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ORIGINAL CONTRIBUTION

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Phytosterol and phytostanol esters are effectively hydrolysed in the gut and do not affect fat digestion in ileostomy subjects

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Summary *Background* Hydrolysis of phytosterol ester (PSTE) and phytostanol ester (PSTA) during fat digestion is not well characterised under controlled dietary conditions. *Aims* The main aims of this study were therefore to quantify the PSTE and PSTA hydrolysis after gut passage and to assess whether or not PSTE and PSTA induce fat malabsorption by measuring the fatty acid excretion following PSTE/PSTA consumption. *Methods* Ileostomy subjects (n = 7) were investigated in a randomised crossover study with one control and two intervention periods, when either 2.5 g of PSTE or PSTA corresponding to 1.5 g free sterol or stanol equivalents were added to a controlled diet. Ileostomy bags were collected and immediately frozen for analysis of nutrients, fatty acids and sterols. *Results* The study showed that 88.4 ± 5.9 % PSTE and 85.7 ± 6.5 % PSTA were hydrolysed following small bowel passage in the ileostomy subjects. The total excretion of fatty acids was similar in all three periods. *Conclusions* A majority of the 2.5 g PSTE and PSTA was hydrolysed during small bowel passage, which did not affect fat absorption as indicated by similar excretions of fatty acids in all periods. Consumption of increasing amounts of esterified phytosterols and phytostanols from enriched food formats should thereby have no adverse effects in this regard.

Key words phytosterols – fatty acids – excretion – ileostomy

Introduction

Phytosterol ester (PSTE) and phytostanol ester (PSTA) are commonly used as ingredients in functional foods to decrease plasma concentrations of low density lipoprotein-cholesterol (LDL-C) [1, 2]. It is well characterised that the blood cholesterol-lowering effect of PSTE and PSTA depends on a relative reduction of cholesterol absorption in the small bowel [3, 4], but the exact mechanisms and effects of PSTE and PSTA during fat digestion are not yet entirely understood.

Esterification of phytosterols increases their lipophilicity and oil solubility, and has commercially been used to incorporate them into various food products [1, 2]. Food or drug components with high lipophilicity may compete with other compounds of a similar lipophilicity for absorption [5, 6]. Several different mechanisms contribute to the LDL-C lowering effect [7]. Co-crystallisation with cholesterol leading to formation of insoluble mixed crystals [8–10], competition with cholesterol for solubilisation in mixed micelles [11, 12], and increased expression of transport proteins for sterol efflux in the gut [13] are the most important steps in the mechanistic pathway. For crystallisation to occur, PSTE and PSTA need however to be hydrolysed [9]. PSTA is known to be hydrolysed following small bowel passage [14], but there are limited data on the split of PSTE. If PSTE is not hydrolysed at a sufficient rate, this highly lipophilic compound could theoretically remain

in the lumen and serve as a lipid pool, in which other lipid components would distribute and thus escape absorption. The degrees of hydrolysis for both PSTE and PSTA are therefore important to be determined during controlled conditions to further elucidate the mechanism of action behind cholesterol-lowering and to determine the potential interaction with specific fatty acids. As the mechanism appears to be exerted by the free counterpart of phytosterols/stanols, and as both PSTE and PSTA reduce cholesterol absorption within the same range [4], it is hypothesised that both esters also are hydrolysed to a similar extent. Interactions between PSTE or PSTA and triacylglycerols (TAG) or free fatty acids have not been described in detail despite the almost 60 clinical studies using varying doses of PSTE or PSTA that have been performed to date [15]. In order to demonstrate the appropriate use of phytosterols/stanols as food ingredients, information about their mechanism of action as well as potential interactions with other lipophilic compounds such as fatty acids are required.

The aims of this study were therefore i) to quantify the hydrolysis of 2.5 g of PSTE and PSTA in the small bowel in human ileostomy subjects and ii) to evaluate their effects on the excretion of individual fatty acids in a well controlled free living situation. Earlier studies include only PSTA [14, 16], and give only partial information about effects on fatty acid excretion, as diets have not been controlled.

Subjects and methods

Subjects

The study design and details about subjects have been described in detail previously [4], but will here be explained in short. In total, 7 proctecolectomized subjects (5 men and 2 women) were studied. The subjects had a mean age of 54 ± 17 y (range: 29–73 y), a mean body mass index (BMI: in kg/m^2) of 25.1 ± 4.4 (range: 18–30), and median time since operation of 14 ± 9 y (2–26 y). Their excretion of bile acids was within the range of 122–956 mg/day, indicating that this was similar to subjects with an intact colon. A higher excretion indicates an impaired bile acid absorption, which ultimately could lead to an impaired fat absorption [17]. Informed consent was sought before the participants agreed to participate in the study and screening took place.

Study design

The study design and consent form were approved by the ethical committee of the Sahlgrenska Academy, Göteborg University according to guidelines of the Declaration of Helsinki. The study had a cross-over design with one control and two intervention periods of 3 days each, which took place during three consecutive weeks. The order of periods was randomised, and there was a minimum washout period of 4 days between study periods. Subjects consumed the same controlled basal diet during all periods, which had an average energy content of 10 MJ per day, of which 37 % came from fat, 17 % from protein and 45 % from carbohydrates. Dietary fibre content was 14 g per day. Individual fatty acids of the diet were not determined.

During the intervention periods, PSTE or PSTA was added to the controlled diet. The daily dose of both esters was 2.5 g, which corresponds to ca 1.5 g of free phytosterol equivalents. In every study period, the first day was an adaptation day, during which subjects only consumed the diet but did not collect any bags. Subjects collected their own ileostomy excreta and froze the bags every 2 hours in portable containers on dry ice during the day. During the night one night bag was used. Contents were freeze-dried and pooled separately in 24-h portions for days 2 and 3.

Test material

The PSTE and PSTA were a gift from the Raisio Group (Raisio, Finland), and had a purity of > 99 % and 98 %, respectively. The sources were mainly soy oil and pine oil, respectively, and the pine oil stanols were obtained by hydrogenation. The esterification degree of the phytosterols and phytostanols was 94 % and 98 %, respectively.

Quantification of sterols and fatty acids in ileostomy excreta

Samples from day 3 of each period were pooled before analysis. The analytical method followed a two-step approach. In the first step the intact sterol esters, free sterols and part of the fatty acids were quantified. Approximately 150 mg of freeze-dried sample was extracted overnight at ambient temperature using 5 mL of hexane containing the internal standards 5α-cholestane, cholesteryl-laurate and tridecanoic acid (C13:0, Sigma, St Louis, Missouri, USA). The hexane extract was isolated after centrifugation and evaporated to dryness under a stream of nitrogen. Prior to gas-liquid chromatographic (GLC, see below) analysis the sample was derivatised using bis(trimethylsilyl)trifluoroacetamide (BSTFA, Pierce, Rockford, IL, USA). Silylation was performed at room temperature for 30 minutes. Quantification of free sterols, sterol esters and fatty acids was performed against their respective internal standards. For the fatty acids, however, a correction was necessary (vide infra) to adjust for partial conversion of

the fatty acids and the fatty-acid internal standard into non-extractable calcium salts. In the second step the residual fatty acids from the first extraction were determined. The sample was saponified by boiling it for 30 minutes with 8 mL of 0.5 M sodium hydroxide. Then, 10 mL boron trifluoride reagent was added (20 % solution in methanol) and the sample was boiled for 4 minutes to yield the fatty acid methyl esters. Finally, the sample was extracted using hexane. The hexane layer was isolated and dried with 1 g of anhydrous sodium sulphate. The dried sample was finally analysed using GLC. The fatty acids were quantified using the internal standard. To correct for the fraction of the internal standard from step 1 that ended up in the extract of step 2, the ratio of the peak areas for the C13:0 internal standard in step 1 and 2 was used to calculate the amount of internal standard from step 1 being transferred into step 2. In this way an accurate concentration for the fatty acid internal standard in step 1 could be established and the amount of fatty acids extracted in step 1 could be calculated accurately.

GLC analysis. All GLC analyses were performed on a Carlo-Erba GLC 5300 (Fisons, Milan, Italy) equipped with an AS-800 auto-sampler (Fisons), an on-column injector with HOT (high oven temperature) option and flame ionisation detection. The column used was a CP-Sil 5 CB column (Varian, Middelburg, the Netherlands) with a length of 10 meters, an inner diameter of 0.32 mm and a film thickness of 0.12 µm. The pre-column was one meter, non-polar deactivated retention gap with an inner diameter of 0.55 mm. Hydrogen at 30 kPa was used as the carrier gas. The injection volume was 0.5 µl. The FID pressures were 50 and 100 kPa for hydrogen and air, respectively. The temperature program started at 80 °C. After an initial hold of 2 minutes, the oven was programmed to 360 °C (15 min) at a rate of 10 °C/min. Data processing was done using Turbochrom (Client-server version 6.1.2, Perkin Elmer, Norwalk, CT, USA).

The GLC method employed could separate all relevant peaks, with the exception of the peaks of cholesterol-C18 esters and sitosterol-C16 esters, which overlapped. Since the sterol composition was known from the analysis of the free sterols, the individual concentrations of cholesterol-C18 ester and sitosterol-C16 ester could be estimated using the ratio of free sitosterol to free cholesterol together with the level of cholesterol-C16.

Method variability. The method variability was assessed by repeated analysis of one of the samples. This sample was analysed 8 times over a 6 week period. The coefficient of variation (CV) obtained for free sterols and fatty acids were 3.4 and 11 %, respectively. The corresponding values for the sterol esters and the total sterol content were 6.9 and 3.8 %, respectively.

Calculations and statistics

The total excretion of cholesterol and phytosterols was calculated as the sum of the analysed free sterols and the analysed sterol esters (esters expressed as free sterol equivalents). The esterified fraction of phytosterols and phytostanols after gut passage was first calculated relative to their consumption, i. e. the fraction of phytosterols and phytostanols present in the esterified form in the excreta was compared with the consumed amounts of PSTE and PSTA. Corrections were made for the purity and esterification degree. The content of PSTE/PSTA in the basal diet was assumed to be zero. Secondly, the esterified fraction of both cholesterol and phytosterols and phytostanols in the excretion was compared to the total amount of cholesterol or phytosterols in the excreta, i. e. the fractions of free phytosterols/stanols expressed as sterol/stanol equivalents were divided by the total amount of phytosterol/stanol excretion.

The excretions of total dry matter, total wet matter, total fat, and total fatty acids were tested for normality by the Shapiro Wilk's test. The data were analysed by ANOVA with subjects as block, treatment as factor, and Tukey's test for posthoc analysis. Results were normally distributed and are presented as means and standard deviations.

Results

PSTE and PSTA hydrolysis during small bowel passage

The excreted amounts of both free and esterified sterols are presented in Table 1. During both periods with PSTE and PSTA, the excretion of free cholesterol and free and esterified phytosterols or stanols was higher compared

Table 1 Excretion of sterols during three periods: Control, plant sterol ester and plant stanol ester periods (means ± SD, n = 7)

	Control	Plant sterol ester	Plant stanol ester
Excretion (mg/d)			
Cholesterol			
Free	654±167[a, b]	978±123[a]	969±70[b]
Esterified[1]	62±40	61±36	66±52
Total	716±143	1039±113	1003±97
Plant sterols/stanols			
Free	96±18[a, b]	1082±154[a]	1112±119[b]
Esterified[1]	14±10[a, b]	152±77a	202±92[b]
Total[2]	110±14	1234±105	1314±57
Ester hydrolysis (%)	–	88.4±5.9	85.7±6.5

Results are significantly different when marked with the same superscript
[1] Expressed as sterol equivalents
[2] Not tested statistically

to the control ($P < 0.001$), but not different from each other. The amount of excreted cholesterol ester remained in the same range and did not change significantly during any of the three periods.

PSTE and PSTA increased the total amount of cholesterol excreted with 48.5 % ± 23.1 % and 42.9 ± 17.5 %, respectively. The proportion of cholesterol that was esterified in the excretion decreased from 11.2 ± 12 % during the control, to 6.5 ± 4 % during PSTE consumption and 7.6 ± 7.4 % during PSTA consumption. The fractional decrease reflects the increased excretion of free cholesterol, since the excreted amount of cholesterol esters did not change.

The portion of phytosterols and phytostanols in the esterified form in the excreta corresponded to 11.6 % ± 5.9 %, and 14.3 % ± 6.5 %, respectively, compared to the total amount consumed. If the portion of phytosterols and phytostanols in the esterified form in the excreta instead is compared to the total amount of phytosterols and phytostanols in the excreta, the values transfer to 12.6 %± 6.8 %, and 15.5 %± 7.2 % respectively. As can be seen from Fig. 1, the variation between subjects was quite large, with a range from approximately 4 % to 25 %.

Effects on fat excretion

The excreted amounts of the individual fatty acids are presented in Table 2. There were no significant differences between any of the periods for the total amount of excreted fatty acids. Individual fatty acids were not tested statistically, due to low power of the study, and anticipated problems with multi-significance. The results were, however, within the same range. This was also reflected by the absence of significant differences in wet weight, dry weight and energy content of the ileostomy excreta between the three periods as described previously (Normén et al. 2000). Compared to the approximately 100 g of fat in the controlled diet, the major part of the dietary fat was absorbed irrespective of whether PSTE or PSTA was consumed additionally, since less than 2 grams were excreted daily during all periods [4].

The ratio between the unsaturated and saturated fatty acids in the excretion remained the same during all three periods. Despite not being tested statistically, also the percentage of saturated fatty acids and the sum of monounsaturated and polyunsaturated fatty acids remained similar between periods. About 2/3 of the excreted fatty acids were saturated, while approximately 30 % were unsaturated.

Table 2 Excretion of individual fatty acids during three periods: Control, plant sterol ester and plant stanol ester periods (means ± SD, n = 7)

	Control	Plant sterol ester	Plant stanol ester
Fatty acids (mg/d)			
C12:0[1]	9.3 ± 7.4	6.2 ± 6.0	9.0 ± 4.4
C14:0[1]	61 ± 30	47 ± 31	51 ± 22
C15:0[1]	7.7 ± 3.0	6.6 ± 3.1	8.7 ± 3.9
C16:0[1]	699 ± 229	642 ± 298	682 ± 183
C16:1/C16:2/C16:3[1]	46 ± 13	40 ± 14	40 ± 9.0
C17:0[1]	8.3 ± 2.5	8.8 ± 5.6	9.6 ± 3.9
C18:0[1]	424 ± 144	364 ± 158	386 ± 102
C18:1/C18:2/C18:3[1]	465 ± 150	427 ± 264	409 ± 109
Total fatty acids	1840 ± 601	1648 ± 806	1696 ± 425
Ratio unsaturated/saturated	0.43 ± 0.05	0.43 ± 0.10	0.40 ± 0.09
SAFA (%)[1, 2]	66 (1.8)	66 (4.7)	68 (4.4)
MUFA + PUFA (%)[1, 2]	28 (2.5)	28 (4.7)	27 (4.3)
Non-spec. FA (%)[1, 3]	6.6 (1.6)	6.5 (1.9)	5.9 (2.6)

Results are significantly different when marked with the same superscript
[1] Not tested statistically
[2] Contribution of fatty acids classified according to their saturation degree (*SAFA* saturated fatty acids; *MUFA* monounsaturated fatty acids; *PUFA* polyunsaturated fatty acids)
[3] Several other fatty acids were present at low levels, in particular in the C20/C24 carbon number range. These were not further identified and explain the difference between the sum of the individual components and the total fatty acid level

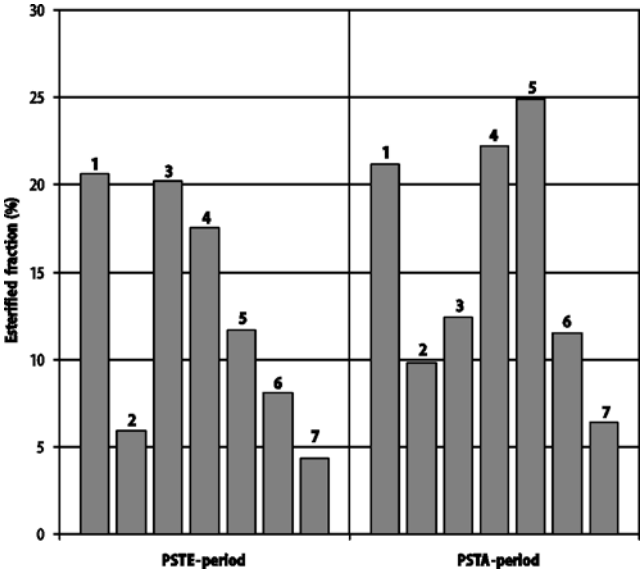

Fig. 1 Relative fraction of plant sterol ester (PSTE) and plant stanol ester (PSTA) in the esterified form compared to the total excreted amount of sterols (expressed as sterol equivalents) in 7 ileostomy subjects (each subject is marked with its respective id number on top of the bar)

Discussion

To our knowledge, this is the first study that evaluates hydrolysis of PSTE and PSTA and their effects on the excretion of fat and fatty acids under controlled dietary conditions. The findings in the present study strengthen the hypothesis that the major part of both PSTE and

PSTA is hydrolysed in the small intestine at a daily intake of 2.5 g. On the other hand, the hypothesis that PSTA and PSTE might impair fat absorption can be refused, as fat excretion remained constant and well within the normal range with both PSTE and PSTA.

To understand the importance of sterol hydrolysis, it is important to assess the latter against the stepwise mechanisms of lipid digestion. Following consumption of fat, lipid droplets of varying size are formed during emulsification in the stomach and gut [18]. When the so formed lipid droplets enter the stomach and duodenum, they are exposed to gastric and pancreatic lipase as well as cholesterol esterase, which catalyse the hydrolysis of TAG and cholesterol ester [19]. TAG and cholesterol esters preferentially occupy the oil phase, i. e. the "core" of lipid droplets [20], while lipolytic products and free cholesterol orient towards the surface [21]. Before free fatty acids and free cholesterol can be absorbed, they interact with bile through micellar formation and are transported from their position at the surface of the lipid droplets via micelles through the water phase [19]. Cholesterol esters need to be hydrolysed to be absorbed [22–24]. PSTE and PSTA also need to be hydrolysed to interact with cholesterol. The results of the present study indicate that PSTE and PSTA are hydrolysed to the free form and can as such enter the water phase, where they can solubilise in or enter micelles [25] and compete with cholesterol for solubilisation [11, 12].

Earlier data of PSTA demonstrate an almost complete hydrolysis. When assessed in 11 colectomised subjects, the fraction of remaining PSTA following small bowel passage was approximately 10 % [14]. This result was based on a comparison with the total amount of phytosterols in the excretion – a calculation, which was also performed in the present study. However, as only a few of the subjects had ileostomies, a somewhat larger degree of hydrolysis could be expected due to bacterial degradation, compared to our subjects. An intubation study with healthy subjects, who consumed a similar dose of PSTA compared to the subjects in this trial, showed that ca 50 % of the ester was hydrolysed already in the intestinal contents of the lower duodenum [16]. This suggests a rapid hydrolysis in the first part of the small bowel, which is probably due to the activities of cholesterol esterase, gastric and pancreatic lipase [19]. Intubation studies, however, have the limitation that the technique in itself shortens the time of gut passage [26]. Our study confirms that PSTE is hydrolysed to the same extent as PSTA.

As shown in this study, neither PSTE nor PSTA interfere with fatty acid absorption. This is consistent with long-term clinical trials, where there is no evidence of side effects such as steatorrhoea [2, 27], which would be an expected result of fat malabsorption. Steatorrhoea is mainly seen in specific clinical conditions such as cystic fibrosis, cholestasis, and short-bowel syndrome [28–30]. An interesting observation in the present study was also that the ratios between unsaturated and saturated fatty acids were similar in all three periods. This indicates that the esterase activity was not affected by PSTE or PSTA, which further strengthens the lack of an effect on fat absorption after supplementation with PSTA and PSTE.

One limitation of the present study is that essential very long-chain fatty acids (n-6 and n-3 fatty acids) were not well represented in the test diet. Normally, a Western-type diet has a relatively low content of omega-3 fatty acids [i. e. in the US: 1, 4 g of α-linolenic acid (ALA 18:3) and 0.1–0.2 g of eicosapentaenoic acid (EPA 20:5) and docosahexaenoic acid (DHA 22:6)] compared to the recommended 2.2 g ALA/d and 0.65 EPA + DHA g/d [31, 32]. Through the deliberate choice of animal (not fish) fats and few plant foods in the controlled diet in the present study, n-3 fatty acids were not present to a high extent. The present study gives therefore limited information about interactions with essential very long chain fatty acids.

In subjects with non-insulin dependent diabetes mellitus, no change of total faecal fat excreted was found following a daily dose of 3 g PSTA [33]. In colectomised subjects, total amounts of fat excreted every day were similar with and without PSTA consumption [14]. Diets were not controlled in these studies, which limits the interpretation of the fat excretion, as the lack of difference could be explained by the common large inter- and intra-individual variation in ad libitum fat intake [34]. In the present study, diet and fat intake were controlled. Here the excretion of individual and total fatty acids did not change with the consumption of PSTE or PSTA, which supports that neither inhibits apparent fat absorption. Again, PSTE gave a similar result to PSTA, demonstrating that despite plant sterols having a slightly different chemical structure than stanols, they are comparable in their action in the human gut.

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