Evaluation of the use of β-sitostanol as a nonabsorbable marker for quantifying cholesterol absorption

James G. Terry, Barbara L. McGill, and John R. Crouse III[1]
Department of Internal Medicine, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27157

Summary For over a decade investigators have quantified cholesterol absorption by comparison of dietary intake and fecal excretion of isotopic cholesterol with that of β-sitosterol as a "nonabsorbable" marker. However, β-sitosterol might not be ideal due to its potential for absorption. We therefore carried out two studies to evaluate a new marker with less potential for absorption, [^{3}H]β-sitostanol. In the first study (Study I, n = 22), we compared absorption of [^{3}H]β-sitostanol and [^{14}C]β-sitosterol in a simultaneous dual-label continuous feeding ("phytosterol absorption") experiment. We observed a consistently higher ratio of [^{3}H]β-sitostanol/[^{14}C]β-sitosterol in the stool relative to diet on the first day of fecal collection (6.1% ± 3.2% loss of [^{3}H]β-sitosterol, range 3–12%), but thereafter, the ratio in stool was similar to that in diet. In Study II (n = 23), we compared cholesterol absorption directly using [^{3}H]β-sitosterol and [^{14}C]cholesterol, and, separately, [^{3}H]β-sitostanol and [^{14}C]cholesterol. We found that mean absorption between the two methods was similar (45% ± 11% versus 44% ± 10%, respectively, P difference = 0.40), and the two methods correlated well with one another (r = 0.83) when samples from all available days were used. Variability between the two methods was greater in individuals who absorbed more than 40% of cholesterol. Cholesterol loss on day 2 estimated from use of β-sitostanol as a nonabsorbable marker was predictive of absorption using ratios from days 4–6 (r = 0.80). These results suggest that, for the majority of subjects, β-sitosterol is a valid nonabsorbable marker for cholesterol absorption.—**Terry, J. G., B. L. McGill, and J. R. Crouse III.** Evaluation of the use of β-sitostanol as a nonabsorbable marker for quantifying cholesterol absorption. *J. Lipid Res.* 1995. **36:** 2267–2271.

Supplementary key words cholesterol • absorption • phytosterols

Cholesterol absorption has recently re-emerged as a potentially important contributor to the regulation of cholesterol metabolism (1, 2). Several methods have been advocated for quantifying absorption including the method of Zilversmit and Hughes (3, 4), the single dose isotopic diet/fecal ratio method (5), and the continuous feeding isotopic diet/fecal ratio method (6). The latter two methods depend on comparison of the excretion of labeled cholesterol with that of a labeled nonabsorbable marker, traditionally β-sitosterol.

Although poorly absorbed, it is recognized that about 5% of β-sitosterol is absorbed (7, 8), and in some patients up to 30% absorption can occur (9). β-Sitostanol, on the other hand, is thought to be nonabsorbable (10).

We therefore compared β-sitosterol and β-sitostanol as markers for cholesterol absorption in human beings.

METHODS

Subjects and diet

We carried out two studies of absorption in 45 healthy individuals. In the phytosterol absorption study (Study I) 22 volunteers were fed radiolabeled [^{14}C]β-sitosterol and [^{3}H]β-sitostanol daily for 6 days and stools were collected. Fecal ratio of isotopes was compared to that in the diet.

In the cholesterol absorption comparison study (Study II) 23 volunteers underwent two tests of cholesterol absorption separated by 1 month in which ^{14}C cholesterol and either [^{3}H]β-sitosterol or [^{3}H]β-sitostanol were dosed. For the two cholesterol absorption studies, 8 patients consumed ad lib diets at home and 15 consumed diets prepared in the Bowman Gray GCRC diet kitchen. Metabolic diets were consumed for 7–11 days and were eucaloric providing 33% of calories from fat, 48% from carbohydrate, and 19% from protein along with 300 mg cholesterol daily. Plasma concentrations of lipids and lipoproteins were measured in the CDC standardized Lipid Laboratory at Bowman Gray School of Medicine (11).

Absorption studies

[4-^{14}C]cholesterol (52 mCi/mmol) and custom synthesized [4-^{14}C]β-sitosterol (55 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, IL. [22,23-^{3}H]β-sitosterol (77 Ci/mmol) and [5,6-^{3}H]β-sitostanol (47 Ci/mmol) were custom synthesized by New England Nuclear, Boston, MA. Both tritiated β-sitosterol and β-sitostanol were produced from tritiated stigmasterol by sequential hydrogenation. Unlabeled β-sitosterol and stigmasterol were purchased form Sigma Chemical Co., St. Louis, MO. Five alpha-cholestane was purchased from Matreya, Inc., Pleasant Gap, PA. Unlabeled β-sitostanol was a gift from Dr. Margo Denke. Unlabeled β-sitostanol was > 90% pure by GLC (Hewlett-Packard 5890A) on a 15 M J&W DB17 column, and retention time was consistent with previous reports (12).

Isotopic purity of radiolabeled sterols was determined by HPLC (ISCO 2350) using a C-18 column (SGE) with isocratic buffer system (acetonitrile–isopropanol 1:1) and absorbance detection at 215 nm. All radiolabeled sterols were HPLC co-chromatographed with cold carriers and fractions were collected as previously described (13). Radioactivity was determined by liquid scintillation counting (Packard CA 2100 LSC). The lack

Abbreviations: GLC, gas–liquid chromatography; HPLC, high performance liquid chromatography; BMI, body mass index; TC, total cholesterol; TG, triglyceride; LDL, low density lipoprotein.

[1]To whom reprint should be addressed.

of a double bond in β-sitostanol prevented absorbance detection at 215 nm, therefore, an additional step using GLC was required. For this, unlabeled β-sitostanol standard was run on HPLC and fractions were collected. The fractions were then subjected to GLC to determine the retention time of β-sitostanol in our HPLC system.

[^{14}C]cholesterol and [^{14}C]β-sitosterol were each > 98% isotopically pure. [^{3}H]β-sitosterol and [^{3}H]β-sitostanol were < 90% isotopically pure and required preparative purification by HPLC. Subsequent HPLC showed that > 96% of the activity from both [^{3}H]β-sitosterol and [^{3}H]β-sitostanol eluted under a single peak consistent with standards.

For Study I a mixture of 0.10 μCi[^{14}C]β-sitosterol and 0.31 μCi[^{3}H]βITC-NewBaskerville"-sitostanol was fed daily to ambulatory volunteers for 6 days (D1–6). Participants were instructed to collect one stool sample per day by the outpatient method of Hoffman, LaRusso, and Hoffman (14) either on D2–6 (n = 10) or only on D4–6 (n = 12).

Duplicate stool samples were saponified and the hexane-extracted neutral lipids were dried onto paper cones prior to combustion by Packard 306 Oxidizer as previously described (15). Samples of administered isotope were included as controls and frequent recovery checks were performed (recovery > 95%).

For quantification of β-sitosterol absorption the formula adopted was:

$$\text{β-sitosterol absorption} = 1 - \frac{\text{fecal β-sitosterol/β-sitostanol}}{\text{dietary β-sitosterol/β-sitostanol}}$$

This formula is a modification of one that has been shown to be reliable for quantifying cholesterol absorption in stool samples from D4–6; differential disappearance of isotope on D2 and D3 is characterized as isotope loss rather than absorption because loss could also occur through isotope exchange.

Study II participants underwent two studies of cholesterol absorption separated by 1 month in which they received either 0.11 μCi [^{14}C]cholesterol and 0.29 μCi [^{3}H]β-sitosterol or alternatively 0.10 μCi [^{14}C]cholesterol and 0.29 μCi [^{3}H]β-sitostanol for 6 consecutive days. Subjects were assigned at random to their sequence of studies and the sample analyst was blinded to the order of assignment.

Daily loss of isotope as well as means for D4–6 were used to determine β-sitosterol absorption or cholesterol absorption as previously described (6). Means and standard deviations and associations were compared between periods and groups using paired *t*-tests and correlation statistics.

RESULTS

Study I

Participants in Study I were 67 ± 6 years of age (range 49–75) and 50% female; they had body mass index (BMI) of 27 ± 4 (range 22–38). They were slightly hypercholesterolemic overall with plasma concentration of total cholesterol (TC) of 236 ± 31 mg/dl (range 186–298 mg/dl) and low density lipoprotein cholesterol (LDL) of 154 ± 22 mg/dl (range 122–203 mg/dl). Plasma concentration of triglycerides (TG) for Study I participants was 218 ± 161 mg/dl (range 69–835 mg/dl).

Twenty-two participants completed Study I that compared [^{14}C]β-sitosterol and [^{3}H]β-sitostanol absorption (**Fig. 1**). All participants were asked to collect stool samples on D4–6 of Study I while a subset of participants (n = 10) was requested to collect samples on D2 and D3 as well. Mean % absorption of β-sitosterol on D4, D5, and D6 was 2.5% ± 3.9%, -1.3% ± 3.6%, and -1.8%, ± 4.6% respectively (mean % absorption D4–6 = -0.1% ± 2.7%, and did not differ from 0% absorption). In those subjects who submitted samples from D2 and D3, preferential loss of β-sitosterol from stool on day 1 was reflected in a ratio of fecal/diet [^{3}H]β-sitostanol/[^{14}C]β-sitosterol that was consistently > 1.0 in stool from D2 (per cent loss [^{14}C]β-sitosterol on D2 = 6.1% ± 3.2%, range 3% to 12.5%). On D3 per cent loss of [^{14}C]β-sitosterol was 0.3% ± 7.0%.

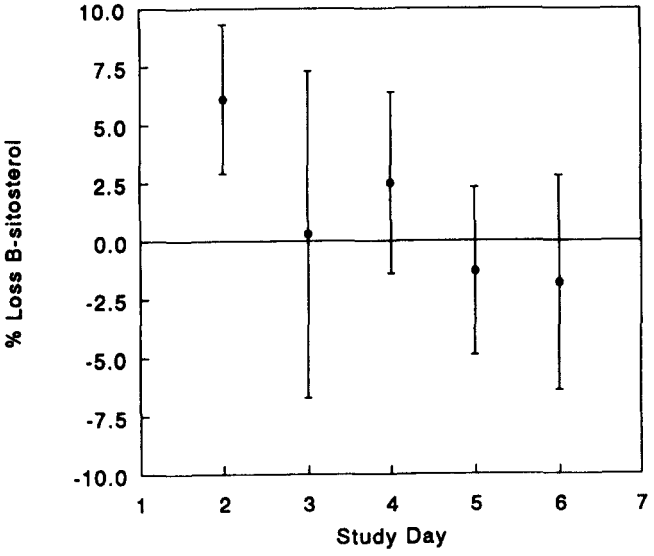

Fig. 1. Mean ± standard deviation percent loss of β-sitosterol relative to β-sitostanol for participants on days 2–6 of phytosterol absorption study (Study I). Days 4–6, n = 23; days 2–3, n = 10. Positive "% loss" indicates greater loss of β-sitosterol than β-sitostanol.

Study II

Study II participants were 65 ± 8 years of age (range 35–73) and were 52% female; they had BMI of 27 ± 4 (range 20–38). These subjects had overall lipids similar to the previous group with TC 226 ± 39 mg/dl (range 159–290 mg/dl), LDLC 151 ± 32 mg/dl (range 85–198 mg/dl), and TG 179 ± 96 mg/dl (range 76–506 mg/dl).

In Study II, cholesterol absorption was compared in 23 individuals using [^{3}H]β-sitostero l, and, on a different occasion, [^{3}H]β-sitostanol as nonabsorbable markers for ^{14}C cholesterol absorption. Comparisons are presented in **Table 1** and the correlation between the two estimates of absorption is illustrated in **Fig. 2.** Overall, there was no consistent difference in absorption as measured by the two methods. Variability was greater in individuals who absorbed more than 40% of cholesterol, but even in these patients, cholesterol absorption measured by the two methods was similar. Three of 23 subjects showed a statistically significant difference in cholesterol absorption using β-sitosterol compared to β-sitostanol (subjects number 8, 9, and 15). There was no consistent pattern to these differences. Overall, the correlation between the two cholesterol absorption methods on D4–6 was $r = 0.76$, $P < 0.0001$. When results from all available days were used (D2–6), the correlation was $r = 0.83$, $P < 0.0001$. Cholesterol loss on D2 as estimated from use of β-sitostanol as a nonabsorbable marker was predictive of absorption using isotope ratios of β-sitosterol and cholesterol from D4–6 ($r = 0.80$, $P < 0.0001$, n = 17). D2 cholesterol loss estimated from use of β-sitosterol as a nonabsorbable marker correlated less well with the published method ($r = 0.56$, $P < 0.02$, n = 18) as previously suggested (6).

Availability of two highly correlated indices of cholesterol absorption allowed us to derive a stable estimate of cholesterol absorption from the 23 individuals. No association was observed between per cent cholesterol absorption and age, or plasma TC or LDLC; statistically significant (negative) associations were found between cholesterol absorption and BMI ($r = -0.46$, $P < 0.03$) and plasma TG ($r = -0.60$, $P < 0.003$).

TABLE 1. Percent cholesterol absorption (mean ± SD) by phytosterol marker for individual participants in cholesterol absorption comparison (Study II)

Subject	β-Sitosterol	β-Sitostanol	*P* Value[a]
1	37±5	36±9	0.932
2	34±4	25±4	0.085
3	52±8	59±9	0.439
4	36±2	31±7	0.425
5	24±6	27±5	0.574
6	42±10	41±9	0.924
7	45±3	42±2	0.317
8	45±1	56±5	0.046
9	52±1	36±5	0.039
10	58±4	43±2	0.069
11	59±3	62±1	0.159
12	40±1	44±4	0.165
13	59±7	60±3	0.808
14	46±2	46±2	0.999
15	72±3	54±3	0.014
16	45±6	44±3	0.844
17	58±9	59±8	0.910
18	36±2	42±4	0.328
19	38±1	41±1	0.732
20	36±8	43±5	0.457
21	34±5	35±13	0.925
22	42±3	37±5	0.259
23	43±2	41±2	0.944
All subjects	45±11	44±10	0.404

[a]Significance level by Student's *t*-test.

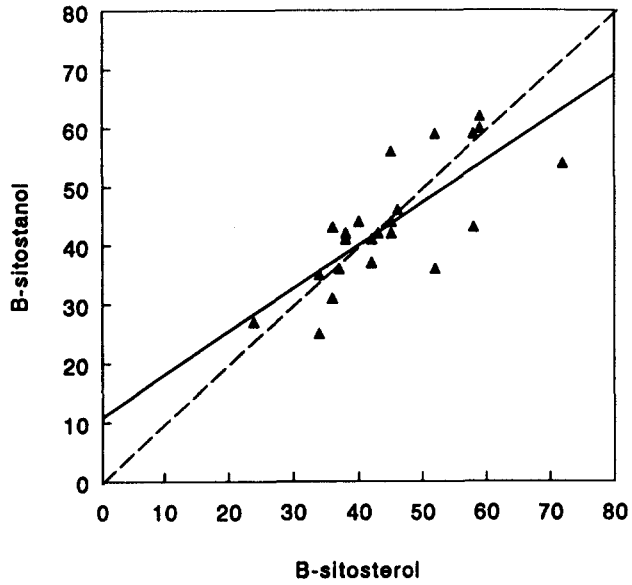

Fig. 2. Percent cholesterol absorption (days 4–6) measured twice in the same 23 individuals using β-sitosterol compared to β-sitostanol as nonabsorbable marker. Solid line, best fit line (equation: y = 0.729x + 10.832); dashed line, line of identity.

DISCUSSION

Several methods have been proposed for measuring cholesterol absorption. The most commonly used of these at present include the Zilversmit method (3) that involves simultaneous intravenous and oral administration of radiolabeled cholesterol (4), the fecal isotope ratio method that involves single dosing of individuals with radiolabeled cholesterol and β-sitosterol with complete fecal collections for 7–10 days thereafter (5), and the continuous oral isotope feeding ratio method that involves continuous dosing over 7 days with radiolabeled cholesterol and β-sitosterol and collection of casual aliquots of stool for analysis (6).

The advantage of the Zilversmit method (3) is its ease of accomplishment, requiring only sampling of plasma after administration of isotope for quantification of cholesterol absorption. The disadvantage of the method, as well as of the single dose ratio method, is that it measures only the cholesterol absorption of the meal with which it is fed. The only method that permits quantification of cholesterol absorption over several days and that provides a mean and standard deviation for cholesterol absorption over that period of time is the continuous isotope feeding method. This method also has an advantage over the single dose isotope ratio method in that casual (not complete) stool collections adequately quantify cholesterol absorption.

A theoretical limitation of the methods using radiolabeled β-sitosterol is the potential for its absorption. Little is known about the variability of β-sitosterol absorption in the population. Approximately 5% of β-sitosterol is thought to be absorbed (7, 8), but certain individuals have been identified who absorb up to 30% of the β-sitosterol in their diets (9). Wide variability among individuals in absorption of β-sitosterol might theoretically prevent accurate measurement of cholesterol absorption. Because only 40–60% of cholesterol is absorbed, small differences in absorption of the "nonabsorbable" marker could lead to larger differences in apparent cholesterol absorption. To the extent that β-sitosterol is absorbed, apparent absorption of cholesterol would be less than "true" percent absorption.

β-Sitostanol is recognized as a nonabsorbable phytosterol (10) and has been used as a nonabsorbable marker in one study in which stable isotopes were fed and fecal collections were obtained (16). The latter method requires isolation and analysis of individual sterols and their metabolites from stool. Use of radiolabeled β-sitostanol as a nonabsorbable marker for cholesterol absorption is attractive as *1)* it is not absorbed; and *2)* complex analysis of stool is not necessary for its quantification.

Accordingly, these studies were initiated to compare β-sitosterol and β-sitostanol with one another for their utility as nonabsorbable markers for cholesterol absorption. In Study I we observed a differential loss of β-sitosterol from the stool compared to β-sitostanol on the first day and extending in some patients to the second day of administration. By the 3rd to 4th day, the isotope ratio in the stool was very similar to that fed, suggesting that in most patients β-sitosterol should be a valid nonabsorbable marker for cholesterol absorption after at least 4 days of isotope feeding. The mechanism for the differential loss of β-sitosterol on day 1 is unclear. There are two possibilities. First, β-sitosterol may exchange with sterol in mucosal cells to a different extent than β-sitostanol and may be lost from the lumen through this mechanism and not appear in the stool until the second or third day of β-sitosterol feeding. Second, β-sitosterol may be absorbed and resecreted very rapidly. The latter is possible in view of the relatively rapid turnover of intravenously administered β-sitosterol as described by Salen, Ahrens, and Grundy (7).

When we quantified absorption of cholesterol through use of β-sitosterol, and, separately, β-sitostanol as nonabsorbable markers, we could show no marked differences between the two isotopes. Although for most subjects cholesterol absorption estimated by the two markers was comparable, eleven individuals had higher apparent absorption with β-sitostanol as the nonabsorbable marker (statistically significant in one individual) and five individuals had higher apparent absorption with β-sitosterol (statistically significant in two

individuals). No consistent pattern of difference was observed between cholesterol absorption measured by either nonabsorbable marker when comparing results from days 4–6. Overall, the difference in cholesterol absorption measured by β-sitosterol and β-sitostanol (mean %-difference 0.4% ± 21.1%) in the present study was similar to that expected for absorption studies separated by 1 month using the same nonabsorbable isotopic marker (2.8% ± 14.2% using [^{3}H]β-sitosterol) (15).

Despite their similar overall results for cholesterol absorption, apparent differences exist between β-sitosterol and β-sitostanol. Cholesterol absorption estimated by isotopic β-sitostanol on day 2 was more reflective of that measured on days 4–6 than was cholesterol absorption as estimated by isotopic β-sitosterol itself on day 2 (r = 0.80 versus 0.56, respectively). This observation, along with the findings of Study I, confirms the notion that isotopic β-sitosterol is only valid as a cholesterol absorption marker after several days of feeding to allow equilibrium in the intestine (6).

In summary, these data support the continued use of β-sitosterol as a nonabsorbable marker for cholesterol absorption. On the other hand it is evident that there are differences in the metabolism of β-sitosterol and β-sitostanol, particularly on the first day of feeding, and uncommon individual patients have previously been shown to absorb β-sitosterol (9). For this reason, we believe that it is also valid to use β-sitostanol as a nonabsorbable marker for cholesterol absorption ■

The authors wish to acknowledge the help of GCRC research dietician Jane Stegner, MS, RD, for the preparation of metabolic diets, and Martha D. Wilson, PhD, for assistance with HPLC purification of isotopic compounds. This study was supported by an unrestricted grant from Pfizer Pharmaceuticals, and by the General Clinical Research Center of the Bowman Gray School of Medicine, grant number M01 RR-07122.

Manuscript received xx May 1995 and in revised form 13 July 1995.

REFERENCES

1. Wilson, M. D., and L. L. Rudel. 1994. Review of cholesterol absorption with emphasis on dietary and biliary cholesterol. *J. Lipid Res.* **35:** 943–955.
2. Spady, D. K., L. A. Woollett, and J. M. Dietschy. 1993. Regulation of plasma LDL-cholesterol levels by dietary cholesterol and fatty acids. *Annu. Rev. Nutr.* **13:** 355–381.
3. Zilversmit, D. B., and L. B. Hughes. 1974. Validation of a dual-isotope plasma ratio method for measurement of cholesterol absorption in rats. *J. Lipid Res.* **15:** 465–473.
4. Samuel, P., J. R. Crouse, and E. H. Ahrens, Jr. 1978. Evaluation of an isotope ratio method for measurement of cholesterol absorption in man. *J. Lipid Res.* **19:** 82–93.
5. Quintão, E., S. M. Grundy, and E. H. Ahrens, Jr. 1971. An evaluation of four methods for measuring cholesterol absorption by the intestine in man. *J. Lipid Res.* **12:** 221–232.
6. Crouse, J. R., and S. M. Grundy. 1978. Evaluation of a continuous isotope feeding method for measurement of cholesterol absorption in man. *J. Lipid Res.* **19:** 967–971.
7. Salen, G., E. H. Ahrens, Jr., and S. M. Grundy. 1970. Metabolism of β-sitosterol in man. *J. Clin. Invest.* **49:** 952–967.
8. Sylven, C., and B. Borgström. 1969. Absorption and lymphatic transport of cholesterol and sitosterol in the rat. *J. Lipid Res.* **10:** 179–182.
9. Salen, G., S. Shefer, L. Nguyen, G. C. Ness, G. S. Tint, and V. Shore. 1992. Sitosterolemia. *J. Lipid Res.* **33:** 945–955.
10. Vanhanen, H. T., S. Blomqvist, C. Ehnholm, M. Hyvönen, M. Jauhiainen, I. Torstila, and T. A. Miettinen. 1993. Serum cholesterol, cholesterol precursors, and plant sterols in hypercholesterolemic subjects with different apoE phenotypes during dietary sitostanol ester treatment. *J. Lipid Res.* **34:** 1535–1544.
11. King, J. M., J. R. Crouse, J. G. Terry, T. M. Morgan, B. J. Spray, and N. E. Miller. 1994. Evaluation of effects of unmodified niacin on fasting and postprandial plasma lipids in normolipidemic men with hypoalphalipoproteinemia. *Am. J. Med.* **97:** 323–331.
12. Miettinen, T. A. 1982. Gas–liquid chromatographic determination of fecal neutral sterols using a capillary column. *Clin. Chim. Acta.* **124:** 245–248.
13. Parks, J. S., and J. R. Crouse. 1992. Reduction of cholesterol absorption by dietary oleinate and fish oil in African green monkeys. *J. Lipid Res.* **33:** 559–568.
14. Hoffman, N. E., N. F. LaRusso, and A. F. Hofmann. 1973. An improved method for faecal collection: the faecal field-kit. *Lancet.* **1:** 1422–1423.
15. Hainer, J. W., J. G. Terry, J. M. Connell, H. Zyruk, R. M. Jenkins, D. L. Shand, P. J. Gillies, K. J. Livak, T. L. Hunt, and J. R. Crouse III. 1994. Effect of the acyl-CoA:cholesterol acyltransferase inhibitor DuP 128 on cholesterol absorption and serum cholesterol in humans. *Clin. Pharmacol. Ther.* **56:** 65–74.
16. Lütjohann, D., C. O. Meese, J. R. Crouse, and K. von Bergmann. 1993. Evaluation of deuterated cholesterol and deuterated sitostanol for measurement of cholesterol absorption in humans. *J. Lipid Res.* **34:** 1039–1046.