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Original article

Sitostanetriol is not formed in vivo from sitosterol in the rat

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Abstract – In a recent study, we observed some oxyphytosterols in the plasma of healthy human subjects. This experiment was effected in order to determine if these compounds could be formed in vivo from phytosterols. Rats were fed with a high level of phytosterols (1% of the diet) and they were compared to rats deprived of phytosterols, using triglycerides purified from phytosterols. Their plasma were analysed for the main oxyphytosterols. The results show that sitostanetriol and campestanetriol were not formed in vivo from phytosterols. Their levels decreased during the experiment. The diet origin is highly probable for the compounds identified in human plasma. In particular, it seems that the sitostanetriol is eliminated very slowly from the organism.

phytosterol / oxyphytosterol / sitostanetriol / plasma

1. INTRODUCTION

Phytosterols are now considered as very important in the diet, due to their action in reducing cholesterol intestinal absorption and then inducing a decrease of LDL cholesterol [1–3]. They are now included in some foods (spreads, yoghurts, …) and their consumption will probably increase in the next years. However, these compounds can be oxidized as well as cholesterol, and the oxyphytosterols can be identified as trace components in food [4–6]. Some oxyphytosterols were also recently identified in the plasma of healthy human subjects [7]. The main identified compounds are sitostanetriol and β-epoxysitosterol (Fig. 1). But, trace levels of α-epoxysitosterol, campestanetriol and 7-ketositosterol were also detected. There are two possibilities concerning the origin of these compounds. First, they could come from the oxyphytosterols present in food. A study demonstrated that little levels of epoxy- and keto- phytosterols pass the intestinal barrier, in rats [8]. Moreover, these compounds were recently identified in hamster tissues, including plasma, when they were present in the diet at sufficient levels [9]. However, a second origin is also possible: plasma oxyphytosterols could come from the in vivo oxidation of little quantities of phytosterols present in the blood. The present study was effected in order to observe if such oxyphytosterols could be

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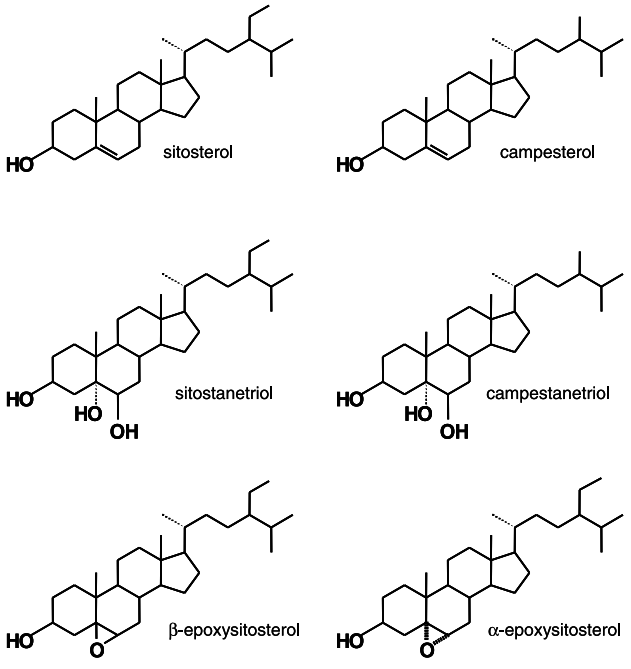

Figure 1. Structure of the main phytosterols and oxyphytosterols studied.

formed in vivo from unoxidized phytosterols in the rat.

2. MATERIALS AND METHODS

2.1. Animal experiment

2.1.1. Phytosterols

The phytosterols included in the diet of rats of group C (as described later) were obtained from ICN Biomedicals (Orsay, France). They were immediately placed under argon and were protected using argon during the entire experiment. They were analyzed for phytosterols and oxyphytosterols using the methods described below. Their composition in phytosterols is noted in Table I. The phytosterols also contained trace levels of oxyphytosterols (7-ketobrassicasterol 52.1 $\mu g \cdot g^{-1}$; 7-ketocampesterol

Table I. Composition of the phytosterols (%) used in the group C diet.

Sterols	% in weight
Cholesterol	0.3
Brassicasterol	3.3
Campesterol	26.7
Campestanol	0.5
Stigmasterol	13.7
n.i.	0.6
24-methylene cholesterol	0.3
Δ7-campesterol	0.4
Clerosterol	0.3
Sitosterol	51.9
Sitostanol + Δ5-avenasterol	1.6
Δ7-sitosterol	0.2
Δ7-avenasterol	0.2

n.i.: not identified.

<table>
<tr><td></td><td>group A
(n = 11)</td><td colspan="2">group B
(n = 11)</td><td>group C
(n = 11)</td></tr>
<tr><td>pre-experimental period (2 weeks)</td><td colspan="4">equilibrated diet including 4% purified triglycerides and 1% saccharose/starch</td></tr>
<tr><td></td><td rowspan="2">sacrifice and blood collection</td><td colspan="2" rowspan="2">idem as above</td><td rowspan="2">equilibrated diet including 4% purified triglycerides and 1% phytosterols</td></tr>
<tr><td>experimental period (2 weeks)</td></tr>
<tr><td></td><td colspan="2">sacrifice and blood collection</td><td>sacrifice and blood collection</td></tr>
<tr><td></td><td>7 samples

normal analysis</td><td>4 samples

adding of phytosterols during analysis</td><td>11 samples

normal analysis</td></tr>
</table>

Figure 2. Experimental design.

54 $\mu g \cdot g^{-1}$; sitostanetriol 27.2 $\mu g \cdot g^{-1}$; 7-ketostigmasterol 39.9 $\mu g \cdot g^{-1}$; 6-ketositostanol 25.7 $\mu g \cdot g^{-1}$; 7-ketositosterol 101 $\mu g \cdot g^{-1}$). This represented an oxidation of 0.027% of the phytosterols.

2.1.2. Purified triglycerides

A blend containing in weight 87% sunflower oil and 13% rapeseed oil was used, in order to supply sufficient polyunsaturated fatty acids amounts, with an adequate ratio between w3 and w6 fatty acids. The phytosterols of this blend were removed, using the method of Ostlund et al. [10]. After purification, the triglycerides contained only 38 $\mu g \cdot g^{-1}$ campesterol, 116 $\mu g \cdot g^{-1}$ sitosterol and some other compounds present as traces (Δ5-avenasterol, Δ7-sitosterol, Δ7-avenasterol, gramisterol, cycloartenol, 24-methylene cycloartanol, citrostadienol). In this fraction, some oxyphytosterols were still identified, but were in such low quantities, that their quantitative evaluation was difficult and imprecise.

2.1.3. Animals

The experimental design is resumed in Figure 2. Thirty-three Wistar four-week old rats were obtained from Janvier (Le Genet St Isle, France). During a pre-experimental period of two weeks, they were all fed with an equilibrated diet containing 4% purified triglycerides and 1% of a blend of starch/saccharose (2/1; w/w). Group A (11 rats), was then sacrificed, after isoflurane anaesthesia. Their blood was collected from the aorta using EDTA and plasma was obtained by centrifugation. The two other groups were fed during two other weeks. Group B (11 rats) received the same control diet as before. Group C (11 rats) received 1% phytosterols and 4% urified triglycerides. At the end of the experimental period, they were sacrificed and their plasma was obtained, as previously described.

2.2. Analyses

2.2.1. Sterol and oxysterol analyses

Five to 7.5 mL of plasma were obtained from each rat and were used for analysis. The lipids of plasma were extracted using the method of Moilanen and Nikkari [11] modified by adding 0.05% butylhydroxytoluene (BHT) as the antioxidant. During all the analytical phases, argon was added when possible, in order to protect the compounds from oxidation. The lipids were saponified under argon, at room temperature, in the

dark, for 16 h, using 10 mL of 1 M ethanolic potassium hydroxide. The unsaponifiable components were then obtained using dichloromethane. A little part (5%) of the unsaponifiable fraction was used directly for phytosterol and cholesterol analysis. They were transformed in trimethylsilyl ether (TMSE) derivatives and analyzed by gas chromatography (GC) and gas chromatography coupled to electron impact mass spectrometry (GC-MS), as described below. The main part of the unsaponifiable fraction was used to determine the oxyphytosterols. They were diluted in 500 μL of hexane/tertio-butyl-methyl-ether (TBME) (90/10 by vol) and purified using solid phase extraction (SPE) on silica cartridges (LC-Si, 3 mL, 500 mg Supelco, L'Isle d'Abeau, France) using successively 35 mL of hexane/TBME (90:10 by vol) and 15 mL of hexane/TBME (80:20 by vol). As recommended by Lai et al. [12], a vacuum manifold (Supelco) was used to ensure a regular solvent flow rate of 0.6 mL·min^{-1} through the cartridge. The oxysterols were then obtained with 10 mL of acetone. Two micrograms of 5α-cholestane were added as the internal standard. After evaporation of the solvent, the samples were redissolved in 200 μL of anhydrous pyridine and 200 μL of BSTFA containing 1% TMCS were added. The TMSE derivatives were obtained by heating 30 min at 55 °C. The reagents were evaporated under nitrogen and the residue was dissolved in hexane for GC and GC-MS analyses.

2.2.2. Gas chromatography

The analyses were effected using a Trace GC 2000 Thermo Finnigan gas chromatograph (Rodano, Italia), equipped with a splitless injector (temperature 290 °C) and a flame ionization detector operated at 300 °C and a DB5-MS, 0.25 μm film thickness, 30 m × 0.25 mm i.d. fused silica capillary column (J&W Scientific, Folsom, CA). Helium was the carrier gas. After 1 min at 50 °C, the oven temperature was raised from 50 °C to 275 °C at 20 °C·min^{-1}, then at 1 °C·min^{-1} until 290 °C. The completion of the analyses was effected at 290 °C. The chromatographic data processing was effected using Diamir software (JMBS Developpements, Fontaine, France).

2.2.3. Gas chromatography coupled to mass spectrometry

Gas-liquid chromatography coupled to electronic impact mass spectrometry was effected using a 6890 Hewlett Packard gas chromatograph coupled to a HP 5973 mass spectrometer (Agilent Technologies, Palo Alto, Ca, USA). The injection was made in the splitless mode. The same column operated under identical temperature conditions as the GC was used. The transfer line was maintained at 300 °C. The mass spectrometer was operated in the electronic impact mode, with an ionization energy of 70 eV.

2.2.4. Monitoring of artifact sterol oxide formation during analysis

The blend of phytosterols already used was submitted to four successive crystallisations in acetone. The product obtained was purified two times on silica cartridges, as described before for oxyphytosterols, in order to remove the eventual oxyphytosterols. It contained 69.3% of campesterol, 28.8% of sitosterol, 1.1% of brassicasterol and 0.7% of stigmasterol and not any oxyphytosterol was measured in this blend. Eight hundred and seventy-two micrograms of this blend were added to each of four samples of group B plasma. This amount allowed to bring to these samples the mean amount of sitosterol already measured in some samples of group C. It also furnished a high level of campesterol. The analysis was then effected as already described.

2.2.5. Statistical analysis

The significance of the differences between groups was evaluated using the analysis of variance and the Newman-Keuls test. A P value of less than 0.05 was considered as significant.

Table II. Phytosterols and cholesterol in rat plasma ($\mu g \cdot mL^{-1}$ plasma).

Diet groups	A	B without addition of phytosterols during analysis (7 rats)	B with addition of phytosterols during analysis (4 rats)	B total group (11 rats)	C
Cholesterol	540.3 ± 84.2[a]			607.5 ± 155.4[a]	563.5 ± 136.5[a]
Campesterol	4.2 ± 1.1[a]	2.8 ± 0.6[b]	90.6 ± 10.5[c]		37.4 ± 7.9[d]
Sitosterol	5.8 ± 1.8[a]	4.1 ± 0.6[b]	41.7 ± 6.0[c]		45.8 ± 9.6[c]

Stigmasterol and Δ5-avenasterol were also identified as trace components, but they were not calculated, due to the coelution with other compounds.
[a,b,c,d] Different superscripts mean that there is a significant difference between groups, at the 5% level of *P*.

3. RESULTS AND DISCUSSION

3.1. Plasma sterols

The plasma cholesterol (Tab. II) was not different between the three groups, probably due to high individual variability. Concerning the phytosterol levels, we can see that the sitosterol and campesterol contents decreased during the two weeks of the experiment (a comparison between groups A and B). So, after four weeks without phytosterols in the diet, the phytosterols were still observed in the plasma. However, their level decreased slowly. It is generally thought that the phytosterols are rapidly metabolised and eliminated from the body [13]. Our results show that a little part of these compounds can remain for some weeks, in the rat.

Group B was the control group. These rats did not receive phytosterols in their diet. However, we decided to add phytosterols in the plasma of four of these animals, in order to observe an eventual formation of artifact oxyphytosterols during analysis. It can be observed that the sitosterol plasma level of these rats was not different from that of group C, fed with phytosterols. This was then a good way to monitor of artifact sterol oxide formation during analysis. A high level of campesterol was also added in the plasma of these four animals. It was better to observe the eventual formation of artifacts.

3.2. Plasma oxyphytosterols

Important progress was recently effected for the identification of oxyphytosterols [6, 14–17]. However, the quantification of each individual oxyphytosterol is still a difficult challenge, due to the fact that several oxyphytosterols are eluted together [6] and that these compounds are not commercial and have to be synthesized. Very numerous syntheses would be necessary. The ideal way would be to obtain individual deuterated compounds and then to use them as standards for quantification using single ion monitoring (SIM) in GC-MS. This is not actually possible, due to the lack of commercial pure deuterated sitosterol. For this study, we restricted the analysis to the compounds which were well separated during the GC analysis, and were present in sufficient amounts. We quantified β-epoxysitosterol, campestanetriol and sitostanetriol. The 7-keto derivatives of sitosterol and campesterol were present as traces, but were not quantified due to coelution with unknown compounds. α-epoxysitosterol was present in little amounts in the peak of 27-hydroxycholesterol, but it was not possible to quantify it, using our analysis conditions. The epoxy-derivatives of campesterol were also identified in some samples, as described later, but they have the same retention time as unknown compounds.

Table III. Oxyphytosterols in rat plasma (ng·mL^{-1} plasma).

Diet groups	A	B without adding of phytosterols during analysis (7 rats)	B with adding of phytosterols during analysis (4 rats)	C
β-Epoxysitosterol	197.8 ± 71.7[a]	37.8 ± 20.3[b]	279.5 ± 162.3[a]	310.0 ± 240.3[a]
Campestanetriol	5.4 ± 7.5	–	–	–
Sitostanetriol	260.1 ± 69.1[a]	21.1 ± 4.3[b]	19.9 ± 6.7[b]	19.0 ± 5.6[b]

[a,b,c,d] Different superscripts mean that there is a significant difference between groups, at the 5% level of *P*.

The results concerning the oxyphytosterols are presented in Table III. The pre-experimental period was effected, in order to wash out the oxyphytosterols eventually present. This was not fully efficient and some important oxyphytosterols were still observed at the beginning of the experiment (group A). The first result is that the amounts of all the measured oxyphytosterols decreased during the two weeks of the experiment (comparison between A and B). The sitostanetriol level fell from 260.1 to 21.1 ng·mL^{-1}. The campestanetriol level was 5.4 ng·mL^{-1} at the beginning of the experiment and was not detectable two weeks after. These phytotriols have been observed in human plasma [7], and sitostanetriol was also observed in tissues of hamsters fed with oxyphytosterols [9]. It is possible that these compounds were issued from oxyphytosterols ingested by the animals before the experiment and that sitostanetriol requires a long time for metabolism or excretion. The amount of β-epoxysterol also decreased from 197.8 to 37.8 ng·mL^{-1}. The same explanation as for phytotriols is possible. However, the plasmas of group B, which were added with phytosterols, presented a high level of β-epoxysitosterol, equivalent to that of the animals of group C, which were fed with phytosterols. Moreover, it was also possible to detect β-epoxycampesterol in these four rats, as well as a smaller level of α-epoxycampesterol. It was not possible to quantify these epoxycampesterols due to coelution with unknown compounds. But, it is evident that a big part of these epoxy-compounds are artefacts. It is then not possible to observe an eventual formation of phytosterol epoxides in vivo. An experiment using the stable isotope of oxygen already did not allow to observe the formation of epoxycholesterols in rats in vivo [18]. In a recent experiment [7], we did not observe artifact formation, using the same method. However, in this case, sitosterol was smaller (more than twenty-one times less). In the present experiment, we used a very high level of phytosterols (1% of the diet), in order to better observe an eventual formation of oxyphytosterols in vivo. In spite of all the precautions used to limit the artifact formation during the analyses, some epoxidation happened and did not allow to observe an eventual formation of epoxides in vivo.

It is, however, evident that the phytotriols were not artifacts. The levels of sitostanetriol and campestanetriol were not different in rats of group C and in those of group B (with the addition of phytosterols during analysis or not). These phytotriols were not formed in vivo from the corresponding phytosterols. There is then a very high probability of a diet origin for the presence of these compounds in human plasma [7]. Some epoxyphytosterols are present as trace compounds in foods. They could be partly transformed into phytotriols in the stomach [19] and a small level could pass the intestinal barrier [8]. A recent experiment [9] also allowed to observe the incorporation of main oxyphytosterols in tissues (plasma, aorta,

liver, kidneys and heart), when they were abundant in the diet, but also to observe sitostanetriol in these tissues, when the level of oxyphytosterols was low in the diet or even in control rats, not receiving any oxyphytosterol in their diet. The metabolism and/or excretion of sitostanetriol is probably rather long. In the present experiment, it was still observed four weeks after the beginning of a purified diet, even if a decrease of the level was evident between the last two weeks.

It would now be interesting to find out if this compound could have physiological effects. The consumption of phytosterols will indeed probably increase in the next years and it will be very difficult to prevent a little oxidation of these compounds in food [20]. Cholestanetriol, the analogous compound issued from cholesterol, is described as cytotoxic [21] and its possible involvement in atherosclerosis was pointed out [22, 23]. It would be very important to study if sitostanetriol presents the same effects.

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