Further validation of the plasma isotope ratio method for measurement of cholesterol absorption in man

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Abstract Recently we evaluated an isotope ratio method (IRM) for measurement of cholesterol absorption in 14 patients (15 experiments) hospitalized in the metabolic ward by comparing it to simultaneous measurements with a fecal radioactivity method (FRM) and found good to excellent agreement between two procedures (Samuel, P., J. R. Crouse, and E. H. Ahrens, Jr. 1978 *J. Lipid Res.* **19:** 82–93). This comparison has now been extended to additional studies carried out in eight hospitalized patients (19 experiments). Of the 34 comparisons between the IRM and the FRM in our hands, 29 were technically acceptable (chromic oxide fecal recovery ≥80%): percent cholesterol absorption was 43.1 ± 12% by the FRM and 46.0 ± 11% by the IRM, exhibiting an accuracy within 3.5% at the 95% and 4.7% at the 99% confidence levels. In addition, various procedural modifications of the IRM were studied in out-patients. The measurement of isotope ratios in a single blood sample (analyzed in sextuplicate) on the third day (or later) following the tests gave identical results to those obtained from six to eight daily blood samplings. Blood samples drawn at any time during the day gave cholesterol absorption values similar to those obtained from samples drawn following an overnight fast. Absorption tests carried out before and 1 hr after breakfast, lunch, or dinner, or giving the oral isotope in three divided daily doses all yielded identical results with tests carried out in the AM in the fasting state. Cholesterol absorption was markedly reduced when the oral radiolabeled cholesterol was administered in orange juice vs. liquid formula, milk or a solution of glucose and amino acids, consistent with the well-known fact that gallbladder contraction is a critical requirement of cholesterol absorption. A meal high in cholesterol consumed on the day of the test did not influence the results of the absorption measurements. Furthermore, addition of three eggs per day (~750mg cholesterol) for 3 weeks to a low-cholesterol polyunsaturated fat diet caused no significant change in percent cholesterol absorption in any of eight patients. We conclude that the isotope ratio method accurately and precisely measures cholesterol absorption in man, and that it is suitable not only for in- but also for out-patient studies.—**Samuel, P., D. J. McNamara, E. H. Ahrens, Jr., J. R. Crouse, and T. Parker.** Further validation of the plasma isotope ratio method for measurement of cholesterol absorption in man. *J. Lipid Res.* 1982. **23:** 480–489.

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The accuracy and precision of the plasma isotope ratio method (IRM) for the measurement of percent cholesterol absorption in man was previously evaluated in this laboratory (1). We found good to excellent agreement between a previously validated fecal radioactivity method (method IV) and the IRM in comparative studies carried out in 14 hospitalized patients (mean absorption by method IV 36.6% and IRM: 42.1%). Method IV is based on the measurement of nonabsorbed dietary cholesterol in the feces over a 6–8 day period after the administration of a single oral dose of radiolabeled cholesterol (2). The IRM, proposed by Zilversmit (3), entails the analysis of plasma cholesterol radioactivity and does not require stool collections. For the IRM measurement ^{14}C- and ^{3}H-labeled cholesterols are administered simultaneously, one orally, the other intravenously, and the ratio of the two-dose-normalized plasma cholesterol specific activities yields the percent cholesterol absorbed (1). The method has been validated in the rat (4), in primates (5, 6), and in man (1).

Our previous report dealt with the comparison of results obtained by method IV and the IRM in 14 hospitalized patients (1); in addition we have reported on the use of the IRM in measuring the effect of clofibrate and/or cholestyramine on cholesterol absorption in 150 out-patients (7). We have shown that the reproducibility of the method, tested in 18 out-patients, was excellent (mean cholesterol absorption: 52.4% in the first and 54.4% in the second test in the same patients) indicating that at the 95% confidence level the inter-test variability was less than 3.6% (7).

In the present study we compare the IRM to method IV in 19 additional experiments and evaluate the accuracy of the method under a variety of procedural mod-

Abbreviation: IRM, isotope ratio method.

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ifications designed to mimic situations which could be encountered in studies of free-living out-patient populations. These include variations in when and how the oral dose of radiolabeled cholesterol is administered, the timing and the number of blood samples drawn for measurement of plasma cholesterol specific activities, and the effect of pre- and post-test dietary cholesterol intake on the measured percent cholesterol absorption. The results demonstrate that of all the modifications tested, only the vehicle for administration of the oral dose will significantly affect the results of the IRM and, as such, the method exhibits characteristics that make it ideally suited for studies in free-living out-patient populations.

METHODS

In-patients

Studies were carried out in eight patients hospitalized on the metabolic ward of the Rockefeller University Hospital Clinical Research Center. Their age, sex, weight, relative body weight, plasma lipid levels, and clinical diagnoses are presented in **Table 1.** Four patients had elevated plasma lipids, and five had coronary artery disease. One patient (No. 8) had partial ileal bypass surgery 3 years before the present study.

Patients 1 and 3–7 were maintained at constant body weights by liquid formula feeding (8); dietary fat contributed 35% (cottonseed oil), protein 15%, and carbohydrate 50% of total caloric intake. The formula contained 90 mg of cholesterol and 62 mg of sitosterol per 500 kcal. Minerals and vitamins were supplemented as described previously (8). In the remaining two in-patients, (Nos. 2 and 8) body weights were maintained constant by the feeding of a rotating menu of solid food in 2- or 3-day repetitive cycles, supplemented by a small amount of formula in patient 8. The percent of calories derived from the major nutrients was calculated from standard food tables and was held constant from one day's menu to the next; fat content was 30 to 35% of total calories (P/S ~ 2.0). The sterol content of each day's food intake was analyzed by gas–liquid chromatography (9). Daily cholesterol intakes were 112 and 167

TABLE 1. Clinical data

Patient: Age (yrs), Sex, Body Weight (kg)	Relative Body weight[a]	Plasma Lipids		n	Diagnosis[c]
		Cholesterol	Triglycerides		
		(mg/dl)[b]			
In-patients					
1. AH: 55, F, 57	1.06	265 ± 16	154 ± 29	32	CAD
2. HS: 62, M, 76	1.15	251 ± 29	586 ± 185	24	HTG
3. PC: 49, M, 74	1.09	579 ± 44	225 ± 43	21	HC, TX
4. NA: 59, F, 46	0.79	338 ± 37	233 ± 72	7	MHL, CAD
5. MC: 60, F, 69	1.13	246 ± 30	105 ± 25	21	CAD
6. JS: 61, M, 66	0.87	192 ± 5	132 ± 10	8	CAD
7. GD: 51, M, 67	0.97	243 ± 19	291 ± 63	18	HTG, CAD
8. SW: 13, F, 44	0.71	362 ± 23	121 ± 11	14	HC, TX, ileal bypass
9. SD: 36, M, 95	1.27	290 ± 8	164 ± 18	4	HC, gout
Out-patients					
10. HK: 46, M, 101	1.35	244 ± 14	214 ± 42	3	HTG
11. PA: 49, M, 87	1.23	264 ± 16	274 ± 46	4	HTG
12. WR: 60, M, 77	1.12	240 ± 13	241 ± 31	4	HTG
13. CA: 43, M, 78	1.18	272 ± 6	150 ± 21	4	HC
14. FO: 57, M, 75	1.07	305 ± 18	193 ± 18	4	HC
15. BS: 52, M, 72	1.00	324 ± 16	212 ± 60	4	MHL, CAD
16. RH: 40, M, 83	1.11	291 ± 9	221 ± 18	4	MHL
17. LG: 59, M, 75	1.09	272 ± 10	254 ± 23	3	HTG
18. YH: 50, M, 93	1.24	376 ± 30	576 ± 90	3	HTG
19. DB: 45, M, 87	1.16	324 ± 18	384 ± 23	4	MHL
20. WR: 25, M, 99	1.21	271 ± 4	116 ± 56	3	HC
21. VD: 40, M, 81	1.16	294 ± 30	652 ± 348	3	MHL
22. YG: 53, M, 78	1.07	242 ± 23	256 ± 22	3	HT
23. DM: 50, M, 91	1.07	175 ± 15	305 ± 29	3	HT
24. GF: 36, M, 76	0.94	310 ± 20	93 ± 22	3	HC, TX
25. YG: 36, M, 92	1.18	272 ± 14	163 ± 5	3	HC
26. VF: 46, M, 99	1.24	309 ± 6	450 ± 12	3	MHL

[a] Relative body weight = weight (kg)/(Height (cm) − 100) × 100.
[b] Data presented as mean ± 1 SD.
[c] Abbreviations: CAD, coronary artery disease; HC, hypercholesterolemic; HT, hypertension; HTG, hypertriglyceridemic; MHL, mixed hyperlipidemic; TX, tendon xanthomatosis.

mg per day and sitosterol content was 128 and 225 mg per day in these two patients. All in-patients were given daily oral doses of chromic oxide to monitor stool recovery (10).

Patient 1 was given 750 mg/day of deoxycholic acid for 6 weeks prior to and during the second absorption test, whereas Patient 2 was given 15 to 30 g of guar gum daily (in three divided doses before meals) during the performance of the second to ninth absorption measurements; these studies were carried out over a period of 10 months of guar gum administration.

Out-patients

Eighteen patients from the Center for the Prevention of Premature Arteriosclerosis (CPPA) at the Rockefeller University Hospital were recruited for this study. This population consisted of hyperlipidemic males ranging in age from 25 to 60 years (Table 1). Each patient received dietary counseling in maintaining a low cholesterol diet ($\leq$250 mg/day), of which 30 to 35% of the total calories consisted of fat (P/S $\sim$ 2.0). The patients maintained 9 consecutive days' food records to evaluate adherence to the dietary regimen and to quantitate daily dietary cholesterol intake (11). Analysis of the records indicated an average daily cholesterol intake of 247 $\pm$ 8 mg/day (n = 18).

The study protocols were approved by the University Institutional Review Board, and informed consent was obtained from each patient.

Radiolabeled sterols

[1,2-^{3}H]Cholesterol and [4-^{14}C]cholesterol were purchased from New England Nuclear Corp. (Boston, MA) and were purified by thin-layer chromatography on Florisil (Floridin Co., Tallahassee, FL) developed with ethyl ether–heptane 45:55 (v:v). Only material that chromatographed with the same R_f value as a cholesterol standard was administered to patients.

The radiochemical reliability of the [1,2-^{3}H]cholesterol was determined by calculation of the dose-normalized plasma cholesterol $^3H/^{14}C$ ratio 1 to 7 days after simultaneous intravenous infusion of [4-^{14}C]cholesterol and [1,2-^{3}H]cholesterol (12). The [1,2-^{3}H]cholesterol used in all but five experiments (Nos. 2H, 2E, 24, 25, and 26) gave a dose-corrected $^3H/^{14}C$ ratio of 1.0. The [1,2-^{3}H]cholesterol used in these five studies was found to give a dose corrected $^3H/^{14}C$ ratio of 0.79 $\pm$ 0.043 in eight tests; this correction value was used to calculate the administered doses of [1,2-^{3}H]cholesterol to these patients.

All dosages of radiolabeled cholesterol were administered in the AM to patients who had fasted overnight; variations from this protocol are detailed in the text. For intravenous administration, [1,2-^{3}H]cholesterol dissolved in 1 ml of ethanol was suspended in 150 ml of saline and immediately infused intravenously. Residual radioactivity in the infusion set was measured after ethanol extraction in order to determine the total dose administered. [4-^{14}C]Cholesterol in ethanol was mixed with liquid formula (except when stated otherwise) and was given orally. After drinking this mixture, patients rinsed the glass with 50 ml of formula, drank it, and the rinse was repeated. Residual radioactivity remaining in the glass was measured following ethanol extraction, and the net amount administered to the patients was determined. Doses of radioactivity varied from 2 to 6 μCi. Radioactivity was measured in a Packard Tri-Carb scintillation counter (model 3380-3390, Packard Instrument Co., Downer's Grove, IL) which quench corrections performed automatically by an absolute activity analyzer (AAA model 544, Packard Instrument Co.).

Analytical methods

Concentrations of plasma cholesterol and triglycerides were determined by the method of Block, Jarrett, and Levine (13) for cholesterol, and of Kessler and Lederer (14) for triglycerides, using the Auto Analyzer II (Technicon Instruments Corp., Tarrytown, NY). Plasma cholesterol specific activities were measured in aliquots of the same plasma used for the determination of concentration, as previously described (9).

Fecal neutral steroids were isolated from 24-hr stool pools collected over a period of 7–8 days following administration of radiolabeled cholesterol; the sterol mass and radioactivity were measured by methods developed in this laboratory (9). Dietary sitosterol was used as an internal standard to correct for losses of cholesterol during intestinal transit (15) and chromic oxide was employed as an internal standard to correct for fecal flow variations and stool recovery (10).

Calculations

Percent cholesterol absorption was calculated from the two plasma cholesterol specific activities expressed in terms of percent dose per gram of cholesterol, as previously described (1).

$$\%\ \text{absorption} = \frac{\%\ \text{dose/g of cholesterol (oral dose)}}{\%\ \text{dose/g of cholesterol (intravenous dose)}} \times 100$$

Clinical procedures

Comparison of method IV to the IRM in in-patients. After an overnight fast, patients were given an intravenous infusion of [1,2-^{3}H]cholesterol as described above, immediately followed by an oral dose of [4-^{14}C]cholesterol.

Patients consuming solid food received the oral dose in liquid formula (eqivalent to 300 calories) in lieu of breakfast; patients maintained on liquid formula diets had the [4-^{14}C]cholesterol mixed with their morning formula.

Daily stools were collected and blood samples were taken for specific activity analysis over the next 7 to 8 days. Cholesterol absorption was calculated by the fecal recovery method as previously described (2) following correction of fecal neutral sterol radioactivity excretion by the recovery of chromic oxide (fecal flow), sitosterol (intraluminal degradation), and for excretion of recirculated absorbed labeled cholesterol (1). Computer analysis of the plasma specific activity decay curves was conducted by Dr. Sidney Lieberman (Department of Mathematics, Queens College, CUNY).

IRM in out-patients. Unless stated otherwise, outpatients were given an oral and an intravenous dose of radioactive cholesterol in the morning after an overnight fast, as stated above. Fasting blood samples were drawn from the third to seventh days after the test for the analysis of isotope ratios. Modifications of this procedure are detailed in the text.

Statistical analysis

All data are presented as the mean ±1 standard deviation for the number of assays presented in parentheses. Statistical analysis was carried out using a Hewlett-Packard 97 calculator and statistical programs in the Hewlett-Packard Stat. Pac.I. Confidence limits and analysis of variance were calculated as described by Glantz (16).

RESULTS

Comparison of the fecal recovery and plasma isotope-ratio measurement of cholesterol absorption

Our previous evaluation of the plasma isotope-ratio measurement of cholesterol absorption was performed in 14 patients by comparison to the established fecal recovery method (1). In the present study, an additional 19 experiments in 8 patients comparing the two methods under metabolic ward conditions are presented in **Table 2.**

Chromic oxide and sitosterol recoveries. In 17 technically acceptable experiments, chromic oxide recoveries equaled or exceeded 80% with the mean excretion for the group being 101.1%; in two experiments, considered technically unacceptable, (2I and 8) only 71–72% of the administered chromic oxide was recovered. We have previously described this non-ideal behavior which occurs in a small percentage of patients (10), and have documented our reasons for discarding any experimental results obtained under such circumstances. Accordingly, experiments 2I and 8 are listed separately in Table 2, and the results of absorption measurements in these two experiments are excluded from the statistical comparison of the two methods. In patients 1 and 2, the excretion of chromic oxide exceeded 100% during the 7 to 8 days of stool collections. However, in patient 1, the mean chromic oxide recovery over a 36-week hospitalization period equalled 100%, and in patient 2, the recovery over an 82-week period was 102%; thus we consider the studies in these two patients to be technically acceptable.

In the patients maintained on formula diets (all except patients 2 and 8) the recovery of the sitosterol marker, corrected with chromic oxide recovery, ranged from 48 to 106%. In patients 2 and 8, who consumed a rotating solid food diet, corrected sitosterol recovery varied from 43 to 122%. As reported previously (1), the low recovery of sitosterol in Experiment 2H is contrary to the commonly held belief that degradation of neutral steroids is negligible in patients eating solid-food diets (17, 18); accordingly, we quantitated sitosterol recoveries in all studies and applied this correction factor in the calculation of fecal radiolabeled cholesterol recovery.

Fecal recovery measurements. The mean percent cholesterol absorption measured by method IV ("double corrected" for fecal flow variations and neutral sterol degradation) was 42.4% ± 11.2 (range 22.8–68.5%, n = 17). When the absorption figures were corrected for the re-excretion of absorbed radiolabeled cholesterol administered orally ("triple corrected"), the mean absorption was 46.7% ± 10.9 (range 24.3–96.7%, n = 17).

Plasma isotope-ratio method. Mean percent absorption by the IRM in the technically acceptable studies was 48.8% ± 9.7 (range 23.3–63.1%, n = 17). The standard deviations for each of the means were small (0.4–4.2), and the coefficients of variation averaged 3.3% (0.2–7.4%). This small variation indicates that the specific activity ratios (after oral cholesterol curves had reached their peaks) were consistently reproducible from day to day, and that the two plasma cholesterol specific activity decay curves (oral and intravenous) were parallel in all studies.

Precision. Total recoveries of orally administered cholesterol were calculated as a measure of the precision of the methods being compared (Table 2). This was determined by summing the unabsorbed radiolabeled cholesterol, determined by fecal analysis, and the absorbed radiolabeled cholesterol, calculated by the isotope-ratio method. In the 17 technically acceptable studies, the mean recovery of administered radioactivity was 102.1% ± 10.3. The widest discrepancy (138.5% recovery) was seen in Patient 3 and resulted from the significant difference between the values obtained by the two methods; the reason for this discrepancy is not apparent.

Comparisons of two sets of absorption data. When the

TABLE 2. Comparison of cholesterol absorption data obtained by two methods

Patient	Study[a]	Recovery of Internal Standards (%)			Fecal Radioactivity Method				Plasma Isotope Method	
			Sitosterol		% Dose Recovered in Feces		% Absorption			
		Cr_2O_3	Uncorr.	Corr.[b]	Uncorr.[c]	Double-corr.[d]	Double-corr.[e]	Triple-corr.[f]	Percent Absorp.[g]	Total Recovery[h]
Technically acceptable studies										
1A	FF + DCA[i]	108	105	97	70.9	70.9	29.1	29.8	23.3 ± 0.1	93.5
1B	FF	115	122	106	42.3	42.3	57.7	58.9	55.9 ± 1.4	97.0
2A	Solid food	82	89	109	25.8	31.5	68.5	69.7	63.1 ± 4.2	93.4
2B	+15 g/day guar gum	103	94	91	62.1	66.1	33.9	45.9	52.2 ± 0.9	106.3
2C	+30 g/day guar gum	114	95	83	60.9	64.1	35.9	47.6	52.7 ± 3.9	105.1
2D	+30 g/day guar gum	108	108	100	48.8	48.8	51.2	53.1	46.5 ± 1.2	93.4
2E	+30 g/day guar gum	117	127	109	60.3	60.3	39.7	44.5	48.1 ± 1.9	103.6
2F	+30 g/day guar gum	119	126	106	51.8	51.8	48.2	49.7	46.0 ± 2.0	96.3
2G	+30 g/day guar gum	101	111	110	59.0	59.0	41.0	42.5	45.5 ± 1.5	103.0
2H	+30 g/day guar gum	103	65	63	42.8	65.9	34.1	35.5	38.5 ± 0.7	103.0
3	FF	104	92	88	71.0	77.2	22.8	24.3	62.8 ± 1.7	138.5
4	FF	94	83	88	49.9	60.1	39.9	42.1	39.9 ± 1.5	97.8
5A	FF	80	70	88	34.4	49.1	50.9	52.9	54.0 ± 2.6	101.1
5B	FF	98	70	71	38.7	55.3	44.7	52.7	57.5 ± 0.5	104.8
5C	FF	95	89	94	76.5	68.1	31.9	38.9	41.6 ± 0.6	102.7
6	FF	83	68	82	35.8	52.6	47.4	50.9	50.9 ± 2.9	100.0
7	FF	95	56	59	31.3	55.9	44.1	54.2	50.5 ± 0.9	96.3
	$\bar{x}$ ± SD	101.0 ± 12.0	92.4 ± 22.0	90.8 ± 15.7	50.7 ± 15.1	57.6 ± 11.2	42.4 ± 11.2	46.7 ± 10.9	48.8 ± 9.7	102.1 ± 10.3
Technically unacceptable studies										
2I	Solid food	72	88	122	17.7	20.1	79.9	85.2	63.6 ± 2.1	78.4
8	Solid food +FF	71	34	48	24.8	72.9	27.1	31.8	51.7 ± 1.5	119.9

[a] Dietary intake during study; FF, formula feeding or solid food rotating diet.
[b] Sitosterol recovery corrected for fecal flow variations using Cr_2O_3 recovery data.
[c] Raw data obtained by method IV (2), uncorrected.
[d] Raw data obtained by method IV, corrected by two internal standards (10, 15).
[e] One hundred-percent dose recovered in feces (corrected by two internal standards).
[f] Percent absorption corrected for re-excretion of absorbed labeled cholesterol (1).
[g] Dose-normalized ratio of $^{14}C/^{3}H$-cholesterol × 100, n = 5–8 determinations for each patient.
[h] Sum of (percent dose recovered in feces (triple correction)) plus (percent absorption by plasma isotope ratio method).
[i] Patient received 750 mg/day deoxycholic acid (DCA) for 6 weeks prior to the absorption test.

percent absorption values obtained by the fecal recovery and plasma isotope-ratio methods are compared (simultaneously tested in all patients), individual data were within 5 percentage points in 12 of 17 experiments, and within 10 percent in 16 experiments. Only in Patient 3 are the comparative results widely discrepant, and in this patient the total recovery of orally administered labeled cholesterol was unacceptable (138.5%), suggesting a technical fault in this study.

Fig. 1 presents the graphic comparison between the two methods; for the experiments carried out in the present study (19 experiments) and those previously reported (15 experiments) (1) (total: 34 studies).

Statistical analysis estimating confidence intervals (16) of the 29 technically acceptable studies (17 from present paper and 12 from ref. 1) shows that we can be 95% confident that the two absorption methods produced results within 3.5 percentage points, and 99% confident that the differences were less than 4.7 percentage points.

Effects of deoxycholic acid and guar gum on cholesterol absorption. Patient 1 was given 750 mg of deoxycholic acid daily for 6 weeks prior to the first absorption test, and the second test was carried out under control conditions 2 weeks later. Absorption during the control test was 58.9% and 55.9% by method IV and the IRM, respectively; this figure decreased to 29.8% (method IV) and 23.3% (IRM) during the administration of deoxycholic acid (Table 2).

Patient 2 was given 15 to 30 g of guar gum, a non-absorbable galacto-mannan-chain fiber, over a period of 10 months. The control cholesterol absorption values were 69.7% and 63.1% by the two methods (2A, Table 2). During the administration of gaur gum, seven technically acceptable absorption tests were done; the percent cholesterol absorption by the fecal recovery method was 40.6 ± 6.8%, n = 7, and by the plasma isotope-ratio method was 47.1 ± 4.8%, n = 7.

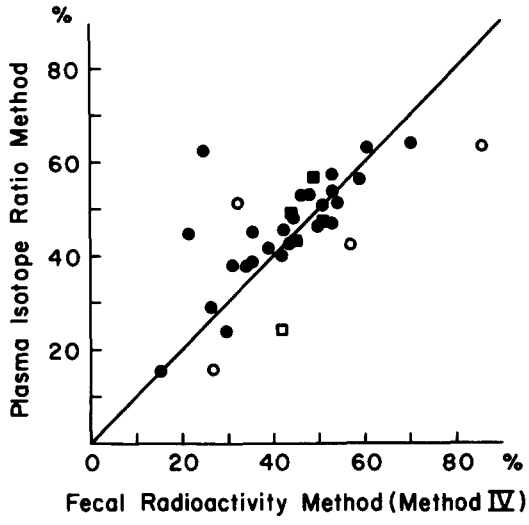

Fig. 1. Percent absorption of cholesterol by two methods in 34 studies (19 in present paper and 15 in ref. 1) plotted against the line of identity. Solid circles or squares, technically satisfactory studies; open circles or squares, unsatisfactory studies (see text). Method IV data shown are those referred to in Table 2 and in Table 4, ref. 1 as triple-corrected (circles) and as double-corrected (squares) (ref. 1, Table 4).

TABLE 3. Percent cholesterol absorption by IRM—all available points vs. single blood assayed in sextuplicate

Patient	Cholesterol Absorption (%) Calculated from: All Available Points	Single in Sextuplicate	Percent Difference	Percentage Points Difference
9	72.2	73.3	1.5	1.1
10	64.1	62.5	2.5	1.6
13A	75.6	75.6	0	1.1
13B	75.6	74.6	1.3	1.0
18	55.0	54.3	1.3	0.7
19	54.7	55.7	1.8	1.0
Mean ± SD	66.2 ± 9.7	66.0 ± 9.7	1.4 ± 0.8	0.9 ± 0.5

Variations in obtaining and assaying plasma cholesterol specific activity

Test of a simplified plasma method. It has become clear that during the performance of the IRM the two plasma cholesterol specific activity decay curves are parallel by day 3 following isotope administration (1). We have tested the feasibility of measuring the plasma isotope ratio at a single time point on the third day (or later) assayed in sextuplicate, and have compared the results obtained to those derived from complete (6 to 8 day) curves in six studies. **Table 3** shows that the mean difference between the results was 1.4% or 0.9 percentage points absorption. In out-patient studies, this procedure has made it possible to schedule two visits to the clinic for the measurement of the percent cholesterol absorption by the IRM, one for dose administration and the second for drawing blood for plasma isotope-ratio measurement (7).

Blood drawn at different times of the day. The conventional procedure of obtaining blood samples in the morning after a 12-hr fast for determining the plasma isotope-ratio was compared in two patients (Nos. 1 and 2) to the results obtained when blood samples were drawn before and after the three meals throughout the day. The results indicate that the mean absorption values were 37.6 ± 0.9% and 53.2 ± 0.9% in the two patients, and the maximum difference in absorption measured on a fasting blood sample and samples obtained during the day were 2.1 and 2.4 percentage points, respectively. Thus, blood samples can be obtained from patients at any time for the measurement of isotope ratios should

TABLE 4. Cholesterol absorption by IRM—oral dose administered before breakfast, lunch, or dinner

Patient	Oral Dose of Cholesterol Administered		
	Before Breakfast	Before Lunch	Before Dinner
	(% absorption)		
9	78.6		83.9
10	67.9	62.6	
11	59.7	60.6	
12	66.1		65.0

it be impossible for them to come to the clinic in the morning in a fasting state.

Variations in the administration of the oral test dose of cholesterol

Studies in the fasting and post-prandial patient. Absorption tests before, and one hr after breakfast were carried out in three patients (Nos. 1, 9, and 10). The data showed essentially no differences between tests carried out in the fasting state and those performed post-prandially. Mean absorption in the three patients in the fasting state was 71.9 ± 5.8% and in the tests done 1 hr post-cibum 69.9 ± 9.5%. The individual differences ranged from zero to 7.2 percentage points.

Single oral dose given before breakfast, lunch, or dinner. In four patients, cholesterol absorption was measured by the IRM following the conventional procedure of administration of the isotopes in the fasting state. A second test in which both the oral and intravenous radiolabeled cholesterol were administered 1 hr before lunch in two of the patients, and 1 hr before dinner in the other two was carried out; these patients consumed three regular meals on the days of the tests. The results, shown in **Table 4,** indicate that the oral dose of labeled cholesterol given before any of the major meals is absorbed equally. The difference in absorption between tests carried out before breakfast (in the fasting state) or before lunch or dinner ranged from 0.9 to 5.3 percentage points (1.5%–7.8%).

Effect of multiple oral dosages. Oral isotope was given in three divided doses with the three meals to two patients (the intravenous isotope was given at noon) and the results obtained by the IRM were compared to tests done with single morning oral and IV doses. The data indicated that the results of the IRM are not significantly changed when the oral isotope is given in three divided doses before the three meals, as compared to a morning dose given in the fasting state. Cholesterol absorption was 63.6 and 63.1% under these two testing conditions in one patient (No. 5) and 54.0% and 57.5% in another (No. 17).

Studies with various oral dose mixtures. The effects of different vehicles of the oral dose on the absorption of cholesterol measured by the IRM were studied in five patients. The oral dose of isotope was administered in milk, formula, orange juice, or a fat-free solution of amino acids and glucose (Vivonex®).

When the oral tracer was given in milk or formula, cholesterol absorption ranged from 63.8 to 73.6% (**Table 5**). However, when orange juice was used as the vehicle, absorption diminished to 6.3 to 21.9% in the four patients studied. It was assumed that the decreased absorption was due to failure of the gallbladder to contract when orange juice was the vehicle of the tracer. To test this possibility, and to demonstrate that it was not the absence of fat from the suspension-fluid that caused this low absorption, we administered the oral isotope in a fat-free solution of glucose and amino acids (Vivonex®), the ingestion of which is known to contract the gallbladder. Tests done in three patients with Vivonex® indicated absorptions of 56.3 to 82.4%, supporting the suggestion that the cause of the low absorption with orange juice was not the absence of fat, but the absence of gallbladder contraction.

Variations in dietary cholesterol intake

Studies varying post-test dietary cholesterol. The effect of a cholesterol meal consumed on the day of the test was studied in five patients (Nos. 9–13). Cholesterol absorption was measured by the IRM during the consumption of a daily diet low in cholesterol (<250 mg/day) and in a second test where patients were given lunch containing three eggs (~750 mg cholesterol) on the day of the isotope administration. The effect of a sudden (and perhaps inadvertant) cholesterol load did not affect the accuracy of the test, mean absorption was 70.5 ± 8.7 during the low- and 70.0 ± 11.5% during the high-cholesterol lunch regimens. The individual differences ranged from 0.4 to 8.3 percentage points.

TABLE 5. Cholesterol absorption by IRM—different vehicles of the oral dose

Patient	Oral Dose of Cholesterol Given in		
	Orange Juice	Milk or Formula	Vivonex®
		(% absorption)	
14	8.7	81.7	
15	21.9	63.8	
16	11.6		61.1
12	6.3		56.3
13		73.6	82.4

TABLE 6. Cholesterol absorption by IRM during a low (<250 mg/dl) and high (~1000 mg/dl) cholesterol diet

Patient	Low Cholesterol Diet			High Cholesterol Diet		
	Plasma Lipids		Cholesterol Absorption	Plasma Lipids		Cholesterol Absorption
	Cholesterol	Triglyceride		Cholesterol	Triglyceride	
	(mg/dl)		*(%)*	*(mg/dl)*		*(%)*
20	271	116	71.9	292	152	76.7
21	294	652	66.0	321	688	59.4
22	242	256	82.9	241	222	74.0
23	175	305	63.6	193	297	52.9
24	310	93	60.8	328	77	62.9
25	272	163	66.4	254	148	63.3
26	309	450	67.1	318	341	53.4
Mean ± SD	268[a] ± 47	291[a] ± 201	68.4[a] ± 7.2	278[a] ± 51	275[a] ± 204	63.2[a] ± 9.3

[a] Plasma cholesterol and triglyceride levels, and percent cholesterol absorption not significantly different.

The effect of low and high cholesterol diets. In seven patients, cholesterol absorption was measured by the IRM during the consumption of a low-cholesterol diet (<250 mg/day); and after 3 weeks of consuming three eggs per day (~750 mg of cholesterol). During the three-eggs-per-day diet period, plasma cholesterol levels increased moderately in four of seven patients (6, 8, 9, 10% respectively); in two subjects there was no change, and in one patient the plasma cholesterol level decreased by 7% during egg feeding (**Table 6**). There were no marked changes in plasma triglycerides. Cholesterol absorption decreased in four of seven patients during the feeding of eggs, the magnitude of the decrease averaged 14.5%; in two patients there was no difference, and cholesterol absorption increased by 7% in one patient. There was no discernable relationship between the changes in plasma cholesterol levels and percent cholesterol absorption. Mean plasma cholesterol for the group was 268 ± 47 mg/dl vs. 278 ± 51 mg/dl and cholesterol absorption was 68.4% vs. 63.2% during low and high cholesterol feeding periods, respectively. Neither of these differences was statistically significant.

DISCUSSION

Our initial evaluation of the measurement of cholesterol absorption by the plasma isotope-ratio method in man was carried out in 14 patients under controlled metabolic ward conditions (1). While it was concluded that the isotope-ratio method afforded results that are concordant with those obtained by established benchmark procedures, we suggested that its suitability for out-patient studies required further evaluation prior to its application in a free-living population. In a later study we demonstrated that the isotope-ratio measurement of percent cholesterol absorption was reproducible in out-patients, and that administration of clofibrate and/or cholestyramine could significantly affect percent cholesterol absorption (7). Of significance was the finding that administration of 8 g of cholestyramine 30 min prior to the absorption test resulted in a 38% decrease in measured absorption (7). This observation suggested that variations in the procedural administration of the test could significantly affect its results. The present study was undertaken to examine the effect of modifications in the test procedure on measured percent cholesterol absorption; our goal was to establish the accuracy of the measurement in out-patient studies where the vagaries of everyday living in an open society do not permit the controls applied under metabolic ward conditions.

Validation of the plasma isotope-ratio method

The plasma isotope-ratio measurement of cholesterol absorption has been compared to the fecal radioactivity recovery method in a total of 34 studies (19 in this report and 15 in reference (1)). Percent cholesterol absorption in the 29 technically acceptable studies was 43.1 ± 12.4% as determined by the fecal recovery method, and 46.0 ± 11.1% by the plasma isotope-ratio method. The agreements were good to excellent, exhibiting an accuracy with 3.5% at the 95% and 4.7% at the 99% confidence limits. Taken together the data demonstrate that the plasma isotope-ratio measurement of cholesterol absorption exhibits a high degree of accuracy, precision (F ratio = 0.927), and reproducibility (16). These data add further support to our original validation of the isotope-ratio method as carried out under metabolic ward conditions.

Studies in free-living out-patients

The isotope-ratio method is ideally suited for studies of cholesterol absorption in out-patients due to its simplicity, its requirement for administration of low levels

of radiolabeled cholesterol, and the derivation of the results from the analysis of plasma, rather than from fecal collections. However, the precision and accuracy of the method in free-living out-patients who are in a non-steady state condition required further investigation.

The data reported in this study have shown that the method can accurately and reproducibly measure percent cholesterol absorption under a variety of conditions that one could expect to encounter during the course of out-patient studies. We have shown that the method's accuracy is not hampered by alterations in the timing of the oral dose, drawing of blood samples, or post-test dietary cholesterol intake. Blood samples for measurement of plasma cholesterol specific activities can be drawn at anytime during the day, not only in the fasting state; furthermore a single plasma sample yields results as precise as those obtained from multiple samples obtained over a 7-day period. This information greatly simplifies the method, inasmuch as only two clinic visits are required to complete the procedure, one for administration of the radiolabeled cholesterols and one for obtaining a blood sample for analysis of the isotope ratio.

The test can also be performed at any time of day, pre- or post-meal. More specifically, our concern was the very nature of the test used for the isotope-ratio method: namely that it measures the absorption of cholesterol from a single bolus, given in the early morning in the fasting state. The data obtained in the present investigation are reassuring: when the oral isotope was given in three divided daily doses, there was no difference in the results of absorption as compared to a single early morning dose in the fasting state; furthermore, when the single dose was given before breakfast, lunch or dinner, cholesterol absorption differed very little. These data suggest that the absorption of cholesterol is much the same throughout the day and thus the single bolus methods (both the isotope-ratio and method IV) give us a valid result of this parameter of cholesterol metabolism.

We have demonstrated that failure to cause gallbladder contraction results in a significant decrease in cholesterol absorption, indicating the requirement for bile acids in the absorption process. The oral dose of radiolabeled cholesterol can be given in a variety of vehicles that do result in effective gallbladder contraction, liquid formula, milk, or Vivonex®.

The process of cholesterol absorption is relatively slow as compared to the absorption of fats or fat-soluble vitamins. Therefore it was important to ascertain whether pre- or post-test dietary intake of cholesterol would affect the test results in out-patients. The data demonstrate that the test is unaffected by either pre-test intake of dietary cholesterol, in the form of three eggs per day, or post-test intake of a cholesterol-containing meal.

The data obtained in the present study suggest that the plasma isotope-ratio method accurately and precisely measures percent cholesterol absorption in man, and that it leaves the clinical investigator with a considerable amount of latitude in the application of this test in studies of cholesterol metabolism in a free-living out-patient population.

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