Absolute Oral Bioavailability and Metabolic Turnover of \( \beta \)-Sitosterol in Healthy Subjects

Guus Duchateau, Brett Cochrane, Sam Windebank, Justyna Herudzinska, Davindera Sanghera, Angela Burian, Markus Müller, Markus Zeitlinger, and Graham Lappin

Unilever R&D, Vlaardingen, The Netherlands (G.D.); Unilever Safety & Environmental Assurance Centre, Colworth, United Kingdom (B.C., S.W.); Xceleron Ltd., York, United Kingdom (J.H., D.S., G.L.); and Medical University of Vienna, Vienna, Austria (A.B., M.M., M.Z.)

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ABSTRACT:

The metabolic turnover, absolute oral bioavailability, clearance, and volume of distribution for \( \beta \)-sitosterol were measured in healthy subjects. \([^{14}C]\beta\)-Sitosterol was used as an isotopic tracer to distinguish pulse doses from dietary sources and was administered by both oral and intravenous routes. The administered doses of \([^{14}C]\beta\)-sitosterol were in the region of 3 to 4 \( \mu \)g, sufficiently low as not to perturb the kinetics of \( \beta \)-sitosterol derived from the diet. Because the plasma concentrations of \([^{14}C]\beta\)-sitosterol arising from such low doses were anticipated to be very low, the ultrasensitive isotope ratio analytical method of accelerator mass spectrometry was used. The limit of quantification for \([^{14}C]\beta\)-sitosterol was approximately 0.1 pg/ml, the oral absolute bioavailability was just 0.41%, clearance was 85 ml/h, volume of distribution was 46 L, and the turnover was 5.8 mg/day. Given the steady-state concentrations of \( \beta \)-sitosterol (2.83 \( \mu \)g/ml), then the dietary load was calculated to be approximately 1400 mg/day.

Introduction

Phytosterols, such as \( \beta \)-sitosterol, have been known for their low-density lipoprotein cholesterol-lowering effects for several decades (Barber and Grant, 1955), with evidence of a dose-response relationship predicting a low-density lipoprotein cholesterol-lowering effect of approximately 9% for the recommended 2 g/day dietary intake (Katan et al., 2003; Panel on Dietetic Products, 2009). The primary molecular mechanism is believed to be physicochemical competition between cholesterol and phytosterols for micellar incorporation and uptake at the gut lumen (Calpe-Berdiel et al., 2009). However, other mechanisms, such as interaction with the transporters involved in cholesterol uptake, their regulation, or the intracellular trafficking of cholesterol and phytosterols, cannot be excluded (Hauser et al., 1998; Plat et al., 2005).

The source of \( \beta \)-sitosterol in the body is known to be dietary, because it is not synthesized in humans (Gordan et al., 1967). The oral bioavailability of phytosterols however is very low, being on the order of 0.5 to 5%, depending on exact sterol structure (Salen et al., 1970). Neither the specific clearance nor volume of distribution for the main dietary phytosterols campesterol and \( \beta \)-sitosterol are currently known. However, plasma concentrations are significantly lower than those commonly observed for cholesterol (approximately 5 mM) and are only in the micromolar range. To gain better insight into the exact bioavailability and the disposition characteristics of xenosterols, using the main \( \beta \)-sitosterol as an example, we designed this human pharmacokinetic study. Understanding the disposition of phytosterols at the gastrointestinal tract and systemic level is important, because they are used as functional food ingredients and this builds further on our mechanistic understanding.

In this article, we describe a healthy volunteer study where tracer quantities of \([^{14}C]\beta\)-sitosterol were administered orally and intravenously along with approximately 1.52 g of \( \beta \)-sitosterol given in Becel/Flora pro.activ margarine. The \( ^{14} \)C tracer amounts of \( \beta \)-sitosterol were administered in as low a dose as feasible (approximately 3–4 \( \mu \)g) so as not to perturb the existing systemic pools of \( \beta \)-sitosterol from dietary sources. The plasma concentration of \([^{14}C]\beta\)-sitosterol therefore was anticipated to be very low, and so the study was designed around the use of the ultrasensitive isotope-ratio analytical method of accelerator mass spectrometry (AMS). AMS was developed originally for radiocarbon dating and can be used to measure \( ^{14} \)C drug concentrations in the range of \( 10^{-15} \) to \( 10^{-18} \) g/ml.

Materials and Methods

Subjects. Twelve healthy male subjects, in two groups of six, 18 to 55 years of age, with a body mass index of 20 to 28 kg/m\(^2\) and a plasma cholesterol concentration of \( <5.5 \) mM were enrolled. They were free from drugs of abuse, smoked no more than five cigarettes per day, and had not taken St. John’s Wort or grapefruit juice for 2 weeks before the start of the study. The subject’s baseline plasma sterol concentrations were analyzed before the study to exclude any abnormalities in sterol homeostasis due to genetic variability of sterol handling transporters. The study was performed in accordance with the
Declaration of Helsinki (as modified October 2008) and the Good Clinical Practice guidelines of the European Commission. Approval from the local ethics committee in Vienna was obtained before study initiation, and written informed consent was obtained from all of the volunteers.

Materials and Test Substance. [14C]-β-sitosterol was obtained from Quotient Bioreresearch (Rushden, UK). The chemical structure of [14C]-β-sitosterol and the position of 14C are shown in Fig. 1. The specific activity was 24.1 nCi/μg, with a purity of >98%, and [14C]-β-sitosterol was shown to be stable over the duration of the study. All of the reagents were of the highest grade available. The AMS calibration standard, Australian National University (ANU) sugar, was obtained from Quaternary Dating Research Centre, Australian National University (Canberra, Australia). Nonlabeled β-sitosterol as an analytical standard was obtained from Sigma-Aldrich (St. Louis, MO).

Non-specific Binding. The [14C]-labeled administered doses were very small, in the microgram range, and therefore losses of the compound due to non-specific binding were assessed before dosing. Significant binding was found in the sterilization filters, and the filter types had to be optimized to minimize binding (see below). The final doses administered were adjusted to account for non-specific binding effects.

Dose Administration. According to the open-label, two-group, single-dose design, volunteers were assigned randomly to two parallel groups of six. A parallel design was preferred given the expected extreme long half-life of elimination. In group A, 15 ml of a drinking solution was swallowed, delivering 3.8 and 4.2 μg and approximately 240,000 dpm (4 kBq) of [14C]-β-sitosterol, depending upon postdose analysis of the dosing apparatus. In group B, 15 ml of an intravenous solution was administered, delivering between 2.9 and 4.2 μg and approximately 4 kBq of [14C]-β-sitosterol, depending upon postdose analysis of the dosing apparatus. The actual doses administered were used in the calculations of the pharmacokinetics. Doses were prepared in the same way irrespective of the route of administration in ethanol (1.5 ml)/physiological saline (0.9% w/v) (13.5 ml) by filtering through a 0.22-μm filter for sterilization (filter type, hydrophilic polytetrafluoroethylene 0.2 μm; Millipore-Millex, Millipore Corporation, Billerica, MA). The intravenous dose was given as an infusion over 15 min. Immediately before both doses, volunteers were given a standardized meal containing 27 g of Becel/Flora pro.activ, equivalent to 2.16 g of phytosterols, of which 1.52 g was β-sitosterol.

Sample Collection. For both dose groups A and B, blood samples were taken at predose, 1, 3, 8, 12, 24, and 48 h and 4, 8, 14, 22, 28, 35, 42, 49, 56, and 63 days after study product administration. Blood was taken from an indwelling catheter in the antecubital vein. For dose group B, additional samples were taken at 15 and 30 min after administration. Whole blood was centrifuged (2000g for 10 min at room temperature) to liberate plasma. Urine samples were collected from subjects 1, 4, and 6 (oral dose) and 7, 10, and 11 (intravenous dose) over 12, 12 to 24, and 24 to 48 h. Urine produced during the 24 h before days 4, 8, 14, 22, 35, 42, 49, 56, and 63 also was collected.

Sample Analysis. Plasma samples were analyzed for total β-sitosterol concentration using gas chromatography–flame ionization detection (Trace GC Ultra; Thermo Fisher Scientific, Waltham, MA) as described previously (Du-chateau et al., 2003). In brief, β-sitosterol was measured after saponification, solvent extraction, and bis(trimethylsilyl)trifluoroacetamide/pyridine silylation. Sterols were separated on a column (CP-sil 13 CB column, 25 m × 0.25 mm, 0.2-μm film and CP-sil 8, 30 m × 0.25 mm, 0.25-μm film; Agilent Technologies, Santa Clara, CA). Sterol identity was verified via retention times of sterol standards.

For the analysis of [14C]-β-sitosterol by high-performance liquid chromatography (HPLC) and AMS, clinical plasma samples and quality control samples were spiked with nonlabeled β-sitosterol as an internal standard (see below), vortexed, and allowed to stand at room temperature for at least an hour. Four times the volume of the plasma sample of NaOH in methanol (0.5 M) was added followed by saponification for 1 h in a water bath at 75°C. After saponification, 3 ml of hexane was added, and the samples were boiled for 6 min, followed by the addition of 10 ml of saturated NaCl and shaking for 5 min. Two phases were allowed to separate, and the upper hexane layer was transferred to a clean vial containing 0.5 g of anhydrous Na2SO4. An aliquot (700 μl) was evaporated to dryness under nitrogen followed by reconstitution in 200 μl of methanol before injection onto the HPLC column. The HPLC column was a Gemini C18 110A (Phenomenex, Macclesfield, UK), 5-μm particle size, 250 × 4.6 mm at 25°C isocratically eluted with acetonitrile and isopropanol (3:2 v/v) at a flow rate of 1.0 ml/min. UV absorbance (208 nm) was used to monitor the retention time (approximately 11.5 min) and for internal standardization of recovery as described previously (Lappin et al., 2008). The HPLC eluent was collected as a series of fractions, and those those fractions corresponding to β-sitosterol were diluted isotopically with the addition of 2.5 μl of liquid paraffin (Sigma-Aldrich) following by the preparation of graphite as reported previously (Lappin and Garner, 2004; Lappin, 2006). Along with the study samples, ANU-certified standards were graphitized along with liquid paraffin controls. The graphite (approximately 2 mg) was placed into the AMS ion source and ionized with a cesium ion beam. The resulting carbon ions were accelerated using a terminal voltage of 4.5 MV and a particle energy of approximately 17.5 to 22.5 MeV. Each sample was measured for 1000 cycles (100.7 s). The operation of the AMS has been described previously (Lappin and Garner, 2004). Resolution of β-sitosterol from any possible coeluting interfering 14C compounds was confirmed by the analysis of individual fractions every 10 s across the chromatographic peak and aligning with nonlabeled β-sitosterol by UV detection for samples taken at 3 and 336 h.

The concentration of β-sitosterol was calculated using Eq. 1, where K is the amount of β-sitosterol in the fraction, R0 is the isotope ratio (14C/12C) measured by AMS, Θ is the amount of carbon added from the isotopic dilutor (liquid paraffin, see above), L is the specific activity of β-sitosterol, and Θ is the recovery of the analyte as calculated from the internal standard (Lappin et al., 2008).

\[
K = \frac{R_0 \times \phi}{L \times \Theta}
\]

In addition to HPLC fractions, 60 μl of whole plasma or urine were graphitized and analyzed by AMS as described above to determine the total 14C concentration (mass equivalents of β-sitosterol). Data acceptance criteria were that the AMS current was ≥1 μAmp, the 13C/14C isotope ratio was 1.1 ± 15%, the ANU controls were 160.61 ± 22% modern carbon (pMC), and the liquid paraffin blanks were <8 pMC (1 modern = pMC/100 = 98 atomole 14C per mg carbon).

For HPLC and AMS analysis, at least 8 of the 12 quality control samples were within ±0% of the true value for high (15 dpm/ml), medium (0.8 dpm/ml), and low (0.37 dpm/ml). These acceptances were within the normally accepted range for HPLC and AMS analysis (Lappin et al., 2011). The LOQ for β-sitosterol after HPLC separation and AMS analysis was defined as 15% above the pMC value for liquid paraffin, which was approximately 0.1 pg β-sitosterol/ml plasma (the value varies slightly from sample to sample due to fraction volume). The LOQ for total 14C measurements was 15 pg Eq/ml. The higher LOQ for total 14C measurement compared with that for unchanged β-sitosterol was due to the higher amounts of interfering background carbon in the former samples.

Pharmacokinetics and Statistics. Pharmacokinetic parameters were calculated using WinNonLin Professional version 5.1 using calculated doses administered for each subject based upon analysis of the dosing apparatus after...
dosing (Tables 1 and 2). Absolute oral bioavailability was calculated from eq.
2, and the metabolic turnover of β-sitosterol was calculated as the product of
clearance and the mean base-level concentration of total β-sitosterol at steady
state.

\[
F = \left( \frac{\text{AUC}_{\text{oral}}}{\text{AUC}_{\text{iv}}} \right) \left( \frac{\text{Dose}_{\text{oral}}}{\text{Dose}_{\text{iv}}} \right)
\]

Results

All of the volunteers enrolled in the study completed blood donations
until the final sample time with no dropouts due to adverse
events related to the study for all individual timings. This resulted in
a complete sample set for further analysis and calculations, with
the exception of the urine samples for one subject for the day 56 and day
63 samples.

The mean total β-sitosterol plasma concentration, measured by gas
chromatography-mass spectrometry (GC-MS), was 2.83 μg/ml (38%
coefficient of variation), as shown in Fig. 2. Plasma concentrations
based on total 14C measurements after intravenous dosing ranged from
2.7 to 306 pg/ml. For the oral dose, all of the samples for total
14C analysis were below the LOQ. After intravenous dosing, plasma
concentrations of [14C]β-sitosterol ranged from 1.8 to 283 pg/ml with
measurements at all time points. After the oral dose, plasma concentra-
tions of [14C]β-sitosterol ranged from 0.1 pg/ml (the LOQ) to 1.5
pg/ml with the last measurable samples collected after 528 h. Data for
total 14C after the intravenous dose and β-sitosterol after intravenous
and oral doses are plotted in Fig. 3. Pharmacokinetic parameters after
intravenous administration of [14C]β-sitosterol, displayed in Table 1,
show that the clearance was relatively low at 85 ml/h and that the
volume of distribution also was low at 46.3 L. Half-life between 24
and 528 h after dosing (t1/2) was 89 h, and the half-life in the second
phase from 528 h after dosing to the end of the study (t1/2) was 372 h.
The metabolic turnover was 5.8 mg of β-sitosterol per day, and the
absolute oral bioavailability was very low with a mean of 0.41%.

Discussion

The method described in this report used a 14C isotopic tracer and
ultrasensitive AMS technology to measure very low concentrations of

Table 1

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<th>t1/2,β</th>
<th>CL</th>
<th>V</th>
<th>Vss</th>
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<td>1.8</td>
<td>0.19</td>
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[14C]β-sitosterol after oral or intravenous administration. To measure
clearance and metabolic turnover, an isotopic label is necessary to
distinguish the pulse dose from the already existing background
plasma concentration of β-sitosterol that arises through dietary input.
The turnover of sterols has been measured in the past using 13C-
labeled compounds, but because of the 14C background (1.1% natural
abundance) the amounts of [13C]sterol that have to be administered
are relatively high and may perturb the pre-existing pools within the
body (Ostlund and Matthews, 1993). In the 1970s, [3H]β-sitosterol
was used as a tracer in human studies, where 21.3 μCi (855 kBq) per
day was administered for 83 days (Salen et al., 1970). These levels are
unlikely to be allowed by ethical, regulatory, and clinical practice
standards nowadays. By using AMS as the analytical technique, it was
possible to administer very low levels of radioactivity (100 nCi), thereby
avoiding the need for dosimetry studies (Lappin and Garner,
2004). In addition, there also are questions of tritium exchange with
this isotope and significant kinetic isotope effects (Wood et al., 2010).

Through the use of AMS analysis, the doses of [13C]β-sitosterol were
minimized (approximately 3 to 4 µg per subject), the levels of radioactivity were minimal (approximately 100 nCi), and yet the assay
sensitivity (approximately 0.1 pg/ml) was sufficient to obtain a good
estimate of the area under the plasma concentration-time curve over
the duration of the study. At these low levels, it was possible to
formulate the intravenous dose in physiological saline, thereby elimi-
nating possible artifacts that sometimes occur due to the formulation
when administering lipophilic compounds (Wheeler et al., 2004).

A total of 0.63% of the 14C administered was recovered in pooled
urine over the 0- to 1512-h period from the subjects administered an
intravenous dose of [14C]β-sitosterol. A total of 0.08% of the 14C
administered was recovered in pooled urine over the 0- to 1512-h
period from the subjects administered an oral dose of [14C]β-sit-
osterol. Urine therefore was not a significant route of elimination for
β-sitosterol or its metabolites.

Log₁₀-linear plots of the mean plasma [14C]β-sitosterol concentra-
tions versus time for both oral and intravenous administrations are
shown in Fig. 3. For the oral administration, exponential elimination of
β-sitosterol from plasma was apparent, although the concentrations
measured were approaching the LOQ (0.1–0.4 pg/ml), and therefore
the data have to be viewed with some caution. For the intravenous
administration, polyexponential elimination was apparent for up to
day 42, then elimination seems to become exponential (i.e., linear on
the semilogarithmic plot). This shape of the curve is consistent with
the expected start of the elimination phase and confirms previously
published reports on the human disposition of β-sitosterol (Salen
et al., 1970).

An unusual finding in the current study was the V shape of the
plasma concentration-time plot for the intravenous administration
over the first 24 h (Fig. 3, inset). This V-shaped plasma concentra-
tion-time curve (Cmin = ~7.6 pg/ml at 1 h, Cmax = ~283 pg/ml at 24 h)
is unusual and has not been reported previously; however, previous studies with β-sitosterol did not take samples at these early time points (Salen et al., 1970), and therefore no good comparison can be made.

After 24 h, the concentration continues to decline to the final sampling time of 1512 h. The effect was seen with each individual subject and with both parent β-sitosterol and total 14C measurements excluding analytical errors. We have no plausible explanation for this phenomenon yet, but it could be related to, for example, solubility changes upon entering the bloodstream due to the poor sterol solubility or binding and distribution phenomena in the blood via erythrocytes or lipoproteins. Although speculative, the proposition that the V-shaped curve was due to the distribution of sitosterol in blood components subsequently removed during the preparation of plasma is consistent with the absence of the V-shaped curve in the oral dose, where the compound had more opportunity to equilibrate.

The total 14C plot (Fig. 3) tracks that of parent sitosterol from the first to the last sampling time point, including the period of the V-shaped curve. This shows that essentially the only circulating chemical species was β-sitosterol, without any metabolites. This apparent lack of metabolism also is a new finding to this study. It is important to realize that this does not mean that β-sitosterol is not metabolized; metabolites might be formed but did not appear in any significant concentrations in plasma. However, they were not excreted via urine, because little 14C was detected in this matrix.

The calculated kinetic parameters (Tables 1 and 2) are very close to those reported previously. As a result of the polyexponential elimination, the half-life ($t_{1/2}$) for the intravenous administration was calculated as $t_{1/2}$...
Values for plasma clearance and volume of distribution for β-sitosterol have not been reported previously, although interestingly the study of Salen et al., 1970 (Salen et al., 1970) included an intravenous administration and so these parameters could have been calculated. Clearance of β-sitosterol from plasma is very low with a mean value of approximately 85 ml/h. Given that β-sitosterol is lipophilic, the expectation was that a significant proportion would be sequestrated in body fat, thus resulting in a large volume of distribution. However, the mean value determined in the current study was just approximately 46 L, close to that of body plasma. The low oral bioavailability is due to either poor absorption at the enterocyte level, very efficient efflux mechanisms, or very high first-pass hepatic extraction. This study could not confirm significant metabolic turnover, excluding that option. Efficient efflux would fit with the activity of the heterodimeric ATP binding cassette efflux transporter ABCG5/G8, which is expressed at the apical site of both enterocytes and hepatocytes. These transporters are known to preferentially handle xenosterols versus cholesterol, resulting in a net efflux of plant sterols into the gut lumen or into bile (Kidambi and Patel, 2008).

The total β-sitosterol plasma concentration was measured using GC-MS for each subject during the conduct of the study. The values thereby obtained represented a “baseline” concentration of β-sitosterol derived from the diet. This baseline level can be assumed to be in steady state; that is, the amount of sitosterol absorbed from the diet equals the amount cleared from the plasma. The baseline concentration depends upon the mass of β-sitosterol entering and leaving the plasma per day, which is known as the turnover rate. The turnover rate is calculated from the product of plasma clearance and the baseline concentration and was found to have a mean value of 5.8 mg/day, which is consistent with that previously reported of 6 to 14 mg/day (Salen et al., 1970), although it is a little low. In the current study, the absolute oral bioavailability was found to be 0.41%. If a mean of 5.8 mg of β-sitosterol was absorbed per day, then the mean total dietary load of β-sitosterol was 1414 mg/day. In the study by Salen et al. (1970), sitosterol dietary input was estimated at between 125 and 6488 mg/day.

In conclusion, the kinetics of β-sitosterol in the present study were consistent with those reported previously, although there were new findings for the shape of the early plasma concentration-time curve, clearance, and volume of distribution. The turnover of sitosterol was 5.8 mg/day, which is consistent with a dietary load of approximately 1400 mg of sitosterol per day. The absolute bioavailability of β-sitosterol was very low (mean 0.41%), and there was no evidence of any metabolites present in plasma.

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Authorship Contributions

Participated in research design: Duchateau, Cochrane, Windebank, Burian, Müller, Zeitzlinger, and Lappin.

Conducted experiments: Sanghera, Burian, Müller, and Zeitzlinger.

Performed data analysis: Duchateau, Herudinzka, Sanghera, and Lappin.

Wrote or contributed to the writing of the manuscript: Duchateau, Cochrane, and Lappin.

References


