Title: PHYTOSTEROL FORMULATIONS TO LOWER CHOLESTEROL ABSORPTION

Abstract

A composition for reducing cholesterol absorption from the intestine comprised of sitostanol (or other phytosterols) and lecithin (or other phospholipids) in proportions of 1:1 to 1:50 of sitostanol (or other phytosterols and lecithin or other phospholipids).
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Phytosterol Formulations to Lower Cholesterol Absorption

Field of Invention

This invention relates to a composition and method for reducing cholesterol absorption and serum cholesterol in humans.

Background of the Invention

Phytosterols are plant sterols structurally similar to cholesterol that have been known for many years to reduce cholesterol absorption and serum cholesterol levels while not being absorbed themselves (1,2,3). Lowering of circulating cholesterol and low density lipoprotein cholesterol is an important part of a strategy to prevent and treat cardiovascular disease and especially coronary heart disease (4). Cholesterol absorption is a critical component of whole body cholesterol metabolism as shown in Fig. 1. Cholesterol derived from the diet and also from endogenous biliary secretion enters the intestine and approximately 50% of the mixed intestinal load is absorbed (5). The failure to absorb cholesterol quantitatively is therefore a key mechanism for the elimination of cholesterol from the body.

Drugs commonly used to treat high cholesterol levels have little or no effect on Cholesterol absorption. The potent new hydroxymethylglutaryl coenzyme A reductase inhibitors have a primary action to reduce cholesterol synthesis rather than increase cholesterol elimination. Bile acid sequestrants such as the ion-exchange resin cholestyramine act within the intestine but do not bind cholesterol and may actually increase cholesterol absorption when given chronically (6). Although orally administered neomycin reduces cholesterol absorption effectively, it is toxic and has the disadvantage of requiring chronic administration of a
potent antibiotic (7). The drug Cytellin, an aqueous suspension of mixed phytosterols, was produced by Eli Lilly Co. for treatment of elevated cholesterol, but its use (if any) apparently is not widespread. New inhibitors of cholesterol absorption would complement currently available treatments for high serum cholesterol.

Since phytosterols are natural products which are non-toxic and inexpensive byproducts of food processing, they may be important in the treatment of individuals with mildly increased serum cholesterol or for the general population in food products or dietary supplements. The use of phytosterols could reduce the need for systemically absorbed drugs.

Despite their potential attractiveness, the usefulness of phytosterols has been limited by small and erratic effectiveness and a large dosage requirement. Doses of 5-18 g sitosterol/day reduced serum cholesterol by 16-20% (8,9). A dose-response study showed that 3-9 g of powdered sitosterol was needed to decrease serum cholesterol levels by 12% (10). To reduce the amount needed recent experiments have used sitostanol instead of sitosterol because it appears to be more potent than other phytosterols and is non-absorbable (11). In subjects with severe hypercholesterolemia sitostanol at 1.5g/day reduced serum cholesterol by 15% (12). However, sitostanol. At 3 g/day had no effect in subjects with moderate hypercholesterolemia (13).

Several investigators have proposed ways to increase the solubility of bioavailability of phytosterols in order to make them more useful. Based on studies in rats and the finding that phytosterol esters are much more soluble in oil than the free sterols, it was proposed to use phytosterol esters in oil to lower
cholesterol absorption (14). U.S. Patent 5,502,045 describes the use of sitostanol ester in oil for the treatment of hypercholesterolemia in humans (15). It was found that 2.8 g sitostanol/day given as sitostanol ester in margarine reduced LDL cholesterol by 16% (16). However, the use of sitostanol ester dissolved in dietary fat has the disadvantage of requiring the administration of 23-50 g/day of dietary fat and of being 21% less effective at reducing cholesterol absorption in humans compared to the unesterified sterol (17).

Additional workers have investigated ways to improve the usefulness of unesterified phytosterols. In International Patent WO 95/00158 a complex of sitosterol and the unabsorbable dietary fiber pectin reduced serum cholesterol by 16.4% when given to hypercholesterolemic humans in a dose of 2.1 g/day (18). However, no measurements of an effect on cholesterol absorption were made and the complex was only about 50% soluble even at strongly alkaline pH suggesting that the bioavailability of the sitosterol component was limited. U.S. Patent 5,244,887 describes the use of stanols including sitostanol in food additives to reduce cholesterol absorption (19). For preparation of the additives, sitostanol is dissolved with an edible solubilizing agent such as triglyceride, an antioxidant such as tocopherol, and a dispersant such as lecithin, polysorbate 80, or sodium lauryl sulfate. However, no experimental data were given to guide one skilled in the art in the selection of the most effective components and their amounts of specific methods of preparation. Effectiveness in reducing cholesterol absorption was also not determined. The preferred embodiment consisted of 25% by weight stanols in vegetable oil, but the solubility of sterols in oil is only 2% (20,14) and that
of sitostanol only 1% (21). Thus, it is not clear whether these food additives can be made.

As shown in Fig. 2 cholesterol is absorbed from an intestinal micellar phase containing bile salts and phospholipids, which is in equilibrium with an oil phase inside the intestine. Delivery of phytosterol as a solid powder or aqueous suspension is not preferred because of the limited rate and extent of solubility in intestinal liquid phases. Esterification of the phytosterol with delivery through the oil phase of foods is an alternative route but has the disadvantage of requiring the administration of 23-50 g/day with the sitostanol ester.

Summary of the Invention

In this application we describe the direct delivery of phytosterol into the micellar phase through combination with phospholipid. Thus, the invention generally features compositions for inhibiting cholesterol absorption from the intestine comprising phytosterols dispersed in phospholipid or other agents.

The phytosterol-phospholipid complex is prepared by vortexing, sonicating or passing through a small orifice a phytosterol: phospholipid mixture in water. The dispersed material is then either used as is or dried by lyophilization or spray-drying. Without wishing to limit myself to a specific molecular mechanism, I conclude that it is useful to avoid self-association of phytosterols into structures that do not readily dissolve in bile.

The composition is useful for reducing cholesterol absorption in humans at doses between 10 and 1000 mg and a preferred dose is 25-300 mg. The dose is less than required by current art. The composition may be used in capsule or tablet form as a drug or dietary supplement. Alternatively it may be used in foods as a food additive.
or substance generally recognized as safe for human consumption.

The current invention has one or more of the following advantages: (1) The dose needed to reduce cholesterol absorption is low, namely 25-300 mg sitostanol. (2) The preferred formulation does not contain triglyceride or oils. The phytosterol is not dissolved in fat but rather is combined with phospholipid to form an aqueous vesicular complex which can enter directly into the intestinal micellar phase (Fig. 2). (3) The agent can be prepared in solid form by drying an aqueous sitostanol/lecithin vesicular formulation with retention of solubility in artificial bile. (4) The agent is effective when consumed separately from cholesterol-containing foods. The principal target of the agent is biliary cholesterol (Fig. 1). (5) The agent can be added to non-cholesterol-containing and fat-free foods and beverages. (6) The agent is prepared in a manner to prevent self-association of sitostanol as it occurs when it is dried from organic solvents containing sitostanol and solubilizing agents. (7) The agent may contain lysolecithin. (8) The agent is bioavailable as assayed with artificial bile in vitro.

Brief Description of the Drawings

Fig. 1 is a schematic depiction of cholesterol transport and storage.

Fig. 2 depicts equilibrium of different forms of cholesterol.

Description of the Preferred Embodiments

In these examples sitostanol is used as an example of a phytosterol and lecithin of a phospholipid. However, other phytosterols and phospholipids might be used provided that the phospholipids increase the
solubility of the phytosterols in bile salt micelles and the phytosterols reduce cholesterol absorption. Phytosterols comprise sterols such as sitostanol, sitosterol, campesterol, stigmasterol, saponins, lignans, aromatic and isoprenoid natural products, and their derivatives and their reduced form with hydrogen. Phospholipids comprise glycerophospholipids and sphingolipids as well as their derivatives such as lysosphospholipids.

Example 1

Sitostanol, tracer amount of $[^3]H$sitostanol, and other compounds that are found in the gut or that are commonly used as food additives were mixed together in chloroform solution at a fixed mole ratio. An aliquot, containing 1.2 μMol of sitostanol, was transferred to an evacuation tube and the solvent was removed under reduced pressure (<50 mtorr). The experiment was initiated by adding 0.5 mL of artificial bile (8mM sodium taurocholate containing 5 mM soy lecithin and 0.15 mM NaCl, pH 7.4) followed by rotation at 8 rev./min for 30 min at 37°C. The tube was then centrifuged for 1 minute at 17,000xg to precipitate any solid material, the supernatant was removed and added to scintillation fluid for measurement of radioactivity, and the percent of radioactivity in the artificial bile supernatant was calculated. The table below summarizes the solubility of sitostanol mixtures in the presence of artificial bile salt.
As shown in line 1, sitostanol alone is poorly soluble in artificial bile salt (2.3%) and the addition of Tween-20, a polysorbate emulsifier used in foods, increases the solubility slightly to 7.8% (line 2.). Sitostanol solubility can be enhanced 25-fold, from 2.3% to 57.7%, if it is dried in the presence of an ionic detergent, such as the bile salt sodium taurocholate (line 3). Since bile salt is a component of the digestive process, other compounds that are found in the gastrointestinal system were also tested. Monoolein and diolein are the products of dietary fat digestion, but as shown in line 4, they only produced a modest enhancement of solubility,
2.3% to 14.5%. Bile contains lecithin and this phospholipid increased sitostanol solubility form 2.3% to 38.2% (line 5). However the reaction product of phospholipase hydrolysis of lecithin, lysolecithin, produced a slight increase in sitostanol solubility, 2.3% to 8.0% (line 6). Surprisingly, when lecithin and lysolecithin were mixed together with sitostanol, the resulting solid mixture produced almost complete solubility of the sterol, 97.9% (line 7). Taken together, these data indicate that solid sitostanol does not readily dissolve in artificial bile, but that it can be made soluble to a varying degree by including other compounds in solid mixture. Moreover, a compound (lysolecithin) that by itself has little effect on sitostanol solubility can have a marked outcome when it is used in combination with other agents (lecithin).

Example 2

Sitostanol, tracer amount of [\(^{3}H\)]sitostanol and lecithin were mixed together in chloroform. Two aliquots containing 1.2\(\mu\)Mol of sitostanol were removed and the chloroform solvent was removed under vacuum as described in Example 1. One aliquot was used without further preparation and to the other 500 \(\mu\)l water was added and the sample mixture was sonicated for 5 minutes on 40% power with a Fisher Sonic Dismembrator Model 300 equipped with a microtip. The sample was then frozen with dry ice acetone and lyophilized to remove water. It is essential to maintain the temperature of the sample below freezing in order to prevent precipitation of sitostanol from the mixture. The solubility of each of these samples in artificial bile was then determined as described in Example 1, and the results are shown in the Table below.
Table 2

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<tr>
<th>Sample Drying Method</th>
<th>Solubility of sterol (weight %)</th>
</tr>
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<tbody>
<tr>
<td>Sitostanol dried from chloroform</td>
<td>2.3</td>
</tr>
<tr>
<td>Sitostanol/Lecithin (1:1 mole ratio) dried from chloroform</td>
<td>38.2</td>
</tr>
<tr>
<td>Sitostanol/Lecithin (1:1 mole ratio) sonicated in water, lyophilized</td>
<td>89.7</td>
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</table>

The data shows the importance of lecithin in solubilizing sitostanol (lines 1 and 2). However, the method of drying the sitostanol/lecithin mixture also affects the subsequent dissolution of the sterol. When the mixture is dried from chloroform 38.2% of the sterol is solubilized by artificial bile. In contrast, when the mixture is sonicated and then lyophilized, solubilization increases to 89.7%. This shows that dispersing sitostanol/lecithin in aqueous medium followed by removal of water is a preferred method for preparing sitostanol/lecithin mixtures.

Example 3

The effectiveness of variable amounts of lecithin to solubilize sitostanol was studied as in example #1 except that after rotation at 37°C for 30 min residual sedimenting sitostanol was re-extracted twice by vortexing with 0.5 ml additional artificial bile and recentrifuging. The following results were obtained:
Table 3

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<th>Mole Ratio</th>
<th>Solubility of sterol (weight %)</th>
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<td>(Sitostanol:Lecithin)</td>
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<tr>
<td>1:1</td>
<td>53.1</td>
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<tr>
<td>1:2</td>
<td>67.9</td>
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<td>1:3</td>
<td>67.6</td>
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These data show that even with repeated extraction and addition of a tenfold excess of lecithin, a significant amount of sitostanol (32%) remained insoluble. When \(^{3}H\)phosphatidylcholine was added as a tracer instead of \(^{3}H\)sitostanol the amount of lecithin solubilized was 93.3%. This indicates that lecithin was nearly quantitatively extracted from the dried sitostanol/lecithin complex whereas a limiting amount of sitostanol remained. Thus, methods to solubilize sitostanol in artificial bile must take into consideration the existence of residual insoluble sitostanol. Drying sitostanol/lecithin mixtures from a more polar solvent such as ethanol or a less polar solvent such as hexane gave similar results.

Example 4
The effect of sonicated sitostanol/lecithin vesicles on human cholesterol absorption was compared to that of solid sitostanol dosed in the presence of sonicated lecithin. Sitostanol was dehydrated by twice dissolving in chloroform and evaporating and was then ground to a powder in a mortar and pestle. To prepare the sitostanol/lecithin vesicles in a 1:3 mole ratio, 2.00 gm of sterol was added to 11.3 gm of purified soy lecithin in a 150 mL glass beaker. Chloroform was added with stirring to solubilize both components and the solvent
was then removed by incubating in a sand bath at 65°C. Soy lecithin (11.3 g) without sitostanol was prepared in the same manner. When all the solvent was removed, the beakers were placed in a lyophilization jar, and the residual chloroform was removed under vacuum for at least 24 hr. The solid in each beaker was then broken up with a spatula, 120mL of deionized water was added, and the suspension was stirred vigorously for one hour. Vesicles were prepared by sonicating the contents of each beaker with a Branson Sonifier (setting 7) equipped with a small tip. During sonication, the beaker was immersed in a room temperature water bath. Vesicles containing lecithin alone were formed in 15-30 min, but those containing both sterol and lecithin required 30-45 min. The samples were then centrifuged at 10,000xg for 10 min and passed through a 5μ filter. The mean diameter of the vesicles determined on a Zetasizer that had been calibrated with a 250 nm standard was 204.7 nm for lecithin vesicles and 247.2nm for the sitostanol/lecithin vesicles. The concentration of sitostanol was measured enzymatically. After preparation and characterization the vesicles were stored overnight in a refrigerator at 4°C. The next day samples were diluted to 60mL with water and 500 mg lemon flavored Crystal Light (Kraft Foods, Inc.) was added. Three U.S.P. stomach capsules were filled with a total of 1 g sitostanol powder or 1 g glucose placebo for each subject. Six normal subjects underwent three cholesterol absorption tests in random order separated by 2 weeks. For each test a National Cholesterol Education Program Step I diet was consumed for 8 days beginning on day 1 of the study. On day 4, a standardized test breakfast was consumed consisting of 240 mL orange juice, 240 mL whole milk, 21 gm corn flakes and a 60 gm bagel saturated with 40 mg [26,26,26,27,27,27-2H₆] cholesterol tracer dissolved
in 2.5 mL corn oil. Each subject also consumed a drink containing either sitostanol/lecithin vesicles or lecithin vesicles and three capsules containing either sitostanol powder or glucose placebo. The concentration of deuterated cholesterol tracer in plasma cholesterol on days 7 and 8 was measured by negative ion methane chemical ionization gas chromatography/mass spectrometry (22). Reduction in cholesterol absorption was determined by dividing the mean deuterated cholesterol concentration on days 7 and 8 by that observed during the test that contained only lecithin vesicles and glucose capsules and expressing it as a percent. The following results were obtained:

<table>
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<th>Treatment Given</th>
<th>% Reduction in cholesterol absorption</th>
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<tr>
<td>1000 mg sitostanol powder</td>
<td>11.3 ± 7.4% (p=0.2)</td>
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<tr>
<td>700 mg sitostanol/lecithin vesicles</td>
<td>36.7 ± 4.2% (p=0.003)</td>
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These results show that, compared to placebo, 1000 mg sitostanol powder did not reduce cholesterol absorption significantly. This is consistent with previous reports showing that only multi-gram sitostanol doses reduce cholesterol absorption. However, 700 mg sitostanol/lecithin vesicles reduced cholesterol absorption by 37% showing that properly formulated sitostanol is active and bioavailable.

**Example 5**

To demonstrate that sitostanol/lecithin reduces cholesterol absorption in a pharmacological dose-response fashion it was given in reduced amount to 5 of the 6 subjects of example 4 during four additional cholesterol absorption tests. A dose of 300 mg sitostanol in
sitostanol/lecithin vesicles was compared to lecithin placebo and a dose of 150 mg sitostanol in sitostanol/lecithin vesicles was compared to another lecithin placebo. No capsules of solid sitostanol or placebo were given. The following results were obtained:

Table 5

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<th>Treatment Given</th>
<th>Reduction in Cholesterol Absorption</th>
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<tr>
<td>300mg sitostanol/lecithin vesicles</td>
<td>34.4</td>
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<tr>
<td>150mg sitostanol/lecithin vesicles</td>
<td>5.8</td>
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Treatment Given Reduction in Cholesterol Absorption
300 mg sitostanol/lecithin vesicles
150 mg sitostanol/lecithin vesicles
15 34.4 (5.8% (p=0.01)
Cholesterol absorption was reduced nearly as much by the 300 mg dose as the 700 mg dose indicating that this dose is saturating. This is consistent with previous work showing that phytosterols do not completely block cholesterol absorption (14).

References


What is claimed is:

1. A composition for reducing cholesterol absorption through the intestine comprising a complex of sitostanol (or other phytosterols) and lecithin (or other phospholipids) in proportions of 1:1 to 1:50 of sitostanol and lecithin (or other phytosterols and other phospholipids).

2. A composition which will reduce cholesterol absorption by 37% at a dose of 700 mg sitostanol and by 34% at a dose of 300 mg sitostanol.

3. A composition as in Claim 1 prepared by dispersing the complex in water by shaking, vortexing, sonication or passage through a small orifice and which is used in liquid form.

4. A composition as in Claim 3 in which the complex is substantially water-free.

5. A composition as in Claim 1 which is added to foods.

6. A composition as in Claim 1 which is used as a dietary supplement.

7. A composition as in Claim 1 which is used as a drug.

8. A composition as in Claim 1 which lysolecithin is incorporated.

9. A composition as in Claim 1 which the complex structure is soluble in artificial bile.
10. A method of treating a patient who is characterized by hypercholesterolemia, comprising administering to the patient a comprising sitostanol (or other phytosterols) and lecithin (or other phospholipids) in proportions of 1:1 to 1:50.

11. The method of claim 10 in which the composition is administered to the patient's intestine.

12. The method of claim 10 in which the composition is administered orally.

13. A method of making a phytosterol/phospholipid complex by dispersing phytosterol and phospholipid in water by shaking, vortexing, sonication or passage through a small orifice.

Figure 1.

Rapid Pool

Slow Pool

Bile 1000mg

Absorption 700 mg

Diet 400mg

Gut

Stool 700mg
Figure 2.

Enterocyte

Micellar Phase

Oil Phase

Sitostanol

Sitostanol Ester
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 31/56, 31/685
US CL. : 514/78, 171, 182
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/78, 171, 182

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>US 5,310,556 A (ZIEGLER) 10 May 1994, see entire document.</td>
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☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

Date of the actual completion of the international search

05 JUNE 1999

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