Oxidized plant sterols in human serum and lipid infusions as measured by combined gas-liquid chromatography-mass spectrometry

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Abstract Some oxidized forms of cholesterol (oxysterols) are thought to be atherogenic and cytotoxic. Because plant sterols are structurally related to cholesterol, we examined whether oxidized plant sterols (oxyphytosterols) could be identified in human serum and soy-based lipid emulsions. We first prepared both deuterated and nondeuterated reference compounds. We then analyzed by gas-liquid chromatography-mass spectrometry the oxyphytosterol concentrations in serum from patients with phytosterolemia or cerebrotendinous xanthomatosis, in a pool serum and in two lipid emulsions. 7-Ketositosterol, 7β-hydroxyisostigmasterol, 5α, 6α-epoxysitosterol, 3β,5α,6β-sitostanetriol, and probably also 7α-hydroxysitosterol were present in markedly elevated concentrations in serum from phytosterolemia patients only. Also, campesterol oxidation products such as 7α-hydroxycampesterol and 7β-hydroxyisocampesterol were found. Interestingly, sitosterol was oxidized for approximately 1.4% in phytosterolemia serum, which is rather high compared with the approximate 0.01% oxidatively modified cholesterol normally seen in human serum. The same oxyphytosterols were also found in two lipid emulsions in which the ratio of oxidized sitosterol to sitosterol varied between 0.038 and 0.041. In conclusion, we have shown that oxidized forms of plant sterols are present in serum from phytosterolemia patients and two frequently used soy-based lipid emulsions. Currently, it is unknown whether oxyphytosterols affect health, as has been suggested for oxysterols. However, 7β-hydroxycholesterol may be one of the more harmful oxysterols, and both sitosterol and campesterol were oxidized into 7β-hydroxyisostigmasterol and 7β-hydroxyisocampesterol. The relevance of these findings therefore deserves further exploration.—Plat, J., H. Brzezinka, D. Lütjohann, R. P. Mensink, and K. von Bergmann. Oxidized plant sterols in human serum and lipid infusions as measured by combined gas-liquid chromatography-mass spectrometry. J. Lipid Res. 2001. 42: 2030–2038.

Supplementary key words atherosclerosis • phytosterols • oxysterols • phytosterolemia • cerebrotendinous xanthomatosis • soy-based lipid emulsions

Plant sterols are nonnutritive compounds that differ from cholesterol only by an additional ethyl (β-sitosterol) or methyl (campesterol) group at the 24-carbon atom of the sterol side chain. Western diets provide about 160–360 mg/day of plant sterols, which consist of approximately 80% β-sitosterol, some campesterol and stigmasterol, minor amounts of brassicasterol, and only traces of delta-5 saturated plant stanols (1).

Cholesterol is susceptible to oxidation, and cholesterol oxidation products, called oxysterols, can be formed by physical processes such as heating and radiation, by nonenzymatic processes involving reactive oxygen and free radical species (2), or enzymatically, by specific cytochrome P450 (CYP450) monoxygenases (3). Consequently, cholesterol oxidation products, as present in the human body, may be derived from absorption of oxidized sterols present in the food, as well as from endogenous origin. The nuclear B-ring of the cholesterol molecule is mainly oxidized by nonenzymatic processes. In this way, 7α-hydroxycholesterol (7α-OH-Chol), 7β-hydroxycholesterol (7β-OH-Chol), 7-ketocholesterol (7=O-Chol), 5α,6β-epoxycholesterol (5α,6β-

Abbreviations: BHT, butylated hydroxy toluene; CTX, cerebrotendinous xanthomatosis; CYP450, cytochrome P450; d-tri-hydroxy-Camp, deuterated tri-hydroxy-Campesterol; d-tri-hydroxy-Sit, deuterated tri-hydroxy-Sitosterol; d-7=O-Sit, deuterated 7-ketocholesterol; 5α,6α-epoxy-Camp, 5α,6α-epoxy-Campesterol; 5β,6β-epoxycholesterol, 5β,6β-epoxycholesterol; 5α,6α-epoxy-Chol, 5α,6α-epoxycholesterol; 5β,6β-epoxy-Chol, 5β,6β-epoxycholesterol; O-Sit, 7-ketositosterol; oxyphytosterols, oxidized plant sterols; oxysterols, oxidized forms of cholesterol; SM, single ion monitoring; TMS, trimethylchlorosilane; 3β,5α,6β-tri-hydroxy-Camp, 3β,5α,6β-tri-hydroxy-Campesterol; 3β,5α,6β-tri-hydroxy-Chol, 3β,5α,6β-tri-hydroxycholesterol; 3β,5α,6β-sitostanetriol.

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been shown in rats that oxyphytosterols [7, 8, 9] are present in wheat flour, and vegetable oils (9, 10). Recently, it has been found in coffee, fried potatoes, and other plant products. Sufficient information is available on the presence of oxyphytosterols in food, but there is a negative feedback from bile acids on cholesterol 7α-hydroxylase due to an increase in bile acid concentrations for health. Further, it was recently found that oxysterols and oxyphytosterols showed similar cytotoxic effects in cultured macrophages (13). In this way, oxidation of plant sterols and the presence of these oxyphytosterols in the circulation might have implications for health.

Phytosterolemia is a rare inherited sterol storage disease characterized by highly elevated serum plant sterol concentrations of up to 65 mg/dl (1.57 mM) (14), tendon and tuberous xanthomas, and by a strong predisposition to premature coronary atherosclerosis (15). Therefore, the primary aim of this study was to examine whether and what kinds of oxyphytosterols are present at all in serum of phytosterolemic patients. In addition, we analyzed serum from patients suffering from cerebrotendinous xanthomatosis (CTX). Patients suffering from CTX, a rare autosomal recessive inherited disease, have decreased concentrations of 27-OH-cholesterol caused by a genetic absence or restriction of the CYP450-dependent 27-hydroxylase, thus increased concentrations of oxysterols and oxysterol-like compounds in serum have been described for these patients. Hence, the primary aim of this study was to examine whether oxysterols and oxyphytosterols showed similar cytotoxic effects in cultured macrophages. In addition, two different soybean oil-based lipid emulsions known to be rich in plant sterols and frequently used as parenteral infusion for short- and long-term nutritive therapy were analyzed for the presence of oxyphytosterols. To address these issues, we first developed a sensitive gas-liquid chromatography-mass spectrometry (LC-MS) method and synthesized all necessary reference compounds and deuterated internal standards.

**METHODS**

All reagents and solvents used were of analytical or HPLC grade. We analyzed by GC-MS a plant sterol mixture purchased from Sigma-Aldrich (Steinheim, Germany) that consisted of 60.19% sitosterol, 36.09% campesterol, and traces (2.72%) of stigmasterol.

Fig. 1. Possible in vivo oxidation of plant sterols, either by enzymatic or nonenzymatic mechanisms. In addition to the structures depicted for oxidation of sitosterol, similar products can be formed from campesterol and other plant sterols.
Chemical synthesis of oxyphytosterols

Chemical synthesis of oxyphytosterols was based on the methods as described for oxygenation of cholesterol by Li et al. (16), with some minor modifications. Several oxygenated plant sterols were synthesized from the plant sterol mixture. In this way, a mixture of 7α- and 7β-OH-Sit, 7α- and 7β-hydroxy-3α(5β,6β)-campesterol (7α- and 7β-OH-Camp, respectively), and 7α- and 7β-hydroxy-stigmasterol (7α- and 7β-OH-stigmasterol, respectively), a mixture of 7α-O-Sit/Camp/stigmasterol, a mixture of 5α,6β-epoxy and 5β,6β-epoxy/Sit/Camp/stigmasterol, and a mixture of 3β,5α,6β-tri-hydroxy-Sit, 3β,5α,6β-tri-hydroxy-campesterol (3β, 5α,6β-tri-hydroxy-Camp), and 3β,5α,6β-tri-hydroxy-stigmasterol was prepared (Fig. 2). We did not separate sitosterol oxidation products from campesterol oxidation products, as they are always present as a mixture in the circulation and can be sufficiently separated by GC-MS. Tentative identification of the synthesized oxyphytosterols and interpretation of the mass spectra was performed by comparison of the spectra with a standard oxyphytosterol mixture, which was kindly provided by Dr. André Grandgirard (INRA, Unité de Nutrition Lipidique, Dijon, France).

Acetylation of plant sterols. All plant sterols were acetylated prior to further use for synthesis of all oxyphytosterols, except for the triols. To this end, 2 g of the plant sterol mixture (Fig. 2, Ia-Ic) was acetylated in toluene after addition of 20 ml acetic anhydride (Fluka Chemika, Buchs, Switzerland), which finally resulted in the acetylated products (Fig. 2, IIa-IIc) as white crystals.

7-Hydroxyphytosterols. Two hundred milligram molecular sieve 0.4 nm (Merck, Darmstadt, Germany) and 3.2 g pyridinium hydride (Fluka Chemika, Buchs, Switzerland), which finally resulted in the acetylated products (Fig. 2, IIa-IIc) as white crystals.

7-Keto plant sterols. 7-Keto plant sterols were formed from 3α,5α,6β-tri-hydroxy-Sit, 3α,5α,6β-tri-hydroxy-Camp, and 3α,5α,6β-tri-hydroxy-stigmasterol prepared (Fig. 2) was did not separate sitosterol oxidation products from campesterol oxidation products, as they are always present as a mixture in the circulation and can be sufficiently separated by GC-MS. Tentative identification of the synthesized oxyphytosterols and interpretation of the mass spectra was performed by comparison of the spectra with a standard oxyphytosterol mixture, which was kindly provided by Dr. André Grandgirard (INRA, Unité de Nutrition Lipidique, Dijon, France).

Fig. 2. In vitro synthesis of oxyphytosterols. Oxyphytosterols were synthesized from a mixture consisting of sitosterol, campesterol, and stigmasterol. All plant sterol standards contained isoforms from sitosterol, campesterol, and stigmasterol, all approximately in the same percentages as the plant sterol distribution of the original plant sterol mixture. Campesterol (a): R = -CH2; sitosterol (b): R = -C2H5; stigmasterol (c): R = -C3H7 (plus a double bond between C22 and C23).
5,6-Epoxy plant sterols. Acetylated phytosterols (Fig. 2, Ia-Ic) were dissolved in chloroform, and by adding m-chloroperoxybenzoic acid (Sigma-Aldrich) and potassium bicarbonate, white crystals (Fig. 2, VIIa-VIIc) were formed, which were saponified as described for the hydroxy plant sterols (Fig. 2, VIIa-VIIc). GC-MS spectra showed a pure mixture of 5α,6α-epoxy-Sit, 5α,6α-epoxy-Camp, 5β,6β-epoxy-Sit, and 5β,6β-epoxy-Camp, and traces of 5β,6β-epoxy-stigmasterol. The ratio of 5α,6α-epoxy-Sit to 5α,6α-epoxy-Camp was 63:37, whereas the ratio of 5β,6β-epoxy-Sit to 5β,6β-epoxy-Camp was 59:41. Mainly the 5α,6α-epoxy forms were formed during synthesis, as the ratio of 5α,6α-epoxy-Sit to 5β,6β-epoxy-Sit was 79:21 and the ratio of 5α,6α-epoxy-Camp to 5β,6β-epoxy-Camp was 76:24. Retention times and m/z used for analysis of epoxy sterols are shown in Table 1.

3α,5β,6α-tri-ols. For plant sterols (Fig. 2, Ia-Ic) dissolved in formic acid (95%) and worked up to the triols (Fig. 2, IXa-IXc), GC-MS spectra showed a pure mixture of 3β,5α,6β-tri-hydroxy-Sit and 3β,5α,6β-tri-hydroxy-Camp. The ratio of 3β,5α,6β-tri-hydroxy-Sit to 3β,5α,6β-tri-hydroxy-Camp was 67:33. Retention times and m/z used for analysis of tri-hydroxy sterols are shown in Table 1.

Internal standard preparation [2,2,4,4,6-2H5]-3β,5α,6β-tri-hydroxy-phytosterols and [2,2,4,4,6-2H5]-7-keto-phytosterols. As internal standards, we synthesized deuterated trihydroxy-phytosterols and deuterated 7-keto-phytosterols from of mixture of d2-2,2,4,4,6-sitosterol/-campesterol/-stigmasterol (Medical Isotopes Inc., Pelham, USA) by similar procedures as described for the unlabeled plant sterols. Identification of the synthesized triols revealed that both deuterated tri-hydroxy-Sit (dTR-hydroxy-Sit) and deuterated tri-hydroxy-Camp (dTR-hydroxy-Camp) were formed in a ratio 66:34, as well as were traces of stigmasterol. Moreover, analyzing the isotope differentiation (corrected for the natural background of 13C) showed the deuterium distribution as d0-tri-hydroxy-Sit, 8.9%; d1-tri-hydroxy-Sit, 3.9%; d2-tri-hydroxy-Sit, 9.2%; d3-tri-hydroxy-Sit, 21.2%; d4-tri-hydroxy-Sit, 29.8%; d5-tri-hydroxy-Sit, 22.4%; d6-tri-hydroxy-Sit, 2.7%; and d7-tri-hydroxy-Sit, 1.9%. Because d4-tri-hydroxy-Sit was the most abundant isotope, we measured on m/z 488 for d4-tri-hydroxy-Sit as internal standard.

The deuterated 7-keto standards were formed in a 60:40 distribution, with some minor traces of stigmasterol as well. Analyzing the isotope differentiation (corrected for the natural background of 13C) showed the deuterium distribution as d0-7-keto-Sit, 6.5%; d1-7-keto-Sit, 4.2%; d2-7-keto-Sit, 7.3%; d3-7-keto-Sit, 20.3%; d4-7-keto-Sit, 31.9%; d5-7-keto-Sit, 22.4%; d6-7-keto-Sit, 2.5%; and d7-7-keto-Sit, 1.8%. Because d4-7-keto-Sit was the most abundant isotope, we measured on m/z 504 for d4-7-keto-Sit as internal standard. The deuterated 7-keto standards were used as internal standard for the calculation of cholesterol oxide products (and also of d2, d6, d26, d26, d27, d27 cholesterol oxide products), which illustrates that interference of oxysterols and oxysteroids in GC-MS identification is not a problem.

Oxysterol analysis in serum

Butylated hydroxy toluene (BHT) and EDTA were added to the blood collection tube prior to sampling and to the serum just before analysis. Final amounts added corresponded with 10 µl BHT (25 mg/ml in methanol) and 20 µl EDTA (10 mg/ml in methanol) for 1 ml serum. Before saponification and extraction, 6 µg each of each internal standard, [2,2,4,4,6-2H5]-3β,5α,6β-tri-hydroxy-phytosterol and [2,2,4,4,6-2H5]-7-keto-phytosterol, were added to the serum sample. Next, the sample was saponified in a closed tube under nitrogen for 2 h at room temperature by adding 10 ml of 0.35 M ethanolic KOH solution. In addition, prior to saponification, the samples were extensively saturated with nitrogen for removal of oxygen to minimize autoxidation. After saponification, 130 µl phosphoric acid 50% in water (v/v) was added to neutralize the solution, followed by addition of 6 ml NaCl solution in water (9 mg/ml), according procedures for oxysterol analysis as described previously (17). After extraction with 2 ml di-chloro-methylene, the bottom layer was transferred into a round bottom flask and evaporated to dryness under vacuum at 50°C. The residue was dissolved in 5 ml ethanol and again evaporated under vacuum to remove all traces of water. The nonsaponifiable lipid samples were dissolved in 1 ml toluene. Silica cartridges (Bond Elut, bonded phase SI, 100 mg, 1 ml; Varian, Harbor City, CA) were equilibrated with cyclohexane before the column fractions were loaded. Neutral sterols (including cholesterol) were eluted from the column with 4 ml 0.5% ethyl acetate-cyclohexane (v/v), whereas the absorbed oxysterols were eluted with 9 ml ethyl-acetate. The oxysterol fraction was dried under vacuum. Finally, the oxidized sterols were dissolved in 100 µl cyclohexane and transferred to an injection vial. Sterols were silylated by addition of 100 µl silylation reagent (dry pyridine–hexamethyl-disilazane–trimethylchlorosilane (TMS) 3:2:1 (v/v/v) and incubation for 1 h at 90°C for GC-MS analysis.

GC-MS analysis

TMS derivates of the oxysterols were analyzed by GC-MS. For this, 2 µl of the TMS derivatives in cyclohexane were injected via an AS2000 autosampler (Thermoquest CE Instruments, Egselsbach, Germany) on a Trace GC2000 (Thermoquest CE Instruments) gas chromatograph equipped with a RTX5MS column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness) coupled to a GCQ-plus ion trap (Thermoquest CE Instruments). Analysis was carried out in single ion monitoring (SIM) mode, making m/z the primary resolving parameter other than retention time. The injector temperature was set at 270°C. Helium was used as carrier gas (constant flow 1 ml/min). The oven temperature gradient was programmed for 150 s at 150°C, then increased by 10°C/min toward 290°C, and then increased by 7°C/min toward 320°C and kept there for 20 min. Thus, one analytical run lasted approximately 42 min. The ions and retention times of all individual compounds are given in Table 1. In addition to plant sterols and oxysterolsterols, we also indicated retention times and m/z of cholesterol oxidation products (and also of d2, d6, d26, d26, d27, d27 cholesterol oxidation products), which illustrates that interference of oxysterols and oxysteroids in GC-MS identification is not a problem.

Patients and lipid emulsions

Sample serum sampled at three different time points from a phytosterolemia patient was used for analysis of oxysterosterols. The patient in 1998 was a 12-year-old girl being treated for her illness with cholestyramine (4 g/day). Two serum samples that were obtained at different time points in 1998 and stored at −20°C with BHT added as well as a recent fresh blood sample from the year 2000 obtained on the day of analysis were used. Furthermore, serum from two CTX patients not treated at the moment of blood sampling was used: The blood samples were taken in 1998 and were stored at −20°C with BHT since that time. In addition, serum from 15 patients not suffering from any sterol metabolism-related disease was pooled and analyzed.

Two frequently used soybean oil-based lipid emulsions in total parenteral nutrition protocols were analyzed for oxysterosterols by the same procedures as described for serum samples.

7α-OH-Chol and plant sterol analysis

7α-OH-Chol (18) and plant sterol concentrations (19) were analyzed as described before.

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RESULTS

Plant sterols and 7α-OH-Chol

Serum plant sterol concentrations from the phytosterolemia patient contained large amounts of plant sterols at all time points (Table 2). Serum plant sterol concentrations (sitosterol plus campesterol) from the phytosterolemia patient were 47.3, 44.5, and 40.3 mg/dl compared with 1.3 mg/dl in the pool serum of nonphytosterolemic patients (Table 3). Because esterified plant sterols are transported in the circulation in lipoproteins, concentrations are usually expressed relative to those of cholesterol. However, because serum cholesterol concentrations were not increased to the same extent as plant sterol concentrations, cholesterol-standardized sitosterol and campesterol concentrations were also still extremely high. Sitosterol concentrations were 19,714 × 10², 18,007 × 10², and 16,714 × 10² μmol/mmol cholesterol, whereas campesterol concentrations were 9,200 × 10², 8,289 × 10², and 8,909 × 10² μmol/mmol cholesterol. Compared with concentrations from the pool serum of nonphytosterolemic patients, cholesterol-standardized plant sterol concentrations were approximately 60 to 70 times increased.

Serum concentrations of 7α-OH-Chol were approxi-
mately 83.3 ng/ml in the phytosterolemia patient receiving 4 g/day of cholestyramine. This concentration was comparable to the value of 76.3 ng/ml, as observed in pool serum (Table 3). As expected, CTX patients had very high concentrations of 7α-OH-Chol, about 10 to 15 times higher than pool- and phytosterolemia serum.

GC-MS assay for oxyphytosterols

For the identification and quantification of oxyphytosterols in serum, we first developed a GC-MS assay, as described in the Methods section. First, standard curves of all individual compounds were made after preparing a standard mixture with the same procedures as described for the serum samples. For this, crystals of all synthesized standards were dissolved in ethanol in a final concentration of 10 mg/100 ml. Correlation coefficients for the different standards, as measured in a range of 0–4 μg/ml, were at least 0.96. The detection limits, as calculated according DIN32645 procedures, were 0.41 μg/ml for 7α-OH-Chol, 0.48 μg/ml for 7α-OH-Sit, 0.67 μg/ml for 7β-OH-Sit, 0.54 μg/ml for 5α,6α-epoxy-Sit, 0.83 μg/ml for 5β,6β-epoxy, and 0.65 μg/ml for 3β,5α,6β-tri-hydroxy-Sit.

Artificial ex vivo oxidation

As indicated above, we were extremely careful to inhibit artificial oxidation of plant sterols during work-up. For example, BHT was added in excess both during serum sampling and again before work-up procedures. Further, EDTA was also added before work-up, and fluids were saturated with nitrogen to remove oxygen before being used. Finally, oxysterols were separated from nonoxidized sterols as quickly as possible. To examine the possibility of ex vivo oxidation during sample preparation, we worked up four different amounts of the plant sterol mixture corresponding to the range of plant sterol concentrations as can be present in phytosterolemia serum (10–40 mg/dl), according the same procedures as described for the serum samples. As shown in Fig. 3, a small amount of 7α-OH-Chol, 7α-OH-Sit, 5α,6α-epoxy-Sit, and 3β,5α,6β-tri-hydroxy-Sit was present in all samples. These amounts, however, were independent of the initial sterol concentration. For 7α-OH-Sit and 3β,5α,6β-tri-hydroxy-Sit, these amounts were derived from d₄-7α-OH-Sit and d₄-3β,5α,6β-tri-hydroxy-Sit as present in the deuterated internal standards. This finding means that the formation of these specific oxysterols in our assay is independent of the sitosterol concentration of the sample. We therefore corrected the serum samples of the patients and the lipid emulsions by subtracting the base amounts of these oxysterols. As shown in Fig. 3, the amount of 7β-OH-Sit increased linearly in relation to the sitosterol concentration of the sample. The correction for ex vivo oxidation for 7β-OH-Sit was therefore based on the sitosterol concentration present in the serum samples of the patients. For campesterol, 7α-OH-Camp and 7β-OH-Camp formation were also related to the campesterol concentration in the sample.

Oxysterols

Serum from the phytosterolemia patient contained significant amounts of oxysterols at all time points (Table

Table 2. Serum plant sterol and oxidized sitosterol concentrations in a phytosterolemia patient treated with cholestyramine (4 g/day)

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Plant sterols (mg/dl)*</td>
<td>47.3</td>
<td>44.5</td>
<td>40.3</td>
</tr>
<tr>
<td>Oxidized sitosterol forms (μg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7α-OH-Chol</td>
<td>0.82</td>
<td>0.99</td>
<td>0.94</td>
</tr>
<tr>
<td>7β-OH-Sit</td>
<td>0.82</td>
<td>0.93</td>
<td>ND</td>
</tr>
<tr>
<td>5α,6α-epoxy-Sit</td>
<td>2.77</td>
<td>2.23</td>
<td>1.90</td>
</tr>
<tr>
<td>3β,5α,6β-tri-hydroxy-Sit</td>
<td>0.08</td>
<td>0.15</td>
<td>1.38</td>
</tr>
<tr>
<td>Total oxidized sitosterol</td>
<td>4.49</td>
<td>4.30</td>
<td>4.21</td>
</tr>
</tbody>
</table>

* Plant sterols are calculated as the sum of sitosterol and campesterol.

Table 3. Serum plant sterol and oxidized sitosterol concentrations in three samples of a phytosterolemia patient versus two CTX patients and pool serum

<table>
<thead>
<tr>
<th></th>
<th>Control Serum</th>
<th>Phytosterolemia</th>
<th>CTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant sterols (mg/dl)*</td>
<td>1.3</td>
<td>44.0 ± 3.5</td>
<td>1.2 and 1.4</td>
</tr>
<tr>
<td>Oxidized sitosterol forms (μg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7α-OH-Chol</td>
<td>ND</td>
<td>0.92 ± 0.09</td>
<td>0.04 and ND</td>
</tr>
<tr>
<td>7β-OH-Sit</td>
<td>ND</td>
<td>0.88 ± 0.08</td>
<td>0.01 and ND</td>
</tr>
<tr>
<td>5α,6α-epoxy-Sit</td>
<td>ND</td>
<td>2.33 ± 0.44</td>
<td>ND and ND</td>
</tr>
<tr>
<td>3β,5α,6β-tri-hydroxy-Sit</td>
<td>ND</td>
<td>0.54 ± 0.73</td>
<td>ND and ND</td>
</tr>
<tr>
<td>7α-OH-Chol (mg/ml)</td>
<td>76.3</td>
<td>83.3 ± 36.9</td>
<td>1,221 and 671</td>
</tr>
</tbody>
</table>

* Values are mean ± SD. ND = not detectable.
CTX patients are characterized by a very high oxidized plant sterol concentration. We analyzed serum from CTX patients. These patients was similar to sitosterol.

For these calculations, it appeared that the percentage of oxidized campesterol in serum of phytosterolemic patients was around 1.4%. If at least a part of the 7α-OH-Sit was comparable to concentrations in pool serum (Table 3).

As expected, 7α-OH-Chol concentrations were increased. We could not, however, identify 7α-OH-Sit in serum from CTX patients. In fact, none of the oxyphytosterols was present in CTX serum in amounts above our detection limits. This might indicate that no enzymatic formation of 7α-OH-Sit occurs in vivo in humans, even in a situation of high 7α-hydroxylase activity. However, it is also possible that serum-oxidized plant sterol concentrations in CTX patients are below our detection limits.

**Lipid emulsions**

Analysis of two different frequently used soybean oil-based lipid emulsions showed that emulsion A contained less plant sterols compared with emulsion B and also contained more cholesterol than emulsion B (Table 4). Oxidized plant sterol concentrations were somewhat higher in emulsion B. The ratios of oxidized sitosterol to sitosterol, however, were comparable: 0.038 in emulsion A; 0.041 in emulsion B. This suggests that the higher oxyphytosterol concentrations in emulsion B were simply due to autoxidation as described above, there was no indication of artificial oxidation of sitosterol during work-up of our samples. Further, most of the nonoxyphytosterols were separated from oxidized sterols during elution on the Bond Elut silica cartridges, making oxidation of plant sterols after this separation impossible. However, because we are not entirely sure that the analyzed 7α-OH-Sit concentration is a real reflection of the in vivo situation, we decided not to use 7α-OH-Sit in the calculations.

The percentage of oxidized sitosterol in serum of phytosterolemic patients was around 1.4%. If at least a part of the 7α-OH-Sit signal is from nonartificial 7α-OH-Sit formation, then this figure is even an underestimation. Also, 7α-OH-Camp and 7β-OH-Camp could be identified in serum, whereas no 7α-Camp, epoxy-Camp, or tri-hydroxy-Camp could be detected. Although a positive identification of oxidized campesterol molecules (7α-OH-Camp and 7β-OH-Camp) was possible, exact concentrations could not be calculated because of difficulties in analyzing the campesterol internal standards. If we used the sitosterol internal standards for these calculations, it appeared that the percentage of oxidized campesterol in serum of phytosterolemic patients was similar to sitosterol.

To examine whether 7α-OH-Sit could be formed endogenously, we analyzed serum from CTX patients. These CTX patients are characterized by a very high 7α-hydroxylase activity, which may also lead to the endogenous formation of 7α-OH-Sit. In these two CTX patients, serum plant sterol concentrations were 1.2 and 1.4 mg/dl, which was comparable to concentrations in pool serum (Table 3).

![Figure 3](https://example.com/fig3.png)

**Figure 3.** To determine ex vivo oxidation and to create correction factors for our further calculations, we worked up four different amounts of a plant sterol mixture. A constant amount of 7α-OH-Sit, 7α-OH-Chol, 5α,6α-epoxy-Sit, and 3β,5α,6β-tri-hydroxy-Sit was present in all samples, which was independent of the sitosterol concentration. This amount was subtracted from the values found in all samples. The 7β-OH-Sit concentration, however, increased in relation to the sitosterol concentration. Therefore, the correction for ex vivo oxidation for 7β-OH-Sit was based on the individual sitosterol concentration present in the serum samples of the patients. The concentration of sitosterol in the sample (mg/dl) on the x-axis is plotted versus the ratio of the peak area oxyphytosterol/peak area internal standard (y-axis).

| TABLE 4. Serum plant sterol and oxidized sitosterol concentrations in lipid emulsions |
|-----------------------------------------------|----------------|----------------|
| Emulsion A | Emulsion B |
| Cholesterol (mg/dl) | 29.8 | 17.8 |
| Plant sterols (mg/dl)* | 11.3 | 27.7 |
| Sitosterol (mg/dl) | 8.6 | 20.1 |
| Campesterol (mg/dl) | 2.7 | 7.6 |
| Oxidized sitosterol forms (μg/ml) | | |
| 7α-OH-Sit | 0.70 | 1.26 |
| 7β-OH-Sit | 0.65 | 0.37 |
| 5α,6α-epoxy-Sit | 2.73 | 9.24 |
| 3β,5α,6β-tri-hydroxy-Sit | 0.16 | 0.49 |
| 7α-OH-Chol (mg/ml) | 19.5 | 9.7 |

* Plant sterols are calculated as the sum of sitosterol and campesterol.
higher plant sterol concentrations. 7α-OH-Chol concentrations in emulsion A were twice as high as those in emulsion B.

DISCUSSION

Feeding LDL receptor (−/−) and apolipoprotein E (−/−) mice oxysterols (a mixture of 7–10% 7α-OH-Chol, 15–20% 7β-OH-Chol, 15–20% 5β,6β-epoxy-Chol, 10–15% 5α,6α-epoxy-Chol, 40–45% 7=O-Chol, and traces of 25-OH-Chol) accelerated fatty streak lesion formation when compared with cholesterol feeding (20). This confirms earlier ideas that oxysterols are atherogenic and may play a role in plaque development in humans (4). Most information about their possible atherogenicity, however, is derived from in vitro studies showing that oxysterols are cytotoxic to several cell types (21) including endothelial cells (22, 23), U937 monocytes (24), and HepG2 liver cells (24), and may induce apoptosis (24, 25, 26). For humans, however, no causal relation so far has been established between oxysterols and lesion formation, but several findings are suggestive. For example, the highest oxysterol/cholesterol ratio was found in the most atherogenic LDL subfractions (LDL−) (27). Furthermore, the ratio of oxysterol to cholesterol is higher in foam cells isolated from atherosclerotic plaques than in plasma (28). Foam cells play a crucial role in early fatty streak formation. Therefore, expanding our knowledge about the presence of oxysterols in the circulation is of great importance not only because of their potential atherogenicity, but also because oxysterols have several other biological effects such as the regulation of gene expression (28). Whether oxysterols also possess atherogenic and/or gene regulatory characteristics needs further study.

It is very difficult to distinguish between oxysterols derived from the diet and those endogenously formed (29). Breuer and Björkhem (30) showed that 7=O-Chol, 7β-OH-Chol, 24-OH-Chol, and 25-OH-Chol are formed endogenously, in contrast to epoxides. In our study, we have not tried to trace the origin of the oxysterols. From the literature, there was only limited evidence on the presence of oxysterols in the circulation (12). We therefore decided first to set up a sensitive GC-MS assay, which enabled us to accurately analyze many different oxysterols in human serum. We showed that similar oxidation products to those formed from cholesterol were present in serum from phytosterolemic patients. We were able to identify 7=O-Sit, 7β-OH-Sit, 5α,6α-epoxy-Sit, 3β,5α,6β-tri-hydroxy-Sit, and most probably 7α-OH-Sit as well. 7α-OH-Camp and 7β-OH-Camp, two campesterol oxidation products, were also found. A recent study found that plant sterol oxides and cholesterol oxides showed the same cytotoxic effects in a cultured macrophage-derived cell line (13).

It is generally accepted that 7α-OH-Chol is derived from both enzymatic and nonenzymatic oxidation, whereas 7β-OH-Chol is entirely derived from nonenzymatic origin (4). Therefore, 7β-OH-Chol may be a better marker for free radical-related lipid peroxidation than 7α-OH-Chol. Two separate studies have now shown that in humans, especially 7β-OH-Chol is associated with the risk for atherosclerosis (31, 32). Interestingly, the in vitro toxicity of 7β-OH-Chol could not be counteracted by antioxidants (33). Rabbit studies also showed no reduction in serum 7β-OH-Chol concentrations after probucol treatment (34), vitamin E supplementation (35), or vitamin E plus vitamin C supplementation (36). In human diabetic patients, however, vitamin E supplementation lowered both 7β-OH-Chol, 7=O-Chol, and triols (37). However, because antioxidant supplementation may lower the risk for atherosclerosis, these findings indicate that a causal relation between 7β-OH-Chol with coronary heart disease is still controversial. Because 7β-OH-Sit and 7β-OH-Camp are also present in serum from phytosterolemic patients, it seems relevant to determine whether these oxysterols are also atherogenic in vivo, and whether antioxidants affect oxysterol concentrations and toxicity.

It has been suggested that sitosterol inhibits cholesterol 7α-hydroxylase activity (38, 39), possibly by stereochemical hindrance of the ethyl group at C24 in sitosterol. Indeed, patients with phytosterolemia have a reduced cholesterol 7α-hydroxylase activity (40) and, consequently, a lower bile acid synthesis (19). However, in cholesterol-free microsomes, 7α-OH-Sit was formed, suggesting that at a relatively high ratio of sitosterol to cholesterol, some conversion of sitosterol may occur (41). Because this ratio is increased in serum from phytosterolemic patients, 7α-OH-Sit might be formed in phytosterolemic patients. This might also explain why Boberg, Einarsson, and Björkhem (38) could not show any conversion of sitosterol into 7α-OH-Sit in human liver microsomes isolated from nonphytosterolemic patients. The fact that we were able to identify 7α-OH-Camp strengthens our assumption that 7α-OH-Sit may be formed to some extent in phytosterolemic subjects.

Interestingly, approximately 1.4% of the sitosterol molecules in phytosterolemic serum was oxidized, which is rather high compared with the approximate 0.01% oxidatively modified cholesterol (37, 42) normally present in human serum. This could indicate that plant sterols are more susceptible to oxidation, as suggested before (8). Another explanation might be that in contrast to cholesterol, plant sterols cannot be converted into bile acids. In pool serum from nonphytosterolemic patients and in CTX patients, no oxysterols could be detected. This suggests that with our assay, rather high serum concentrations are needed to find oxidized forms of these plant sterols. It should be mentioned that others recently reported oxysterol concentrations of 0.3 μg/ml, but no details on the type of analysis and the origin of the serum were provided (12). It therefore seems worthwhile to lower the detection limits of our assay, for example, by using Cl-negative-ion-GC-MS.

Soy-based lipid emulsion used in parenteral nutrition protocols may be associated with a higher prevalence of cholestasis, especially in children (43, 44). Indeed, a 3-year-old patient with total parenteral nutrition-associated cholestasis showed a high serum plant sterol concentra-
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REFERENCES

34. Stalenhoef, A. F. H., H. A. Kleinveld, T. G. Kosmeijer-Schul, P. N. M. Plat et al. Oxidized plant sterols in human serum 2037


