Phytosterol Glycosides Reduce Cholesterol Absorption in Humans

Xiaobo Lin
Lina Ma
Susan B. Racette
Catherine L. Anderson Spearie
Richard E. Ostlund, Jr.

1Division of Endocrinology, Metabolism and Lipid Research, Department of Medicine, 2Program in Physical Therapy, and 3Center for Applied Research Sciences, Washington University School of Medicine

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Corresponding Author:
Richard E. Ostlund, Jr. M.D.
Division of Endocrinology, Metabolism and Lipid Research
Washington University School of Medicine
660 South Euclid Ave., Campus box 8127
St. Louis, MO 63110.
E-Mail: ROstlund@dom.wustl.edu
Abstract

Dietary phytosterols inhibit intestinal cholesterol absorption and regulate whole-body cholesterol excretion and balance. However, they are biochemically heterogeneous and a portion is glycosylated in some foods with unknown effects on biological activity. We tested the hypothesis that phytosterol glycosides reduce cholesterol absorption in humans. Phytosterol glycosides were extracted and purified from soy lecithin in a novel two-step process. Cholesterol absorption was measured in a series of three single-meal tests given at intervals of two weeks to each of 11 healthy subjects. In a randomized crossover design, participants received approximately 300 mg of added phytosterols in the form of phytosterol glycosides or phytosterol esters, or placebo in a test breakfast also containing 30 mg cholesterol-d7. Cholesterol absorption was estimated by mass spectrometry of plasma cholesterol-d7 enrichment 4-5 days after each test. Compared to the placebo test, phytosterol glycosides reduced cholesterol absorption by 37.6 ± 4.8% (P<0.0001) and phytosterol esters 30.6 ± 3.9% (P=0.0001). These results suggest that natural phytosterol glycosides purified from lecithin are bioactive in humans and should be included in methods of phytosterol analysis and tables of food phytosterol content.
**Introduction**

Phytosterols are cholesterol-like compounds that occur naturally in plant foods and reduce cholesterol absorption (11, 17-19) and plasma LDL-cholesterol (7, 12). Thus, the U.S. National Cholesterol Education Program Adult Treatment Panel III has recommended adding 2.0 g/day of phytosterols to the diet of adults to reduce LDL-cholesterol and coronary heart disease risk (1). Phytosterols at levels present in natural foods have also been shown to reduce cholesterol absorption (17, 19).

Phytosterols occur in diverse forms in food matrices and not all have been tested for bioactivity (10). The form of the phytosterols is critical for bioactivity because crystalline phytosterols are not readily soluble in bile and do not reduce cholesterol absorption (20). Properly formulated with lecithin or other emulsifiers, free phytosterols do reduce cholesterol absorption and LDL cholesterol (4). Phytosterol esters solubilized in the triglyceride phase of margarines are also bioactive (6, 22). However, the amphipathic structure of phytosterol glycosides (Fig. 1) raises questions about the degree of solubility in intestinal bile salt micelles and reactivity with pancreatic enzymes. Although literature on the physiology of phytosterol glycosides is sparse, previous workers have shown that fatty acids are cleaved from glycosylated phytosterols *in vitro* by pancreatin but the sugar moiety itself is not removed (9). This was confirmed by another study where $^{14}$C-sitostanol glucoside was not cleaved in the stomach or small bowel after intragastric administration (23). If cleavage of the sugar from glycosylated
phytosterols is needed for bioactivity, or if poor solubility limits physiological interactions, then phytosterol glycosides might not necessarily be biologically active.

Because phytosterol glycosides comprise a significant proportion of naturally-occurring phytosterols in some foods (2, 13, 14), we tested the effect of phytosterol glycosides in a clinical trial in which cholesterol absorption was measured in repeated single-meal tests. In a randomized crossover study design, we compared phytosterol glycosides to placebo using deuterated cholesterol as a tracer with detection by mass spectrometry. Phytosterol esters were included as an experimental positive control, based upon reduction in cholesterol absorption observed with phytosterol esters (15). Because large amounts of phytosterol glycosides are not commercially available, we first developed a method for the purification of gram quantities from soy lecithin.
Materials and Methods

Subjects

Twelve subjects were recruited by the Volunteer for Health Program at Washington University School of Medicine. Inclusion criteria were healthy individuals aged 18-80 years with plasma LDL cholesterol below 190 mg/dL, triglycerides lower than 250 mg/dL, and no active medical or surgical illnesses. Subjects also were required to have stable body weight within 5 pounds during the preceding two months and be willing to maintain their body weight throughout the study. Exclusion criteria included lipid abnormalities requiring treatment under current guidelines, history of clinical lactose intolerance, breastfeeding, and anticipated or current pregnancy. Four of the participants took birth control pills but no other medications potentially affecting lipid metabolism. Written informed consent was obtained on forms approved by the Washington University Human Research Protection Office. One subject dropped out before receiving any treatments and 11 completed the entire protocol.

Materials

[25,26,26,27,27-2H7]Cholesterol was purchased from CDN Isotopes (Quebec, Canada). Soybean oil (Bakers & Chefs™, Sam’s Club) was purified as described in order to reduce the content of phytosterols (19). Hunt’s Snack Pack® fat-free pudding was purchased from a local supermarket in vanilla or chocolate flavor. Lecithin was purchased from The Vitamin Shoppe (North Bergen, NJ). Magnesol® (a food-grade magnesium trisilicate) was purchased from Dallas Group of America, Inc. (Whitehouse, NJ). CoroWise™ Phytosterol
esters containing 60.6% phytosterol moiety in phytosterol esters were a gift from Cargill (Minneapolis, MN).

**Purification of phytosterol glycosides from lecithin**

Soybean-derived commercial lecithin containing 3% glycosylated phytosterols was used as a starting material. Glycosides are of intermediate polarity compared to high-polarity phospholipids and low-polarity neutral lipids (triglycerides, phytosterol esters, unesterified phytosterols). The unique polarity of phytosterol glycosides was used to isolate them using hexane, ethyl acetate, solid-phase magnesium trisilicate and acetone (Fig. 2).

Two steps were employed to purify glycosylated phytosterols from lecithin. Solvent-resistant plasticware was used and the procedure was performed in a clean room. The first step was removal of phospholipids. Fifty grams of lecithin (which is slow to dissolve in ethyl acetate but readily soluble in hexane) was dissolved in 313 mL hexane and diluted to 25% hexane with 938 mL ethyl acetate. Next, Magnesol® was added at 187.5 g/L and the suspension was mixed for 10 minutes and then filtered under vacuum. Under these conditions, phospholipids bound to Magnesol® whereas phytosterol glycosides and neutral lipids did not. Residual phospholipids were removed from the filtrate by repeating the treatment with 120 g/L Magnesol® (not shown in Fig. 2). The filtrate was then dried.

The second step was removal of neutral lipids. Since these contaminants are much more soluble than phytosterol glycosides in acetone, they were removed by extraction. Ice-cold acetone equal in weight to the dried filtrate was added with intermittent swirling on ice for 30
minutes, followed by decanting the acetone. This process was repeated 5 times. The solid glycosylated phytosterols were lyophilized and analyzed.

**Preparation of test meals**

Cholesterol-d$_7$ was first disinfected by dissolving in ethanol followed by filtration through a 0.22 micron solvent-resistant FG filter and evaporation of the solvent. Lecithin was dissolved in purified soybean oil by heating at 100°C for 1 hour which was then cooled to room temperature. The lecithin/oil mixture was centrifuged at 1,600 x g for 30 minutes at room temperature. The supernatant was added to the dried cholesterol-d$_7$. Phytosterol esters or glycosides were added to 15% lecithin in purified soybean oil containing cholesterol-d$_7$, warmed at 65°C until completely dissolved and then cooled to room temperature. Lecithin was added to the oil in order to increase the solubility of phytosterol glycosides. Each test meal consisted of 240 g of pudding and 12 g of 15% lecithin in purified soybean oil with or without phytosterol additions. The oil was heated again in a 65°C water bath for 10 minutes before weighing into a bowl and mixing with room temperature pudding just prior to serving the test meal. The meal was consumed within 10 minutes and the subjects had nothing else to eat for 4 hours. All test meals contained 303 kcal, 44 g carbohydrate, 2.4 g protein, 12.5 g fat, 82 mg of background phytosterols derived from pudding, oil and lecithin, and 30 mg cholesterol-d$_7$. To the meals were added one of the following: 1) 300 mg phytosterols from the phytosterol glycoside preparation (PG, experimental treatment), 2) 325 mg phytosterols added in the form of phytosterol esters (PSE, positive control treatment), or 3) no addition (placebo treatment). Since phytosterols given in single meal tests at a dose of 300 mg have a nearly maximum effect on
cholesterol absorption in humans (20), we chose an experimental dose of 300 mg provided either by phytosterol esters or phytosterol glycosides.

**Clinical protocol**

Eleven subjects received three cholesterol absorption tests each, which were performed 2 weeks apart in a randomized crossover design. For each test they reported to the Clinical Trials Unit after an overnight fast, and a baseline plasma sample was drawn. The test meal was consumed and fasting blood samples were drawn 4 and 5 days later for analysis.

**Analyses**

Plasma samples were saponified, extracted, converted to pentafluorobenzoyl esters and analyzed for natural cholesterol and cholesterol-d$_7$ using NCI GC/MS (negative ion methane chemical ionization gas chromatography/mass spectrometry) with an Agilent Technologies 5973 positive/negative ion mass spectrometer in the Washington University Mass Spectrometry Resource as described (19). The cholesterol area ratio of m/z 587 (M) for tracer to m/z 581 (M+1) for natural cholesterol was calculated and converted to mole ratio of cholesterol-d$_7$ to cholesterol by reference to a standard curve. The mole ratio in the plasma before each test meal was subtracted from that of day 4 and day 5. The net mole ratios of the two days were averaged and reported. Reduction in the mole ratio for a given treatment compared to the value during the placebo test was used as a measurement of reduction in percent cholesterol absorption as described previously (20). The sensitivity of this method was calculated from the m/z 587/581 area ratios of the standard curve point containing no added cholesterol-d$_7$ run on different days. The between-assay standard deviation of these measurements was converted to a mole ratio of
cholesterol-d7/natural cholesterol (0.0000055), multiplied by 2, and then divided by the mean change in mole ratio observed in plasma cholesterol during the placebo test (0.00038), yielding a value of 0.029. This represents an estimate of the fraction of average cholesterol absorption or change in cholesterol absorption that can be detected by the mass spectrometric method with approximately 95% confidence.

The phytosterol glycoside fraction was analyzed in two stages. First, total phytosterols were determined after acid hydrolysis followed by alkaline hydrolysis (double hydrolysis), a procedure that liberates free sterols from both phytosterol esters and phytosterol glycosides (2). Then, in a separate analysis, the sum of free and esterified phytosterols was determined after alkaline hydrolysis only (single hydrolysis). Phytosterols contained in phytosterol glycosides were calculated as the difference between total phytosterols obtained from double hydrolysis and free plus esterified phytosterols from single hydrolysis. The hydrolysates were derivatized to pentafluorobenzoyl esters and analyzed by NCI GC/MS using pentadeuterated soy sterols as an internal standard (16).

**Statistical Analyses**

Statistical analyses were conducted using SAS (version 9.2, SAS Institute Inc., Cary, NC). The independent effects of sequence of treatment, period, and treatment on the net enrichment of cholesterol-d7 tracer were analyzed using the Proc Mixed of SAS with patient within sequence as the random effect. The significance of multiple comparisons among the treatments was adjusted with the Tukey procedure. The Proc Mixed procedure also was used to analyze possible
carryover effects from one period to the next, with the previous treatment encoded as a separate class variable. Values represent mean ± SEM.
Results

Purification of glycosylated phytosterols from lecithin

We have developed a protocol to purify glycosylated phytosterols from lecithin in gram quantities. The final product contained principally acylated phytosterol glycoside as shown by thin layer chromatography (Fig. 3). Most neutral lipids and phospholipids had been removed. The total phytosterol content was determined by acid hydrolysis (to break glycosidic bonds) followed by alkaline hydrolysis and yielded a value of 41.6% phytosterols by weight. Residual free and esterified phytosterols determined after alkaline hydrolysis were only 1.1% by weight, demonstrating that phytosterol glycosides contributed 97.3% of the total phytosterols present. Assuming a molecular weight of 852 for sitosterol glucoside linolate as a principal form, the preparation had a phytosterol glycoside content of 73% by weight.

Effects of phytosterol esters and glycosides on cholesterol-d7 enrichment in the plasma

The baseline measurements for the eleven subjects are listed in Table 1. Phytosterol glycosides were solubilized in purified soybean oil containing 15% lecithin and cholesterol-d7 tracer and then mixed with low-phytosterol commercial pudding and consumed as a test breakfast. The resulting enrichment of plasma cholesterol-d7 was determined 4 and 5 days later during the time of peak plasma concentration by GC/MS. Each subject received three cholesterol absorption tests in random order that included phytosterol glycosides, phytosterol esters or no addition (placebo). Plasma cholesterol-d7 enrichment was highest after the placebo test and it was reduced by 37.6 ± 4.8 % by phytosterol glycosides \((P<0.0001)\) and 30.6 ± 3.9 % by phytosterol esters \((P=0.0001)\) (Fig. 4). There was no significant difference between
phytosterol glycosides and phytosterol esters ($P=0.39$). No carryover effects were found from the different supplementations on the net cholesterol-d7 tracer enrichment.
Discussion

Plants have a tendency to glycosylate many cell components and this modification needs to be taken into account in dietary studies for which bioavailability is a concern. In particular, phytosterols are glycosylated in many plants (10, 13, 14) and may even be the predominant form encountered in some foods such as potatoes where phytosterol glycosides have been reported to comprise 82% of phytosterols (2). However, only a few studies have addressed the potential biological activity of these compounds, due in part to the lack of commercial availability in the amounts needed.

Clinical studies usually require several grams of phytosterols. While it is possible to prepare analytically-pure material at a milligram scale using techniques such as high performance liquid chromatography, the low throughput and solvent consumption makes these methods impractical or expensive at gram scale. To obviate these difficulties, we used simple adsorptive methods beginning with soy lecithin where phytosterol glycosides constitute about 3% of the total mass (Fig. 2). Phospholipids, the principal contaminant, were removed by selective adsorption to magnesium trisilicate. Then neutral lipids, including most free and esterified phytosterols, were removed by extraction into cold acetone. Contaminating free phytosterols and phytosterol esters comprised only 2.7% of phytosterols in the final phytosterol glycoside fraction, which was suitable for use in human studies.

In order to approximate the natural delivery of phytosterol glycosides, we dissolved them in soybean oil containing 15% lecithin, a condition associated with improved solubility at room
temperature. Phytosterol glycosides were compared with placebo in a series of three single-meal cholesterol absorption tests performed in each of 11 normal subjects. The subjects consumed 30 mg cholesterol-d$_7$ tracer and plasma enrichment was measured several days later at the peak of appearance in the plasma by negative ion mass spectrometry. The results show that phytosterol glycosides reduced cholesterol absorption by 37.6%, a typical reduction observed during other studies of conventional forms of phytosterols (22), and comparable to the 30.6% reduction observed simultaneously with phytosterol esters. Thus, phytosterol glycosides are biologically active in humans.

We are not aware of previous studies of the effect of phytosterol glycosides on cholesterol absorption. However, glycosides of related non-phytosterol compounds have been shown to reduce cholesterol absorption and provide a framework in which to consider the present results. Synthetic glycosides of spirostane derivatives (saponins) reduced cholesterol absorption in monkeys (8). Similarly, spirostane di-glycosides reduced cholesterol absorption in hamsters (3) and humans (5). Thus, there is precedent suggesting that glycosylated steroids might also be expected to reduce cholesterol absorption. The mechanism by which such glycosides are active would need to take account of the reports that phytosterol glycosidic bonds are not cleaved by mammalian pancreatic enzymes in vitro (9). Furthermore, a study in rats showed that [4-$^{14}$C]sitosteryl β-D-glucoside remains unhydrolyzed and unabsorbed in rat stomach and small intestine (23). This suggests that phytosterol glycosides may be active with the glycosidic bond intact and not necessarily hydrolyzed to yield free phytosterols. If this is the case, then phytosterol glycosides would necessarily need to enter intestinal micelles and exert actions there. Further work is needed to test this hypothesis.
The biological activity of soy phytosterol glycosides has implications for both analytical methods and for nutrition databases. Traditional protocols for extracting and analyzing phytosterols from foods employ only alkaline hydrolysis and do not measure glycosylated forms (24). To include phytosterol glycosides in the results, an acidic hydrolytic step must be included in order to cleave the phytosterol-glycosidic bond so that the free phytosterol is liberated and measured (2, 10). Alternatively, phytosterol glycosides have been measured directly by gas chromatography of the intact material (21). Further work is needed to validate these methods to be sure that phytosterols are adequately extracted, not destroyed by the hydrolytic conditions, and are included in the final analytical method.

In the current work phytosterol glycosides were solubilized in lecithin and triglyceride before study. Since our material was derived from soy lecithin and ultimately from crude soybean oil this appears to be a reasonable formulation. However, phytosterol glycosides in other foods may not necessarily be associated with lecithin with unpredictable effects on cholesterol absorption. Bioavailability might also be adversely affected by different food matrices, and this needs further experimental work. While the measured reduction of cholesterol absorption by phytosterol glycosides and phytosterol esters was similar, only a single dose was analyzed and a much larger study would be needed to establish bioequivalence.

Currently our nutritional databases include relatively little information about phytosterols despite their demonstrated ability to lower cholesterol absorption and LDL cholesterol. Our
work suggests that food tables and databases that report phytosterol amounts may have underestimated the true values by failing to measure and report glycosylated phytosterols. Additional studies on bioactivity of glycosylated phytosterols are needed in order to understand the actions of this important class of nutrients.
Acknowledgments

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Figure legends

Figure 1. An example of glycosylated phytosterol structure. Sitosterol on the right is bonded to glucose which is then attached to a fatty acid, FA. Potential structural variability is present in the phytosterol moiety, the orientation of the bond from phytosterol to the sugar, the structure of the sugar, and the presence and type of fatty acid. When a fatty acid is present the material is referred to as acylated phytosterol glycoside.

Figure 2. Flow chart for purifying phytosterol glycosides from lecithin. Lecithin [containing phospholipids (PL), phytosterol glycosides (PG), phytosterol esters (PSE), unesterified phytosterols (PS), and triglycerides (TG)] was dissolved in 25% hexane in ethyl acetate followed by mixing with Magnesol® (187.5 g/L). PL bound to Magnesol® and were removed by vacuum filtration. The rest of the components remained in the solvents. After distillation to remove the solvents, the neutral lipids (NL: PSE, PS, and TG) were removed by cold acetone extraction. The precipitated PG was dried and analyzed.

Figure 3. Analysis of lecithin and the purified phytosterol glycosides (PG) by TLC. Lecithin or the purified PG [120 µg each (3 µL of 40 µg/µL in hexane)] was spotted on a TLC slide, run in pure ethyl acetate, and developed in iodine. Lecithin contained mostly phospholipids (PL) and neutral lipids (NL: PSE, PS, and TG); whereas purified PG was enriched with acylated sterol glycosides (ASG).
Figure 4. Effects of phytosterol glycosides (PG) and phytosterol esters (PSE) on cholesterol absorption. Eleven patients were randomly assigned in a crossover design to receive either placebo, PG, or PSE in three single-meal cholesterol absorption tests given 2 weeks between treatments. The net increase in plasma cholesterol tracer enrichment was determined 4 and 5 days after the test and is reported as a mole ratio of tracer to natural cholesterol.
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References


Step 1: Removing PL

Lecithin (PL, PG, PSE, PS, & TG) + 25% hexane in ethyl acetate & Magnesol®

Filtration → PL in Magnesol®

Filtration

PG, PSE, PS, & TG in solvents

Distillation

PG, PSE, PS, & TG + Cold acetone

Extraction → PSE, PS, & TG in acetone

Residue

Precipitated PG

Step 2: Removing NL by acetone extraction