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Compound-Specific Carbon Isotope Ratio Determination of Enriched Cholesterol

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On-line gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) was evaluated for use in stable isotope studies of human cholesterol metabolism. Calibration curves were constructed by analyzing underivatized and TMS-cholesterol over a range of ^{13}C abundances from natural abundance to 0.05 mol of [3,4-^{13}C]cholesterol per mole of unlabeled cholesterol (δ ^{13}C −24 to 270‰). The calibration curves were highly linear (r^2 = 0.99) with slopes of 0.98 and 0.88 for underivatized and TMS-cholesterol, respectively. Higher precision of $^{13}C/^{12}C$ ratio determination was obtained using TMS-cholesterol, with a detection limit (2 SD) of 0.3‰ or 0.004 mol% of [3,4-^{13}C]-cholesterol. By comparison standard GC/MS had a detection limit of 0.06 mol% for [3,4-^{13}C]cholesterol. A 57-day study of cholesterol elimination was conducted in one human subject with oral administration of 50 mg of [3,4-^{13}C]cholesterol. Peak enrichment was 9‰ above baseline (40 times the detection limit) and could be followed for 40–50 days. The results indicate that GC/C/IRMS provides 15-fold lower detection limits for [3,4-^{13}C]-choleseterol than GC/MS and is useful for human studies of cholesterol metabolism.

INTRODUCTION

Abnormalities in cholesterol metabolism are implicated in the development of cardiovascular disease in humans.[1] As such, investigators have developed a wide range of tracer methods to study the absorption, synthesis, and turnover of cholesterol.[2,3] These methods, however, typically rely on radioisotopic tracers, which limit their applications for human studies. Recently several methods using stable isotope tracers for cholesterol synthesis have been reported;[4–7] however, there are very few stable isotope techniques for the study of cholesterol distribution and elimination kinetics.

A limiting factor in the use of stable isotope techniques for the study of cholesterol metabolism is the large size of the cholesterol pool in humans. The total body pool of cholesterol is estimated to be about 80 g, with a rapidly miscible central pool of 24 g.[8] The large pool requires a sizable tracer dose for precise measurement of the stable isotope enrichment using standard mass spectrometric techniques. This presents problems for physiological delivery of the tracer as well as cost.

To reduce the dose requirement, several investigators have used differential isotope ratio mass spectrometry (IRMS) because it can detect and measure much smaller excesses of ^{13}C than organic mass spectrometry (MS).[9,10] IRMS, unfortunately, requires laborious sample purification and up to 1 mg of cholesterol. The recent development of on-line gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) has the potential to overcome these limitations.[11–13] With this technique, the effluent of the gas chromatograph is passed through a small combustion furnace and all carbon is oxidized. The resulting CO_2 is directly introduced into an IRMS for dynamic ^{13}C analysis. The precision of this method for the analysis of natural carbon isotope abundance of cholesterol was recently demonstrated by Jones et al.[14] and has been applied to ^{13}C tracer studies for fatty acids[15] and glucose.[16] We have investigated the use of GC/C/IRMS for human ^{13}C cholesterol tracer studies.

EXPERIMENTAL PROCEDURES

Chemicals. Unlabeled cholesterol and cholestane were purchased from Sigma (St. Louis, MO); [3,4-^{13}C]cholesterol (99% ^{13}C) was from MSD Isotope (Montreal, Canada); derivatizing reagent bis(trimethylsilyl)trifluoroacetamide (BSTFA, 99%), trimethylchlorosilane (TMCS, 98%), and anhydrous dichloromethane (99%) were from Aldrich (Milwaukee, WI).

Preparations for Calibration Curve Determination. Unlabeled and [3,4-^{13}C]cholesterol were prepared as solutions in hexane (1 mg/mL). Solutions of labeled cholesterol were diluted on a molar basis with unlabeled cholesterol with ratios of 1 part labeled cholesterol to 6510, 651, 217, 65, and 21.7 parts unlabeled cholesterol to make stock mixtures containing 0.015, 0.154, 0.46, 1.54, and 4.62 mol% labeled cholesterol. These dilutions were chosen to make stock mixtures corresponding to increases in ^{13}C of about 1, 10, 30, 100, and 300‰ relative to unlabeled cholesterol. Dilutions were made using an Eppendorf pipet. Two 1-mL aliquots of each of these mixtures were transferred to test tubes and dried under N_2 at room temperature. One of the duplicates

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was derivatized to TMS-cholesterol as described below. The other was left underivatized. Both underivatized and TMS-cholesterol were reconstituted with 1 mL of hexane (HPLC grade) before GC analysis.

Derivatization of Cholesterol. Cholesterol derivatization procedure was adopted from Kinter et al.[17] with minor modification. BSTFA and TMCS were prepared as a mixture in a ratio of 9:1 (v/v). A 100-μL aliquot of the mixture was added to the test tube containing 1 mg of cholesterol, followed by 100 μL of dichloromethane. The samples were heated to 60 °C for 30 min. After cooling to room temperature, the derivatizing reagents were evaporated with N_2. The TMS-cholesterol residue was taken up with 1 mL of hexane for GC/C/IRMS analysis.

Separation of Cholesterol from Human Plasma. For measurement of cholesterol elimination, one subject ingested 50 mg of [3,4-^{13}C]cholesterol (99 atom % excess) dissolved in 10 mL of ethanol and mixed with a 250-kcal liquid breakfast (Ensure, Mead Johnson, Evansville, IN). The subject was a 44-year-old male without history of chronic disease. The subject's total cholesterol level was 4.8 mmol/L. A 10-mL fasting blood sample was collected by forearm venipuncture before and 1, 2, 4, 8, 12, 14, 22, 35, and 57 days after the dosing. Plasma was separated and stored at 4 °C. The total plasma cholesterol was isolated by extraction after saponification. Ethanol (1 mL) and 0.4 mL of ethanolic potassium hydroxide solution (3.5 M) were added to 0.5 mL of plasma. The samples were heated to 37 °C for 3 h. Hexane (4 mL) was then used to extract the free cholesterol three times. An aliquot containing 0.1–0.2 mg of the extracted cholesterol was passed through an NH_2-SPE column (Supelco, Inc., Bellefonte, PA) by gravity flow. The column had been preconditioned with 3 mL of hexane. The eluate was dried with N_2 at room temperature. The TMS derivative was prepared as described above.

Separation of Cholesterol from Foods. A 1–2-g sample was homogenized and mixed with 15 mL of 2-propanol and 2 mL of 50% (w/w) aqueous KOH in screw-stopper heavy-duty tubes. The samples were shaken overnight, and 10 mL of 10% NaCl (w/w) was added to facilitate formation of two phases. The cholesterol was then extracted with 5 mL of hexane–ether (85:15, v/v) three times. The upper layer containing cholesterol was filtered through a paper filter layered with 10 g of anhydrous Na_2SO_4. An aliquot of the filtrate was passed through an NH_2-SPE column to purify the cholesterol. The effluent was dried under N_2 at room temperature and the cholesterol residue reconstituted with hexane for injection into GC. This procedure was adopted from Oles et al.[18] and Beyer et al.[19]

GC/C/IRMS Conditions and Operation. The commercial system consisted of a Finnigan MAT Delta S isotope ratio mass spectrometer (IRMS) (Finnigan MAT, San Jose, CA), a Varian 3400 gas chromatograph (GC) (Varian Analytical Instruments, San Fernando, CA), and a ceramic microcombustion furnace. The GC was equipped with an on-column injector and fitted with a Ultra-1 nonpolar capillary column (25 m × 0.32 mm i.d., 0.5-μm stationary-phase coating on fused-silica support, Hewlett Packard, Kenneth Square, PA) except as indicated below. The column temperature was programmed from 50 to 290 °C at a rate of 40 °C/min. The helium carrier gas flow was 2 mL/min. An additional 2 mL/min He was added to the gas flow after the combustion furnace. A He flow of 0.5 mL/min was introduced into the ion source of the IRMS. Each injection introduced about 500 ng of cholesterol.

The combustion furnace consisted of an alumina ceramic tube filled with copper and platinum wires to act as catalysts of oxidation. The Cu was activated by heating to 600 °C overnight in the presence of O_2 (0.5 mL/min). The Cu was reoxidized after every 16–32 h of use. The operating temperature of the reactor was set to 900 °C for cholesterol analysis. Water of oxidation was removed by passing the effluent from the reactor through a water-permeable Nafion tube with a countercurrent He flow of 5 mL/min. During the period of solvent elution, the solvent was prevented from entering the reactor by backflushing with He.

The operating conditions of the ion source were as follows: pressure 4×10^{-6} mbar; ionizing potential −80 eV; ion accelerating voltage 3 kV. The integration time was preselected as 0.25 s per point. The minimal ion current intensity for peak detection was set to 50 mV. Background ion currents just prior to the cholesterol peak were recorded and subtracted from the cholesterol ion currents in subsequent calculations of isotope ratios. Prior to analysis, focusing of the ion beam was optimized by manually adjusting the voltages and symmetry applied to the source focusing lenses for maximal signal responses with standard CO_2 admitted through the inlet system.

Isotope abundance (expressed as per mil, ‰) was calculated using CO_2 as reference gas admitted from a standard viscous leak inlet. The CO_2 (−37.25‰) had been calibrated relative to Peedee belemnite limestone carbon (PDB) using Solenhofen limestone. The range for integration was defined automatically by the ISODAT system, except for highly enriched samples (≥100‰). For these samples, the software tended to continue integration well beyond the time of cholesterol elution. We manually set the integration end point to about 1 min after the cholesterol elution time.

Plasma cholesterol in the cholesterol turnover study was derivatized to TMS-cholesterol and separated using a DB-5 capillary column (15m × 0.32 mm i.d., 0.25-μm film thickness, J&W Scientific, Folsom, CA). The GC oven temperature was programmed from 50 to 275 °C at a rate of 45 °C/min. IRMS conditions were the same as above.

GC/MS Conditions and Operations. The TMS derivative was also analyzed on an HP-5985 quadrupole mass spectrometer. The GC separation was performed on a 60 cm × 2 mm glass column packed with 3% SE-30 on 100/120 Gas Chrom Q (Alltech, Inc., Deerfield, MI). The GC was programmed from 235 to 250 °C at 10 °C/min. The injector was maintained at 270 °C, and the carrier was helium at a flow rate of 23 mL/min. EI spectra were obtained at −70 eV, and the M − 90 ion cluster between m/z 367 and 376 was monitored. The ion intensities were normalized to that of m/z 368 and corrected for natural abundance; mass isotopomer distribution was calculated by a least-squares method as previously described.[6] The mole percent [3,4-^{13}C]cholesterol was calculated from the ratio of the [3,4-^{13}C]cholesterol relative to total cholesterol.

Calculation and Expression. The notation ‰ is used for ^{13}C enrichment expression. It is defined as

$$\delta\ (‰) = (R_{sample} - R_{std})/R_{std} \times 1000$$

where R stands for $^{13}C/^{12}C$ ratio. R_{std} used here is the international standard PDB, 0.011 237.

For comparison of ^{13}C enrichment of calibration mixtures between GC/C/MS and GC/MS, the δ values determined by GC/C/IRMS are transformed to atom percent excess (APE):

$$APE = 100R/(1 + R)$$

$$R = (\delta - \delta_{na})(0.011237/1000)$$

δ and δ_{na} are the per mil abundance of the enriched and natural-abundance cholesterol samples, respectively. Mole percent excess (MPE) of the cholesterol isotopomer determined by GC/MS is transformed to APE: APE = MPE × 2/27; i.e., 27 carbons of which 2 were labeled.

When TMS-cholesterol is analyzed on GC/C/IRMS, the apparent δ ^{13}C values are corrected for dilution by extra carbons of the TMS moiety in the derivative to obtain the δ ^{13}C values of cholesterol using the mass balance equation of Goodman and Brenna.[15] The equation used is

$$30\delta_{TMS\text{-}C} = 27\delta_c + 3\delta_{TMS}$$

where $\delta_{TMS\text{-}C}$ is the apparent ^{13}C abundance of TMS-cholesterol, δ_c that of cholesterol, and δ_{TMS} the ^{13}C abundance of TMS moiety. The constants are corresponding numbers of carbons in these compounds.

To determine the ^{13}C abundance of the TMS carbon, underivatized natural cholesterol and the TMS derivative were analyzed on GC/C/IRMS (−24.50 and −26.27‰, respectively),

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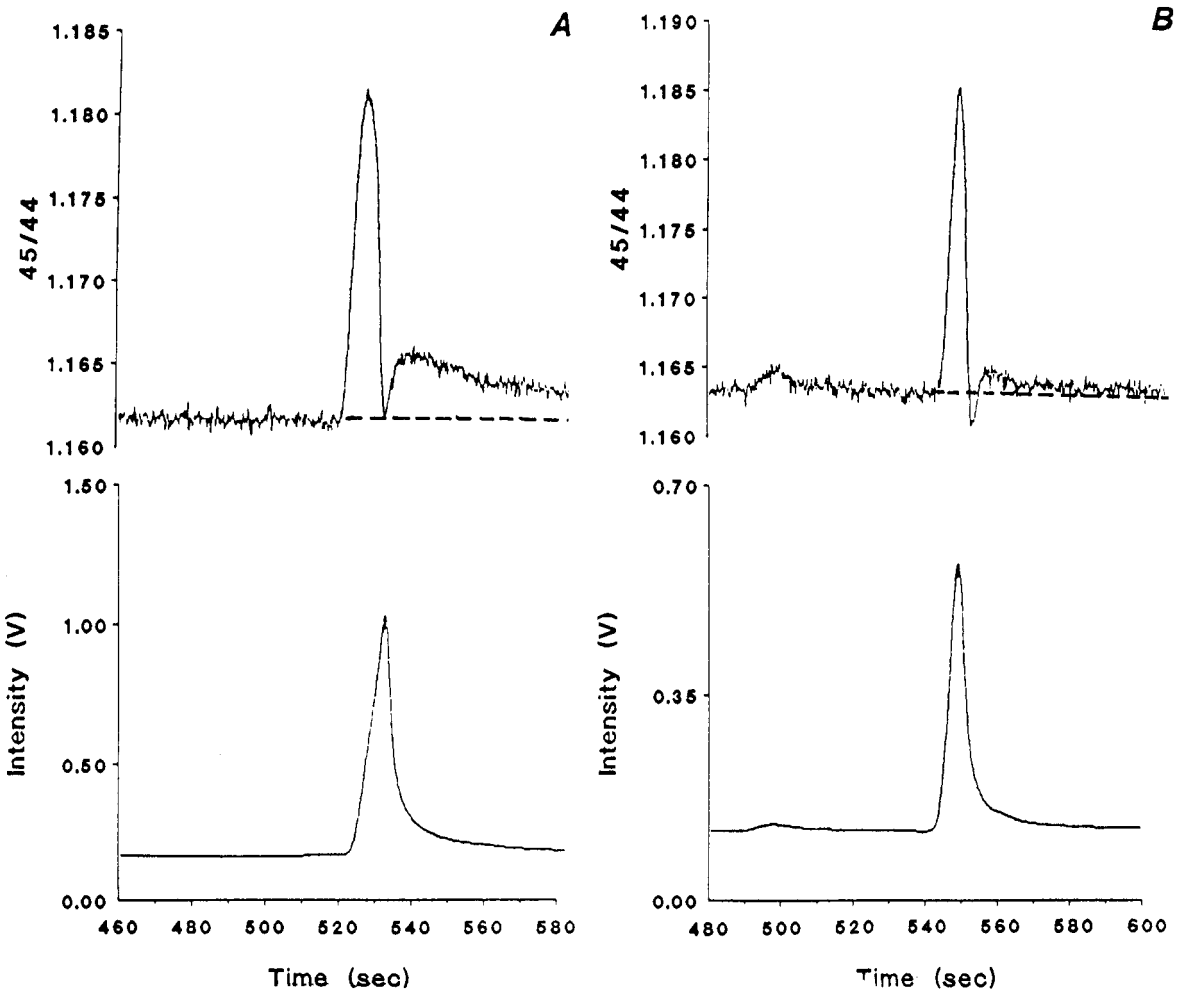

Figure 1. The *m/z* 44 ion chromatogram and 45:44 ratio plot for natural-abundance cholesterol without (A) and with (B) TMS derivatization.

and the respective results were substituted for $\delta_{TMS\text{-}C}$ and δ_c in the above equation. The calculated abundance of the TMS carbon was −42.2‰, which is similar to the −41.4‰ reported by Jones et al.[14]

The calculated value was confirmed by isotope analysis using off-line combustion and dual-inlet IRMS. A 50-μL aliquot of derivatization reagent BSTFA and TMCS (9:1, v/v) was transferred to a quartz tube filled with 100 mg of CuO and an Ag wire. After solvent evaporation, the quartz tubes were flame-sealed under vacuum and heated at 750 °C for 90 min to complete oxidation. The resulting CO_2 gas was separated from H_2O and transferred to vacuum-transfer bulbs. The CO_2 sample was analyzed for ^{13}C abundance by IRMS. The result was −42.9‰. Subsequently, the calculated δ TMS (−42.2‰) was used for calculation of δ ^{13}C of cholesterol from TMS-cholesterol.

RESULTS AND DISCUSSION

Chromatographic Behaviors. The chromatographic properties of underivatized cholesterol and TMS-cholesterol were compared on the GC/C/IRMS system (Figure 1). Both were highly retained on the nonpolar column. The underivatized cholesterol demonstrated considerably more tailing than the TMS counterpart. At natural abundance, the tailing of the underivatized cholesterol disrupted the commonly observed sinusoidal pattern in the ratio trace. Instead of the ratio dropping below the background ratio, the tailing produced a positive shoulder on the backside of the trace (Figure 1A). This shoulder was still evident when the TMS derivative was used, but it was reduced (Figure 1B). At high enrichments, the tailing and resulting shoulder in the ratio trace were even more apparent (Figure 2), as the ratio trace remained positive relative to background well beyond the center of the peak. TMS derivatization of cholesterol reduced the tailing as previously reported by Jones et al.[14]

The broad elution profile of cholesterol required that the signal be integrated over a relatively large time window. This was especially true for the analysis of ^{13}C-enriched mixtures (Table I). At natural abundance, changes in integration time did not introduce significant differences. This is because the cholesterol carbon had an isotopic abundance similar to that of the background carbon and thus contributed little to the integrated area of the ion intensities. At high enrichments, however, it was difficult to identify an optimal time to stop the integration because of the enhanced tailing of the ratio plot. This enhanced tailing was previously noted for ^{13}C-enriched fatty acids.[15] Reduction of tailing through the use of TMS derivatization generally reduced the sensitivity of the apparent ^{13}C abundance to changes in integration time, but did not eliminate the effect. In relative terms—i.e., as a percent of enrichment above natural abundance—these offsets are 1 or 2% and generally acceptable. Further efforts in defining optimal integration windows for highly enriched compounds, however, will improve performance.

Accuracy. To evaluate the accuracy of the GC/C/IRMS method, ^{13}C abundances were compared with traditional off-line combustion isotope ratio mass spectrometry (Table II). The GC/C/IRMS values were systematically lighter than the traditional off-line combustion method. The difference was 0.6‰ for both underivatized cholesterol and cholestane. The on-line combustion analyses of highly enriched TMS-cholesterol (100 and 300‰ excess relative to natural-abundance cholesterol) were also 0.9‰ lighter than the value calculated from the weighted sum of the cholesterol and TMS carbons.

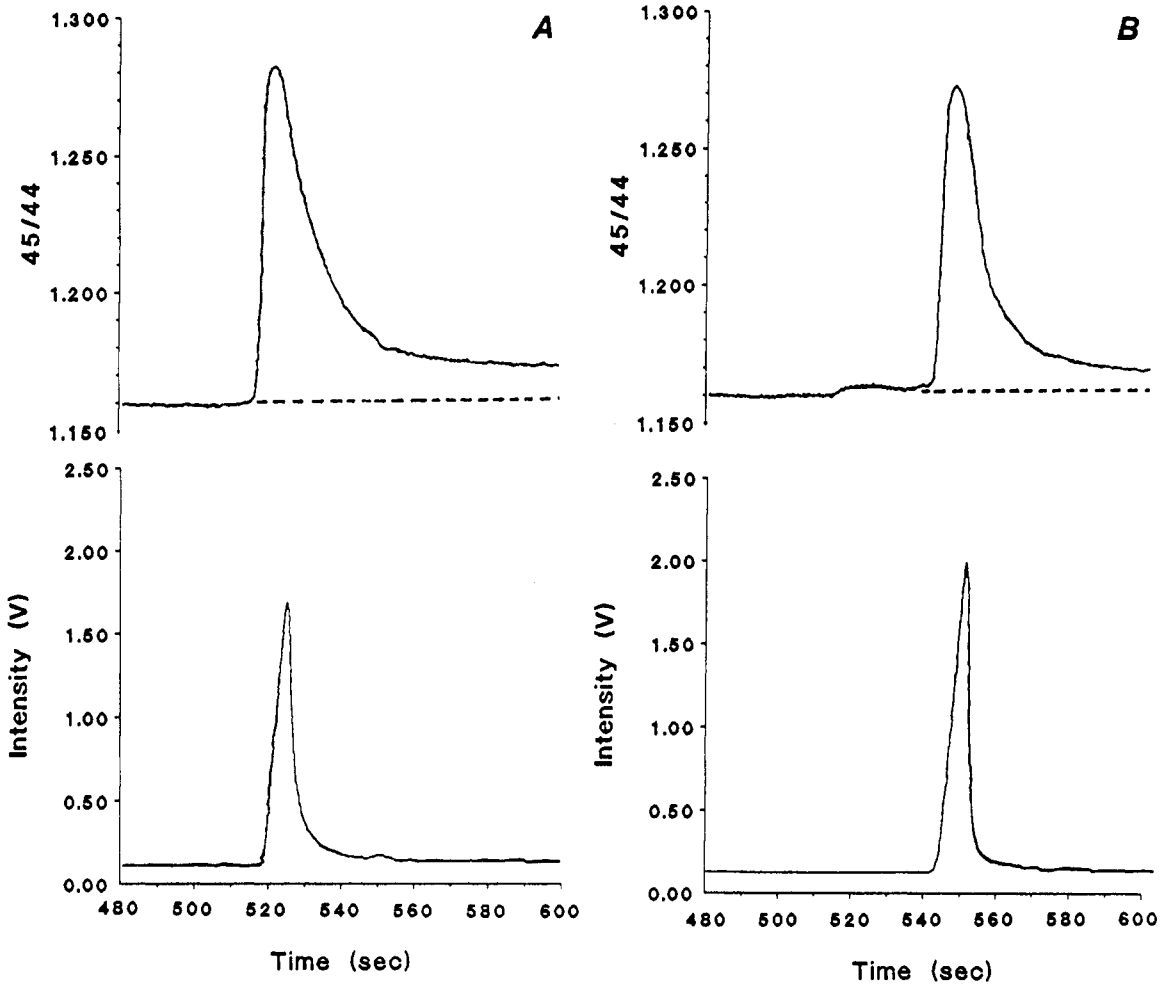

Figure 2. The *m/z* 44 ion chromatogram and 45:44 ratio plot for cholesterol enriched to ca. 100‰ above natural abundance with [3,4-^{13}C]-cholesterol without (A) and with (B) TMS derivatization.

Table I. Effects of Using Different Time Windows on Apparent ^{13}C Abundances of Underivatized and TMS-Cholesterol

	natural abundance (mean ‰ ± SD)		100% excess (mean ‰ ± SD)	
time from peak center (s)	cholesterol	TMS-cholesterol	cholesterol	TMS-cholesterol
+20	+0.18 ± 0.16	−0.19 ± 0.17	+3.53 ± 0.24[a]	+1.69 ± 0.23[a]
+30	+0.07 ± 0.14	−0.21 ± 0.13	+2.27 ± 0.22[a]	+1.24 ± 0.16[a]
+40	+0.01 ± 0.07	−0.10 ± 0.06	+1.09 ± 0.12[a]	+0.81 ± 0.09[a]
[+60	−22.77 ± 0.50	−25.79 ± 0.92	73.33 ± 1.68	60.18 ± 0.48][b]
+90	+0.07 ± 0.11	+0.10 ± 0.09	−0.74 ± 0.04[a]	−1.41 ± 0.27[a]
software defined	+0.02 ± 0.03	+0.02 ± 0.07	2.90 ± 0.11[a]	0.71 ± 0.22[a]

[a] Significantly different from the 60-s value. [b] The 60-s integration was chosen as the reference ratio and the δ ^{13}C entries for 20, 30, 40, and 90 s are relative to those for 60 s. The values for TMS-cholesterol are without correction for TMS carbons; n = 3, mean ± SD.

Table II. Accuracy of Correction of ^{13}C Abundance of TMS-Cholesterol for Isotopic Dilution by TMS Carbon[a]

obsd δ ^{13}C TMS-cholesterol (n)	corr δ ^{13}C cholesterol	obsd δ ^{13}C cholesterol (n)	error
−26.27 ± 0.08 (4)	−24.50	−24.50 ± 0.14 (4)	0
−25.40 ± 0.06 (3)	−23.53	−23.44 ± 0.09 (3)	−0.09
−16.85 ± 0.13 (4)	−14.03	−13.95 ± 0.28 (3)	−0.08
61.41 ± 0.18 (3)	72.92	73.57 ± 0.80 (3)	−0.65

[a] Values are mean δ ^{13}C (‰) ± SD.

This inaccuracy is significant compared to the precision of the instrument, but is still relatively small as it corresponds to an addition of 1 part ^{13}C in 1 × 10^5 parts ^{12}C. The similarity in the offsets between on-line and off-line analyses for underivatized and TMS-cholesterol indicates that isotope fractionation during the derivatization procedure was not detectable as does the similarity of TMS carbon abundances determined by direct combustion and by difference between derivatized and underivatized cholesterol.

The factor(s) causing the 0.6‰ inaccuracy by GC/C/IRMS is (are) unknown. It is unlikely that it results from GC column bleed as the background was quite stable and corrections were made using the background measured just before the cholesterol eluted from the column. Furthermore, application of an interpolated background using both pre- and postelution background measures did not eliminate the offset. Finally, the offset was noted on both an Ultra-1 and a DB-5 column. It is also unlikely the offset was due to coeluting material because it was observed for cholestane and cholesterol. Although this issue of other carbon sources does not appear to explain the isotopic offset observed in the present study, it may be limiting for GC/C/IRMS under some circumstances. Unlike GC/MS all GC/C/IRMS monitoring is done at *m/z* 44 and 45 and the mass selectivity of GC/MS is lost.

Table III. Comparisons of Variations in $^{13}C/^{12}C$ Ratio Analyses of Calibration Mixtures by GC/C/IRMS and GC/MS[a]

theoretical		observed: GC/C/IRMS underivatized			observed: GC/C/IRMS TMS-derivatized[b]			observed: GC/MS TMS-derivatized[b]		
(mol %)	per carbon (atom %)	ratio (atom %)	SD (atom %)	CV (%)	ratio (atom %)	SD (atom %)	CV (%)	ratio (atom %)	SD (atom %)	CV (%)
0	1.085 18	1.085 18	0.000 15	0.014	1.08518	0.00009	0.008	1.0983	0.0022	0.20
0.015	1.086 29	1.086 35	0.000 10	0.009	1.08635	0.00007	0.006	N/A[c]		
0.154	1.096 28	1.096 89	0.000 31	0.028	1.09680	0.00014	0.013	1.1076	0.0015	0.14
0.461	1.118 48	1.116 02	0.000 20	0.018	N/A			1.1308	0.0015	0.13
1.560	1.196 18	1.194 04	0.000 89	0.075	1.19332	0.00020	0.017	1.1954	0.0032	0.27
4.831	1.418 18	1.418 37	0.003 35	0.236	1.40888	0.00289	0.205	1.4190	0.0007	0.05

[a] Isotope ratios from GC/C/MS and GC/IRMS transformed to atom percent as described under Experimental Procedures. CV (coefficient of variation) is the ratio of SD to the mean atom percent × 100. [b] The entries for TMS-cholesterol include correction for isotopic dilution by the TMS carbons. [c] Not analyzed.

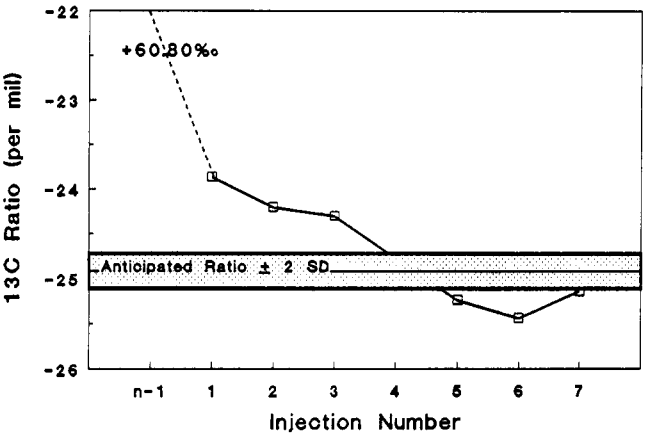

Figure 3. Effect of memory on the ^{13}C abundance of the TMS-cholesterol +1‰ enriched standard when analyzed directly after the +100‰ enriched standard. The gray area represents the mean ± 2 SD of the +1‰ standard when analyzed after natural-abundance cholesterol.

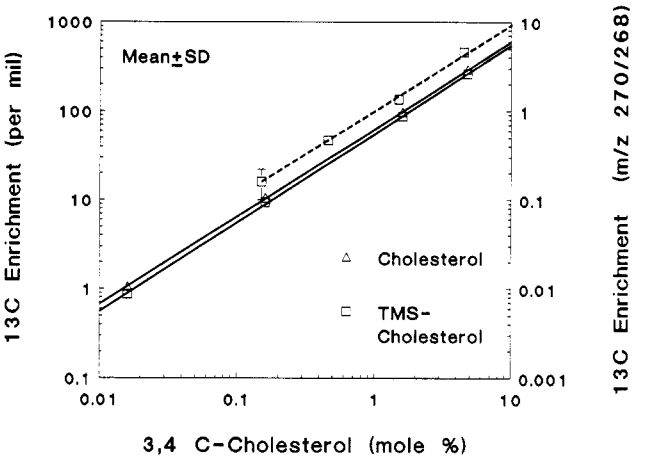

Figure 4. Calibration curve for underivatized and TMS-cholesterol by GC/C/IRMS (—) and standard GC/MS (- - -). Enrichments are expressed as the arithmetic difference from natural abundance (per mil) or the ion current ratios at *m/z* 368 and 370, respectively. The SDs for the GC/C/IRMS analyses are smaller than the symbol and thus not visible.

Isotopic memory within the GC/C/MS was investigated by analyzing a TMS-cholesterol standard of 1‰ excess relative to natural abundance immediately after a 61‰ enriched standard. Memory was defined as $(\delta_{obsd} - \delta_{low}) \times 100/(\delta_{high} - \delta_{low})$, where high and low refer to the ^{13}C abundances of the highly enriched and near-natural-abundance samples, respectively. Memory was 1.5%, but continued at the 1% level for next two or three injections after the enriched sample, suggesting that the memory was not due to a simple first-order exchange (Figure 3).

Detection Limit for Enriched Samples. Calibration mixtures for both underivatized and TMS-cholesterol were prepared over the range of natural abundance to 5 mol% of [3,4-^{13}C]cholesterol in unlabeled cholesterol. The calibration curve is very linear throughout this range (Figure 4). Greater enrichments were not tested. The TMS-cholesterol calibration was offset to the right of the underivatized cholesterol. The difference was expected because of the addition of three carbons from the TMS moiety. The slopes of the calibration curves, when analyzed without log transformation, were 0.98 and 0.88. The 10% decrease in the slope of the line was not different from the expected dilution resulting from the addition of three carbons (3/30 = 10%). The coefficients of variation, expressed as the ratio of the SD for each calibration preparation to the mean atom percent × 100, are compared in Table III. The coefficient of variation for ^{13}C by GC/C/IRMS analysis of TMS-cholesterol is somewhat lower than that for the underivatized and that of GC/C/IRMS is considerably lower than that for GC/MS. The difference disappears at the highest enrichment tested (δ ^{13}C = 270‰).

The detection limit (2 SD) for the GC/IRMS was similar for both underivatized cholesterol and TMS-cholesterol, 0.7 and 0.3‰, respectively, which corresponded to 0.01 and 0.004 mol % for [3,4-^{13}C]cholesterol. For comparison, a TMS-cholesterol calibration curve was also prepared on a standard GC/MS. The detection limit for the GC/MS method was 0.06 mol % or 6–15 times worse than GC/C/IRMS.

Due to the difference in ^{13}C abundances of cholesterol and the TMS reagent carbon, the observed δ values must be manipulated to obtain the δ values of cholesterol carbon per se. A simple arithmetic equation[15] was used for this purpose as described under Experimental Procedures. A comparison of the corrected results to those of the directly observed with unlabeled cholesterol is presented in Table II. The corrected values were constantly lighter than those observed with unlabeled cholesterol. The average discrepancy was −0.2‰ (-2.2×10^{-4} APE).

Biological Applications. The separation of cholesterol from plasma and foods produced a very clean chromatogram with no detectable peaks other than cholesterol. The ^{13}C abundance of human plasma cholesterol was intermediate between those of the foods (Table IV). The median precision of these analyses is slightly worse than previously reported by Goodman and Brenna[15] for comparable amounts of carbon loaded onto the column (500 ng), but slightly better than previously reported for underivatized cholesterol.[14]

The time course of ^{13}C enrichment in plasma cholesterol of one subject who ingested [3,4-^{13}C]cholesterol is shown in

Table IV. ^{13}C Abundance of Cholesterol from Human Plasma and Different Foods vs PDB

source	^{13}C abundance (‰)	SD (‰)
human plasma	−21.0	0.1
food		
turkey	−18.7	0.3
salmon	−25.6	0.2
egg yolk	−19.2	0.1
cheese	−27.1	0.2
beef	−26.3	1.3
chicken	−21.6	0.3

Figure 5. The peak enrichment of 8.3‰ (above baseline) appeared 2 days after the dosing. The elimination rate constant between days 2 and 35 was 0.087^{-day}. This was quite similar to results obtained after oral ingestion of [^{14}C]-cholesterol except that we were not able to identify a second component in the elimination curve.[20] We estimate that a 4 times greater dose is needed to obtain sufficient precision to identify this second component. The ingested labeled cholesterol was no longer detectable after 8 weeks at this dose.

The precision of $^{13}C/^{12}C$ ratio analyses was preserved in analyses of plasma cholestrol by GC/C/IRMS. The average variation of $^{13}C/^{12}C$ ratio determinations with plasma cholesterol was 0.08‰ (n = 31), which was even better than that observed during the analysis of standards and suggested a detection limit of 0.2‰ (0.003 mol % [3,4-^{13}C]cholesterol). The low detection limit permits kinetic studies with a physiological dose of expensive ^{13}C-labeled cholesterol and will facilitate studies of cholesterol metabolism in a population where radioactive isotopes are contraindicated.

(20) Hellman, R. L.; Rosenfeld, R. S.; Eidenoff, M. L.; Fukushima, D. K.; Gallagher, T. F. *J. Clin. Invest.* **1955**, *34*, 48–60.

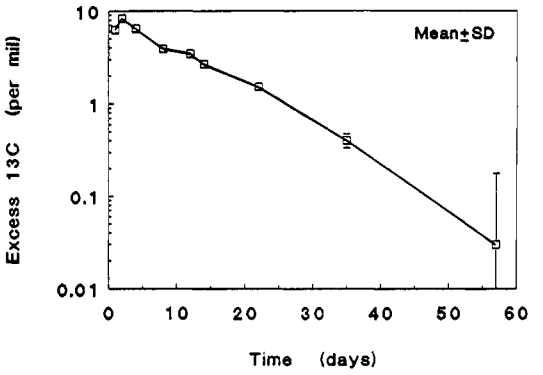

Figure 5. Appearance and disappearance of excess ^{13}C in plasma cholesterol from a 44 year-old adult male after ingestion of 50 mg of [3,4-^{13}C]cholesterol.

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