의학석사 학위논문

기체 크로마토그래피 질량분석법을 이용한 피토스테롤 검사 및 소아에서 고콜레스테롤혈증과 고피토스테롤 농도의 관련성

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2019년 2월

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ABSTRACT

Study on the Development of a Multiplex Phytosterol Assay utilizing GC-MS, and the Association between Hypercholesterolemia and High Phytosterol Levels in Children

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Background: Phytosterols are increased in rare, inherited lipid storage disorders including cerebrotendinous xanthomatosis and sitosterolemia. Early diagnosis can improve disease outcomes, but phytosterols cannot be readily measured by traditional chemical or enzymatic assays. In this study, we developed a multiplex phytosterol assay utilizing gas chromatography-mass

spectrometry (GC-MS), and also evaluated the association between hypercholesterolemia and high phytosterol levels in children.

Methods: Serum mixed with internal standard (epicoprostanol) was prepared, and cholestanol, sitosterol, and campesterol was measured using a HP 6890N GC coupled to a HP 5975 MS (Agilent Technologies, Santa Clara, CA, USA) with a HP-5MS capillary column. Analytical parameters including imprecision, linearity, lower limit of detection (LLOD) and quantification (LLOQ), ion suppression, recovery and carryover effect, were evaluated for the assay. Phytosterol levels of hypercholesterolemic patients (cholesterol >250 mg/dL) and normal controls (cholesterol <200 mg/dL) under the age of 18 were measured using the multiplex phytosterol assay, and the results between the two groups were compared.

Results: All three phytosterols and the IS showed clear differentiation upon GC-MS analysis. The validation results showed excellent precision, with within-run imprecision determined by 5 replicated analyses and between-run imprecision measured on 5 consecutive days below 10.9% of coefficients of variation (CV) for all compounds. The linearity of all three plant sterols had r^2 >0.999 over the full range of measured concentrations (0 - 20.0 mg/dL). The LLOD was 0.1 mg/L for all three phytosterols. The LLOQ was 0.5 mg/dL for sitosterol, and 0.3 mg/dL for cholestanol and campesterol. Ion suppression was not observed, with good recovery and process efficiency. No significant carryover effect was observed. A total of 220 hypercholesterolemic patient samples and 109 normal control samples were collected and tested for phytosterols. The

concentrations of cholestanol, sitosterol and campesterol were significantly higher in hypercholesterolemic children.

Conclusion: The multiplex plant sterol assay using GC-MS showed excellent analytical performance in all evaluated areas. This assay can be utilized in the clinical laboratory for the accurate diagnosis of inherited lipid storage disorders which can ultimately lead to better treatment outcomes and prognosis. Furthermore, the detection of high phytosterol levels in hypercholesterolemic children should be carefully evaluated and adequately treated when indicated.

Keywords: Phytosterols; Xanthomatosis, Cerebrotendinous; Sitosterolemia; Hypercholesterolemia; Gas Chromatography-Mass Spectrometry

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INTRODUCTION

Phytosterols, which encompass plant sterols and stanols, are increased in lipid storage disorders including cerebrotendinous xanthomatosis (CTX) and sitosterolemia. Both are autosomal recessive diseases with heterogeneous clinical manifestations such as tendon xanthomas, premature cardiovascular disease, cataracts, and progressive neurologic abnormalities. Mutations of the *CYP27A1* gene[1] leads to the accumulation of cholestanol due decreased bile acid synthesis in CTX[2], and mutations in either *ABCG5* and/or *ABCG8*[3] causes increased intestinal absorption and decreased biliary excretion of sitosterol and campesterol in sitosterolemia[4].

If appropriately treated, the outcomes of both diseases can dramatically improve. Replacement therapy with chenodeoxycholic acid (CDCA) is an effective treatment for CTX and can prevent disease progression via correction of the biochemical phenotype[5,6]. The current treatment-of-choice for sitosterolemia is ezetimibe, an inhibitor of intestinal sterol absorption, which has been proven to both lower phytosterol levels and alleviate symptoms[4,7,8].

However, it is difficult to measure phytosterols using standard diagnostic assays. For example, enzymatic colorimetry, which reacts to the C-5 double bond or presence of 3β-hydroxyl group, cannot differentiate between cholesterol and phytosterols because these groups are present on both types of sterols[7,9]. Among the methods which can properly measure phytosterols, mass spectrometry (MS) is deemed superior due to its high analytical sensitivity and specificity.

The technical difficulties of measuring phytosterols with common assays, along with the fact that these rare, inherited lipid storage disorders show a wide range of clinical presentations with occasional overlapping with familial hypercholesterolemia, many claim that these disease are prone to underdiagnosis[1,3,4,10]. We developed a triplex phytosterol assay utilizing gas chromatography-mass spectrometry (GC-MS), and also evaluated the association between hypercholesterolemia and phytosterol levels in children.

METHODS

Materials

GC-grade methanol (Burdick and Jackson, Muskegon) and water (Mallinckrodt Baker) were used. Cholestanol, sitosterol and campesterol were purchased from Sigma-Aldrich. Epicoprostanol (USP, Rockville, MD, USA) was used as the Internal Standard (IS). All other reagents were of research grade or better and were purchased from Sigma Chemical. The institutional review board of Seoul National University Hospital and Seoul National University Bundang Hospital approved the study (E-1901-001-998).

Standard Solutions

Stock samples containing 50.0 mg/dL sitosterol, 50.0 mg/dL campesterol, and 100.0 mg/dL cholestanol were stored at −70 ° C. 400 μL of stock sitosterol, 400 μL of stock campesterol and 200 μL of stock cholestanol were mixed to form 1 mL of working 20.0 mg/dL standard solution. The 20.0 mg/dL standard solution was serially diluted in 90% ethanol to produce working standards of different concentrations. Calibration curves were constructed using 6 concentrations of the respective plant sterols (0, 0.5, 1.0, 5.0, 10.0, and 20.0 mg/dL). Stock solution of 10.0 mg/dL epicoprostanol was prepared in 90% ethanol and stored at -70° C. 1.0 mg/dL of working IS solution was produced by 10-fold dilution using 90% ethanol. 4% potassium hydroxide (KOH) solution was prepared by dissolving 0.4g of KOH crystals in 1 mL of distilled water (DW), and then mixing with 9 mL of

100% ethanol.

Sample Preparation

10 μL of serum sample and 60 μL of working IS (1.0 mg/dL epicoprostanol in 90% ethanol) was inserted into glass tubes. After adding 1 mL of 4% KOH in 90% ethanol, the mixture was incubated without light at 65° C for 60 minutes. After addition of 1 mL distilled water, liquid-liquid extraction was performed 3 times: 2 mL of hexane was added, the mixture was vortexed and centrifuged at 4000 rpm for 7 minutes, with subsequent extraction of supernatant into a separate tube, and the procedure was repeated twice, resulting in approximately 6 mL of supernatant hexane. The separated hexane was dried completely using nitrogen gas. After adding 100 μL bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 10% trimethylchlorosilane (TMCS) (Regis techologies, inc., Morton Grove, IL, USA), the mixture was incubated without light at 65° C for 60 minutes. After incubation, the final sample was injected into the gas chromatography-mass spectrometer (GC-MS).

GC-MS Instrument and Conditions

The GC-MS system consisted of a HP 6890N GC coupled to a HP 5975 MS (Agilent Technologies, Santa Clara, CA, USA) with a HP-5MS capillary column (30 m x 0.25 mm i.d, 0.25 μm stationary phase, J&W, Agilent Technologies). The column temperature gradient was programmed from 150° C (hold for 2 min) to 270° C at 30° C /min, to 290° C at 10° C /min (hold

for 7 min), to 300° C at 10° C /min (hold for 2 min). The run-time of the assay was 18 minutes. Quantitative data was obtained via selected ion monitoring analysis (m/z 306, 343, 357 and 370 for cholestanol, campesterol, sitosterol and IS respectively).

Method Validation

Various parameters of analytical performance, such as imprecision, linearity, lower limit of detection (LLOD) and quantification (LLOQ), ion suppression, recovery and carryover effect, were evaluated for the assay. Within-run precision was evaluated via 5 replicated analyses at low (1.0 mg/dL) and high (5.0 mg/dL) standard concentrations. Between-run precision was evaluated using low and high standard concentrations for 5 consecutive days. Linearity was evaluated using 6 concentrations levels (0, 0.5, 1.0, 5.0, 10.0, and 20.0 mg/dL) and repeated 4 times in accordance with the CLSI EP06-A guidelines. LLOD was determined as the lowest concentration with a signal-to-noise (S/N) ratio of > 3.0. LLOQ was determined as the lowest concentration with a precision coefficient of variant (CV) <20% and an accuracy within ±20%. The quantitative assessment of the absolute matrix effect, recovery and process efficiency associated with ion suppression was performed according to the protocol described by Matuszewski et al[11]. Evaluation of carryover was done by injection of a reconstitution solvent blank immediately after the ULOQ of standard curve.

Patient Samples (Assay Evaluation)

Normal control (NC) samples were obtained from healthy individuals with no significant medical diagnoses. Positive control samples were obtained from known cerebrotendinous xanthoma (for cholestanol) and sitosterolemia (for sitosterol and campesterol) patients. Phytosterol levels measured by the multiplex GC-MS assay between negative and positive controls were compared using a Mann-Whitney U test.

Patient Samples (Phytosterols in Normocholesterolemic and Hypercholesterolemic Patients)

Normocholesterolemic (<200 mg/dL) pediatric samples were obtained from healthy children under the age of 18 with no significant medical diagnoses. Hypercholesterolemic (>250 mg/dL) pediatric samples were obtained from children under the age of 18. Patients with malignant diseases and severe congenital disorders, such that an incidental finding of increased phytosterols would not alter their mainline treatment, were excluded from the study. Phytosterol levels measured by the multiplex GC-MS assay between normocholesterolemic and hypercholesterolemic children were compared using a Mann-Whitney U test. A value of median+5SD of the normocholesterolemic patients was used to determine an arbitrary "abnormal" cutoff for sitosterol and evaluate those patients above the cutoff.

RESULTS

The three phytosterols and the IS were clearly separated upon GC-MS analysis (Fig. 1). At low (1.0 mg/dL) concentrations, within-run imprecision results ranged from 1.64% to 4.37% for the measured phytosterols. Between-run imprecision results at the same low concentration ranged from 7.32% to 10.94%. At high (5.0 mg/dL) concentrations, within-run imprecision results ranged from 0.82% to 2.57%, and between-run imprecision results ranged from 4.92% to 10.23%. Detailed imprecision results for each phytosterol are noted in Table 1.

Reproducible linearity was observed over a concentration of 0 - 20.0 mg/dL, with a correlation coefficient, r2, >0.999 for all three phytosterols (Fig. 2). The LLOD was 0.01 mg/dL for all three phytosterols. The LLOQ was 0.5 mg/dL for sitosterol, and 0.3 mg/dL for campesterol and cholestanol (Table 1). No significant carryover effect was observed over the full evaluation period with all solvent blank concentrations measured as less than 20% of the LLOQ.

Plant sterols in lipid disorder patients were significantly higher than normal controls (Fig. 3). Cholestanol was increased in cerebrotendinous xanthoma patients (mean cholestanol 3.5 mg/dL) compared to normal controls (mean cholestanol 0.5 mg/dL), whilst sitosterol and campesterol were increased in sitosterolemia patients (mean sitosterol 13.1 mg/dL, mean campesterol 5.5 mg/dL) compared to normal controls (mean sitosterol 0.2 mg/dL, mean campesterol 0.3 mg/dL) (Table 2).

An additional 109 normocholesterolemic and 220 hypercholesterolemic pediatric patients were enrolled in the

study to evaluate the association between phytosterol levels and total cholesterol levels. Basic patient characteristics are shown in Table 3. There was no significant difference in the sex distribution between the two groups.

Phytosterol levels were significantly higher in hypercholesterolemic patients with mean values of 1.5, 0.9 and 1.1 mg/dL, for cholestanol, sitosterol, and campesterol respectively, compared to normal controls with mean values of 0.6, 0.4 and 0.5 mg/dL for cholestanol, sitosterol, and campesterol respectively (Table 4, Fig. 4a-d). However, the phytosterol/cholesterol ratios were not significantly different between the two groups (Table 4, Fig. 5a-c).

Fourteen hypercholesterolemic patients (6.4%) and one normal control (0.9%) had a sitosterol value above the arbitrary median+5SD cutoff of 1.48 mg/dL (Fig. 6). Amongst these hypersitosterolemic findings, three patients showed extremely high phytosterol levels, with sitosterol levels higher than 11.5 mg/dL in all three patients, campesterol levels higher than 10.7 mg/dL in two patients, and cholestanol levels higher than 5.6 mg/dL in all three patients. These phytosterol levels are in the pathogenic range shown by the known lipid storage disorder patients. Brief clinical data of all fifteen hypersitosterolemic patients are shown in Table 5.

Tables

Table 1. Imprecision (CV), matrix effect, recovery, process efficiency, lower limit of detection, lower limit of quantification of each analyte

	Within-run imprecision (%)		Between-run imprecision (%)		ME (%)		Recovery (%)		PE (%)		LLOD (mg/dL)	LLOQ (mg/dL)
	Low	High	Low	High	Low	High	Low	High	Low	High		
Cholestanol	2.13	0.82	8.64	4.92	150.8	118.7	90.0	80.2	135.8	95.2	0.01	0.3
Sitosterol	4.37	2.57	7.32	9.06	119.0	107.4	94.9	83.5	112.9	89.7	0.01	0.5
Campesterol	1.64	1.16	10.94	10.23	114.8	103.3	95.5	82.2	109.6	84.9	0.01	0.3

Abbreviations: CV, coefficient of variation; ME, matrix effect; PE, process efficiency; LLOD, lower limit of detection; LLOQ, lower limit of quantification

Table 2. Mean sterol concentrations between normal and positive controls

Mean concentration (mg/dL)	Cholestanol	Sitosterol	Campesterol
NC (n=18)	0.5	N/A	N/A
CTx (n=5)	3.5	N/A	N/A
NC (n=14)	N/A	0.2	0.3
Sitosterolemia (n=4)	N/A	13.1	5.5

Abbreviations: NC, negative control; CTx, cerebrotendinous xanthoma

Table 3. Patient characteristics

	Normal controls (N=109)	Hypercholesterolemia (N=220)	p-value
Sex			0.833
Male	65 (59.6%)	127 (57.7%)	
Female	44 (40.4%)	93 (42.3%)	
Age (years)	6.8 ± 4.4	9.8 ± 4.9	<0.001
Cholesterol (mg/dL)	155.6 ± 21.7	324.1 ± 100.3	<0.001

Table 4. Phytosterol levels and phytosterol/cholesterol ratios of normal controls and hypercholesterolemic subjects

Analyte	Normal controls (N=109)	Hypercholesterolemia (N=220)	p-value
Cholestanol (mg/dL)	0.8 ± 0.2	1.5 ± 1.1	<0.001
Sitosterol (mg/dL)	0.5 ± 0.2	0.9 ± 0.2	0.002
Campesterol (mg/dL)	0.6 ± 0.3	1.0 ± 1.3	<0.001
Cholestanol/cholesterol (μg/mg)	5.0 ± 1.0	4.7 ± 2.7	0.095
Sitosterol/cholesterol (μg/mg)	3.2 ± 1.3	2.8 ± 6.0	0.363
Campesterol/cholesterol (μg/mg)	3.8 ± 1.7	3.2 ± 4.0	0.064

Table 5. Clinical data of hypersitosterolemic children (in decreasing order)

Patient	Sex	Age (yr)	Cholesterol (mg/dL)	Cholestanol (mg/dL)	Sitosterol (mg/dL)	Campesterol (mg/dL)	Clinical diagnosis
HC1	F	18	340	5.64	19.32	15.58	NS
HC2	F	7	288	6.99	17.52	10.70	Hypercholesterolemia (on statin therapy)
HC3	F	0	317	8.71	11.49	3.27	BPD, R/O TPN-associated cholestasis
HC4	F	11	266	2.24	3.42	1.78	Encephalomyelitis
HC5	M	11	454	2.99	3.08	3.08	ESRD
HC6	F	10	363	3.75	3.03	2.14	Alagille syndrome
HC7	M	7	256	2.34	3.01	3.34	Hypercholesterolemia
HC8	M	16	956	4.24	2.61	3.78	NS
HC9	F	18	415	3.40	2.08	2.85	Alagille syndrome
HC10	F	13	406	1.90	1.96	2.93	LFT abnormality
HC11	F	13	288	1.82	1.93	1.11	Juvenile dermatomyositis
HC12	F	6	632	2.99	1.85	2.36	NS
HC13	M	8	508	2.82	1.53	2.31	NS
HC14	M	3	256	2.33	1.53	1.76	Encephalitis
NC1	M	1	173	1.36	1.52	1.81	Concealed penis

Abbreviations: NS, nephrotic syndrome; BPD, bronchopulmonary dysplasia

Table 6. Sitosterol cutoff levels of normal, patients, and obligate heterozygotes of various studies (shown in chronological order)

Reference	Subjects (Normal-Patients-Obligate heterozygotes)	Analysis method	Normal mean±SD (median & interquartile range in some studies)	Patient mean±SD	Obligate heterozygote mean±SD
Bhattacharyya 1974	0-2-2	GLC		27.1±2.3 / 17.7±1.6 (two sisters, measured 3 times each)	0.19(father) 0.27(mother)
Hidaka 1990	10-3-4	HPLC	0.71±0.13	25.9±11.6	1.33±0.44
Salen 1992	20-8-4	GLC	0.22±0.20	24.5±14.11	0.60±0.30
Lembcke 2005	49-0-0 (All <24 yrs)	APPI-LC-MS/MS	0.24±0.10		
Assman 2006	318-159 (controls/patients with CAD in 10yr FU)	GC-MS	0.17±0.10(controls) 0.2±0.14(CAD)		
Pinedo 2007	758-373 (controls/patients with CAD in 5yr FU)	GLC	0.21[0.17-0.29](controls) 0.21[0.15-0.28](cases) *median [IQR]		
Z. Wang 2013	10-13-10	HPLC	1.12±0.22	58.43±22.46	2.45±1.45
Bastida 2017	X-1-0	GLC	<0.40	20	
Khan 2017	17-40 (controls/type 2 DM	GLC	0.25±0.10(controls) 0.47±0.22(cases)		

W. Wang 2017	X-1-X	GC	0.1~1.5	62.77±30.59 (measured 3 times)	
Brinton 2018	205,825-1992-109-2 (<99th/>99th percentile/ frank sitosterolemic levels/parents of proband	GC-MS	0.25 [0.16] (<99th) 0.91 [0.19] (>99th) 1.87 [0.66] (frank) *median [IQR]	5.35 (proband)	0.31 (father) 0.42 (mother)

Abbreviations: X, exact number not given; CAD, coronary artery disease; GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography, APPI-LC-MS/MS, atmospheric pressure photoionization-liquid chromatography mass spectrometry; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry

Figures

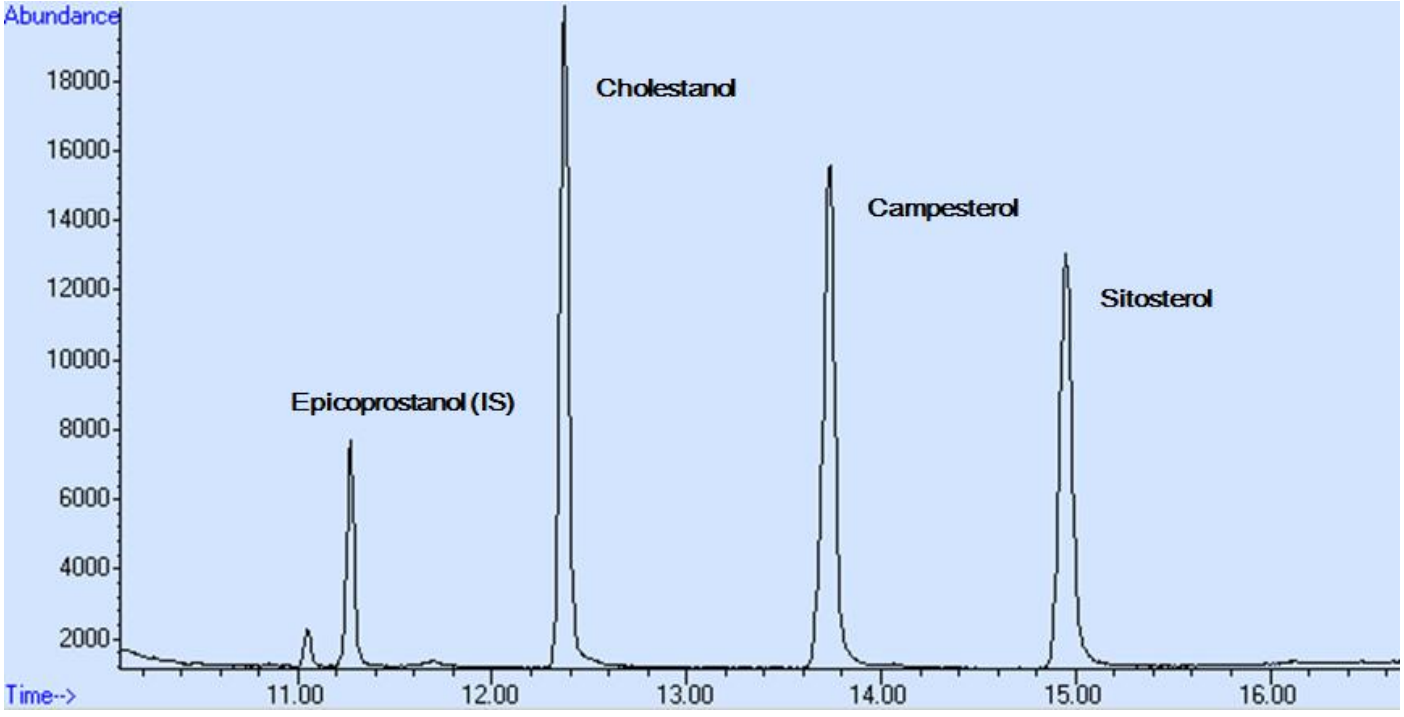


Figure. 1 Cholestanol ($C_{27}H_{48}O$, Molecular weight: 388.67), Sitosterol ($C_{28}H_{48}O$, Molecular weight: 400.68), Campesterol ($C_{29}H_{50}O$, Molecular weight: 414.71) and Epicoprostanol ($C_{27}H_{48}O$, Molecular weight: 388.67) are clearly separated upon GC-MS analysis

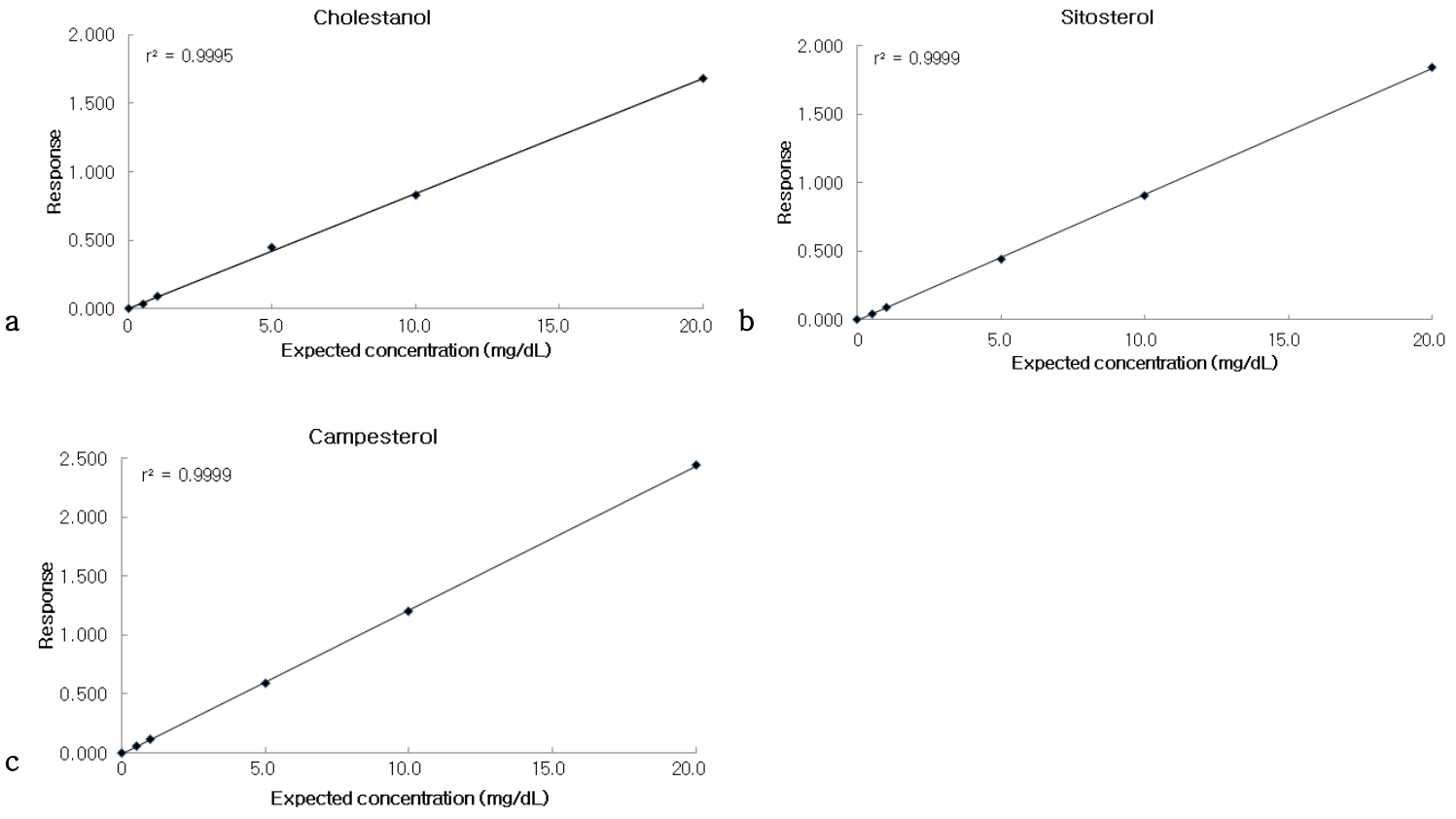


Figure 2. Linearity curves for (a) cholestanol, (b) sitosterol, and (c) campesterol

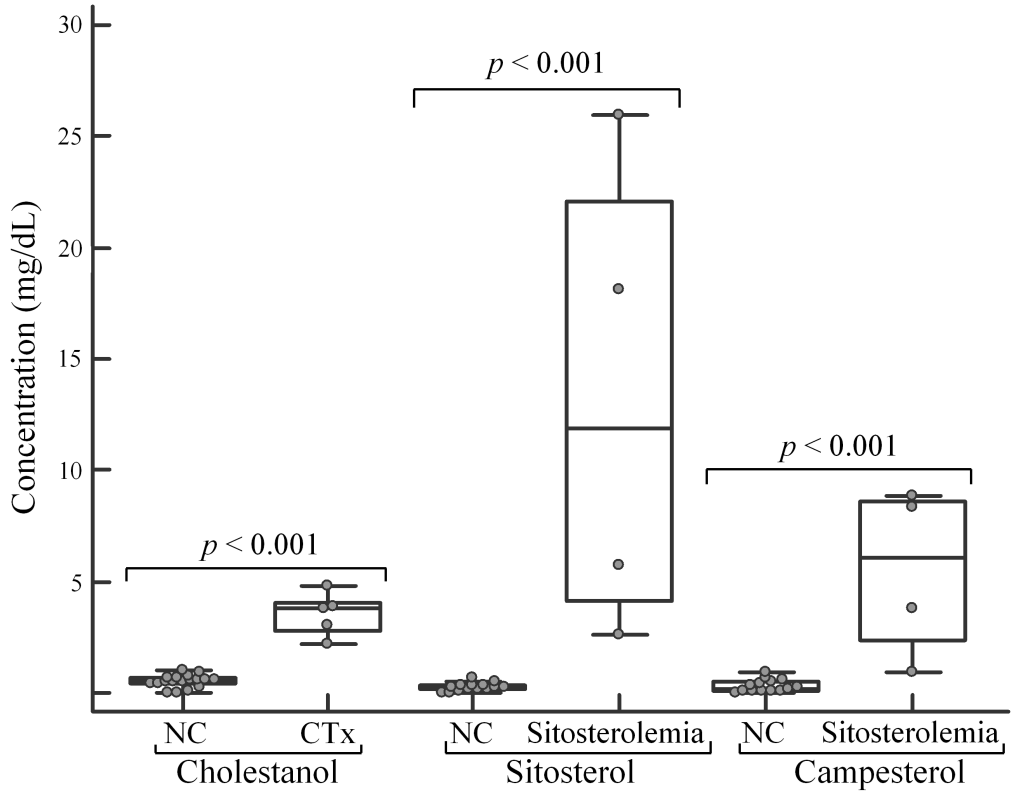
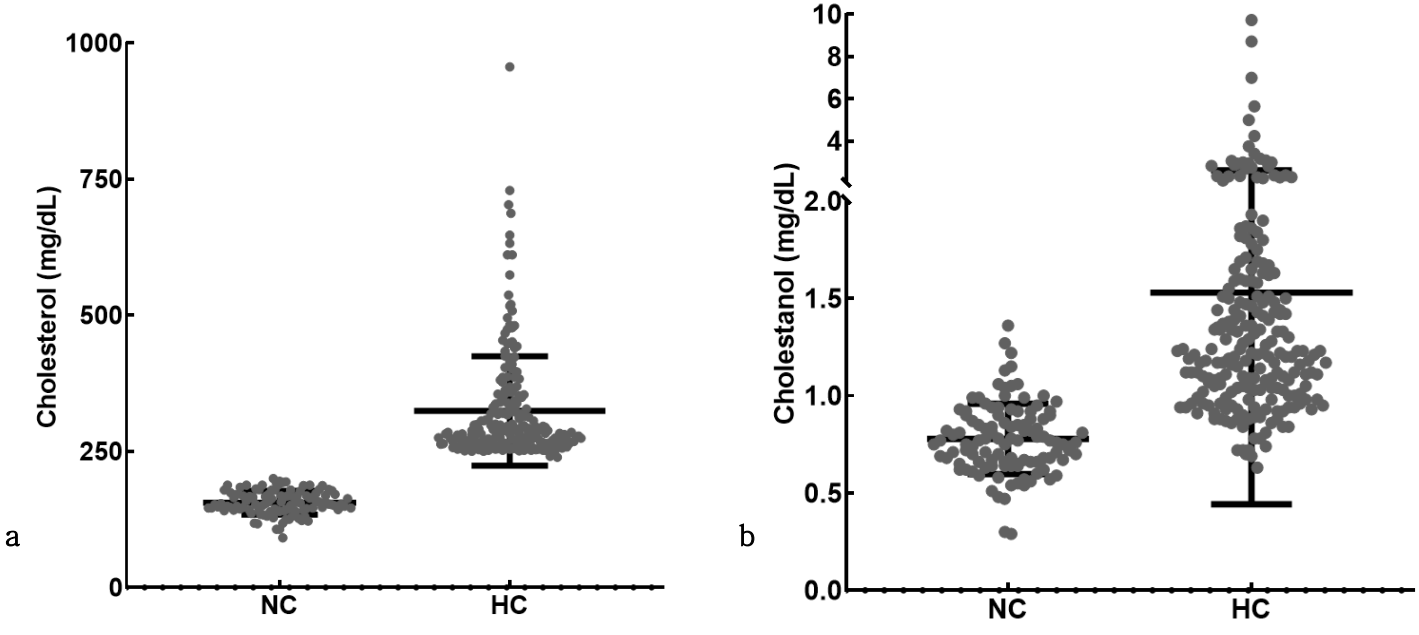


Figure 3. Comparison of phytosterol levels between normal controls (NC) and known cerebrotendinous xanthomatosis (CTx) and sitosterolemia patients

Figures 4a-d. Cholesterol (a) and phytosterol (b-d in order of cholestanol, sitosterol, campesterol) comparison between normocholesterolemic and hypercholestolerolemic children



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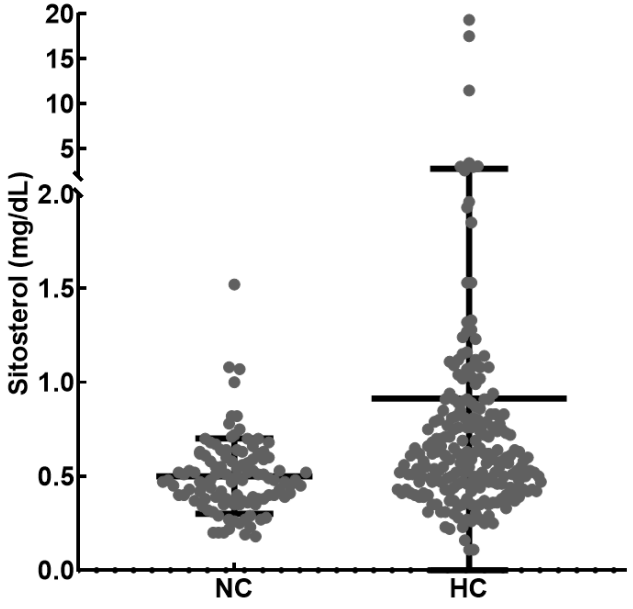
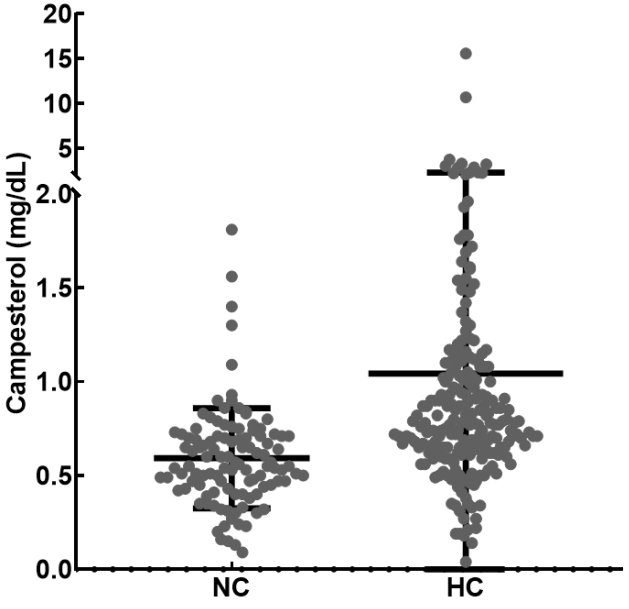
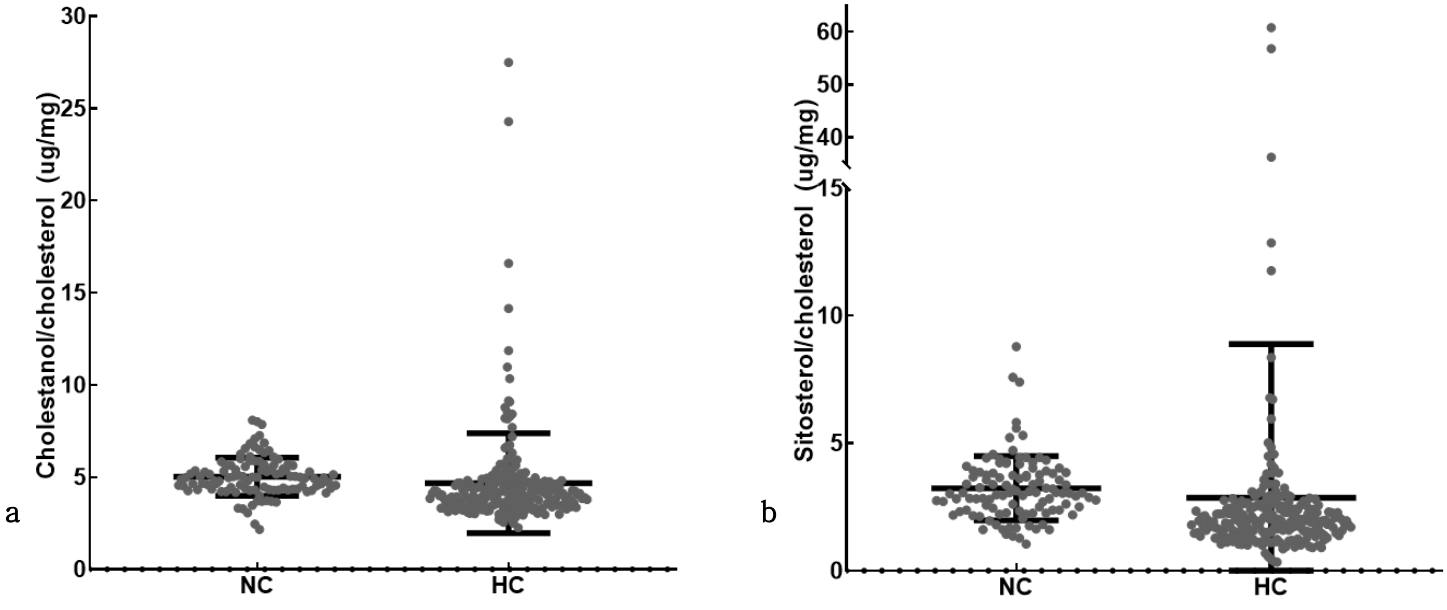

Sitosterol (mg/dL)
20
15
10
5
2.0
1.5
1.0
0.5
0.0
NC
HC

d


Campesterol (mg/dL)
20
15
10
5
2.0
1.5
1.0
0.5
0.0
NC
HC

Figures 5a–c. Phytosterol/cholesterol ratio comparison between normocholesterolemic and hypercholestolerolemic children



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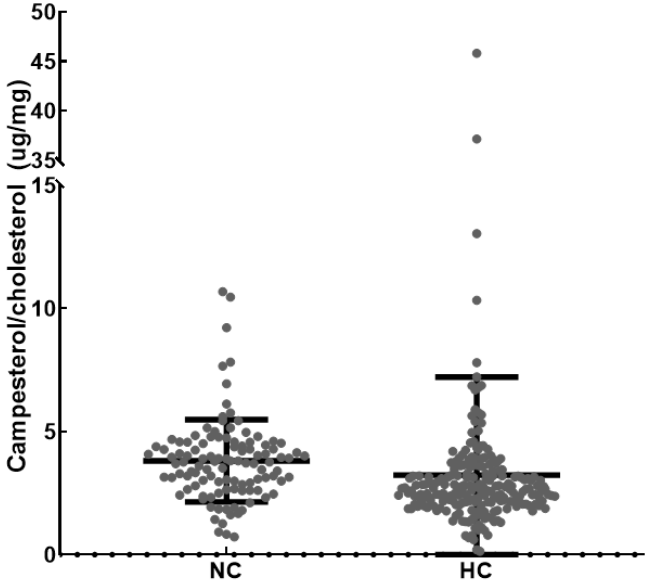
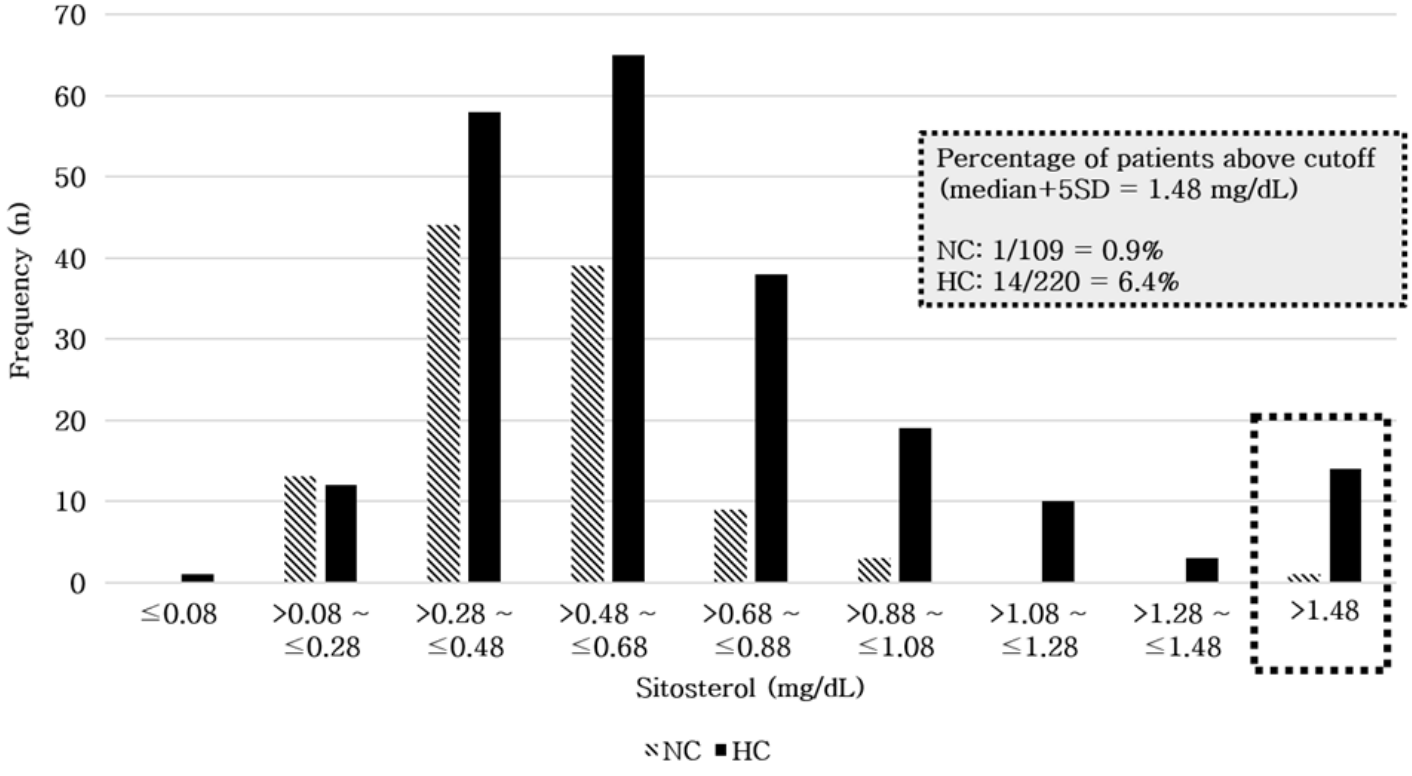


Figure 6. Comparison of sitosterol levels between normocholesterolemic and hypercholestolerolemic children



Abbreviations: NC, normocholesterolemic patients; HC, hypercholesterolemic patients

DISCUSSION

Phytosterols are similar to cholesterols but differ in their carbon side chains and/or presence/absence of a double bond. Phytosterols are broadly classified into two types: sterols and stanols. Sterols have a double bond in the sterol ring, meaning that they are unsaturated, whilst stanols do not have a double bond, meaning that they are saturated compounds. Unlike cholesterols, of which approximately 50% of dietary intake is absorbed, the biological absorption of phytosterols is very low (<5%)[12,13]. The most common dietary phytosterols are sitosterol and campesterol, which are structurally equal to cholesterols with an additional ethyl and methyl group respectively[14].

Phytosterols have been shown to be able to lower cholesterol[13,15,16] and LDL[17] levels. Due to these properties, a large proportion of cholesterol lowering products contain various levels of phytosterols[18,19]. However, the exact amount of phytosterols required to show clinically significant improvements have yet to be determined, and in animal models, the accumulation of phytosterols was proven to show cytotoxicity[20,21]. In humans, there have been reports of increased phytosterol levels been associated with artherosclerosis, cardiovascular disease and stroke[3,22-25]. Furthermore, rare, inherited lipid storage disorders show increased levels of specific phytosterols.

In cerebrotendinous xanthomatosis, mutations of the *CYP27A1* gene cause deficiencies of the mitochondrial enzyme sterol 27-hydroxylase[1]. This leads to reduced bile acid synthesis, and subsequent upregulation of cholesterol 7α-hydroxylase, which is

the rate-limiting enzyme in the classic bile acid pathway. Metabolism involving cholesterol 7α-hydroxylase leads to an accumulation of 7α-hydroxy-4-cholesten-3-one, a precursor to cholestanol[26], and finally leads to increased peripheral blood cholestanol levels in CTX patients.

Normally, cholesterol is absorbed from the intestinal lumen via the Nieman Pick C1 Like 1 (NPC1L1) transporter[12], followed by esterification by acetyl-sterol O-acyltransferase 2 (SOAT2)[27]. Unesterified cholesterols are secreted back into the lumen via ABCG5/ABCG8 efflux transporters[28]. Sitosterol shares the same influx/efflux enzymes as cholesterol, but NPC1L1 and SOAT2 have a much lower affinity for sitosterol, which means that sitosterol is more likely to be pumped out rather than be absorbed. In sitosterolemia, mutations in the *ABCG5* or *ABCG8* genes mean that sitosterol is not properly pumped out of the enterocytes[3], thus sitosterolemia patients absorb 15-60% of their sitosterol intake[14,29].

Though not exclusively pathognomonic, cholestanol and sitosterol are currently used as biomarkers for cerebrotendinous xanthomatosis and sitosterolemia respectively. Due to difficulties in measuring plant sterols with basic chemical or enzymatic assays, MS techniques are required for accurate measurement. We developed a multiplex plant sterol assay using GC-MS that measures cholestanol, sitosterol, and campesterol. The assay showed excellent analytical performance in all evaluated areas, including imprecision, linearity, LLOD & LLOQ, ion suppression, and carryover effect. Also, with relatively short sample preparation times (~several hours, including incubation) and a run-time of 18 minutes, the assay has the advantage of being

less complicated than other MS techniques.

As depicted in Fig. 3, phytosterol measurements using the multiplex assay showed clear discrimination between normal controls and known patients. Thus, we believe this assay can be utilized in the clinical laboratory for the accurate diagnosis of inherited lipid storage disorders.

No consensus has yet been reached with regards to the true prevalence of the aforementioned inherited lipid storage disorders such as cerebrotendinous xanthomatosis and sitosterolemia, with varying reports depending on the region and ethinicity. Due to heterogeneous clinical presentations, different symptoms can appear at different timeframes among patients. Even hypercholesterolemia, initially deemed as a excluding criterion, has been increasingly observed in these diseases, thus acting as a major confounder and making it even more difficult to obtain a definite diagnosis.

The latest reports estimate the prevalence of cerebrotendinous xanthomatosis as 5/100,000 worldwide[10], and sitosterolemia as an even lower rate of <1/1,000,000[30]. Due to both lipid profile testing not being routinely undergone in all children, and the requirement for specialized techniques to detect plant sterols, many claim that these diseases are particularly vulnerable to underdetection[3,31]. Current pediatric guidelines recommend screening children first at 9–11 years and then at 17–21 years of age[32]. However, these guidelines are primarily focused on hypercholesterolemia, meaning that the risk of underdetection of the rarer, inherited disorders still remain.

After development of the multiplex phytosterol assay, we conducted further studies to evaluate the hypothetical association

between hypercholesterolemia and phytosterol levels in children. Our study results showed hypercholesterolemic children to have higher phytosterol levels than normal controls. Unlike cholesterol, which is both ingested from dietary sources and produced from endogenous synthesis, phytosterols cannot be produced by humans, meaning that all phytosterols must come from dietary ingestion. Thus, the higher phytosterol levels in the hypercholesterolemic patients must either be due to increased absorption or decreased excretion. Due to the rarity of studies regarding phytosterol metabolism, there is no known mechanism which can properly explain this observation. However, as high phytosterol levels are reported to be associated with increased risks of cardio- and neurovascular diseases, further studies will eventually be required to elucidate the metabolic pathway.

In an ideal study population, a cutoff value would be determined by utilizing subjects above the 99.9th percentile. However, the relatively small size of our study population meant that the 99.9th percentile would not be a reliable cutoff. Instead, an arbitrary value of median+5SD of the 109 normocholesterolemic patients was utilized to calculate a cutoff value of 1.48 mg/dL. This cutoff is in concordance with previous studies regarding sitosterolemia (Table 6), and identified a total of fifteen hypersitosterolemic patients. Among these patients, three showed exceptionally high sitosterol levels, which were similar to the levels reported in sitosterolemia patients.

The first patient (HC1) was a 18-year old girl under treatment for nephrotic syndrome. Hypercholesterolemia is a common finding in nephrotic syndrome[33,34], due to both increased synthesis compensating for hypoproteinemia and decreased lipid

clearance from the kidneys. As phytosterols cannot be synthesized and are not known to be significantly affected by renal clearance, we believe that further investigation into this finding may be of benefit to the patient.

The second patient (HC2) was a 7-year old girl, undergoing statin therapy for familial hypercholesterolemia. She had a mild family history, with her paternal grandmother treated for dyslipidemia and hypertension, paternal grandfather treated for hypertension, and her mother having modestly increased cholesterol levels (197 mg/dL). As clinical records mentioned poor medical compliance, we cannot clearly determine whether her hypercholesterolemia is unresponsive to statin therapy. We also noted that the patient had modest anemia (Hb 11.0 g/dL). Hemolytic anemia has been previously reported in sitosterolemia[7], and hypercholesterolemia with poor response to statins is also an indication for phytosterol testing[4]. Therefore, especially considering the patient's family history of hypertension, further phytosterol testing would be warranted in this case.

The third patient (HC3), 6-month old baby girl, was being treated for bronchopulmonary dysplasia whilst on total parenteral nutrition (TPN). She also showed signs of cholestasis, and the etiology was undecided upon whether it was due to her underlying problems, or due to TPN. High incidence of cholestatic liver disease was reported in infants who received long-term TPN[35,36], meaning that the patient's nutritional intake must be evaluated. Nevertheless, even considering the possibility of increased phytosterol intake due to TPN, the patient's phytosterol levels are much too high to ignore.

Currently, we cannot definitely affirm that the hypercholesterolemic patients with extremely high sitosterol levels are undetected sitosterolemia patients or that those with moderately increased sitosterol levels are obligate heterozygotes, but we believe that these incidental findings are of the level that at least require clinical inquisition. After evaluating the patients' disease context, further molecular studies may be warranted in those with unexplained increase of sitosterol. The observation rate of high sitosterol (14/220) is a hitherto unreported finding, and when considering the reported incidence of sitosterolemia (<1/1,000,000), the suspicions of underdiagnosis of lipid storage disorders will definitely continue.

Some limitations to our study include the relatively small sample size of both normal controls and positive controls. The extreme rarity of lipid storage disorders makes it very difficult to obtain a significant number of patient samples. Also, due to the study being conducted in a tertiary teaching hospital setting, there is an innate difficulty associated with obtaining medically normal pediatric samples. A longer study period will partially compensate for these limitations.

Furthermore, important clinical data on the hypercholesterolemic patients were unavailable in many patients, such as complete lipid profiles, bilirubin, etc. A prospective, systemic cohort study with complete medical records will definitely be more informative from a research viewpoint.

Finally, the phytosterol levels of all measured patients were determined from a single sample obtained at unknown fasting conditions, and a nonuniform time of day, meaning that diurnal variation cannot be ruled out. However, intraindividual variation

of non-cholesterol sterols are reported to be insignificant[37]. A longitudinal study involving repeated phytosterol measurements of the same hypercholesterolemic patients will help exclude falsely-elevated phytosterol levels.

Although further studies are required to validate the efficacy, we believe that, based on current findings and considering the potential risks associated with increased phytosterols, specialized lipid profile testing at an earlier age, including phytosterol testing, may help diagnose patients whom would have previously gone undetected. Likewise, more clinical suspicion with regards to the hitherto neglected phytosterol levels seems to be required when managing hypercholesterolemic children.

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초록

기체 크로마토그래피 질량분석법을 이용한 피토스테롤 검사 및 소아에서 고콜레스테롤혈증과 고피토스테롤 농도의 관련성

배경: 피토스테롤은 뇌건성 황색종과 시토스테롤혈증과 같은 선천성 지질축적질환에서 증가된다. 조기에 발견을 하여 치료를 시작하면 예후가 매우 좋아질 수 있으나, 전통적인 화학법 혹은 효소법을 이용한 검사만으로는 피토스테롤을 제대로 측정할 수 없다는 문제가 있다. 본 연구에서는 기체 크로마토그래피 질량분석법을 이용하여 콜레스타놀, 시토스테롤, 그리고 캄페스테롤을 측정할 수 있는 다항목 피토스테롤 검사를 개발 및 평가하였다. 더불어, 소아에서 고콜레스테롤혈증과 피토스테롤 농도와의 관계에 대해서도 고찰하였다.

방법: 대상자의 혈청에 내부표준물질을 (에피코프로스타놀) 첨가 후 처리하여, HP 6890N 기체 크로마토그래피 및 HP 5975 질량분석기와 (Agilent Technologies, Santa Clara, CA, USA) 함께 HP-5MS 캐필러리형 컬럼을 이용하여 콜레스타놀, 시토스테롤, 그리고 캄페스테롤을 측정하였다. 정밀도, 선형성, 측정 및 정량한계, 이온 억제, 회수율, 및 이월효과 등의 항목을 평가하였다. 추가적으로 18세 미만의 정상콜레스테롤 (콜레스테롤 <200 mg/dL) 환아 및 고콜레스테롤 (콜레스테롤 >250 mg/dL) 환아들에 대해 피토스레롤 수치를 측정하고 그 결과를 비교하였다.

결과: 기체 크로마토그래피 질량분석을 시행하였을 때, 측정한 세가지

피토스테롤과 내부표준물질 모두 정확히 분리되는 것을 확인하였다. 개발한 검사에 대한 유효성 결과는 우수하였다: 5회의 반복측정에 의한 검사 중 정밀도 및 5일의 연속된 검사에 의한 검사 간 정밀도는 모든 항목에 대해 변동계수가 10.9% 미만이었다. 측정된 범위 (0−20.0 mg/dL) 내에서 세가지 피토스테롤 모두 결정계수(r^2)가 >0.999로 선형성을 보였다. 세가지 피토스테롤의 측정한계는 0.01 mg/dL였다. 시토스테롤의 정량한계는 0.5 mg/dL였고, 캄페스테롤 및 콜레스타놀의 정량한계는 0.3 mg/dL이었다. 이온 억제효과는 발견되지 않았으며, 검사 회수율 및 효율성 모두 우수하였다. 이월효과 또한 관찰되지 않았다. 총 220명의 고콜레스테롤 환아 및 109명의 정상 환아에 대해 피토스테롤을 추가적으로 측정하였을 때, 고콜레스테롤 환아들에서 유의하게 피토스테롤 농도가 높은 것으로 확인되었다.

결론: 기체 크로마토그래피 질량분석법을 이용한 다항목 피토스테롤 검사는 모든 평가항목에서 우수한 결과를 보였다. 이 검사를 이용하여 보다 정확하게 선천성 지질축적질환을 진단할 수 있을 것으로 기대하며, 이로 인해 궁극적으로 치료효과와 예후가 향상될 것으로 생각된다. 더불어, 고콜레스테롤 환아에서 높은 피토스테롤 농도가 측정될 경우, 추가적인 임상적인 평가가 필요하며 해당사항이 있을 경우 이에 대해 치료를 하는 것이 권장되는 바이다.

주요어: 피토스테롤; 뇌건성 황색종; 시토스테롤혈증; 고콜레스테롤혈증; 기체 크로마토그래피 질량분석법

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