Evaluation of deuterated cholesterol and deuterated sitostanol for measurement of cholesterol absorption in humans

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Abstract The continuous isotope feeding method of Crouse and Grundy (1978. *J. Lipid Res.* **19:** 967–971) for measurement of dietary cholesterol absorption has been modified by using markers labeled with stable isotopes ([2,2,4,4,6-$^2H_5$]cholesterol or [25,26,26,26,27,27,27-$^2H_7$]cholesterol or [26,26,26,27,27,27-$^2H_6$] cholesterol and [5,6,22,23-$^2H_4$]sitostanol) quantified by gas–liquid chromatography–selected ion monitoring. Tracing of the isotope distribution of the authentic markers and after their intestinal passage, including the microbiological products (coprostanol and coprostanone) revealed stability of the labels. The new method was evaluated in six monkeys on two occasions by comparison with the original method using radioactively labeled cholesterol and sitosterol. The results obtained by the two different methods were in excellent agreement, and absorption ranged from 49% to 73% (mean 60%) for the stable isotope method and from 51% to 69% (mean 62%) for the radioactive method. The coefficient of variation of cholesterol absorption in animals ranged from 3.9% to 15.1% (mean 7.1%) for stable isotopes and 1.9% to 13.6% (mean 5.7%) for radioactive isotopes. In twelve subjects cholesterol absorption was measured by the new method from total fecal samples frozen immediately and compared to results obtained from small fecal aliquots ($\approx$ 1 g) sent by ordinary mail to the laboratory. A significant correlation of cholesterol absorption between the two different sample handlings was obtained (r = 0.981, $P < 0.001$). In addition, measurement of cholesterol absorption twice in seven volunteers 2 weeks apart revealed identical results. Thus, the new method is extremely safe and reproducible without radioactive exposure to the subjects and laboratory staff and can be used on women of child-bearing age.—**Lütjohann, D., C. O. Meese, J. R. Crouse III, and K. von Bergmann.** Evaluation of deuterated cholesterol and deuterated sitostanol for measurement of cholesterol absorption in humans. *J. Lipid Res.* 1993. **34:** 1039–1046.

Supplementary key words stable isotopes • mass spectrometry • selected ion monitoring

Measurement of cholesterol absorption gives important information on the relationship between diet and plasma cholesterol. For this purpose, a variety of different methods have been developed for estimating absorption of cholesterol in humans (1–12). Most of these methods use radioactive markers ([^{14}C]- or [^{3}H]cholesterol and/or [^{14}C]- or [^{3}H]sitosterol) or intestinal intubation. Neither the radioactive tracer methods nor the intestinal intubation procedures are available for routine and repeated measurements of cholesterol absorption. Crouse and Grundy (13) used a simplified continuous feeding method with a low dose of radioactivity, which has been used extensively by Miettinen and coworkers (14–18). However, the use of radioactive tracers for this purpose is not allowed in all countries, and it cannot be used in children or young females. In the present study we describe a continuous stable isotope marker feeding method for measurement of cholesterol absorption. This new method utilizes deuterium-labeled [^2H_x]cholesterol (x = 5, 6, or 7) and [$^2H_4$]sitostanol as the nonabsorbable marker. Estimates of cholesterol absorption are calculated from fecal samples analyzed by gas–liquid chromatography–selected ion monitoring (GLC–SIM). The results show that deuterated cholesterol and deuterated sitostanol are reliable markers for measuring cholesterol absorption in humans under various experimental conditions; they are applicable for studies in children, in women of child-bearing age, and can be repeated as often as necessary without hazard to the subject.

MATERIALS AND METHODS

Chemicals

Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO), 5α-cholestane from Serva Feinbiochem-

Abbreviations: TMSi, trimethylsilyl; GLC, gas–liquid chromatography; GLC–SIM, gas–liquid chromatography–selected ion monitoring.

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ica (Heidelberg, Germany), coprostanol and coprostanone from Steraloids, Inc. (Wilton, NH), and sitostanol from Delalande Arzneimittel GmbH (Köln, Germany). Purities of the sterols were checked by gas-liquid chromatography (GLC) as their trimethylsilyl (TMSi)- ether derivatives and purities of the sterols were better than 98%, except of the sitostanol preparation which contained 8% of campestanol. All other chemicals and solvents used for sample preparation and derivatization were of grade recommended in the literature.

Synthesis of [2,2,4,4,6-$^2H_5$]cholesterol was performed according to a previously published method (19). Sixty mg of [25,26,26,26,27,27,27-$^2H_7$]cholesterol was kindly provided by L. Siekmann (Department of Clinical Biochemistry, University of Bonn, Germany), [26,26,26,27,27,27-$^2H_6$]cholesterol was purchased from Medical Isotopes, Inc. (Concord, NH). The isotopic compositions of the compounds determined by GLC-SIM are given in **Table 1.** [5,6,22,23-$^2H_4$]sitostanone was synthesized by oxidation of [5,6,22,23-$^2H_4$]sitostanol according to Brown, Gard, and Lin (20). A yield of 88% was obtained after recrystallization.

Synthesis of [5,6,22,23-$^2H_4$]sitostanol

A modified procedure of a previously described hydrogenation reaction (21) was used to reduce stigmasterol to tetradeuterated sitostanol. Briefly, stigmasterol (15.0 g, 36.3 mmol, purity > 98%, Fluka, Neu-Ulm, Germany) was dissolved under an atmosphere of argon in a rapidly stirred suspension of 10% Pd/C (4.0 g, Aldrich-Chemie GmbH, Steinhein, Germany, product No. 20569-9) in 3000 ml of ethyl acetate. The apparatus was then flushed with deuterium gas (99.7% $^2H_2$) and the deuteration

TABLE 1. *Isotope distribution of deuterated authentic markers and the markers and their metabolites after intestinal transit*

m/z	[$^2H_5$]Cholesterol Authentic	*m/z*	[$^2H_5$]Cholesterol Feces	*m/z*	[$^2H_5$]Coprostanol Feces	*m/z*	[$^2H_5$]Coprostanone Feces
			%				
461	6.20 ± 0.15	461	5.92 ± 0.38	373	6.01 ± 0.15	389	6.12 ± 0.17
462	26.44 ± 0.06	462	27.20 ± 0.27	374	26.30 ± 1.20	390	26.93 ± 0.92
463	44.29 ± 0.36	463	45.12 ± 0.14	375	43.27 ± 0.83	391	43.65 ± 0.72
464	17.59 ± 0.23	464	15.38 ± 0.43	376	16.18 ± 1.07	392	16.29 ± 1.04
465	5.48 ± 0.20	465	6.38 ± 0.31	377	8.54 ± 0.31	393	7.01 ± 0.93
m/z	[$^2H_6$]Cholesterol Authentic	*m/z*	[$^2H_6$]Cholesterol Feces	*m/z*	[$^2H_6$]Coprostanol Feces	*m/z*	[$^2H_6$]Coprostanone Feces
			%				
463	4.12 ± 0.21	463	3.69 ± 0.36	375	4.81 ± 0.42	391	3.79 ± 1.19
464	62.29 ± 0.79	464	64.73 ± 0.17	376	64.03 ± 1.13	392	64.31 ± 0.87
465	24.27 ± 0.93	465	25.60 ± 0.94	377	26.17 ± 0.79	393	25.72 ± 0.34
466	7.72 ± 0.26	466	5.92 ± 0.82	378	4.99 ± 0.16	394	6.19 ± 0.93
m/z	[$^2H_7$]Cholesterol Authentic	*m/z*	[$^2H_7$]Cholesterol Feces	*m/z*	[$^2H_7$]Coprostanol Feces	*m/z*	[$^2H_7$]Coprostanone Feces
			%				
464	3.86 ± 0.04	464	4.03 ± 0.09	376	3.97 ± 0.11	392	3.81 ± 0.07
465	65.39 ± 0.37	465	64.90 ± 0.43	377	64.30 ± 1.43	393	65.02 ± 0.30
466	23.89 ± 0.10	466	22.93 ± 0.17	378	23.18 ± 0.97	394	24.20 ± 0.62
467	6.75 ± 0.45	467	8.14 ± 0.10	379	8.55 ± 0.38	395	6.97 ± 0.13
		m/z	[$^2H_4$]Sitostanol Authentic	*m/z*	[$^2H_4$]Sitostanol Feces		
			%				
		488	7.03 ± 0.07	488	6.82 ± 0.03		
		489	19.42 ± 0.02	489	20.31 ± 0.12		
		490	24.64 ± 0.03	490	27.01 ± 0.63		
		491	23.52 ± 0.27	491	22.36 ± 1.01		
		492	17.24 ± 0.04	492	16.32 ± 0.25		
		493	6.15 ± 0.14	493	7.18 ± 0.18		

Each value represents the average of three measurements ± SD.

proceeded at room temperature and atmospheric pressure over a 5-h period. Thereafter the catalyst was filtered off, and the residue, after evaporation of the solvent, was recrystallized twice from boiling ethanol to give colorless crystals of [5,6,22,23-$^2H_4$]sitostanol in 63% yield; mp 133°C, $[\alpha]^{22}_D$ +22.8 (c= 1.25, $CHCl_3$). The proton decoupled carbon-13 NMR spectrum (not shown) displayed a resonance pattern identical with unlabeled sitostanol, with the exception of missing signals at 26.3, 28.8, 34.5, and 45.0 ppm (referred to TMS as internal standard; solvent $CDCl_3$) which correspond to the labeled carbon positions C-23, C-6, C-22, and C-5, respectively. Deuterated sitostanol can also be purchased from Medical Isotopes, Inc.

Animals

Six monkeys (cynomolgus macaques), mean body weight 3.8 ± 0.5 kg were kept under special conditions at the Bowman Gray School of Medicine, Winston-Salem, NC. All monkeys received 0.1 μCi [4-^{14}C]cholesterol and 0.3 μCi [1,2-$^3H_2$]sitosterol t.i.d. together with 2 mg [$^2H_5$]cholesterol and 2 mg [$^2H_4$]sitostanol t.i.d. for 7 days on two occasions, one during control and one during administration of acarbose (3 mg/kg) for 1 month. The markers were divided into three equal daily doses and were administered in grapes. Fecal samples were collected from day 5 to 7 and after homogenization 2 g was extracted after alkaline hydrolysis into n-hexane. The ratios of $^{14}C/^3H$ were measured by liquid scintillation counting. Five ml of the n-hexane extract was sent to Bonn for measurement of deuterated sterols by GLC-SIM. Cholesterol absorption using radioactive tracers was then calculated by the equation published previously (13), and by the following equation using deuterium labeled markers.

$$\% \text{ Absorption} = 100 \times \left[1 - \frac{\text{Fecal}\left(\frac{[^2H_x]\text{cholesterol} + [^2H_x]\text{coprostanol} + [^2H_x]\text{coprostanone}}{[^2H_4]\text{sitostanol}} \right)}{\text{Dietary}\left(\frac{[^2H_x]\text{cholesterol}}{[^2H_4]\text{sitostanol}} \right)} \right] \quad \textit{Eq. 1)}$$

where x is equal to 5, 6, or 7, corresponding to the deuterium label of the marker.

Preparation of deuterated capsules

A mixture (1:1; w/w) of [$^2H_5$]cholesterol or [$^2H_7$]cholesterol or [$^2H_6$]cholesterol and [$^2H_4$]sitostanol was dissolved in sunflower oil by gently heating and stirring. After complete dissolution 0.4 ml (containing 2 mg or 3 mg of each compound) was dispensed into small stomach-soluble gelatine capsules. One aliquot was kept for measurement of the respective deuterated cholesterol/[$^2H_4$]sitostanol ratio.

Volunteers

In the first study twelve volunteers participated in cholesterol absorption measurements. They received one capsule t.i.d. for 7 days. Total fecal samples were collected in plastic boxes from day 5, 6, and 7 and immediately stored at −20°C in portable deep-freeze boxes (Engel/O, model MH FD-010-A, Sawataj; Electric Co., Tokyo, Japan). A small aliquot (≈ 1 g) of each bowel movement was placed in small plastic tubes containing a small spoon (Saarstedt Nr. 80623022, Nümbrecht, Germany) and sent to the laboratory by ordinary mail (transport time 1 to 5 days). In all consecutive studies cholesterol absorption measurements were performed from small aliquots of fecal samples (see results).

One volunteer received one capsule of 3 mg [$^2H_5$]cholesterol and 3 mg [$^2H_4$]sitostanol t.i.d. for 1 week and cholesterol absorption was determined as described above. After a washout period of 1 week the volunteer received three capsules of 3 mg [$^2H_7$]cholesterol together with 3 mg [$^2H_4$]sitostanol a day for 1 week and measurements of cholesterol absorption were repeated.

In a second set of experiments five patients with mild hypercholesterolemia received one capsule t.i.d. of deuterated markers as described above and collected fecal samples from day 5 to 7. Thereafter, sitosterol (0.5 g t.i.d.) was administered for the following 6 weeks. During the last week patients took deuterated markers for 1 week and collected fecal samples again from day 5 to 7.

In a third set of experiments cholesterol absorption was measured twice in seven normolipemic male volunteers 2 weeks apart. For this purpose they received 3 mg t.i.d. of [$^2H_6$]cholesterol together with [$^2H_4$]sitostanol [3 mg t.i.d.] for 7 days. The volunteers kept a 7-day food diary from which their cholesterol intakes were calculated by a computer program (22).

All subjects had normal liver function and no signs of renal disease, thyroid dysfunction, or diabetes mellitus, and informed consent was obtained from each subject. The study was in accordance with the principles of the Helsinki Declaration and approved by the local ethical committee.

Sample preparation

Total frozen fecal samples from each day and aliquots from the same fecal specimen sent by mail were thawed overnight and homogenized with distilled water (1:1; w/w). Fifty μl of fecal homogenate was dispensed into a 10-ml glass tube. After a 1-h mild alkaline hydrolysis with 1 ml of 1 N NaOH in 90% ethanol in a water bath at 67°C, the samples were cooled to room temperature and 1 ml of distilled water was added. The neutral sterols were extracted three times with 3 ml cyclohexane. The combined cyclohexane phases were evaporated to dryness under a stream of dry nitrogen at 50°C and the neutral sterols were converted to trimethylsilyl (TMSi)-ethers by adding

1.5 ml TMSi-reagent (pyridine–hexamethyldisilazan–trimethylchlorosilane 9:3:1, vol/vol/vol). After heating the samples to 70°C for 1 h, the silylating reagent was evaporated to dryness under nitrogen. The TMSi-derivatives were dissolved in 2.0 ml of n-decane, and after a 10-min centrifugation at 2000 rpm, 1 ml was transferred into vials for GLC–MS; 2 μl was injected.

Gas–liquid chromatography–mass spectrometry

Combined GLC–SIM was performed on a Hewlett-Packard gas chromatograph (HP 5890) equipped with an automatic sample injector (HP 7673 A) using an on-column injection technique. The gas chromatograph was connected with a direct capillary inlet system to a quadrupole mass selective detector (HP 5970). The combined equipment was controlled by an HP 9000/300 computer. The TMSi-derivatives of neutral sterols were separated on a 50-m fused silica capillary column (CS-FS-OV-101, inner diameter 0.32 mm; Chrompack, Middleburg, Netherlands), with a 2-m uncoated pre-column (inner diameter 0.53 mm). Helium was used as a carrier gas with an inlet pressure of 10 psi. The oven temperature was set to an initial value of 150°C for 1 min followed by a temperature program (30°C/min to 250°C, thereafter 5°C/min to 280°C). The temperature of the transfer line was kept at 280°C. Electron impact ionization was applied with 70 eV. Selective ion monitoring (SIM) was performed by cycling the quadrupole mass filter between different *m/z* at a rate of 3.7 cycles/sec. In the SIM mode, the ion of *m/z* 372 (M^+) was scanned for 5α-cholestane, *m/z* 375 (M^+–90) for TMSi-derivative of [$^2H_5$]coprostanol, *m/z* 391 (M^+) for [$^2H_5$]coprostanone, *m/z* 463 (M^+) for TMSi-derivative of [$^2H_5$]cholesterol, *m/z* 492 (M^+) for TMSi-derivative of [$^2H_4$]sitostanol, and *m/z* 418 [M^+] for [$^2H_4$]sitostanone. In the one experiment where [$^2H_7$]cholesterol was administered for measurement of cholesterol absorption, the TMSi-derivatives were scanned for [$^2H_7$]cholesterol on *m/z* 465 (M^+), for [$^2H_7$]coprostanol on *m/z* 377 (M^+–90), and for [$^2H_7$]coprostanone on *m/z* 393 (M^+). In the third experiment where [$^2H_6$]cholesterol was used, the TMSi-derivatives were scanned for [$^2H_6$]cholesterol on *m/z* 464 (M^+), for [$^2H_6$]coprostanol on *m/z* 376 (M^+), and for [$^2H_6$]coprostanone on *m/z* 392 (M^+). The selected ions were monitored at least for 25 scans as a minimum sampled over the peak of the eluting compound.

Standards

Standard curves of the TMSi-derivatives of [$^2H_5$], [$^2H_7$], [$^2H_6$]cholesterol, [$^2H_4$]sitostanol, cholesterol, coprostanol, sitostanol, and coprostanone were prepared over the range from 5 to 200 nmol/ml using 5α-cholestane as internal standard. Linear regression analysis revealed coefficients of correlation from 0.978 for cholesterol to 0.993 for [$^2H_4$]sitostanol. Because of lack of deuterated coprostanol and coprostanone as standards, three samples of exact identical concentrations of TMSi-derivative of coprostanol (*m/z* 370), TMSi-derivative of cholesterol (*m/z* 458), and coprostanone (*m/z* 386) were analyzed during each run. The peak area of the corresponding deuterated TMSi-derivative of coprostanol and coprostanone in fecal samples was then corrected for the ratio of coprostanol to cholesterol and coprostanone to cholesterol. The area of deuterated cholesterol was added to the corrected areas of deuterated coprostanol plus deuterated coprostanone for calculation of cholesterol absorption according to equation 1.

RESULTS

Stability of deuterium label in the different sterols

Isotope distribution of TMSi-derivatives of deuterium-labeled authentic compounds ([$^2H_5$]cholesterol, [$^2H_6$]cholesterol, [$^2H_7$]cholesterol, and [$^2H_4$]sitostanol) are given in Table 1. Repeated measurements of the isotope distribution of the authentic compounds fed orally compared with those excreted in feces including the main bacterial products of deuterated cholesterol ([$^2H_5$], [$^2H_6$], and [$^2H_7$] deuterated coprostanol and coprostanone) in humans and animals revealed no differences in distribution of deuterium label, indicating the stability of the deuterium label in the sterols during intestinal transit (Table 1). No bacterial oxidation product of [$^2H_4$]sitostanol ([$^2H_4$]sitostanone) could be detected in fecal samples.

Cholesterol absorption in monkeys using radioactive and stable isotope labeled markers

A comparison of radioactive and stable isotope-labeled markers for measuring cholesterol absorption in six monkeys on two occasions is given in **Table 2.** Comparison of

TABLE 2. Cholesterol absorption in six monkeys on two occasions (control and acarbose 3 mg/kg × day) measured with stable and radioactive isotope-labeled markers

	Cholesterol Absorption			
	Control		Acarbose	
Animal	SA[a]	RA[b]	SA[a]	RA[b]
	%		%	
1	61 ± 3	68 ± 1	62[c]	69 ± 2
2	62 ± 2	61 ± 2	53 ± 8	63 ± 6
3	49 ± 4	60 ± 4	73 ± 10	64 ± 2
4	49 ± 4	59 ± 1	54 ± 6	66 ± 5
5	64 ± 3	60 ± 6	62 ± 8	51 ± 7
6	57 ± 5	63 ± 3	69 ± 3	65 ± 4
Mean ± SD	57 ± 7	62 ± 3	62 ± 8	63 ± 6

Values represent the mean ± SD of 3 consecutive days.
[a]SA, calculated with stable isotope markers.
[b]RA, calculated with radioactive labeled markers.
[c]One vial broke in the centrifuge.

the methods revealed that the results obtained with the stable isotope-labeled markers were not different from those calculated from the radioactive tracers. For some animals the agreement was extremely close, whereas for others, a greater difference was noted. The coefficients of variation for cholesterol absorption over 3 days whether determined with stable or radioactive isotopes were not different and ranged from 4% to 15%, and from 2% to 14%, respectively.

Cholesterol absorption in twelve normal volunteers determined from frozen fecal samples and samples sent by mail

No differences could be detected in cholesterol absorption in twelve subjects, no matter whether the samples were analyzed from total frozen specimens or from small aliquots sent by mail (**Table 3**). Cholesterol absorption ranged from 24% to 62% and coefficients of correlation for cholesterol absorption in the twelve subjects between the two different sample handlings showed excellent agreement (**Fig. 1**).

Comparison of [$^2H_5$]- and [$^2H_7$]cholesterol in one subject

Cholesterol absorption in one volunteer determined 2 weeks apart using first [$^2H_5$]- and then [$^2H_7$]cholesterol averaged 59% and 58%, respectively.

Effect of sitosterol on cholesterol absorption

Feeding of sitosterol in a dose of 0.5 g t.i.d. for 6 weeks to five patients with mild hypercholesterolemia resulted in a reduction of cholesterol absorption in all patients (**Table 4**). The reduction in cholesterol absorption ranged from 30% to 57% and averaged 40%.

TABLE 3. Results of cholesterol absorption using stable labeled isotope markers determined from fecal specimens frozen immediately and small aliquots sent by mail in twelve normolipemic volunteers

Subject	Cholesterol Absorption	
	F	M
	%	
1	42[a]	42
2	52 ± 9	47 ± 6
3	36 ± 6	36 ± 3
4	24 ± 8	25 ± 2
5	58 ± 4	60 ± 6
6	28 ± 1	28 ± 3
7	58 ± 3	55 ± 8
8	52 ± 3	48 ± 3
9	46 ± 14	45 ± 3
10	55[a]	55
11	54 ± 4	53 ± 5
12	62 ± 2	56 ± 2
Mean ± SD	47 ± 12	46 ± 11

Results from frozen (F) and mailed (M) samples. Values for each subject represent the average of 3 consecutive days ± SD.

[a] Fecal samples from only 2 days could be obtained from subjects 1 and 10.

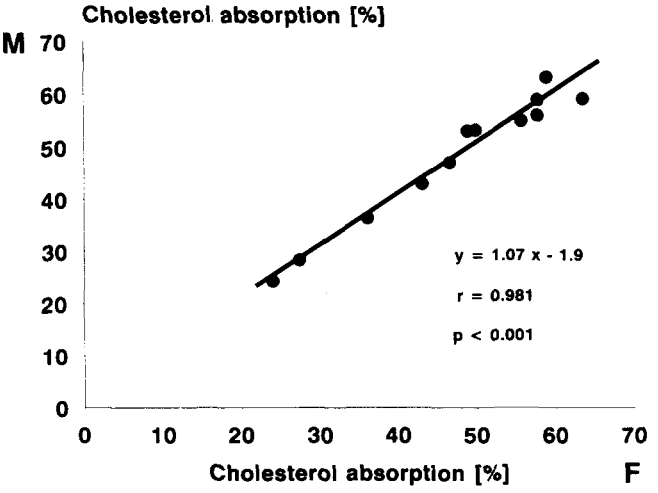

Fig. 1. Effect of sample handling on measurement of cholesterol absorption. Comparison of twelve volunteers measured from frozen specimens (F) and specimens sent by mail (M).

Repeated measurements of cholesterol absorption in volunteers

Intraindividual comparison of cholesterol absorption in seven volunteers on two occasions 2 weeks apart on a constant dietary cholesterol intake revealed no differences (**Table 5**).

DISCUSSION

Previous methods for measurement of cholesterol absorption have used ^{14}C- or ^3H-labeled cholesterol and/or ^{14}C- or ^3H-labeled sitosterol for this purpose. The radioactive tracers were administered intravenously and/or orally. At least seven different methods have been validated for measurement of dietary cholesterol absorption using radioactive tracers with or without simultaneous measurement of fecal excretion of neutral sterols (1–9, 13). Only two methods were developed without the use of radioactive tracers. One method used intestinal intubation and the other one intestinal intubation together with fecal excretion of neutral sterols (11, 12). Both methods determine mostly biliary cholesterol absorption. Neither the radioactive tracer methods nor the intestinal intubation procedures are available for routine and repeated measurements of cholesterol absorption, and they cannot be used in children or young females. The purpose of the present study was to develop a safe and accurate method for cholesterol absorption measurements that can be used routinely without radioactive exposure. The increased availability of good reliable mass selective detectors for quantification of stable isotope-labeled compounds

TABLE 4. Effect of sitosterol feeding (0.5 g t.i.d.) on cholesterol absorption in five subjects with moderate hypercholesterolemia

Subject	Cholesterol Absorption	
	Control	Sitosterol
	%	
1	67 ± 4	29 ± 8
2	44 ± 1	23 ± 11
3	74 ± 5	52 ± 3
4	60 ± 7	27 ± 15
5	70[a]	37 ± 13
Mean ± SD	63 ± 12	34 ± 11

Values for each subject represent the average of 3 consecutive days ± SD.
[a] Fecal samples from only 2 days could be obtained from subject 5 during control.

prompted us to modify the continuous radioactive isotope feeding method of Crouse and Grundy (13) into a continuous stable isotope feeding procedure.

For measurement of dietary cholesterol absorption using stable isotope-labeled markers, it is important to trace and quantitate all labeled metabolites formed by bacterial degradation during intestinal transit. Therefore, it is necessary to measure not only cholesterol in feces but also the corresponding labeled metabolites (coprostanol and coprostanone) which together with cholesterol represent more than 98% of all neutral sterols in feces. As previous studies have shown that some tritium-labeled cholesterol may be radiochemically unstable (23, 24) it was important to assess also the stability of the deuterium label in the different sterols after intestinal passage. The deuterium atoms were located either at the nucleus in those positions where no bacterial enzymatic oxyreductive products occur or in the side chain. The stability of the isotope label of deuterated neutral sterols excreted in feces (cholesterol, coprostanol, coprostanone, and sitostanol) was checked by selected ion monitoring and the isotope distributions were compared with the authentic deuterium-labeled cholesterol and sitostanol. The stability of the deuterated label could always be confirmed; this is in agreement with previous studies in animals and humans using different deuterium-labeled cholesterols (25–30). These results indicate that deuterated cholesterol whether labeled in the nucleus or the side chain can be used for this method.

The isotope distribution of [$^2H_4$]sitostanol was also stable during intestinal transit in different species. Furthermore, it was important to demonstrate that sitostanol is not transformed to its 3-oxo metabolite by intestinal microbes. Thus, [$^2H_4$]sitostanol is an ideal marker for measurement of cholesterol absorption because it is neither metabolized nor absorbed to any extent (31–35) and can be synthesized relatively easily from stigmasterol (see Materials and Methods).

The method was validated in monkeys by direct comparison of cholesterol absorption during simultaneous administration of radioactive labeled tracers and deuterated markers. Some differences could be observed; for the group, however, mean values were not significantly different. This direct comparison indicates that absorption estimated by deuterated markers is comparable to that estimated by radioactive tracers as described in the original method (13). In addition, cholesterol absorption measured in the volunteers and patients was in the same range as values previously obtained by other techniques (1–13).

The new method for measurement of cholesterol absorption has also been validated from samples whether frozen immediately or sent by mail. Therefore, this method can be combined with measurements of cholesterol and bile acid synthesis as described previously (34) and much more information on cholesterol metabolism in a greater number of subjects can be obtained.

The inhibitory effect of sitosterol on cholesterol absorption with the new method was of the same order of magnitude observed previously (35, 36), confirming that the new method probably can be used to evaluate different diets and drugs for their ability to influence cholesterol absorption.

Cholesterol absorption measurements 2 weeks apart in the same group of volunteers gave identical rates of cholesterol absorption. Therefore, the results indicate that the present method can be used in short-term intervals in the same subject. This might be desirable in short-term metabolic studies.

In summary, we have shown that deuterated cholesterol and sitostanol can be used for measurement of cholesterol absorption in humans. This method shares the advantages of the previously described method (13) of not requiring complete fecal collections, simplicity of administration of isotope, and provision of repeated daily estimates of ab-

TABLE 5. Results of cholesterol absorption using stable labeled isotope markers during two different occasions in healthy volunteers on a constant dietary cholesterol intake

Subject	Cholesterol Absorption[a]		Dietary Cholesterol[b]	
	A	B	A	B
	%		*mg/day*	
1	31 ± 7	38 ± 7	572	510
2	50 ± 5	52 ± 2	465	470
3	45 ± 1	37 ± 8	507	423
4	54 ± 2	55 ± 1	553	497
5	27 ± 8	29 ± 7	475	534
6	40 ± 5	37 ± 6	554	574
7	36 ± 5	30 ± 8	407	407
Mean ± SD	40 ± 10	40 ± 10	505 ± 60	488 ± 59

Results from the first week (A) and third week (B).
[a] Values for each subject represent the average of 3 consecutive days ± SD.
[b] Mean of 7 days dietary record.

sorption. In addition the new method has advantages over previous methods for measuring cholesterol absorption in that it is extremely safe and avoids radioactive exposure to subjects and laboratory staff, and can be used in outpatients basis. It is therefore ideal for studies of cholesterol metabolism even in children and women of child-bearing age.

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REFERENCES

1. 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.
2. Borgström, B. 1969. Quantification of cholesterol absorption in man by fecal analysis after the feeding of a single isotope-labeled meal. *J. Lipid Res.* **10:** 331–337.
3. Grundy, S. M., E. H. Ahrens, Jr., and G. Salen. 1971. Interruption of the enterohepatic circulation of bile acids in man: comparative effects of cholestyramine and ileal exclusion on cholesterol metabolism. *J. Lab. Clin. Med.* **78:** 94–121.
4. Sodhi, H. S., L. Horlick, D. J. Nazir, and B. J. Kudchodkar. 1971. A simple method for calculating absorption of dietary cholesterol in man. *Proc. Soc. Exp. Biol. Med.* **137:** 277–279.
5. 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.
6. Zilversmit, D. B. 1972. A single blood sample dual isotope method for the measurement of cholesterol absorption. *Proc. Soc. Exp. Biol. Med.* **140:** 863–865.
7. Grundy, S. M., and E. H. Ahrens, Jr. 1969. Measurements of cholesterol turnover, synthesis, and absorption in man, carried out by isotope kinetic and sterol balance methods. *J. Lipid Res.* **10:** 91–107.
8. Kudchokar, B. J., H. S. Sodhi, and L. Horlick. 1973. Absorption of dietary cholesterol in man. *Metabolism.* **22:** 155–163.
9. Sodhi, H. S., B. Kudchodkar, P. Varughese, and C. H. Weber. 1974. Validation of the ratio method for calculating absorption of dietary cholesterol in man. *Proc. Soc. Exp. Biol. Med.* **145:** 107–111.
10. Simmons, W. J., A. F. Hofmann, and E. Thodor. 1967. Absorption of cholesterol from a micellar solution: intestinal perfusion studies in man. *J. Clin. Invest.* **46:** 874–890.
11. Grundy, S. M., and H. Y. I. Mok. 1977. Determination of cholesterol absorption in man by intestinal perfusion. *J. Lipid Res.* **18:** 263–271.
12. Mok, H. Y. I., K. von Bergmann, and S. M. Grundy. 1979. Effects of continuous and intermittent feeding on biliary lipid outputs in man: application for measurements of intestinal absorption of cholesterol and bile acids. *J. Lipid Res.* **20:** 389–398.
13. 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.
14. Kesäniemi, Y. A., C. Ehnholm, and T. A. Miettienen. 1987. Intestinal cholesterol absorption efficiency in man is related to apoprotein E phenotype. *J. Clin. Invest.* **80:** 578–581.
15. Kesäniemi, Y. A., and T. A. Miettinen. 1987. Cholesterol absorption efficiency regulates plasma cholesterol level in the Finnish population. *Eur. J. Clin. Invest.* **17:** 191–195.
16. Gylling, H., and T. A. Miettinen. 1992. Cholesterol absorption and synthesis related to low density lipoprotein metabolism during varying cholesterol intake in men with different apoE phenotypes. *J. Lipid Res.* **33:** 1361–1371.
17. Vuoristo, M., H. Väänänen, and T. A. Miettinen. 1992. Cholesterol malabsorption in pancreatic insufficiency: effects of enzyme substitution. *Gastroenterology.* **102:** 647–653.
18. Tilvis, R. S., and T. A. Miettinen. 1986. Serum plant sterols and their relation to cholesterol absorption. *Am. J. Clin. Nutr.* **43:** 92–97.
19. Gruenke, L. D., and L. C. Craig. 1978. The synthesis of cholesterol-2,2,4,4,6-d5. *J. Labelled Compd. & Radiopharm.* **16:** 495–500.
20. Brown, H. L., W. Gard, and K. T. Lin. 1971. Saturated secondary alcohols also give nonepimerised ketones in good yield when ether is used instead of acetone. *J. Org. Chem.* **36:** 387–390.
21. Kircher, H. W., and F. U. Rosenstein. 1972. Hydrogenation of stigmasterol. *Lipids.* **8:** 101–105.
22. Souci, S. W., W. Fachmann, and H. Kraut. 1990. Food Composition and Nutrition Tables 1989/1990. Wissenschaftliche Verlagsgesellschaft mbH. Stuttgart, Germany.
23. Davidson, N. O., E. H. Ahrens, Jr., D. J. Bradlow, D. J. McNamara, T. S. Parker, and P. Samuel. 1980. Unreliability of tritiated cholesterol: studies with [1,2-^{3}H]- and [24,25-^{3}H]cholesterol in man. *Proc. Natl. Acad. Sci. USA.* **77:** 2255–2259.
24. Grundy, S. M. 1982. Role of isotopes for determining absorption of cholesterol in man. *In* Lipoprotein Kinetics and Modeling. M. Berman, B. Howard, and S. M. Grundy, editors. Academic Press, New York. 363–371.
25. Ferezou, J., and F. Chevallier. 1982. Comparative methods of quantifying fecal neutral sterols in rats and humans after intravenous [^{14}C], [^{3}H] or [^{2}H]cholesterol labeling. *Biochim. Biophys. Acta.* **713:** 678–683.
26. Ferezou, J., J. Rautureau, T. Coste, E. Gouffier, and F. Chevallier. 1982. Cholesterol turnover in human plasma lipoproteins: studies with stable and radioactive isotopes. *Am. J. Clin. Nutr.* **36:** 235–244.
27. Taylor, C. B., B. Mikkelson, J. A. Anderson, and D. T. Forman. 1966. Human serum cholesterol synthesis measured with the deuterium label. *Arch. Pathol.* **81:** 213–231.
28. Ferezou, J., T. Coste, and F. Chevallier. 1981. Origins of neutral sterols in human feces studied by stable isotope labeling (D and ^{13}C). Existence of an external secretion of cholesterol. *Digestion.* **21:** 232–243.
29. Ferezou, J., J. C. Sulpice, T. Coste, and F. Chevallier. 1982. Origins of neutral sterols in human feces studied by stable isotope labeling (D and ^{13}C). *Digestion.* **25:** 164–172.
30. Virelizier, H., and R. Hagemann. 1980. Use of stable isotopes in the study of human cholesterol metabolism. *In* Advances in Mass Spectrometry. A. Quale, editor. Heyden & Son Ltd., London, GB. **8B:** 1155–1158.

31. Ikeda, I., and M. Sugano. 1978. Comparison of absorption and metabolism of beta-sitosterol and beta-sitostanol in rats. *Arteriosclerosis.* **30:** 227–237.
32. Hassan, A. S., and A. J. Rampone. 1979. Intestinal absorption and lymphatic transport of cholesterol and β-sitostanol in the rat. *J. Lipid Res.* **20:** 646–653.
33. Heinemann, T., O. Leiss, and K. von Bergmann. 1986. Effect of low-dose sitostanol on serum cholesterol in patients with hypercholesterolemia. *Atherosclerosis.* **61:** 219–223.
34. Czubayko, F., B. Beumers, S. Lammsfuss, D. Lütjohann, and K. von Bergmann. 1991. A simplified micro-method for quantification of fecal excretion of neutral and acidic sterols for outpatient studies in humans. *J. Lipid Res.* **32:** 1861–1867.
35. Heinemann, T., G. A. Kullack-Ublick, B. Pietruck, and K. von Bergmann. 1991. Mechanisms of action of plant sterols on inhibition of cholesterol absorption. *Eur. J. Clin. Pharmacol.* **40Supl1:** 59–63.
36. Mattson, F. H., S. M. Grundy, and J. R. Crouse. 1982. Optimizing the effect of plant sterols on cholesterol absorption in man. *Am. J. Clin. Nutr.* **35:** 697–700.