Evaluation of a continuous isotope feeding method for measurement of cholesterol absorption in man

John R. Crouse[1] and Scott M. Grundy

Department of Medicine, School of Medicine, University of California, San Diego, and Veterans Administration Hospital, San Diego, CA 92161

Abstract A new method has been developed for measurement of cholesterol absorption. Seven patients were fed [^{14}C]cholesterol together with [^{3}H]-β-sitosterol three times daily for 10 days. Stools were collected on days 3–10. The ratio of isotopes in stools became essentially constant after the first 3 days of isotope administration. Percentage absorption of cholesterol was calculated from the difference between the dietary and fecal isotope ratios. Percentage absorption of cholesterol in these seven patients ranged from 47 to 65% (mean 54%). The coefficient of variation on individual samples for all the patients ranged from 3.2 to 15.0% (mean 7.8%). This method was evaluated in five additional patients by comparison with a standard sterol balance method (Method II, see Quintao, E., S. M. Grundy, and E. H. Ahrens. 1971. *J. Lipid Res.* **12:** 221–232). The results obtained by the two methods were essentially identical. The new method offers the advantages of simplicity of administration of isotope, ease of analysis of stool samples, and daily repeatability of analysis.

Supplementary key words [^{14}C]cholesterol · [^{3}H]β-sitosterol

At least eight different methods have been reported for measuring cholesterol absorption in man (1–6) (**Table 1**). Most of these methods make use of radioactive cholesterol either with or without simultaneous measurement of cholesterol excretion. Generally, they estimate percentage absorption of radioactive cholesterol which can be converted to an estimate of mass absorption if the intake of dietary cholesterol is known. Although these methods have afforded considerable information about cholesterol absorption in man, most suffer from one of two drawbacks, i.e., either they afford one measurement for the absorption of a single dose of radioactive cholesterol (which may not accurately estimate mean absorption over a period of time), or they require laborious laboratory procedures and hospitalization of patients on a metabolic ward. In the present study we describe a method for estimating absorption of dietary cholesterol that overcomes many of the drawbacks of previous techniques. Our method is based on measurement of isotope ratios in casual stool specimens during feeding of [^{14}C]cholesterol and [^{3}H]β-sitosterol for 10 days. To validate the technique we have compared estimates of absorption with those derived by a technique of cholesterol balance, previously designated as Method II (1), that combines oral administration of radioactive cholesterol for several weeks with simultaneous mass measurements of fecal excretion of neutral steroids. This latter technique was shown previously to provide the most consistent and reliable estimates of absorption of exogenous cholesterol (1).

METHODS

Twelve male patients were studied on the Special Diagnostic and Treatment Unit (metabolic ward) at the Veterans Administration Hospital, San Diego, CA. Most patients had clinical evidence of atherosclerotic disease, but none had intestinal, hepatic, or other clinical disease that might alter cholesterol absorption. Patients' ages ranged from 45 to 65. Informed consent was obtained from each patient. Two separate studies were carried out as described below.

Study A

Study A comprised seven patients who were maintained at steady weight and consumed ad lib solid-food diets (restricted only in that eggs and organ meats were not allowed). Each day for 1–4 weeks, patients were fed 0.1 μCi of [4-^{14}C]cholesterol and 0.3 μCi of [^{3}H]β-sitosterol. Both isotopic sterols were obtained from New England Nuclear Corporation, Boston, MA; [4-^{14}C]cholesterol was shown to be greater than 95% pure by TLC, and β-sitosterol was purified by reisolation on reverse phase TLC (7). The latter procedure consistently showed that at least 25% of radioactivity was not contained in the β-sitosterol fraction, but ran with an R_f of campesterol. However, rechromatography of the isolated β-sitosterol showed that greater than 95% of radioactivity migrated in the β-sitosterol zone.

A mixture of the two isotopes was dissolved in

Abbreviation: TLC, thin-layer chromatography.

[1] Address reprint requests to John R. Crouse, M.D., Department of Medicine, Veterans Administration Hospital, 3350 La Jolla Village Drive, San Diego, CA 92161.

Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 1. Measurement of cholesterol absorption in man by various methods

Method[a]	Isotope (Route & Duration of Administration)	Analyses[b]	Comments	References
I	[^{14}C]- or [^{3}H]Cholesterol (I.V., single dose)	(1) FNS mass (TLC & GLC) and S.A. (2) Plasma cholesterol S.A.	(1) Measures daily variation in absorption (2) Measures mass absorbed	1
II	[^{14}C]- or [^{3}H]Cholesterol (P.O., daily × 10–30 days)	(1) FNS mass (TLC & GLC) and S.A. (2) Plasma cholesterol S.A.	(1) Measures daily variation in absorption (2) Measures mass absorbed	1
III	[^{14}C]- or [^{3}H]Cholesterol (P.O. daily to steady state)[c]	(1) FNS mass (TLC & GLC) and S.A. (2) Plasma cholesterol S.A.	(1) Exceedingly long time to reach steady state (months) makes method infeasible	1
IV (Fecal isotope ratio)	[^{3}H]Cholesterol & [^{14}C]β-sitosterol (P.O., single dose)	(1) Total stool collection (2) FNS separation by TLC & quantitation of isotope excretion	(1) Measures absorption in bolus fed (2) Measures % absorption	1, 2
IVa	[^{14}C]Cholesterol & [^{3}H]β-sitosterol (P.O.) one dose	(1) FNS separation by TLC isotope ratio single stool	(1) Measures absorption in bolus fed (2) Measures % absorption	3
V	[^{14}C]- and [^{3}H]Cholesterol (^{14}C I.V. one dose, ^{3}H P.O. × 10–30 days)	(1) FNS mass (TLC & GLC) and S.A.	(1) Measures daily variation in absorption (2) Measures mass absorbed (3) Special technique for cases of rapid cholesterol synthesis	4
VI (Plasma isotope ratio)	[^{14}C]- and [^{3}H]Cholesterol ([^{3}H]cholesterol, I.V., one dose; [^{14}C]cholesterol, P.O., one dose)	(1) Plasma cholesterol isotope ratio	(1) Measures absorption in bolus fed (2) Measures % absorption	5
VII Grundy–Mok	Cholesterol (via duodenal tube × 8 hr)	(1) Biliary neutral steroid secretion (GLC)	(1) Measures mass absorbed of endogenous separate from exogenous cholesterol	6
VIII Present	[^{14}C]Cholesterol, [^{3}H]β-sitosterol (P.O. × 0–10 days)	(1) FNS separation by TLC and isotope ratio. Single or many stools.	(1) Measures daily variation in absorption (2) Measures % absorption	

[a] Methods I–V and the method of Grundy and Mok require metabolic ward conditions. The others are suitable for outpatients.
[b] Abbreviations: FNS, fecal neutral steroids; S.A., specific activity; TLC and GLC, thin-layer chromatographic separation and gas–liquid chromatographic analysis of FNS.
[c] Steady state defined as time when amount of isotope excreted equals amount administered.
[d] Carmine administered with isotope and single stool with most carmine analyzed.

alcohol and added to about 50 ml of a formula feeding (8) divided into three equal doses administered with meals. Stools were collected daily, and fecal neutral steroids were isolated by hexane extraction and TLC, as described by Miettinen, Ahrens, and Grundy (9). Ratios of ^{14}C/^{3}H were determined by scintillation counting.

Using this method, cholesterol absorption was calculated as follows:

$$\% \text{ Absorption} = 100\left(1 - \frac{\text{fecal } ^{14}\text{C}/^{3}\text{H}}{\text{dietary } ^{14}\text{C}/^{3}\text{H}}\right) \qquad \text{Eq. 1}$$

Study B

Five patients were fed a metabolic diet of mixed solid-food formula (8, 10). They were also fed daily 1.0 μCi of isotopic cholesterol and 450 mg of unlabeled β-sitosterol for 20–40 days. For these five patients absorption of cholesterol calculated by Method II (1) was compared with a slight modification of the current method described in Study A, i.e., unlabeled β-sitosterol was employed instead of the isotopic sterol. For Method II measurements, specific activities of plasma cholesterol and fecal neutral steroids were determined as described previously (1). The measurement of cholesterol absorption by this technique depends on differentiating endogenous and exogenous fecal neutral steroids by comparing fecal, dietary, and plasma specific activities of cholesterol during continuous isotope feeding. No additional measurements were required for the presently described method since it depends only on calculation of the ratio of isotopic cholesterol to unlabeled β-sitosterol in the diet and stool.

Calculation of cholesterol absorption

Calculations of cholesterol absorption by the two techniques were determined as follows. For Method

Downloaded from www.jlr.org by guest, on June 19, 2012

II, cholesterol absorption was calculated during oral administration of [^{14}C]cholesterol by two simultaneous equations [these equations were shown previously to be valid at any time after 4 days of isotope feeding (1)]:

$$Z = X + Y \qquad \text{Eq. 2}$$

where: Z is total fecal neutral steroids (mg/day); X is fecal neutral steroids of endogenous origin (mg/day); and Y is unabsorbed dietary cholesterol.

$$Z \cdot SA_{(Z)} = X \cdot SA_{(X)} + Y \cdot SA_{(Y)} \qquad \text{Eq. 3}$$

where $SA_{(Z)}$ is the specific activity of total fecal neutral steroids; $SA_{(X)}$ is the specific activity of plasma cholesterol; and $SA_{(Y)}$ is the specific activity of dietary cholesterol. Equation 2 states that fecal neutral steroids are composed of two components: endogenously derived neutral steroids and unabsorbed dietary cholesterol. Equation 3 is a restatement of this fact, expressing the radioactivity of each component as its mass times its specific activity. Solving for Y (unabsorbed dietary cholesterol), absorbed dietary cholesterol is obtained as the difference between dietary intake and unabsorbed dietary cholesterol.

For the present method (using unlabeled β-sitosterol as marker) cholesterol absorption was calculated as follows.

$$\% \text{ Absorption} = 100\left(1 - \frac{\text{fecal } ^{14}\text{C dpm/plant steroids (mg)}}{\text{dietary } ^{14}\text{C dpm/}\beta\text{-sitosterol (mg)}}\right) \qquad \text{Eq. 4}$$

Equations 2 and 3 can be shown to be identical to equation 4 as long as $SA_{(X)}$ is very small.

RESULTS

Fig. 1 shows estimates of percentage absorption of cholesterol following simultaneous administration of [^{14}C]cholesterol and [^{3}H]β-sitosterol to seven patients of Study A. The ratio of the two isotopes in feces attained by the second day remained essentially constant over the succeeding 10 days. In **Table 2,** mean values for percent absorption (±SD) over the first 10 days are presented for the seven patients; values ranged from 40 to 65%. When the two isotopes were fed for longer than 10 days, the fecal ^{14}C/^{3}H ratio increased somewhat (about 10%) over 10–30 days, producing an apparent decline in absorption.

In **Fig. 2,** data obtained by the current technique were compared with results calculated by Method II. The present ratio technique gave values for absorption that paralleled those of Method II, but were

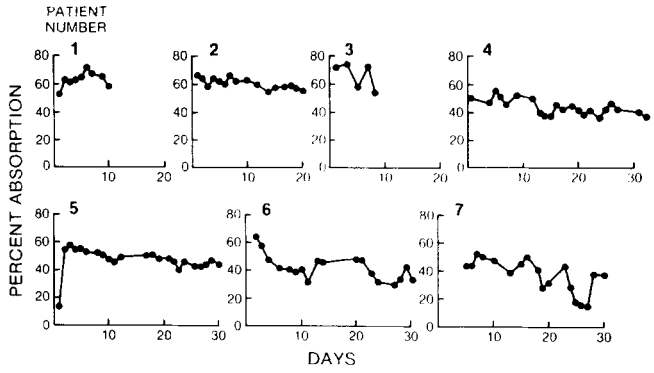

Fig. 1. Percent absorption for seven patients of Study A. (●), Individual daily stool collections.

slightly lower. This difference is most likely explained by internal recycling of radioactive cholesterol so that previously absorbed cholesterol is returned to the intestine for excretion into feces. Because of the continuous input of small amounts of labeled endogenous cholesterol, with time a simple comparison of fecal to dietary ratios of radioactive cholesterol to β-sitosterol led to an underestimation of cholesterol absorption by the new method. The quantity of recycled radioactive cholesterol should be a function of the number of days after starting isotope feeding; the longer the period of feeding, the greater the amount of absorbed isotope in plasma, and the greater the influence of the $SA_{(X)}$ component of equation 3 on the fecal excretion of radioactive cholesterol. In other words, for the first few days the amount of recycled label in feces should be small, but it would increase thereafter. The data of this study were in accord with this concept; thus, for six stool samples of three patients from whom stools were collected within 2 weeks of feeding radioactive cholesterol, results by the new method averaged 2.5% (range 2–5%) less than those by Method II, but in 19 stool samples collected from 14–40 days after the start of isotope feeding, the Ratio Method gave results that averaged 6.4% less than Method II (range 2–

TABLE 2. Percent absorption by continuous isotope feeding method

Patient	n^a	Cholesterol Absorption[b]	Coefficient of Variation (%)
		x% ± *S.D.*	
1	7	65 ± 3	4.6
2	7	62 ± 2	3.2
3	4	64 ± 10	15.0
4	5	50 ± 4	8.0
5	7	53 ± 3	5.7
6	5	40 ± 4	10.0
7	5	47 ± 4	8.5

[a] *n* = stools over days 3–10.
[b] Mean percent absorption ± SD.

Downloaded from www.jlr.org by guest, on June 19, 2012

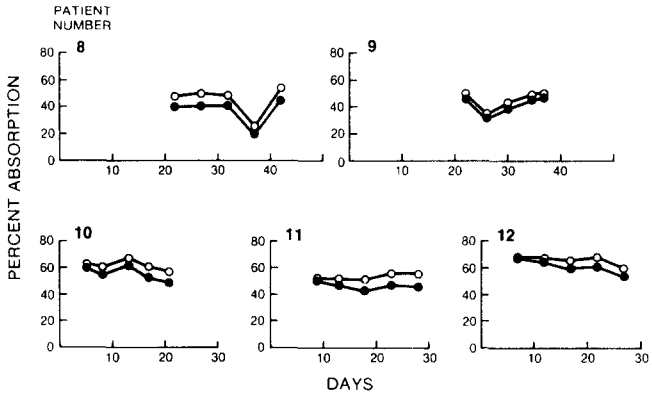

Fig. 2. Comparison of Method II (Table 1) measurements (○) with continuous isotope feeding measurements (●) of percent absorption. Points represent 4- or 5-day stool pools.

10%). This gradually increasing discrepancy between the two methods can be seen in results for patients 10, 11, and 12.

DISCUSSION

All the various methods described previously for measuring cholesterol absorption in man have their particular advantages and disadvantages (1–6) (Table 1). The cholesterol balance technique (1) affords one approach to estimation of absorption of exogenous cholesterol. This method estimates absorption as the difference between dietary cholesterol and fecal exogenous neutral steroids. Differentiation of fecal endogenous from exogenous steroid requires administration of radioactive cholesterol to a subject. The radioactivity is administered either as a single dose intravenously (Method I) or by continuous daily intake of labeled cholesterol over several weeks (Method II); the intravenous dose labels endogenous steroids whereas oral administration of radioactivity labels exogenous cholesterol and its steroid products. Both methods gave similar results, although those of Method II were somewhat more steady and consistent from day to day.

Two other balance methods have been described for measuring cholesterol absorption (Methods III and V, ref. 1). Method III has been found to be unsuitable for such measurements because of the exceedingly long time required to reach the isotopic steady state, and Method V is only appropriate for cases of very rapid cholesterol synthesis (e.g., cholestyramine feeding).

In an attempt to simplify the measurement of absorption of exogenous cholesterol in man, two other methods have been designed to estimate the percentage absorption of a single oral dose of radioactive cholesterol. Both require the use of another isotopic compound as a reference standard. One technique (Method IV, ref. 1) was described first by Borgstrom (2) and subsequently was studied and refined by Quintao, Grundy, and Ahrens (1) and other investigators (4). With this procedure [^{3}H]β-sitosterol is employed as a reference compound; i.e., both [^{14}C]-cholesterol and [^{3}H]β-sitosterol are given as a single dose by mouth. Assuming that β-sitosterol is not absorbed, cholesterol absorption is estimated from the quotient of the ratios of the two isotopes in the oral dose and in stools. In theory, with Method IV any stool sample obtained after administration of the isotope should be suitable for determining percent absorption. In practice, however, the isotope ratios vary markedly from stool to stool in the days following isotope adminstration. This led Quintao et al. (1) to conclude that "isotope exchange" stood in the way of the use of casual stool samples for determining percent absorption by Method IV; they felt that isotopic cholesterol (in contrast to β-sitosterol) exchanged for unlabeled cholesterol in the intestinal mucosa and then reappeared in the stool at a later date. Thus they were unable to use the ratio in any single stool to calculate absorption, but rather were forced to determine a ratio for the total isotope excreted (formed by summing values for fecal excretion over 5–7 days after isotope administration).

A second method that aimed at determining the percent absorption of a single dose of radioactive cholesterol was introduced by Zilversmit (11) and has been validated in man (5). With this method the reference compound is [^{3}H]cholesterol given intravenously and simultaneously with an oral dose of [^{14}C]cholesterol. The resulting ^{14}C/^{3}H ratio in the plasma compartment then gives an estimate of the percentage absorption of [^{14}C]cholesterol from the intestine. These procedures have the advantage of simplicity and convenience, and can even be used in outpatient studies; nevertheless, both have several drawbacks. *a*) The absorption of a single bolus of radioactivity may not be a true reflection of mean absorption over a 24-hr period. In previous studies[2] the percent absorption of a single dose of radioactive cholesterol depended to a certain extent on the composition of the test meal fed with the isotope; thus, when the isotopic cholesterol was fed with orange juice, absorption was low, whereas when fed to the same patient with milk or formula it was higher. *b*) Both methods yield only a single measure of ab-

[2] Samuels, P., J. R. Crouse, and E. H. Ahrens, Jr. Unpublished observations.

Downloaded from www.jlr.org by guest, on June 19, 2012

sorption. To obtain an index of fluctuation in absorption the isotope administration and fecal or blood collections must be repeated after a suitable delay for biologic decay of isotope. *c*) The methods give little indication of the total mass of absorbed cholesterol.

A third approach has been to measure absorption of cholesterol over a long segment of small intestine (6). This is the only method that attempts to quantitate absorption of endogenous as well as exogenous cholesterol, and it has the additional advantage of producing results rapidly. However, the method does require intestinal intubation and hospitalization for the day of intubation and does not assess fluctuations in absorption.

In the present method isotope is fed three times daily with meals, and stools can be collected over one or several days; thus sequential stool samples automatically afford a measure of the fluctuation in absorption. The method is valid provided there is no resecretion of absorbed isotopic cholesterol, since that would cause a falsely high estimate of "exogenous" cholesterol in stool and thus an artificially low value for percent absorption. We believe that the relative constancy of the ratio of the two isotopes in stools from days 3–10 is evidence that continuous exposure of the mucosa to labeled cholesterol minimizes the problem of isotope exchange.

This study provides two lines of evidence that there is little recirculation of absorbed isotopic cholesterol into fecal samples in the first 10 days after administration of the isotope. First, the fecal isotope ratio representative of the first 10 days is obtained very early, by the time of excretion of the first or second stool after administration of the isotopic markers. This ratio remains essentially constant for 10 days and only begins to decline after 2 weeks of continuous oral administration of isotopes. Second, comparison studies of the present method with Method II show divergences in percent absorption by the two methods that appear only after about 2 weeks of continuous isotope administration. Thus, the present method gives an accurate measure of percent cholesterol absorption as long as the stool samples chosen for analysis are obtained within the first 10 days of isotope feeding. Only the ratio of isotopes in the stool need be measured during this period (no quantitation of fecal mass is necessary), and therefore the method should be suitable for outpatient studies. The opportunity to sample multiple stool specimens over these 10 days affords an index of fluctuations in the measurements over this period of time. Thus, the method combines the convenience and simplicity of the outpatient method with the ability to define fluctuations in percent absorption usually found only in inpatient techniques.

We are grateful to Ms. Marjorie Whelan, Mrs. Joan Rupp, and others from the nursing and dietetic services of the Special Diagnostic and Treatment Unit, Veterans Administration Hospital in San Diego for their assistance on this project. Excellent technical assistance was provided by Mr. Christopher Ritchlin and Mr. Delbert Henninger.

The conceptualization of this project developed in part from conversations with Dr. E. H. Ahrens, Jr., to whom we extend our thanks.

This project was supported by the Medical Research Service of the Veterans Administration and by NIH Research grants No. HL-14197 awarded by the National Heart, Lung, and Blood Institute and No. AM-16667 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, PHS/DHEW.

Manuscript received 7 November 1977; accepted 14 April 1978.

REFERENCES

1. Quintao, E., S. M. Grundy, and E. H. Ahrens. 1971. An evaluation of four methods for measuring cholesterol absorption by the intestine in man. *J. Lipid Res.* **12:** 221–232.
2. Borgstrom, 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, and J. 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., B. J. Kudchodkar, P. Varughese, and D. Duncan. 1974. Validation of the ratio method for calculating absorption of dietary cholesterol in man. *Proc. Soc. Exp. Biol.* **145:** 107–111.
5. Samuel, P., J. R. Crouse, and E. H. Ahrens. 1978. Validation of an isotope ratio method for measurement of cholesterol absorption in man. *J. Lipid Res.* **19:** 82–94.
6. Grundy, S. M., and H. Y. I. Mok. 1977. Determination of cholesterol absorption in man by intestinal perfusion. *J. Lipid Res.* **18:** 263–271.
7. deSouza, N. J., and W. R. Nes. 1969. Improved separation of sterols by reversed-phase thin-layer chromatography. *J. Lipid Res.* **10:** 240–243.
8. Grundy, S. M. 1975. Effects of polyunsaturated fats on lipid metabolism in patients with hypertriglyceridemia. *J. Clin. Invest.* **55:** 269–282.
9. Miettinen, T. A., E. H. Ahrens, and S. M. Grundy. 1965. Quantitative isolation and gas–liquid chromatographic analysis of total dietary and fecal neutral steroids. *J. Lipid Res.* **6:** 411–424.
10. Grundy, S. M., and H. Y. I. Mok. 1977. Colestipol, clofibrate, and phytosterols in combined therapy of hyperlipidemia. *J. Lab. Clin. Med.* **89:** 354–366.
11. Zilversmit, D. B. 1972. A single blood sample dual isotope method for the measurement of cholesterol absorption in rats. *Proc. Soc. Exp. Biol. Med.* **140:** 862–865.

Downloaded from www.jlr.org by guest, on June 19, 2012