A Single Blood Sample Dual Isotope Method for the Measurement of Cholesterol Absorption in Rats[1] (36568)

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The quantitative measurement of cholesterol absorption from the gastrointestinal tract has been carried out in a variety of species, but until now the measurement of fecal sterols has been required. Although the use of thin-layer and gas–liquid chromatography, as well as radioactive isotopes and fecal markers, have improved the accuracy of these methods, the collection and analysis of fecal samples is difficult and time consuming. Sodhi *et al.* (1) have described a method in which ^{3}H-cholesterol and ^{14}C-β-sitosterol were given to patients together with carmine red. The $^{3}H/^{14}C$ in the first fecal sample compared to that of the administered dose was taken as a measure of cholesterol absorbed. Quintão *et al.* (2) compared this procedure with three other methods of cholesterol absorption. They concluded that it was necessary to collect feces for as long as significant amounts of radioactivity appeared in feces.

The present paper presents a method for measuring the absorbed fraction of an oral dose of ^{14}C-cholesterol from a ^{14}C to ^{3}H-cholesterol ratio in a single sample of blood plasma (3).

Experimental. Materials. Cholesterol-1-2-^{3}H, cholesterol-4-^{14}C and β-sitosterol-4-^{14}C were obtained from Amersham/Searle (Arlington Heights, IL). They were purified by thin-layer chromatography on silica gel H with the solvent system hexane:diethyl ether (50:50 v/v). The purified cholesterol samples were then tested on silica gel G $AgNO_3$ (2% w/w) plates with chloroform as the developing system (4) and by reverse phase chromatography on kieselguhr G (5). In both systems more than 99% of the radioactivity migrated with a digitonin-precipitated, dibromide-purified cholesterol preparation which was used for carrier.

Preparation of isotope doses. ^{3}H or ^{14}C-cholesterol (2–7 μCi) containing 0.025–0.18 μmoles of sterol was dissolved in 0.025 ml of 95% ethanol. To this was added 1 ml of 0.9% NaCl. The resulting mixture was injected intravenously within 1 hr.

The oral dose of ^{3}H or ^{14}C-cholesterol (2–7 μCi) consisted of 18 μmoles of cholesterol dissolved in 200 μmoles of triolein (Sigma Chemical Co., St. Louis, MO, grade II). This was suspended by sonication in 0.8 ml of 6.8% skim milk powder in water according to a procedure described by Borgström (6).

Animals. Male rats weighing 200–400 g were obtained from Chordata Corp. (Ontario, NY). The animals were fed a casein–cerelose diet (7) with a fat content of 10% triolein (Nutritional Biochemicals, Cleveland, OH). The diet was fed for 30 hr and was followed by a 16–18 hr fast after which the isotopes were given. Fasting continued for 6 hr more and the semisynthetic diet was then resumed *ad libitum*.

The oral isotope dose was given by stomach tube to the unanesthetized rats. This was followed immediately by the other isotope dose which was given into the external jugular vein of rats under ether anesthesia.

Immediately after isotope dosage, fecal collection cups were put into place (8). The cups were replaced daily and feces were collected in methanol.

Isotope measurements. Heparinized blood,

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collected from the tail, or abdominal aorta (terminal sample) was centrifuged. Plasma samples were saponified according to Abell *et al.* (9) and sterol was extracted into hexane. Feces were treated with chloroform:methanol (1:1), homogenized, boiled, filtered and an aliquot was dried for saponification according to the procedure of Borgström (6). Sterols were extracted with hexane. In a single partition between hexane and the ethanol phase, 99% of isotopic cholesterol was recovered.

^{3}H and ^{14}C-cholesterol were determined by liquid scintillation counting in a toluene cocktail with 0.4% PPO and 0.01% of POPOP. The samples were counted with internal standards to a standard deviation of less than 0.6%.

Results and Discussion. Borgström (6) showed that 45.2% of a single dose of orally administered cholesterol was recovered in the feces of rats and that after the first 2 days only small amounts of label are present in feces. Our results in Table I confirm these findings for 5 animals in which daily fecal collections were made and analyzed separately. In subsequent experiments we have used pooled feces, collected during the 4 days following isotope administration, to validate the data obtained from the plasma isotope ratio.

Two corrections were applied to the fecal sterol data: (a) corrections for the presence of labeled cholesterol that had been absorbed and subsequently excreted into the intestine, and (b) corrections for losses of sterol as a result of bacterial degradation (10). The first correction factor was estimated from the presence in feces of isotopically labeled sterol that had been administered by the intravenous route. This correction lowered the value of the uncorrected nonabsorbed isotopic cholesterol from 44.1 ± 9.6 to 42.8 ± 9.8% of the oral dose. The losses caused by bacterial degradation, as measured by the recovery of ^{14}C-β-sitosterol in 5 animals in a separate experiment,[3] raises the value for the nonabsorbed isotopic cholesterol to 45.1 ± 10.3% of the oral dose. The two corrections thus nearly cancel each other, but were applied nevertheless to calculate the percentage of cholesterol absorbed in column 6 of Table III.

TABLE I. Orally Administered ^{3}H Neutral Sterols in Feces.[a]

Rat no.	Days after dosage 1	2	3	4	Total
1	34.2	18.3	3.0	1.6	57.1
2	32.9	12.7	0.8	1.5	47.9
3	23.8	22.4	2.8	0.8	49.8
4	25.9	14.1	2.2	0.5	42.7
5	22.7	11.5	1.8	0.8	36.8

[a] Numbers are expressed as a percentage of the orally administered dose.

TABLE II. $^{3}H/^{14}C$ Cholesterol in Rat Plasma × 100.[a]

Rat no.	Hours after isotope dosage 24	48	96
1	36.2	32.8	34.6
2	47.7	51.6	47.3
3	48.6	50.2	49.7
4	32.7	36.3	38.3
5	48.4	46.2	46.9

[a] Rats were given one oral dose of ^{3}H-cholesterol in skim milk and one intravenous dose of ^{14}C-cholesterol in a colloidal suspension. The ratios are adjusted for equal radioactivity in the oral and intravenous doses.

In Table II are shown the ratios for ^{3}H to ^{14}C-cholesterol in plasma of rats 24, 48 and 96 hr after dosage with a collodial suspension of ^{14}C-cholesterol by the intravenous route and of ^{3}H-cholesterol given orally. Previous work has shown that within 1 hr of administering a collodial cholesterol suspension by vein the cholesterol is removed from the bloodstream by phagocytes primarily in liver (11). This cholesterol is returned to the bloodstream within 24 hr and appears to be metabolized in a manner similar to lipoprotein cholesterol. The data in Table II show

[3] In 5 animals dosed orally with 0.065 μmole β-sitosterol-^{14}C and 18 μmole cholesterol-^{3}H the recovery of ^{14}C in a 4-day pool of feces was 91.1 ± 4.3%. The $^{14}C/^{3}H$ in plasma, 4 days after isotope dosage, was 0.079. Since cholesterol absorption on the average is 48.5% (Table III) the percentage of sitosterol absorbed was 0.079 × 48.5 = 3.8%. Bacterial degradation of sitosterol-^{14}C was, therefore, 100 − (91.1 + 3.8) = 5.1%.

TABLE III. Comparison of Cholesterol Absorbed by Fecal (F) and Plasma Isotope Ratio (P) Methods.

	Cholesterol dose		Unabsorbed cholesterol (%)		Absorbed cholesterol[c] (%)		Recovery[d]
Rat	Oral	Intravenous	Uncorrected[a]	Corrected[b]	F	P	%
1	^{3}H	^{14}C	57.1	59.2	40.8	34.5	93.7
2	^{3}H	^{14}C	47.9	48.2	51.8	48.9	97.1
3	^{3}H	^{14}C	49.8	50.3	49.7	49.5	99.8
4	^{3}H	^{14}C	42.7	43.5	56.5	35.8	79.3
5	^{3}H	^{14}C	36.8	37.2	62.8	47.2	84.4
6	^{3}H	^{14}C	34.9	34.5	65.5	57.9	92.4
7	^{14}C	^{3}H	53.8	55.5	44.5	44.1	99.6
8	^{14}C	^{3}H	58.6	61.1	38.9	37.0	98.1
9	^{14}C	^{3}H	51.7	54.3	45.7	48.7	103.0
10	^{14}C	^{3}H	34.9	34.9	65.1	52.6	87.5
11	^{14}C	^{3}H	36.4	35.8	64.2	63.6	99.4
12	^{14}C	^{3}H	30.9	30.7	69.3	59.9	90.6
13	^{14}C	^{3}H	40.4	41.7	58.3	50.4	92.1
Av ± SD			44.1 ± 9.6	45.1 ± 10.3	54.9 ± 10.3	48.5 ± 9.0	93.6 ± 6.9

[a] Fecal labeled neutral sterol collected in 4 days after dosage, as a percentage of the oral dose.

[b] Corrected for orally dosed neutral sterol which had been absorbed and reexcreted and for bacterial loss of neutral sterols.

[c] Absorbed cholesterol as a percentage of oral dose: F, calculated from fecal excretion; P, calculated from plasma isotope ratio × 100, adjusted for differences in radioactivity of oral and intravenous doses.

[d] Recovery: The sum of absorbed cholesterol calculated from plasma ratio and corrected neutral sterol in 4-day feces pool.

that the ratios of ^{3}H to ^{14}C-cholesterol in blood plasma are independent of time for the 24–96 hr interval.

In Table III are shown the results of 6 experiments in which ^{3}H was given orally and ^{14}C by vein and 7 experiments in which the isotopes were reversed. Cholesterol absorption was calculated in two ways. Column 6 shows that the average percentage of cholesterol absorbed, calculated from fecal isotope analysis, is 54.9 ± 10.3%. Column 7 shows that the average percentage of cholesterol absorbed, as reflected by the plasma isotope ratio is 48.5 ± 9.0%. Column 8 shows that the sum of cholesterol absorbed, as measured from the plasma ratio, and the cholesterol not absorbed, as measured by fecal analysis, is on the average 93.6 ± 6.9% of the orally administered dose. One may conclude, therefore, that the cholesterol absorption as measured from the isotope ratio in a single plasma sample agrees rather well with that calculated from fecal samples. Moreover, the value of 48.5% absorption reported here agrees also rather closely with the value of 42% absorption reported by Borgström with rats on a similar diet (6).

The advantages of the plasma ratio absorption method are several. Only a single blood sample is needed for analysis. The time of sampling is unimportant as long as it is beyond the point at which absorption of cholesterol from the gastrointestinal tract and release of cholesterol from the reticuloendothelial cells are complete. The method allows repeated determinations when the isotope left from the first experiment is small compared to the isotope used the second time. The method does not require fecal collections and is not affected by sterol degradation in the lower gut. Whether or not coprophagy, which was prevented in our study, might alter the plasma isotope ratio sufficiently to require corrective action is not known as yet.

The method should, in principle, be applicable to man. The isotope ratio in plasma would be expected to give the correct figure for cholesterol absorption when the labeled cholesterol absorbed from the gut and that released from the reticuloendothelial cells reach the blood stream during the same time interval. Calculations, based on the two-pool

model of Goodman and Noble (12), show that as much as a 24 hr difference between these time intervals should affect the plasma isotope ratio by less than 5% if the ratio is determined 10 days or more after isotope dosage (Table IV). The assumption of a 24 hr difference between the entry of orally and intravenously administered isotopic cholesterol into the circulation is probably unrealistically large, since the intravenous dose does not mix instantaneously with blood but is taken up by reticuloendothelial cells and, in the rat at least, is released at a slow rate (11), so that oral and intravenous doses enter the blood over a similar period of time. No information on this point appears to be available in man.

It should be noted that the plasma isotope ratio method presented here complements cholesterol turnover studies carried out with the two-pool model (12). From the latter one obtains a production rate of cholesterol, which is the sum of cholesterol synthesis and of dietary cholesterol absorbed. By the use of the double isotope method, as presented here, and extending the measurements to obtain plasma cholesterol specific activity–time curves, one could measure simultaneously cholesterol absorption and cholesterol synthesis in the same individual.

TABLE IV. Calculated Isotope Ratio in Plasma of Man after Simultaneous Intravenous Colloidal ^{3}H-Cholesterol and Oral ^{14}C-Cholesterol Administration.[a]

Days after dosing:	5	10	20	50	100
$^{14}C/^{3}H$	1.06	1.05	1.03	1.01	1.01

[a] The ratio $^{14}C/^{3}H$ is calculated from the turnover data of Goodman and Noble (12) by the use of the equation:

$$\text{Specific activity of serum cholesterol (dpm/mg)} = 2000\ e^{-0.15t} + 500\ e^{-0.02t}$$

(t in days).

For clarity it is assumed that the oral and intravenous dose of radioactive cholesterol contain the same dpm and that *all* of the oral dose is absorbed. Thus the ideal $^{14}C/^{3}H$ should be 1.00. The equation predicts that this is so from zero-time on, when the oral and intravenous isotope doses enter the circulation at the same rates. The figures in the table are calculated for conditions in which the absorption of the oral dose takes 24 hr longer than the release of ^{3}H-cholesterol into the bloodstream after its initial uptake by phagocytic cells. The ratio then approaches unity within 5% after day 10 of isotope dosage.

Summary. The determination of cholesterol absorption in rats from a single blood sample is described. The method consists of dosing the rats with a collodial suspension of cholesterol-^{3}H intravenously and with cholesterol-^{14}C orally. The ratio of ^{14}C to ^{3}H in plasma, 24 hr or more after isotope dosage, gives the fraction of the oral dose absorbed. The method was tested by comparison to fecal ^{14}C-cholesterol excretion corrected for recirculated sterol and sterol degradation. The results of the two methods are in good agreement. Calculations based on a two-pool model show that the plasma ratio method should be applicable to studies in man, and would allow calculation of dietary cholesterol absorption and endogenous cholesterol synthesis from plasma cholesterol specific activity data.

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