Comparison of the intestinal uptake of cholesterol, plant sterols, and stanols in mice

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Abstract The recent identification of the aberrant transport proteins ABCG5 and ABCG8 resulting in sitosterolemia suggests that intestinal uptake of cholesterol is an unselective process, and that discrimination between cholesterol and plant sterols takes place at the level of sterol efflux from the enterocyte. Although plant sterols are structurally very similar to cholesterol, differing only in their side chain length, they are absorbed from the intestine to a markedly lower extent. In order to further evaluate the process of discrimination, three different sterols (cholesterol, campesterol, sitosterol) and their corresponding 5α-stanols (cholestanol, campestanol, sitostanol) were compared concerning their concentration in the proximal small intestine, in serum, and in bile after a single oral dose of deuterated compounds. The data obtained support the hypothesis that i) the uptake of sterols and stanols is an extremely rapid process, ii) discrimination probably takes place on the level of reverse transport back into the gut lumen, and iii) plant stanols are taken up, but not absorbed to a measurable extent, and iv) the process of discrimination probably also exists at the level of biliary excretion. The range of structural alterations that decrease intestinal absorption and increase biliary excretion is: 1) campesterol, 2) cholesterol-sitosterol, and 3) campestanol-sitostanol.

Supplementary key words phytosterols • phytostanols • stable isotope • gas chromatography-mass spectrometry • ABC-transporters

Investigators have been intrigued for more than a decade with the concept that there is a protein-mediated transport system responsible for intestinal uptake of cholesterol (1). The recent identification of the molecular defects underlying phytosterolemia (2, 3) and the lines of evidence supporting the existence of a specific transport protein located in the brush border membrane that possibly mediates intestinal sterol absorption (4–6) has shed new light on the cellular transport of cholesterol and plant sterols. Based on the characterization of sterol uptake in brush border membranes, it is currently hypothesized that the process of intestinal sterol uptake is protein mediated and that the pumping of sterols from the intestine into the enterocyte is unselective. In phytosterolemia, a rare autosomal recessive disorder caused by mutations in the tandem ABC genes, either ABCG5 or ABCG8, affected individuals hyperabsorb and retain not only cholesterol but also plant sterols (7–11). Consequently, it is hypothesized that the ABC transporters G5 and G8 are able to discriminate between cholesterol and other sterols. They are pumping the noncholesterol sterols out of the intestinal cell back into the gut lumen and also into bile, and they are differentiating between the side-chain length and probably also between the stereoselective 5α saturation of the Δ5 double bond to stanols (12, 13).

Plant sterols are structurally related to cholesterol and differ in their chemical structure only due to the presence of an additional methyl (campesterol) or ethyl (sitosterol) group at the C-24 position of the side chain. Stanols differ from the corresponding sterols due to saturation of the Δ5 double bond to the 5α position (cholestanol, campestanol, sitostanol). Apparently, the structural differences of these sterols-stanols seem to be only of minor extent. Nevertheless, previous studies in animals and humans revealed that the efficacy of their intestinal absorption differs markedly from cholesterol (10, 14–16). However, data comparing these compounds concerning their intestinal uptake have not been experimentally verified. In order to further evaluate this hypothesis, we compared three deuterated sterols (cholesterol, campesterol, and sitosterol) and their corresponding 5α-stanols (cholestanol, campestanol, and sitostanol).

MATERIALS AND METHODS

Mice

Male C57BL/6OlaHsd mice were purchased from Harlan-Winkelman (Borchen, Germany). All experiments were carried out in accordance with the institutional guidelines for use of animals.
out with four animals per group aged 8 to 11 weeks. They were fed a standard laboratory diet (1320N, Altromin, Lage, Germany), had free access to food and water, and were caged individually. Before starting the experiments, all animals were adapted to the environment and to the feeding procedure by stomach tube for 2 weeks. The Principals of Laboratory Animal Care were followed, and approval for the treatment protocol was obtained from the committee for animal care of the local administration.

Synthesis of deuterated stanols

Deuterated cholesterol, campesterol, and sitosterol were purchased from Medical Isotope, Inc. Deuterated cholesterol, campesterol, and sitostanol were synthesized by hydrogenation of the corresponding deuterated unsaturated sterols using microwaves (17).

Application of deuterated sterols and stanols


Collection of serum and bile

Fifteen minutes, 30 min, 60 min, 120 min, and 240 min after the administration of deuterated sterols-stanols, mice were anaesthetized with diethyl ether. The chest cavity was opened and blood collected by heart puncture. Serum was obtained by centrifugation, immediately frozen in liquid nitrogen, and stored at −20 °C. The gall bladder was removed. Due to the extremely small volume of bile in the gall bladder, measurement of the volume of bile was not possible. Thus, the whole gall bladder was immediately frozen in liquid nitrogen and stored at −20 °C. The relations of the different sterols and stanols in bile are therefore expressed as ratios to cholesterol.

Preparation of small intestine

After the administration of deuterated sterols-stanols, the small intestine was removed at the above mentioned time points. A section of the proximal jejunum starting 3 cm distal from the Papilla Vateri and measuring 3 cm in length was prepared. The specimens were dissected, thoroughly washed in phosphate buffered saline in order to remove any intestinal content, immediately frozen in liquid nitrogen, and stored at −20 °C.

Sample preparation

Before starting the extraction of lipids, the small intestine specimens were dried by vacuum, the dry weights determined, and the specimen homogenized. Lipids were extracted for 48 h in 5 ml chloroform-methanol (2:1, v/v) evaporated to dryness under a stream of dry nitrogen at 63 °C, and hydrolyzed for 1 h with 1 ml of 1 M NaOH in 90% ethanol. Extraction of lipids from bile was performed directly in the vial containing the gall bladder by adding 1 ml chloroform-methanol (2:1, v/v).

Measurement of deuterated sterols-stanols by gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) was performed on a Hewlett-Packard system (5890 series II GC combined with a 5971 mass selective detector) equipped with a DB-XLB column (J and W Scientific); 30 m; 0.25 mm ID; 0.25 μm film thickness. Samples were monitored for [3H2]cholesterol (m/z 464), [4H2]campesterol (main fragment M+4 m/z 476), [4H2]sitosterol (main fragment M+4 m/z 490), [4H2]cholesterol (m/z 466), [4H2]campestanol (m/z 479), and [4H2]sitostanol (m/z 495).

Calculation of deuterated sterols-stanols in small intestine

The concentrations of deuterated sterols-stanols measured in the specimen of the small intestine were corrected for the different amounts of deuterated sterol-stanol applied by stomach tube. Concentrations are expressed in ng per mg of dried intestine specimen.

Statistical methods

Data are given as mean ± SD. Probability values were calculated using Student’s t-test.

RESULTS

The concentrations of sterols and their respective 5α-stanols in the small intestine at different time points are given in Table 1. All deuterated sterols and stanols were already detectable in the intestinal mucosa 15 min after administration into the stomach. An almost uniform pattern of the concentration of sterols and stanols was seen until 240 min. In general, the concentrations of cholesterol at every time point were higher than those of campesterol, which in turn were higher than sitosterol. A similar pattern was observed with the stanols (cholestanol > campestanol > sitostanol). The concentration of cholesterol in the intestine samples increased continuously up to 7-fold during the first 120 min (Table 1). Thereafter, the concentration of cholesterol decreased markedly (P = 0.041). The concentration of campesterol was only 10% lower compared with cholesterol after 15 min (P = 0.049). After 30 min, the concentration of campesterol was twice as high compared with the concentration after 15 min, but already 39% lower than

<p>| TABLE 1. Concentrations of deuterated sterols and stanols in the upper small intestine in mice |
|---------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>15 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>240 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>110 ± 18</td>
<td>327 ± 208</td>
<td>444 ± 388</td>
<td>775 ± 224</td>
<td>472 ± 69</td>
</tr>
<tr>
<td>Campesterol</td>
<td>99 ± 12</td>
<td>200 ± 96</td>
<td>213 ± 100</td>
<td>206 ± 60</td>
<td>96 ± 25</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>81 ± 5</td>
<td>123 ± 51</td>
<td>124 ± 43</td>
<td>51 ± 18</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>76 ± 7</td>
<td>168 ± 97</td>
<td>204 ± 166</td>
<td>160 ± 55</td>
<td>89 ± 42</td>
</tr>
<tr>
<td>Campesterol</td>
<td>56 ± 4</td>
<td>86 ± 45</td>
<td>88 ± 30</td>
<td>17 ± 12</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Sitostanol</td>
<td>45 ± 4</td>
<td>61 ± 28</td>
<td>58 ± 17</td>
<td>6 ± 3</td>
<td>4 ± 0.5</td>
</tr>
</tbody>
</table>

Each data point represents the mean of four animals. The data represent means ± SD of four animals and are corrected for the different dosages of deuterated sterols and stanols administered.
the concentration of cholesterol. The concentrations of campesterol remained constant up to 120 min and declined thereafter significantly by 53% (P = 0.015). However, the differences between the concentration of cholesterol and that of campesterol increased to 52% after 60 min and to 73% after 120 min (P = 0.001). The concentrations of sitosterol after 15 min were lower compared with the concentrations of cholesterol and campesterol. The differences at this time point were 26% and 19% lower compared with cholesterol and campesterol, respectively (P = 0.040 for both). Maximal concentrations of sitosterol were found between 30 and 60 min. The differences after 30 and 60 min to cholesterol were 62% and 72%, and to campesterol 39% and 42%, respectively. Thereafter, the concentration of sitosterol increased to 52% after 60 min and to 73% after 120 min (P < 0.040). The percent difference between cholesterol and campesterol as well as between campesterol and sitosterol were almost similar at every time point. The concentrations of the stanols in the identical intestinal specimen showed a similar pattern, although on a lower level than the respective sterols. Just as with cholesterol, cholestanol was the most abundant stanol found at all time points. Maximal concentrations were reached after 60 min. The decrease that followed was less pronounced compared with the other stanols. After 240 min, the concentration of cholestanol was almost identical to and not significantly different from the level found after 15 min. The concentration of campestanol after 15 min was significantly lower compared with cholestanol (P = 0.002). Between 15 and 30 min, it increased slightly and remained at this level until 60 min. The differences to cholestanol were 49% and 57%, respectively. Thereafter, the concentration of campestanol decreased markedly more than 5-fold (P = 0.005), and disappeared almost completely after 240 min. The drop in the concentration of campesterol was highly significant (P = 0.005). The same pattern was observed with sitostanol, albeit on an even lower level. Between 15 and 30 min, its concentration increased only 1.4-fold, remained close to this level until 60 min, declined thereafter nearly 10-fold (P = 0.001), and was almost undetectable after 120 min. The percent difference of sitostanol to cholestanol at every time point was greater than the difference of campestanol to cholestanol.

The range of the ratios of plant sterols and stanols to cholesterol in the small intestine is given in Table 2 and Fig. 1. After 15 min, the highest ratio was that of campesterol to cholesterol (0.91 ± 0.05). This ratio declined markedly between 15 and 30 min (0.67 ± 0.14, P = 0.019), and between 60 and 120 min (P = 0.014). After 240 min, it was 0.2 ± 0.04. The ratio of sitosterol to campesterol after 15 min (0.75 ± 0.11) was lower compared with campesterol (P = 0.022), and it declined thereafter more rapidly (Fig. 1). The difference to campesterol was significant at every later time point (P ≤ 0.009). The ratio of cholestanol to cholesterol was 0.7 ± 0.08 after 15 min. It declined in the same manner as the ratio of campesterol. A highly significant difference to the ratio of campesterol was seen after 15 min (P = 0.004), and another marked difference after 120 min (P = 0.028). The ratios of campestanol and sitostanol to cholesterol showed the most rapid decline and were almost zero after 120 min.

Deuterated sterols and cholestanol in serum could only be traced accurately after 120 and 240 min. The concentrations of deuterated campestanol and sitostanol in serum were below the limit of detection. The concentrations resembled the same pattern as seen in the small intestine (Table 3). The highest concentration found after 120 min and 240 min was for cholesterol, followed by campesterol, cholestanol, and sitosterol. The differences between the four substances at both time points were all significant (P ≤ 0.013 after 120 min, and P ≤ 0.021 after 240 min), except for the difference between campesterol and cholestanol. Concerning the increase between 120 min and 240 min, a different range was found. Cholesterol showed the highest increase of almost 2.4-fold, followed by cholestanol (2-fold), campesterol (1.5-fold), and

![Fig. 1](https://i.imgur.com/3Q5Q5Q5.png)

Fig. 1. Ratio of plant sterols and stanols to cholesterol in small intestine of mice.

**Table 2.** Ratio of deuterated plant sterols and stanols to cholesterol in the upper small intestine in mice

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>ng/ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.91 ± 0.05</td>
</tr>
<tr>
<td>30</td>
<td>0.67 ± 0.14</td>
</tr>
<tr>
<td>60</td>
<td>0.60 ± 0.19</td>
</tr>
<tr>
<td>120</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>240</td>
<td>0.20 ± 0.04</td>
</tr>
</tbody>
</table>

The data represent means ± SD of four animals and are corrected for the different dosages of deuterated sterols and stanols administered.
The most rapid decline was that of sitosterol (1.4-fold). The difference between 120 min and 240 min was not significant for the plant sterols.

The ratios to cholesterol in serum are given in Table 3. The most rapid decline was that of sitosterol (−44%), followed by campesterol (−37%). The differences were significant ($P = 0.009$ and $P = 0.011$, respectively). In contrast, only a slight and insignificant change was noticed for the ratio of cholestanol to cholesterol in serum (−14%) between 120 min and 240 min.

The ratios of plant sterols and cholestanol to cholesterol in bile are given in Table 4. Until the time point of 60 min, the amounts of deuterated sterols and cholestanol in bile were below the limit of detection. After 120 min, the concentrations of both plant sterols and cholestanol in bile exceeded the concentration of cholesterol. Between 120 min and 240 min, the ratios of sitosterol and campesterol showed a much higher decrease (−54% and −49%; $P = 0.047$ and $P = 0.028$, respectively) compared with the ratio of cholestanol (−6%), which did not differ significantly. The differences between the ratio of campesterol to cholesterol and that of sitosterol to cholesterol at 120 min and at 240 min were both significant ($P = 0.024$ and $P = 0.004$, respectively).

### DISCUSSION

The present data indicate that the uptake of cholesterol, plant sterols, and their corresponding 5α-stanols from the intestinal lumen into the enterocyte is an extremely rapid process in mice. As early as 15 min after application by stomach tube, all compounds are detectable in the small intestine. The differences seen in their concentrations and in their ratios to cholesterol suggest that the process of discrimination between the different compounds had already started to take place before the first measurement was performed after 15 min. Several possible explanations concerning the level of discrimination could be discussed. The first explanation that cannot be ruled out by our experiments might be that there are several, yet unknown, separate transporters that differentiate between cholesterol, plant sterols, and stanols concerning their uptake into enterocytes. Accordingly, the differences seen would result from the saturation of these transporters that is obtained with plant sterol and stanols at lower levels than cholesterol in the intestine and that might also result from the dosages of sterols and stanols applied. Second, cholestereryl ester synthesis in the enterocyte via ACAT might be the crucial step of discrimination in that cholesterol is preferentially esterified. Consequently, less free cholesterol would be available for transport out of the cell back into the intestinal lumen or into bile. Finally, the currently favored explanation for the differences seen after 15 min assumes that sterols and stanols are almost completely taken up into enterocytes and that the process of discrimination takes place on the level of reverse transport from the enterocyte into the intestinal lumen. Consequently, this would imply that there should be no differences between the compounds at an earlier time point than 15 min. Unfortunately, measurements at an earlier time point were not possible to perform due to the necessary handling of the animals and specimens. Therefore, the hypothesis that the process of discrimination relies on the efflux rather than the uptake or esterification is only indirectly supported by the ratios to cholesterol that decrease markedly between 15 min and 30 min (Table 2), and by the extrapolation of the trend lines of the ratios over time (Fig. 1).

The pattern of discrimination obviously depends on the chemical structure of the sterols and stanols. This pattern could be described as follows: the addition of one methyl group at the C-24 position of the side chain (campesterol) is the smallest alteration. The saturation of the Δ5 double bond (cholesterol) and the addition of an ethyl group at the C-24 position (sitosterol) result in even more structural discrepancy and potency of discrimination. Alterations of both the C-24 position of the side chain and the Δ5 double bond (campestanol, sitostanol) leads to structural differences, which further increase the power of discrimination, especially concerning the transport into the plasma compartment.

Thirty and 60 min after their application, the process of discrimination in the small intestine between the different sterols and stanols becomes more and more obvious, and
creased 18-fold on a high-cholesterol diet compared with hepatic cholesterol levels of ABCG5/8 knock-out mice in- and wild-type animals (20); and in fecal neutral sterol excretion in mice (18);

ABCG5 and ABCG8 express the transgene primarily in the liver and small intestine (18); ABCG5 and ABCG8 express the transgene in intestine but also in cells of the liver (3); 2) transgenic mice overexpressing human ABCG5 and ABCG8 show a much lower increase in their concentrations and a more rapid decrease of their ratios than cholestanol. Furthermore, the ratios of cholestanol to cholesterol in serum do not alter significantly. These data suggest that alterations of the side chain favor the elimination from serum over the saturation of the double bond. The data from bile could support the hypothesis that a process of discrimination exists between plant sterols and cholestanol on the level of clearance from serum. Plant sterols are eliminated faster and to a greater extent than cholestanol. Thus, the different alterations of the side chain would determine the rate of elimination, as indicated by the difference between the two plant sterols. On the other hand, it cannot be ruled out that the differences in bile are due to differences in uptake by the liver, although transport systems that discriminate between different compounds concerning their uptake into liver are not described. The speculation that the discrimination also applies to sterol-stanol excretion into bile is supported by the finding that 1) ABCG5 and ABCG8 in humans are not only expressed in intestine but also in cells of the liver (3); 2) transgenic mice overexpressing human ABCG5 and ABCG8 express the transgene primarily in the liver and small intestine (18); 3) coexpression of ABCG5 and ABCG8 results in sorting of ABCG5 to the apical, canalicular surface of hepatocytes (19); 4) overexpression of ABCG5 and ABCG8 results in biliary cholesterol concentrations more than 5-fold, and a 3- to 6-fold increase in fecal neutral sterol excretion in mice (18); 5) hepatic cholesterol levels of ABCG5/8 knock-out mice increased 18-fold on a high-cholesterol diet compared with a 3-fold increase in wild-type animals (20); 6) the fractional absorption of dietary plant sterols and cholestanol increased 2- to 3-fold in ABCG5/8 knock-out mice, although the fractional absorption of dietary cholesterol was not significantly different between the ABCG5/8 knock-out mice and wild-type animals (20); and 7) patients with sitosterolemia excrete only a small fraction of the ingested plant sterols into bile, although the concentration of plant sterols in serum and bile is markedly higher compared with unaffected individuals (2, 18).

The discrimination capacity of the small intestine between cholesterol, plant sterols, and corresponding stanols, in that cholesterol is preferentially absorbed from the intestinal lumen, is well documented in the literature (10, 15, 21–26). Only traces of campestanol and sitostanol are detectable in serum under physiological conditions (22, 26–28). In contrast, high serum concentrations of both plant sterols and stanols are present in patients with sitosterolemia (7, 9). The identification of the mutations in the tandem ABC genes, ABCG5 and ABCG8, has given credence to the hypothesis that there is a molecular pathway regulating dietary sterol and stanol absorption and biliary excretion (2, 3). Further support for this hypothesis comes from patients with Tangier disease (29–33), suggesting that even more ABC transport proteins could be involved in the regulation of sterol absorption in the intestine and from the identification of orphan nuclear hormone receptors, such as the retinoid X receptor, the liver X receptors, and the farnesoid X receptor (34) that are involved in the regulation of these transporters. These findings form the basis of the current hypothesis regarding sterol metabolism that the discrimination process does not involve the uptake from the intestinal lumen, but rather the efflux from the enterocyte back into the intestinal lumen. Consequently, the process of discrimination would require some time to build up. This hypothesis can be supported by the data obtained here, as the degree of discrimination depicted by the ratios of plant sterols and stanols to cholesterol gradually increases over time. Furthermore, the data presented here imply that the process of discrimination is an extremely rapid one that probably takes only minutes to start. Significant differences in the concentrations of cholesterol, plant sterols, and their corresponding stanols are found as early as after 15 min. Unfortunately, measurements at an earlier time point are difficult due to the design of the experiment, and probably show a much higher variability. However, in vitro data might distinguish early mechanisms of the process of discrimination.

In summary, the data show for the first time a comparison of intestinal uptake, absorption, discrimination, and biliary excretion of cholesterol, plant sterols, and the corresponding 5α-stanols at short time intervals, and support the current hypothesis that the efflux rather than the uptake of sterols is the main process discriminating in favor of the absorption of cholesterol over plant sterols and stanols. Finally, the ratios of plant sterols to cholesterol in the intestine and bile at the two different time points (120 min and 240 min) indicate that the fast efflux from the intestine back into the gut lumen as well as the elimination into bile is probably regulated by the same transporter(s). Nevertheless, further studies are needed utilizing the properties of investigational drugs and genetically altered mice, as well as in vitro experiments, to clarify this process.
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