor for various diseases including hyperpiesia, neurodegenerative disorders, and cardiovascular diseases [1–4]. As a result, in recent studies, the metabolic pathway of cholesterol has become an important issue clinically. Analyses of cholesterol by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) have typically been performed after forming the trimethylsilyl (TMS) derivative [3,5–7]. However, *tert*-butyldimethylsilyl (TBDMS) derivatives have superior GC and MS properties and higher stability against hydrolysis compared with TMS derivatives [8]. Despite this, the TBDMS derivatization method has rarely been applied to the analysis of cholesterol [9,10]. In this study, simultaneous profiling analysis of cholesterol and its precursors, such as lanosterol and 7-dehydrocholesterol [11], in rat plasma was performed using GC-MS in selected ion monitoring (SIM) mode (GC-SIM-MS).

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2. Materials and methods

2.1. Chemicals and reagents

Cholesterol, lanosterol, 7-dehydrocholesterol, epicoprostanol as an internal standard (IS), and ammonium iodide were purchased from Sigma-Aldrich (St. Louis, MO). Potassium hydroxide, ammonium iodide, sodium chloride, hexane, toluene, methanol, diethyl ether, and dichloromethane of spectroanalytical grade were purchased from Kanto (Tokyo, Japan). *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was from Pierce (Rockford, IL). All other chemicals were of analytical reagent grade and were used as received.

2.2. Preparation of standard solutions

Stock solutions of cholesterol, lanosterol and 7-dehydrocholesterol in free form were prepared in hexane at 5.0 mg/ml. Working standard solutions at 2 different concentrations, 1.0 and 50 μg/ml, for each compound was prepared by taking appropriate aliquots of each stock solution and diluting with hexane. The stock solution of IS at 5.0 mg/ml in hexane was used to prepare the IS working solution at 50 μg/ml in hexane. The mixed calibration samples were prepared at seven different concentrations, ranging from 0.001 to 2.0 μg/ml, by mixing appropriate aliquots of each working solution. All standard solutions were stored at 4 °C.

2.3. Gas chromatography-mass spectrometry

GC-MS analyses in SIM mode were performed with an Agilent 6890N gas chromatograph interfaced to an Agilent 5975B mass-selective detector (70 eV, electron

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Table 1
GC-SIM-MS method for the assay of three cholesterols as *tert*-butyldimethylsilyl derivatives

Compound	Selected ion (*m/z*)	Linearity[a] *r*	LOD[b] (ng/ml)	Added (μg/ml)	Precision (% RSD)	Accuracy (% RE)	Recovery[c] (%)	Added (μg/ml)	Precision (% RSD)	Accuracy (% RE)	Recovery (%)	Levels in serum (Mean ± SD, μg/ml)
Cholesterol	367, **443**, 485	0.9994	0.06	0.05	3.1	7.7	93.3 ± 2.3	0.2	2.2	−0.02	95.4 ± 3.8	500.8 ± 32.5
7-Dehydrocholesterol	325, 351, **441**	0.9995	0.02	0.05	6.8	6.1	89.5 ± 3.6	0.2	4.2	−1.2	93.8 ± 2.3	0.6 ± 0.1
Lanosterol	393, **483**, 540	0.9999	0.07	0.05	7.5	−5.6	90.2 ± 3.7	0.2	3.7	1.5	94.2 ± 4.1	1.1 ± 0.3
Epicoprostanol (IS)	369, **445**, 487											

Ultra-2 capillary column (25 × 0.20 m I.D., 0.11 m d_f), from 240 °C (1 min) and programmed to rise to 300 °C (10 min) at 20 °C/min in SIM mode with 100 ms dwell time and 1100 V electron multiplier.
Bold ion was selected for quantitation.
[a]Correlation coefficient in the calibration range of 0.01–2.0 μg/ml for cholesterol standards.
[b]LOD, limit of detection.
[c]Recovery rate, calculated at 0.05 and 0.2 μg for cholesterol and its precursors with IS (0.5 μg), as (extracted peak area ratio/non-extracted peak area ratio) × 100.
All measurements were made in triplicate.

ionization mode) and equipped with an Ultra-2 (5% phenyl–95% methylpolysiloxane bonded phase; 25 m × 0.20 mm I.D., 0.11 μm film thickness) cross-linked capillary column (Agilent Technologies, Atlanta, GA, USA). The temperatures of the injector, interface, and ion source were 260, 300 and 230 °C, respectively. Helium was used as the carrier gas, at a flow rate of 0.5 ml/min in constant flow mode. Samples were introduced in split-injection mode (10:1) and the oven temperature was set initially at 240 °C (1 min) and then programmed to rise to 300 °C (10 min) at a rate of 20 °C/min. The mass range scanned was 50–600 u at a rate of 0.99 scans/s. In SIM mode, three characteristic ions for each compound were used for peak identification, while one bold ion was selected for quantification (Table 1). A dwell time of 100 ms was chosen for all ions. The relative voltage of the electron multiplier was set to 200 V higher than that in the scanning mode (1200 V) for each ion monitored. Each peak in the rat plasma samples was identified by matching the area ratios of three ions with those of the standards. All the GC-SIM-MS runs were performed in triplicate.

2.4. tert-Butyldimethylsilylation and method validation

A mixed standard solution (0.5 μg) containing cholesterol, lanosterol, 7-dehydrocholesterol and IS (0.5 μg) was used to determine the optimal reaction time at 60 °C. The mixed standard was saponified with 0.5 ml of 5 mol/l potassium hydroxide solution in methanol at 60 °C for 1 h. After cooling to room temperature, 0.5 ml distilled water was added and saturated with sodium chloride, followed by solvent extraction with 3 ml hexane and 3 ml diethyl ether, in sequence. The combined extracts were evaporated to dryness under a gentle stream of nitrogen (40 °C). Toluene (20 μl) and a mixture (20 μl) of MTBSTFA/NH_4I (1000:4, v/w), to form the TBDMS derivative, were added to the residue containing standards and the mixture was heated at 60 °C for 60 min for the GC-SIM-MS analysis.

Calibration samples at seven different concentrations (0.001 to 2.0 μg/ml) were added with the IS (0.5 μg/ml) and subjected to saponification and the TBDMS reaction in the same manner. All samples were individually prepared in triplicate and were analyzed on the same day to assess repeatability, accuracy, and linearity. Linearity was tested by least-squares regression analysis on the peak area ratios against increasing concentration ratios to plot calibration curves. The limit of detection (LOD) for each analyte was estimated based on the lowest concentration giving a signal greater than the sum of the mean blank signal plus three times the standard deviation of the blank signal obtained *via* three blank measurements. The repeatability, expressed as a percentage of the relative standard deviation (% RSD), and accuracy, as a percentage of the relative error (% RE), of the method were determined from the calibration samples, in triplicate. Recovery rates were assessed by comparing the percentages of peak area ratios of extracted samples to those of non-extracted counterparts (representing 100% recovery), prepared by direct TBDMS derivatization in dry, neat form at the same nominal concentrations.

2.5. Sample preparation for assay of cholesterol and its precursors in rat plasma

The use of animals in this study was approved by the Animal Care and Use Committee of Ajou University, and all procedures were carried out in accordance with institutional guidelines. Normal rat plasma samples were collected from 5 healthy male Sprague–Dawley rats (weighing 250–300 g). A 1-μl sample of normal rat plasma containing 0.5 μg of IS was saponified and extracted with hexane and diethyl ether, in sequence. The supernatant layer was subjected to the TBDMS reaction described above before GC-SIM-MS analysis. Blank sample using in this work was distilled water and distilled water spiked with standards was used for method validation process.

3. Results and discussion

3.1. tert-Butyldimethylsilylation and MS properties of cholesterol and its precursors

Cholesterol, lanosterol, and 7-dehydrocholesterol, and the IS, were simultaneously converted into their respective single TBDMS derivatives (Fig. 1). The optimal reaction time for TBDMS derivatives was determined by heating for various times (10, 20, 30, 40, 60, 80, 100, and 120 min) at 60 °C. For all analytes, 60 min showed a higher peak area ratio than 30 min, while it was very similar to those at 80, 100, and 120 min. Thus, 60 min was selected as the optimal reaction time for forming the TBDMS derivative. All TBDMS derivatives were found to be stable for several weeks when they were tightly capped and refrigerated.

The electron ionization (EI) mass spectral data of the *O*-TBDMS derivative are shown in Fig. 2. The pair of $[M\text{-}15]^{+*}$ and $[M\text{-}57]^{+*}$ ions known to be characteristic of TBDMS derivatives facilitated peak identification of cholesterol and related compounds. These were formed by the loss of the labile $-CH_3$ and *tert*-butyl functions from the molecular ions, respectively. The base peaks for cholesterol and 7-dehydrocholesterol were seen as *m/z* 443 and 351 (the $[M\text{-}57]^{+*}$ ion), respectively. In this study, mass spectral data sets for lanosterol and the IS were newly established as TBDMS derivatives. Their base peaks were *m/z* 75 $[(CH_3)_2SiOH]^{+*}$. The prominent peaks of lanosterol were *m/z* 393 $[M\text{-}147]^{+*}$, 483 $[M\text{-}57]^{+*}$, 525 $[M\text{-}15]^{+*}$, and 540 (the molecular ion), while those of IS were *m/z* 445 $[M\text{-}57]^{+*}$ and *m/z* 369 $[M\text{-}133]^{+*}$. Each mass spectral pattern was characteristic of the TBDMS derivative.

3.2. Optimal GC-SIM-MS conditions

In SIM mode, 3 characteristic ions for each molecule were selected for peak identification, while one bold ion, $[M\text{-}57]^{+*}$, for all compounds was used for quantification (Table 1). Among the dwell times tested, 100 ms was found to yield the highest ion abundances for most of the cholesterol-related compounds.

Plasma matrix effects on SIM detection were examined using plasma extracts prepared from blank plasma and showed that the SIM ion sets were very selective for cholesterol analysis. The cholesterols (including epicoprostanol, the IS) were simultaneously detected with good sensitivity and excellent selectivity from plasma, with no major interference (Fig. 1).

3.3. Method validation for cholesterol and its precursors

When the whole procedure of TBDMS derivatization and subsequent GC-SIM-MS analysis was validated for the assay of cholesterols in rat plasma samples, each calibration curve measured at 0.001 to 2.0 μg/ml was linear (r = 0.9994–0.9999), and the LODs varied from 0.02 to 0.07 ng/ml (Table 1). This overall linearity demonstrated the suitability of the present method for quantitative assays in biological samples. The ranges of precision (% RSD) and accuracy (% RE) of the overall procedure at 2 different added amounts (0.05 and 0.2 μg/ml) in triplicate within 1 day varied from 2.2 to 7.5 and from −5.6 to 7.7, respectively (Table 1). The recoveries ranged from 89.5 to 95.4% (Table 1). Thus, the overall precision, accuracy, and recovery appear to be satisfactory for the quantification of cholesterols in unknown plasma samples.

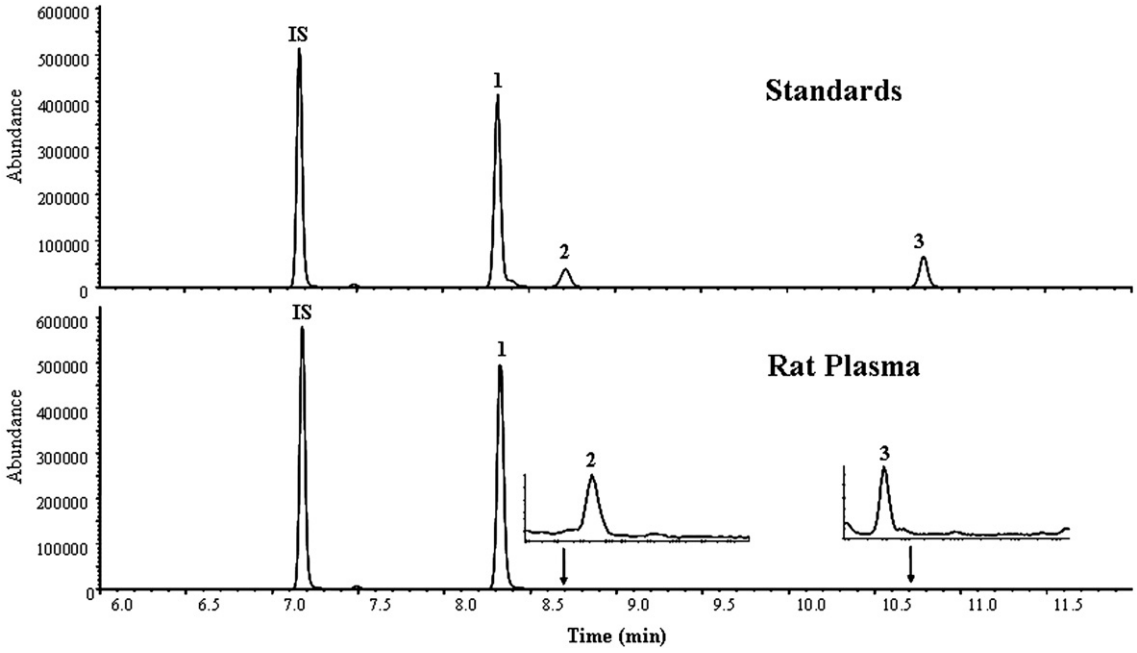

Fig. 1. Selected ion monitoring profiles of standards and rat plasma of three cholesterols as *tert*-butyldimethylsilyl derivatives, separated on Ultra-2 capillary columns (25 m×0.20 mm I.D., 0.11 μm film thickness). The helium flow rate was set to 0.5 ml/min, in constant flow mode, and samples (~1.0 μl) were injected in split mode (10:1). The oven was held at 240 °C (1 min) and then programmed to rise to 300 °C (10 min) at a rate of 20 °C/min. Peaks: (1) cholesterol, (2) 7-dehydrocholesterol, (3) lanosterol, (IS) epicoprostanol.

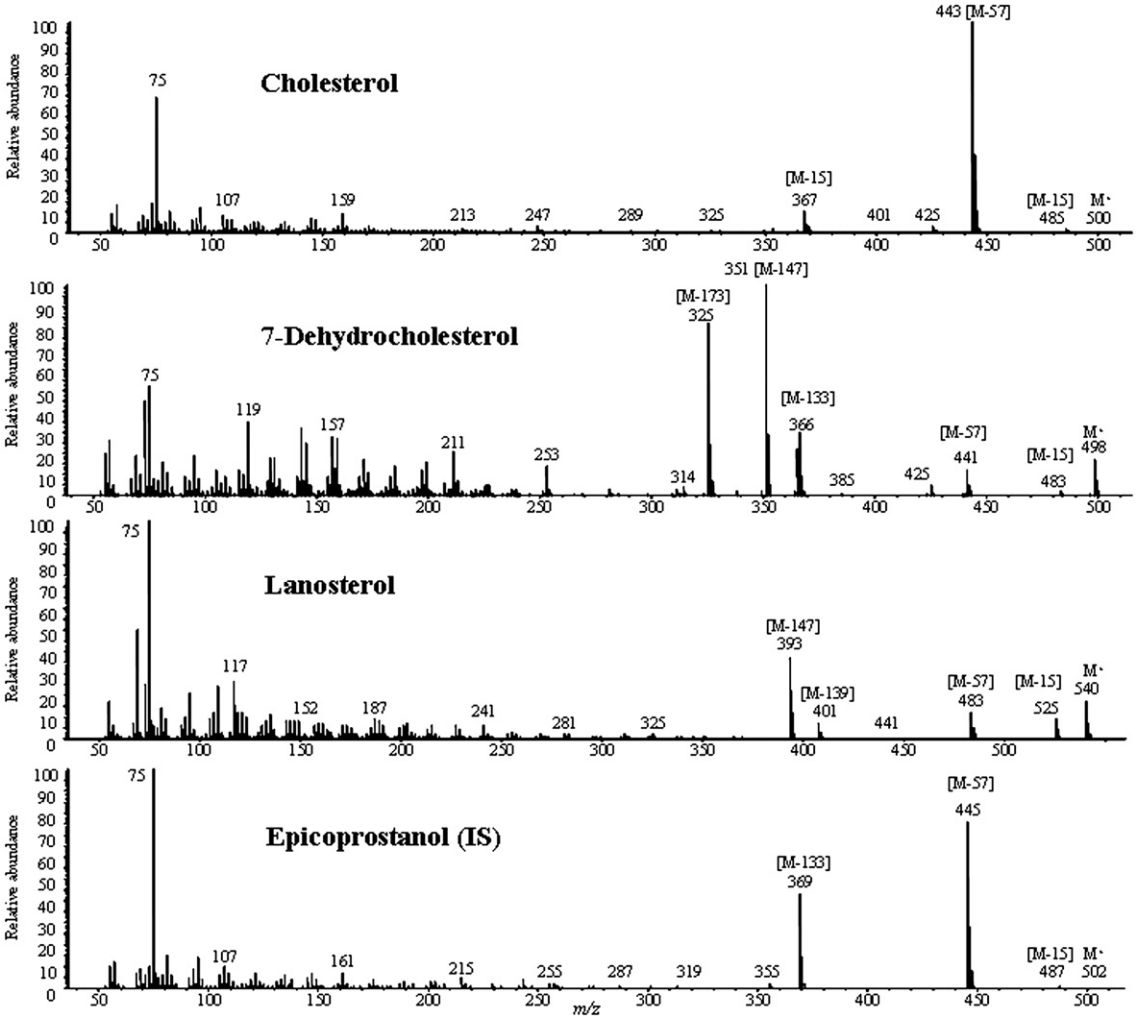

Fig. 2. Electron ionization mass spectra of cholesterol, 7-dehydrocholesterol, lanosterol, and epicoprostanol (IS) as *tert*-butyldimethylsilyl derivatives.

3.4. Levels of cholesterol and precursors in normal rat plasma

The present method was applied to 5 normal rat plasma samples of 1 μl. Fig. 1 shows a SIM chromatogram of these compounds in rat plasma. The concentrations of lanosterol, 7-dehydrocholesterol, and cholesterol were 1.1 ± 0.3, 0.6 ± 0.1, and 500.8 ± 32.5 μg/ml, respectively. These levels were similar to those previously reported [12,13]. These results indicate that the method is accurate and reliable for the determination of cholesterol and its precursors in rat plasma samples. Thus, the present TBDMS derivatization method, combined with GC-SIM-MS, and using a minimal sample volume (1 μl) will be useful for simultaneous, rapid screening and monitoring of cholesterol and its precursors in animal studies and possibly clinical research also.

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