Reference Intervals of Serum Non-Cholesterol Sterols by Gender in Healthy Japanese Individuals

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Aims: The present study was conducted to establish a practical method for measuring non-cholesterol sterols and reference intervals of serum levels.

Methods: Healthy subjects (109 men and 151 women), four patients with sitosterolemia, and 10 heterozygous mutation carriers of ABCG5/ABCG8 genes were investigated. Then, three non-cholesterol sterols (sitosterol, campesterol, and lathosterol) of fasting serum samples were measured via a practical and highly sensitive gas chromatography (GC) method with 0.2 µg/mL as the lower limit of quantification. The coefficient of variation (CV) values for within-run reproducibility were 3.06%, 1.89%, and 1.77% for lathosterol, campesterol, and sitosterol, respectively. The CV values for between-run reproducibility were 2.81%, 2.06%, and 2.10% for lathosterol, campesterol, and sitosterol, respectively.

Results: The serum levels of sitosterol and campesterol were significantly higher in women than in men, whereas the serum levels of lathosterol were significantly higher in men than in women. Because of these gender difference, the determination of reference intervals of the three sterol values was performed by considering gender. The reference intervals of sitosterol, campesterol, and lathosterol were 0.99–3.88, 2.14–7.43, and 0.77–3.60 µg/mL in men and 1.03–4.45, 2.19–8.34, and 0.64–2.78 µg/mL in women, respectively. The serum levels of sitosterol and campesterol were higher in patients with sitosterolemia (94.3 ± 47.3 and 66.3 ± 36.6 µg/mL, respectively) than in healthy subjects.

Conclusion: These results demonstrate a practical and highly sensitive GC method to measure non-cholesterol sterol levels and gender-segregated reference intervals of sitosterol, campesterol, and lathosterol in Japanese healthy subjects.

Key words: Non-cholesterol sterol, Sitosterolemia, Reference interval, Gas chromatography
Introduction

Sitosterolemia is a rare autosomal recessive disease involved with increased concentrations of non-cholesterol plant sterols, including sitosterol and campesterol. Sitosterolemia was first reported in 1974 by Bhattacharyya and Connor in two young sisters with tendonous xanthomas in a similar manner as familial hypercholesterolemia (FH) and markedly elevated plasma plant sterol regardless of having total cholesterol (TC) levels of approximately 200 mg/dL. This disease is caused by gene mutations in either the ATP-binding cassette (ABC) subfamily G member 5 (ABCG5) or ABCG8. Although sitosterolemia with double mutations in those genes has long been considered extremely rare, the frequencies of this situation can be approximately 1 in 200,000 based on large-scale sequencing projects. Patients with sitosterolemia show extreme phenotypic heterogeneity, ranging from almost asymptomatic patients to severe hypercholesterolemic patients with accelerated atherosclerosis, including premature coronary heart disease (CHD).

Sitosterolemia often involves elevated levels of sitosterol and low-density lipoprotein-cholesterol (LDL-C). In such cases, statins sometimes insufficiently decrease LDL-C, but ezetimibe can substantially decrease LDL-C. In addition, patients with sitosterolemia can have either normal, moderately elevated, or exceedingly high concentrations of TC and LDL-C. Brinton et al. reported that approximately 4% of patients with LDL-C concentrations ≥ 190 mg/dL have plasma sitosterol concentrations above the 99th percentile (≥ 8 mg/L) and approximately 0.3% have concentrations consistent with those of sitosterolemia (≥ 15 mg/L). The diagnosis of sitosterolemia should be considered in patients with LDL-C levels ≥ 190 mg/dL, allowing for the optimal diet and drug therapy, including ezetimibe and statins.

Ezetimibe is one of the appropriate drug therapies for patients with sitosterolemia. A recent study, the Heart Institute of Japan-PROper level of lipid IOwering with Pitavastatin and Ezetimibe in acute coRonary syndrome (HIJ-PROPER), has shown that serum sitosterol level, which is a surrogate marker of cholesterol absorption, can be a good biomarker for CHD prevention efficacy under ezetimibe therapy. In a sub-analysis of HIJ-PROPER, the aggressive lipid-lowering treatment with ezetimibe had a preventive effect on clinical outcomes in the high sitosterol subset (≥ 2.2 µg/mL) of dyslipidemic patients with acute coronary syndrome.

Aim

The measurement of serum sitosterol is useful for the diagnosis of sitosterolemia and is helpful for the management of CHD risk. However, the measurement of non-cholesterol sterols is not currently covered by the health insurance in Japan. A practical and certain method to measure non-cholesterol sterol fractions (sitosterol, campesterol, and lathosterol) and the established reference intervals of these sterols should be basically required for clinical use. Accordingly, an ad-hoc project was organized in the Japan Atherosclerosis Society. In Japan, a gas chromatography (GC) method is commonly used for clinical and research settings. The improvement of the GC method to measure non-cholesterol sterols was launched in SRL Inc. (Hachioji, Tokyo, Japan) of the Miraka Group. Here, we show a practical and highly sensitive GC method to measure the serum levels of the three non-cholesterol sterols and their reference intervals.

Subjects and Methods

Subjects

In the present study, 260 healthy Japanese subjects (109 men and 151 women, including 16 postmenopausal women) and 14 sitosterolemic patients were investigated. The healthy subjects, aged 20 to 70 years, from the Jikei University Kashiwa Hospital, Ochanomizu University, and Shizuoka Tobu Medical Center were invited to join this study. The residual samples of annual medical check-up and samples from volunteers were used to measure serum sterol fractions, and the medical check-up record data were retrospectively investigated. The patients, who took supplements and medications for dyslipidemia, hypertension, diabetes, and dysfunctions of the liver and kidney, were excluded from the present study. Current cigarette smokers and subjects with alcohol consumption habit were also excluded. In addition, subjects with body mass index (BMI) < 17 or ≥ 30 kg/m² were excluded from this study with reference to the BMI classification of the World Health Organization. The study was conducted in accordance with the principles of the Declaration of Helsinki. The institutional
Patients with sitosterolemia with double pathogenic mutations in ABCG5/ABCG8 gene (four women, aged 9.5 years) and subjects (six women, aged 47.5 years; four men, aged 41.5 years) with a heterozygous mutation in ABCG5/ABCG8 gene from Kanazawa University Hospital were invited to participate in this study. Subjects with CHD, diabetes, hypertension, impaired function of the liver and kidney; with habits of smoking and alcohol drinking; and taking supplements were excluded from the present study. This study was approved by the Ethics Committee of Kanazawa University School of Medicine (approval number: 2017-268), and the patients were enrolled with written informed consent. Sitosterolemia (homozygous) was definitely diagnosed by tendon xanthomas, increased serum sitosterol (≥10 μg/mL), and the presence of ABCG5/ABCG8 mutations after the differential diagnosis, including FH, in Kanazawa University Hospital.

**Laboratory Analyses**

Blood samples were collected after a 10-hour fasting, and sera were subsequently separated. Serum concentrations of TC and triglyceride (TG) were enzymatically measured, and LDL-C and high-density lipoprotein-cholesterol (HDL-C) were measured via homogenous methods. These data and the characteristic information of the study subjects were retrospectively collected from medical laboratory records. The laboratory analysis for three non-cholesterol sterol measurements by the GC method was exclusively performed at SRL Inc. (Hachioji, Tokyo, Japan) of the Miraka Group. The three non-cholesterol sterols are sitosterol and campesterol, biomarkers of cholesterol absorption from the intestine, and lathosterol, a biomarker of cholesterol synthesis in the body. Serum samples were cryopreserved until the measurement of the serum non-cholesterol sterols. The freeze storage period was an average of 10 days. Differences of non-cholesterol sterol values between fresh samples and frozen samples were generally negative (data not shown). The measurement procedure is briefly described as follows.

**Preparation of Standard Solutions and the Internal Standard (IS) Solution**

Serial dilutions of lathosterol, campesterol, and β-sitosterol were prepared in differently labeled solutions. Mixtures of all chemicals were prepared at a concentration for the calibration standard (20.0 μg/mL) and stored at −20°C. The IS solution was 5α-cholestane (50 μg/mL), which was diluted to 100 mL.

**Sample Pretreatment**

At first, 200 μL of serum was dispensed and transferred to a glass tube. Then, the mixed solution of 2.5 mL of 1 M NaOH and ethanol was added to the tube, mixed, and saponified. After cooling, 50 μL of 5α-cholestane (50 μg/mL) was added as an IS, and then 7.0 mL of n-hexane was added and mixed for 5 minutes. After the mixture was centrifuged, 6 mL of n-hexane layer was fractionated and evaporated to dryness under a nitrogen stream. After drying, 100 μL of N, O-bis (trimethylsilyl) acetamide was added to the tube and mixed. Then, silylation was performed. After silylating, the phase was evaporated to dryness under a nitrogen stream. Then, 0.6 mL of ethyl acetate was added to the tube and mixed. To measure the non-cholesterol sterols, 1.5 μL of the upper phase was injected to a GC.

**GC Conditions**

A GC machine (GC-2010) with flame ionization detector (FID) and manufactured by Shimadzu Corporation (Japan) was utilized in this study. The GC analysis was carried out in an InertCap 5MS/Sil capillary column (5% diphenyl (equiv.)–95% dimethylphenylenesiloxane; GL Sciences, Japan) (40 m × 0.18 mm × 0.18 μm) under the following conditions: 60°C for 2 min to 320°C and 40°C/min; temperature of the FID, 320°C; sample injection temperature, 310°C; sample introduction pressure, 200 kpa; and splitless mode sampling, 1.5 μL. Furthermore, helium was used as the carrier gas.

**Determination of the Measurement Values of the Three Non-Cholesterol Sterols, Cholesterol Synthesis, and Absorption Markers**

GC-2010 GC data were used for image acquisition by a data processing device (C-R7A, Shimadzu Corporation, Japan). The concentration of each marker was determined by the IS curve method. The GC measurement protocol provides 0.2 μg/mL as the lower limit of quantification. Typical GC chromatograms for a healthy subject and a patient with sitos-
terolemia are shown in Fig. 1. Coefficient of variation (CV) values for within-run reproducibility were 3.06%, 1.89%, and 1.77% for lathosterol, campesterol, and sitosterol, respectively. CV values for between-run reproducibility were 2.81%, 2.06%, and 2.10% for lathosterol, campesterol, and sitosterol, respectively.

Statistics

In all the statistical analyses, a value of $p<0.05$ was considered statistically significant. Normality evaluations for the measured data of the healthy subjects were performed by the Shapiro–Wilk test. Original values were not normally distributed, but the logarithmic conversion data of sitosterol, campesterol, and lathosterol values by gender were normally distributed ($p>0.05$). Then, reference intervals (mean ± 1.96 standard deviation [SD]) of the three non-cholesterol sterol levels were determined in accordance with the method recommended by the Clinical & Laboratory Standards Institute\(^6\). After normalizing all data through the logarithmic conversion, the mean and SD were calculated, and samples exhibiting measured values outside the mean ± 2.58 SD were excluded. This process was repeated until exception data were not found any longer. Finally, the reference intervals were expressed after restoring logarithmic data to real numbers. In addition, Mann–Whitney $U$ test was used to compare data between the two groups.

Results

The three non-cholesterol sterol values of the whole study subjects are summarized in Table 1. The levels of LDL-C and HDL-C (≤ 140 mg/dL and ≥ 40 mg/dL) and TG (≤ 150 mg/dL) were within normal limits in all the subjects. The levels of sitosterol, campesterol, and HDL-C were significantly higher in women than in men, whereas lathosterol and LDL-C, TG, and BMI were significantly higher in men than in women. Therefore, gender differences were found in the three non-cholesterol sterol values. Subsequently, the determination of reference intervals of the three sterol values was performed with gender consideration. Samples exhibiting sterol values outside the mean ± 2.58 SD were excluded. In men, the same sample was excluded for the analysis of sitosterol and campesterol, and another sample was excluded for the analysis of lathosterol. Even in women, the same two samples were excluded from the analysis of sitosterol and lathosterol, and the same two samples plus another sample were excluded from the analysis of campesterol. Table 2 shows the data of the three non-cholesterol sterol values for the analysis of reference intervals by gender. In the same way as the whole study subjects, gender differences were found in the three non-cholesterol sterol values.

Table 3 shows the gender-segregated reference intervals of sitosterol, campesterol, and lathosterol. Presumable cardiovascular high-risk subjects with relatively high sitosterol values (≥ 2.2 µg/mL) were found in 39 of the 108 healthy men and 76 of the 149 healthy women. In the present study subjects, the median values of serum sitosterol levels were 1.9 µg/mL in men and 2.2 µg/mL in women.

Table 4 shows the characteristics and serum lipid data of the patients with gene mutations of $ABCG5/ABCG8$. The patients with sitosterolemia (four women with homozygous gene mutations of $ABCG5/ABCG8$) demonstrated markedly higher levels of serum sitosterol and campesterol ($p<0.01$) compared with female healthy subjects shown in Table 2. However, lathosterol values were significantly lower in sitosterolemic patients than in the healthy subjects ($p<0.01$). As for the subjects with a heterozygous gene mutation of $ABCG5/ABCG8$ (Table 4), lathosterol values were not different from those in the healthy subjects (Table 2) in both genders. But, the sitosterol and campesterol levels were significantly higher in the heterozygous subjects than in the healthy subjects ($p<0.01$).

Discussion

The present study shows the gender-segregated reference intervals of sitosterol, campesterol, and lathosterol measured by the established GC method. The difference of sitosterol and campesterol levels between men and women was previously reported, and the levels are similar in the present study results\(^7\). However, the definite gender difference of lathosterol levels has never been reported and remains to be cleared. The reference intervals of sitosterol, campesterol, and lathosterol measured by the GC method are shown by gender in Table 3. To the best of our knowledge, the definite demonstrations on the reference intervals of non-cholesterol sterols have not been reported. A large cohort of American patients constituting a clinical laboratory database ($n=667,718$) showed GC mass spectrometry (MS)-measured values of sitosterol and campesterol (sitosterol, 2.45 ± 1.39 µg/mL; campesterol, 3.3 ± 1.83 µg/mL) regardless of gender. These mean ± SD values can implicitly provide the tentative reference intervals (sitosterol, 0–5.23 µg/mL; campesterol 0–6.09 µg/mL) through simple arithmetic although this large cohort study may include diabetes and other metabolic diseases. As compared with these values, the
Fig. 1. Typical chromatograms of gas chromatography (GC) for non-cholesterol sterols

Typical chromatograms of GC for non-cholesterol sterols were derived from a healthy subject (A) and a patient with sitosterolemia (B).
Table 1. Characteristics and serum lipid data of whole study subjects

| (µg/mL)                            | Men (n = 109) | Women (N = 151) | p. value |
|------------------------------------|---------------|-----------------|----------|
| Age (years)                        | 35.8 ± 10.7   | 35.9 ± 11.0     |          |
| Body mass index (kg/m²)            | 22.1 ± 3.3    | 20.8 ± 2.1      | <0.01    |
| Total cholesterol (mg/dL)          | 189 ± 22      | 191 ± 26        |          |
| LDL-cholesterol (mg/dL)            | 105 ± 19      | 100 ± 17        | <0.05    |
| HDL-cholesterol (mg/dL)            | 65 ± 14       | 75 ± 14         | <0.01    |
| Triglyceride (mg/dL)               | 85 ± 31       | 68 ± 24         | <0.01    |
| Non-cholesterol sterols (µg/mL)    |               |                 |          |
| Sitosterol                         | 2.12 ± 0.83   | 2.41 ± 1.09     | <0.05    |
| Campesterol                        | 4.26 ± 1.57   | 4.79 ± 2.04     | <0.05    |
| Lathosterol                        | 1.84 ± 0.84   | 1.44 ± 0.54     | <0.01    |

Mann-Whitney U test was used to compare data between the two groups. A value of $p < 0.05$ was considered as statistically significant.

Table 2. Non-cholesterol sterol values of healthy subjects applied for the analysis of reference intervals by gender

| (µg/mL)                            | Men | Women | p. value |
|------------------------------------|-----|-------|----------|
| Non-cholesterol sterols            |     |       |          |
| Sitosterol                         | 2.09 ± 0.77 (n = 108) | 2.30 ± 0.89 (n = 149) | <0.05 |
| Campesterol                        | 4.20 ± 1.46 (n = 108) | 4.53 ± 1.59 (n = 148) | <0.05 |
| Lathosterol                        | 1.80 ± 0.72 (n = 108) | 1.42 ± 0.52 (n = 149) | <0.01 |

Mann-Whitney U test was used to compare data between the two groups. A value of $p < 0.05$ was considered as statistically significant.

Table 3. Gender-segregated reference intervals of serum values of sitosterol, campesterol, and lathosterol

| (µg/mL) | Men | Women |
|---------|-----|-------|
| Sitosterol | 0.99-3.88 | 1.03-4.45 |
| Campesterol | 2.14-7.43 | 2.19-8.34 |
| Lathosterol | 0.77-3.60 | 0.64-2.78 |

Table 4. Characteristics and serum lipid data of patients with gene mutations of ABCG5/ABCG8

|               | Homozygous | Heterozygous |
|---------------|------------|-------------|
|               | men        | women       |
| Numbers       | 4 (women)  | 4           | 6           |
| Average age (year) | 9.5 ± 8.2   | 41.5 ± 25.5 | 47.5 ± 13.8 |
| Body mass index (kg/m²) | 18.5 ± 0.8  | 22.1 ± 1.0  | 23.0 ± 2.2 |
| Total cholesterol (mg/dL) | 311 ± 144    | 218 ± 20    | 227 ± 58   |
| HDL-cholesterol (mg/dL) | 54.5 ± 11.9  | 47.3 ± 13.7 | 69.3 ± 24.3 |
| Non HDL-cholesterol (mg/dL) | 257 ± 156    | 171 ± 33    | 158 ± 34   |
| Non-cholesterol sterols (µg/mL) |         |             |
| Sitosterol | 94.3 ± 47.3 | 7.85 ± 1.29 | 8.98 ± 4.56 |
| Campesterol | 66.3 ± 36.6 | 12.9 ± 1.79 | 15.4 ± 7.48 |
| Lathosterol | 0.53 ± 0.15 | 2.18 ± 0.93 | 1.40 ± 0.45 |
upper reference limits determined by the present study are slightly lower albeit just indirect comparisons. These differences may be presumably attributed to the adequacy of the present study analysis resulting from using the apparently healthy subjects.

The present study demonstrates the non-cholesterol sterol values of sitosterolemia. Sitosterol and campesterol values were markedly higher in patients with sitosterolemia than in healthy subjects, and patients with heterozygous mutations of genes (ABCG5/ABCG8) also provided significantly higher values of sitosterol and campesterol than the healthy subjects. Viewed from this perspective, these reference intervals are markedly lower as compared with the serum values of patients with sitosterolemia and heterozygous mutations of genes (ABCG5/ABCG8). The GC method is considered appropriate as a measurement method for the non-cholesterol sterols and diagnosis of sitosterolemia although the reference interval is essentially different from clinical decision criteria. In addition, serum sitosterol level ≥ 10 µg/mL may be one of the diagnosis criteria for sitosterolemia. However, its validation needs further studies with a number of patients with sitosterolemia. In this study, all the four sitosterolemic patients had serum sitosterol levels over 30 µg/mL, and among the 10 patients with heterozygous mutations of genes (ABCG5/ABCG8), three had serum sitosterol levels around 10 µg/mL. A wide range of conditions need to be considered in identifying the underlying causes of sitosterolemia. Comprehensive analyses of sitosterolemia-related genes in conjunction with measuring serum sitosterol levels of many samples by the present method are required to explore the optimal cut-off level of serum sitosterol for the clinical diagnosis of sitosterolemia.

As described above, serum levels of sitosterol and campesterol were obviously higher in the patients with sitosterolemia and heterozygous gene mutations of ABCG5/ABCG8 than in the healthy subjects. Interestingly, serum lathosterol levels were significantly lower in the patients with sitosterolemia than in the healthy subjects. Previous papers have reported that serum lathosterol levels are related to the overall cholesterol synthesis in the body but inversely associated with the intestinal absorption of cholesterol. In other words, the accumulation of non-cholesterol sterols may account for the low rates of cholesterol synthesis in patients with sitosterolemia.

This study has several limitations. First, the present study was performed by considering gender, not age group bracket. A previous study demonstrated that serum levels of sitosterol and campesterol were affected not only by gender but also by age. In women, these levels increased with advancing age. These trends were found after most women experienced menopause. However, the upper limits appear to be less than 3.0 µg/mL in sitosterol and 3.5 µg/mL in campesterol. These levels are within the reference intervals of sitosterol and campesterol shown in the present study including 16 postmenopausal women. Consequently, the influence of fluctuation of serum sitosterol and campesterol levels on their reference intervals is trivial, but larger-sized studies are needed to verify that. In addition, levels of desmosterol, another biomarker of cholesterol synthesis, decreased with advancing age in men, showing no data about lathosterol. Therefore, the influence of age on the reference interval of serum lathosterol should be checked in the future although the range of fluctuation of serum desmosterol levels was small.

Second, apolipoprotein E (APOE) isoforms differentially affect plasma lipid and lipoprotein concentrations, and the prevalence of cholesterol hyperabsorption was higher in APOE ε4 allele carriers as compared with the other APOE genotypes. Although APOE genotypes were not investigated in the present study, the presence of APOE ε4 allele carriers may have a little effect on the results and conclusion because the present study subjects were healthy and not dyslipidemic. The APOE genotypes of most healthy subjects in the present study might not presumably carry APOE ε4 allele.

Third, the GC method instead of GC/MS and liquid chromatography (LC)/MS–MS was used in the present study. Extraction and derivatization of non-cholesterol sterols differ between research groups, as do separation techniques, which include GC and LC systems. In addition, the quantification of non-cholesterol sterols is divided between FID and MS detection. GC/MS method with selective ion monitoring (SIM) and LC/MS–MS are used worldwide, but in Japan, the GC method with FID is commonly used in clinical studies. The quantification of non-cholesterol sterols is divided between GC with FID and MS detection with SIM, and currently, considerable variability exists between measurement techniques. According to our preliminary study, a GC/MS method with a removal of cholesterol from samples during the pretreatment process can be at risk of lowering retrieval rates. Another GC/MS method addressing the avoidance of cholesterol effects using cholesterol-existing samples provided difficulties of the selection of appropriate ion strength to deal with the separation capacity and measurement sensitivity simultaneously. Whether the preferred usefulness of LC/MS–MS surpassing the property of the present GC method with FID is found remains unclear because we did not perform the comparison investiga-
tion between LC/MS–MS and our GC method.

Fourth, non-fasting changes in serum levels of non-cholesterol sterols are uncertain because fasting samples were used in the present study. Previous papers showed the postprandial increase in plasma sitosterol levels after the consumption of plant sterol-enriched diet24, 25). Therefore, postprandial samples will be investigated for the significance of measurement of non-cholesterol sterol values in the future.

Fifth, differences of non-cholesterol sterol values between serum and plasma are a critical issue for clinical laboratory medicine. In some parts of previous papers, plasma samples were used for the measurement of non-cholesterol sterol values24, 26). However, serum samples, instead of plasma samples, were used in the present study. Further studies are needed to investigate the differences of non-cholesterol sterol values between serum and plasma.

Conclusion

In conclusion, the present study shows a practical and highly sensitive GC method to measure non-cholesterol sterol levels and gender-segregated reference intervals of sitosterol (0.99–3.88 µg/mL in men; 1.03–4.45 µg/mL in women), campesterol (2.14–7.43 µg/mL in men; 2.19–8.34 µg/mL in women), and lathosterol (0.77–3.60 µg/mL in men; 0.64–2.78 µg/mL in women) in Japanese healthy subjects.

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Conflict of Interest

None had conflict of interest to be disclosed in terms of the present study.

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