methods

Oxazolinone derivative of leucine for GC-MS: a sensitive and robust method for stable isotope kinetic studies of lipoproteins

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Abstract **Stable isotope labeled amino acids are commonly used as endogenous tracers to study the metabolism of lipoproteins. The determination of isotopic enrichment of particular amino acids in apolipoproteins is carried out by gas chromatography mass spectrometry (GC-MS). This report describes a robust and sensitive derivative for analysis of d_3-leucine by GC-MS and its utility in studying the metabolism of human lipoproteins. The trifluoromethyloxazolinone (oxazolinone) derivative of leucine was formed in a rapid single step procedure using a mixture of trifluoroacetic anhydride (TFAA) and trifluoroacetic acid (TFA). Analysis of the oxazolinone by negative ion chemical ionization GC-MS gave excellent sensitivity and precision, which enabled accurate determination of low levels of isotopic enrichment from small amounts of protein. For example, enrichments between 0.05% and 100% in 100 pg leucine can be measured with a coefficient of variation of <3%. To demonstrate the utility of this procedure, we measured d_3-leucine enrichment in apolipoprotein B (apoB) isolated from VLDL and LDL as well as apoA-I isolated from HDL by gel electrophoresis and western blotting. The derivatization procedure gave excellent enrichment data from a single intravenous bolus dose of 5 mg/kg, from which the fractional catabolic rate and production rate of the lipoproteins were calculated. In conclusion, the oxazolinone derivative provides a robust and simple procedure for the sensitive analysis of isotopic enrichment for metabolic studies of human lipoproteins.**—Dwyer, K. P., P. H. R. Barrett, D. Chan, J. I. Foo, G. F. Watts, and K. D. Croft. **Oxazolinone derivative of leucine for GC-MS: a sensitive and robust method for stable isotope kinetic studies of lipoproteins.** ***J. Lipid Res.*** **2002.** 43: **344–349.**

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The use of isotopically labeled amino acids as endogenous tracers to study the metabolism of apolipoproteins in vivo has become widely accepted (1–3). Endogenous labeling with stable isotopes has several advantages over the use of exogenously radiolabeled lipoproteins. Apart from patient safety, there are no concerns about lipoprotein modification during isolation and labeling. Furthermore, since all newly synthesized proteins will become labeled following administration with the labeled amino acid, the rate of production of a number of different proteins can be studied simultaneously (4).

In metabolic studies using stable isotope tracers, gas chromatography mass spectrometry (GC-MS) is used to measure isotopic enrichment of amino acids in apolipoproteins. Several different amino acids, such as leucine, valine, and lysine, have been used to determine lipoprotein kinetics (4, 5), with deuterated leucine being favored because this essential amino acid is not converted to other amino acids and is commercially available in quantities required for in vivo human studies. In lipoproteins with rapid turnover, such as VLDL, the isotopic enrichment of leucine in apoproteins, such as apolipoprotein B (apoB), can be easily measured. However, in lipoproteins with a slow rate of turnover, such as apoB in LDL and apoA-I in HDL, adequate precision for determining very low levels of enrichment of the apoproteins can be difficult. Enrichment must be measured against the isotopomer background of the natural compound. The background increases with the number of carbon atoms in the ion monitored and decreases with the mass shift of the labeled ion. The three mass unit shift of trideuterated leucine is therefore advantageous. Silylated derivatives, on the other hand, increase the isotopomer background by increasing the number of carbon atoms and by introducing the isotopes of silicon. Currently, the most commonly used sensitive derivative for GC-MS using negative chemical ionization is the *n*-propyl,*N*-heptafluorobutyramide (5).

Abbreviations: CV, coefficient of variation; GC-MS, gas chromatography mass spectrometry; FCR, fractional catabolic rate; NCI, negative chemical ionization; oxazolinone, trifluoromethyloxazolinone; TFA, trifluoroacetic acid; TFAA, trifluoroacetic anhydride; TTR, tracer/tracee ratio.

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In order to increase the enrichment of stable isotope into lipoproteins, primed-constant infusion of the labeled amino acid is given for up to 15 h, but there are theoretical and logistic advantages in using a small bolus dose of labeled amino acid (3). Therefore, we sought a derivative of low molecular weight that would also be capable of detection in the small amounts of protein available following isolation by gel electrophoresis. The excellent chromatographic properties and robust preparation method of the trifluoromethyloxazolinone (oxazolinone) derivatives of amino acids have been reported (6). We recognized that this derivative would have the low isotopomer background required and speculated that this fluorinated heterocyclic compound could be measured with good sensitivity by negative ion chemical ionization mass spectrometry.

In this report we show that the oxazolinone derivative of leucine allows excellent precision and limits of detection for isotopic enrichment in apoB and apoA-I.

MATERIALS AND METHODS

Materials

[5,5,5-$^2H_3$]L-leucine (d_3-leucine) with isotopic purity of >98% was obtained from Cambridge Isotope Labs (Woburn, MA). Polyacrylamide precast gels and PVDF membranes were obtained from Novex (San Diego, CA). Trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) were of analytical grade purchased from Sigma Chemicals (Castle Hill, Australia). Ion exchange resin AG 50W-X8 was obtained from Biorad. All solvents were AR grade or better.

Human study design

After fasting for 14 h, subjects were given an intravenous bolus injection of d_3-leucine (5 mg/kg). Blood samples were obtained before and 5, 10, 20, 30, 40, 60 min, 1.5, 2, 2.5, 3, 4, 5, 6, 8, and 10 h following injection of d_3-leucine. During the study period patients rested quietly and consumed only water. Patients returned at 24, 48, 72, and 96 h after the initial injection for a further blood sample. In each case subjects fasted overnight. The study was approved by the Human Ethics Committee of Royal Perth Hospital, and participants gave their informed consent.

Isolation, hydrolysis, and derivatization of apolipoproteins

Blood samples were collected into EDTA (0.1%) and plasma obtained by centrifugation at 1000 g for 10 min at 4°C. Plasma for isolation of apoB was used fresh while plasma samples for isolation of HDL apoA-I and free amino acids were stored at −80°C until required. Isolation of VLDL (density < 1.006 kg/l) and LDL (density = 1.019–1.063 kg/l) was by sequential ultracentrifugation, and apoB was separated from each lipoprotein fraction by precipitation with isopropanol as previously described (7–9). The precipitated protein was heated at 110°C with 1 ml 6 M HCl for 24 h. The hydrosylate was diluted to 8 ml and a portion (50 μl) was evaporated to dryness at 120°C. For the isolation of apoA-I, apoB containing lipoproteins were precipitated with heparin/manganese chloride, then 200 μl of supernatant plasma was mixed with 60 μl of CsCl (64%, w/w) and transferred to a Beckman 42.2 rotor. Following centrifugation at 42,000 rpm for 16 h at 4°C, a portion of the supernatant (containing ~1 μg/μl apoA-I) was applied to a Novex precast polyacrylamide mini gel according to the manufacturer's instructions (Novex, San Diego, CA). Following electrophoresis at constant voltage of 200 v for 35 min, the apoA-1 was blotted onto a PVDF membrane and located by staining with amidoblack. The protein band was cut from the membrane and hydrolyzed with 200 μl of 6 M HCl for 24 h at 110°C. For plasma, amino acids were extracted from 20 μl plasma by Dowex ion exchange after precipitation of proteins with 6% perchloric acid. For derivatization, samples were evaporated to dryness at 120°C and a mixture of TFA/TFAA (100 μl, 50:50 v/v) was added. Samples were capped with a teflon coated silicone septum and heated at 110°C for 5 min. Benzene (500 μl) and water (1 ml) were added, and the tube shaken vigorously and centrifuged briefly to separate the phases. The benzene layer was removed for direct analysis by GC-MS.

GC-MS analysis of isotopic enrichment

Oxazolinone derivatives (**Fig. 1**) were analyzed by negative ion chemical ionization GC-MS using a Hewlett-Packard 5989B instrument with methane as the reagent gas. The equivalent of 100 pg of leucine was injected onto the column. Chromatographic separations were performed on a 23 m × 0.18 mm Perkin Elmer 5MS column. The helium carrier gas flow rate was 0.7 ml/min, and the temperature was programmed, following an initial hold at 50°C for 1 min, to rise from 50°C to 90°C at 6°C/min and then ramped to 280°C at 30°C/min. The ion source pressure was 1.8 torr and the source temperature was 200°C. The ions *m/z* 209 [M] and 212 [M for d_3-leucine] were monitored in the selected ion mode. The isotopic enrichment of leucine and protein samples was determined as the tracer/tracee ratio (TTR), calculated as:

$$TTR = 100(IR_t - IR_0)$$

where IR_t is the isotopic ratio of 212 (derived from d_3-labeled leucine)/209 (derived from unlabeled leucine) for the sample at time t and IR_0 is the isotope ratio of the baseline sample at zero time. The ISOPRO v2.1 program was used to calculate theoretical isotopomer distribution (M. Senko, National High Magnetic Field Laboratory, Florida State University).

The SAAM II program (SAAM Institute, Seattle, WA) was used for compartmental modeling of the tracer data. A four-compartment model was used to describe the plasma kinetics of the d_3-leucine following the bolus i.v. dose. The apoB model used to fit the VLDL and LDL apoB tracer has been described previously (10).

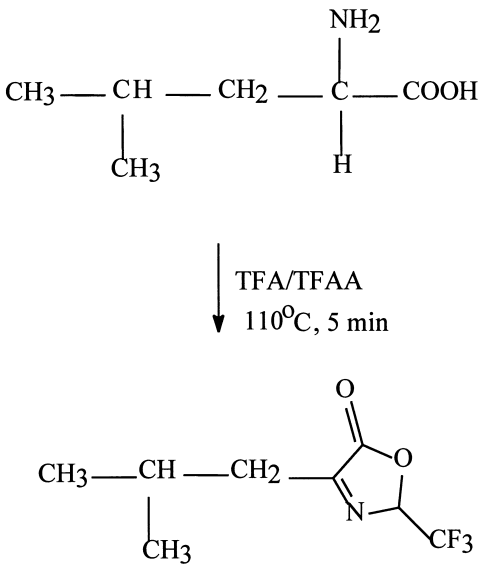

Fig. 1. Chemical structure of leucine and the oxazolinone derivative.

HDL apoA-I kinetics were described using a single plasma compartment that exchanged with an extravascular compartment. Tracer in the apoA-I pool was derived from the same plasma leucine model. The model also included a delay compartment to account for the time associated with the synthesis and secretion of apoA-I into plasma. The compartment model was fit to the apoB and apoA-I tracer data. Metabolic parameters, such as fractional catabolic rate (FCR) and production rates, are derived following fitting.

Determination of optimal sample derivatization conditions

Optimal conditions for the time and temperature for formation of the oxazalinone derivative were studied using authentic leucine standards. The yield of the leucine derivative was quantified against an internal standard of dichlorobenzene (m/z 145) added to the solvent after derivatization. Temperatures from 70°C to 130°C and reaction times of 5–60 min were tested.

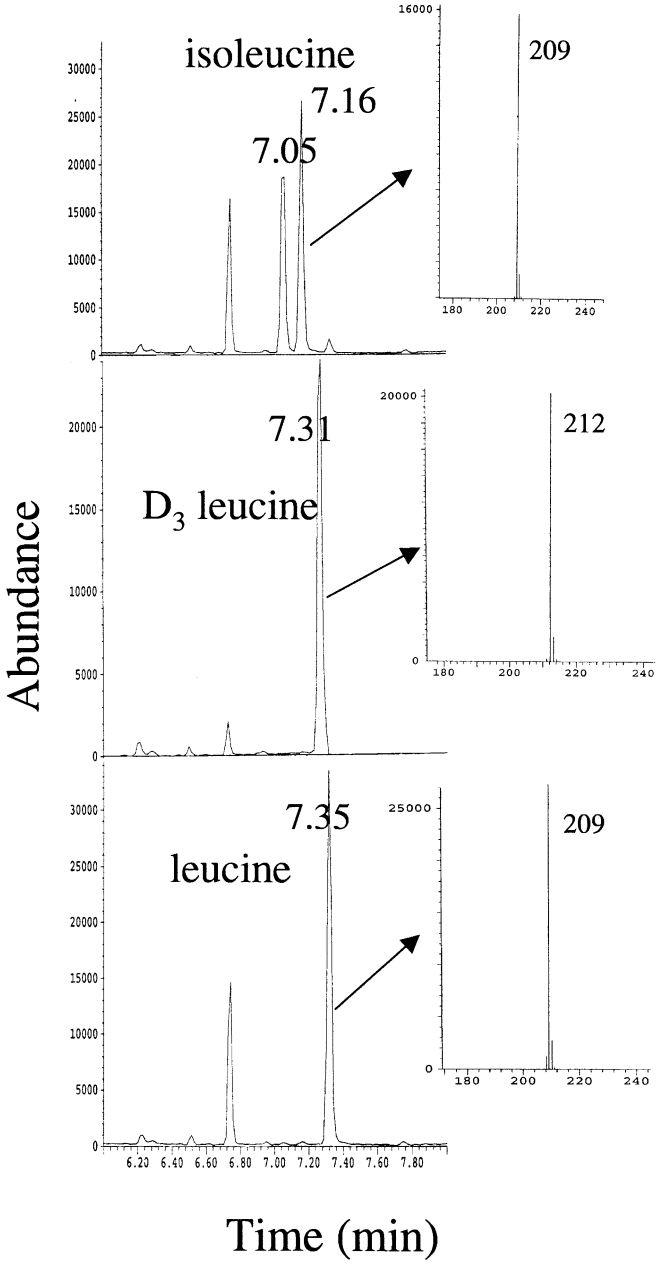

Fig. 2. Total ion chromatograms from injection of 300 pg of leucine, d_3-leucine, and isoleucine standards. Inserts show the negative chemical ionization (NCI) molecular ion spectra. The small peak at 7.35 min in the isoleucine trace is an unknown contaminant with major mass of m/z 208. Samples were run in negative ion mode using methane reagent gas with ion source pressure of 1.8 torr.

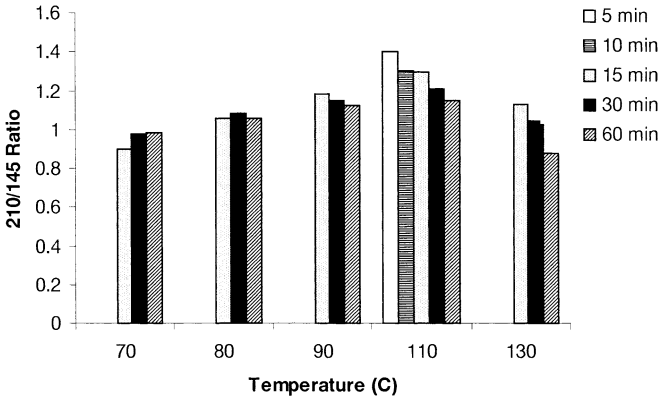

Fig. 3. Yield of leucine oxazolinone derivative following incubation with TFA/TFAA as described in Materials and Methods section at temperatures ranging from 70°C to 130°C and times from 5 to 60 min. Yield was measured relative to dichlorobenzene internal standard (m/z 145); the m/z 210 ion was used for the leucine derivative as it was of similar abundance to the internal standard.

Preparation of isotope enrichment standards

Stock solutions of the pure d_3-leucine tracer and the unlabeled leucine tracee were prepared to known weight percent composition. Tracer and tracee solutions were mixed gravimetrically in doubling dilutions to give TTRs that spanned the range of enrichments found in the in vivo studies. Enrichment standards were dried and derivatized in the same manner as the lipoprotein derived samples.

Reproducibility and precision of the method

Reproducibility for the GC-MS method was determined from values obtained by repeated injections of samples from apoA-I, VLDL apoB, and plasma leucine to cover the range of enrichments from 0.01% to 70%. The mean and coefficient of variation for the ratios of 212/209 were calculated from the chromatographic peak area counts.

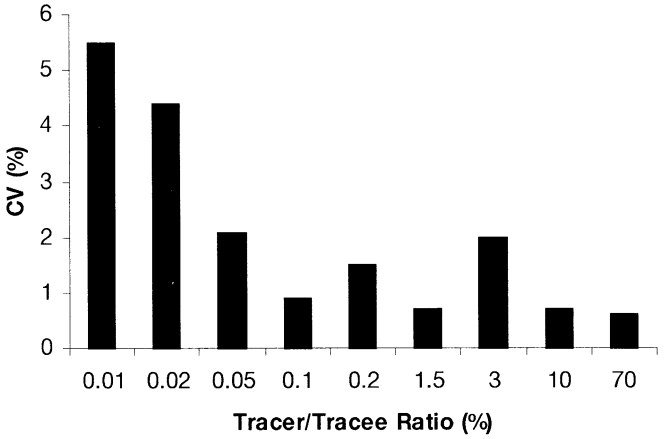

Fig. 4. The coefficient of variation (CV%) of the leucine tracer/tracee ratio determined at different enrichment levels. Data are based on five repeat injections for each sample.

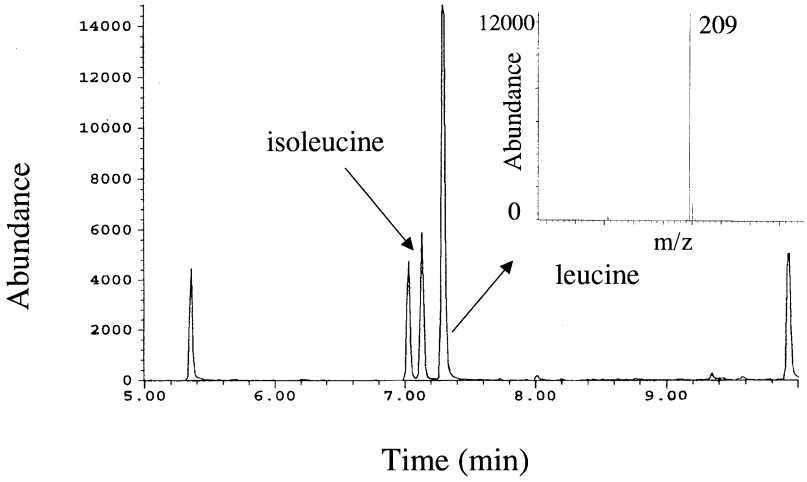

Fig. 5. Total ion chromatogram of the oxazolinone derivative of leucine isolated from LDL apolipoprotein B (apoB). Insert is the NCI molecular ion for the leucine peak eluting at 7.35 min. Gas chromatography mass spectrometry (GC-MS) conditions are the same as those in Fig. 2.

Minimization of contamination

The femtogram sensitivity of the method can lead to problems of contamination. This was particularly evident in the detection of small amounts of isoleucine in apoA-I, a protein that does not contain isoleucine. As contamination arises from a number of sources, such as reagents, gels, and glassware, it cannot be eliminated entirely. We therefore reduced its significance by using relatively large amounts (1 μg) of protein during the hydrolysis and derivatization. Glass tubes used for hydrolysis and derivatization were efficiently cleaned by heating in a muffle furnace at 500°C for 2 h. Necessary dilutions were made of the benzene extract following water washing after the derivatization.

RESULTS

Figure 2 shows the total ion chromatogram and NCI molecular spectrum of derivatized standards of leucine, d_3-leucine, and isoleucine. The derivative has good chromatographic properties, and on a capillary column the deuterium labeled leucine is separated from unlabeled leucine. The heterocyclic ring of the derivative (Fig. 1) contains an asymmetric carbon so that a pair of diastereoisomers would be expected from amino acids with asymmetric side chains. This is the case with isoleucine, which is detected with similar sensitivity to leucine as a pair of peaks, each with the same isotopomer distribution as leucine, but with retention times 11 and 18 s shorter (Fig. 2). Figure 2 also shows that there is no interference from isoleucine with either leucine or d_3-leucine.

The derivatization process was studied over a range of temperatures and times. The results indicate that the oxazolinone is formed in greatest yield at 110°C for the relatively short reaction time of 5 min (**Fig. 3**). Once formed, the derivative is stable for several months after extraction into benzene. Negative ion chemical ionization GC-MS is very sensitive, being able to detect the oxazolinone derivative of d_3-leucine at less than 20 fg (TTR of 0.02% of 100 pg injected). Levels of enrichment of 0.01% can be obtained with a coefficient of variation (CV) of <6%, whereas enrichment levels above 0.1% can be determined with a CV of <2% (**Fig. 4**). Since GC-MS methods can exhibit some degree of concentration dependency, where the ion ratios vary with the quantity of sample injected (11), we measured TTR compared with the theoretical TTR over a range of sample concentrations injected (55–440 pg/μl). We observed with injections of 220 pg/μl or less a linear response across the full range of enrichment from 0.01% to 100% (slope = 0.97, correlation coefficient = 1.00). Only at concentrations of 440 pg/μl did we observe a nonlinear dose response, which corresponded to the manufacturer's quoted upper limit of abundance for linearity of the MS detector.

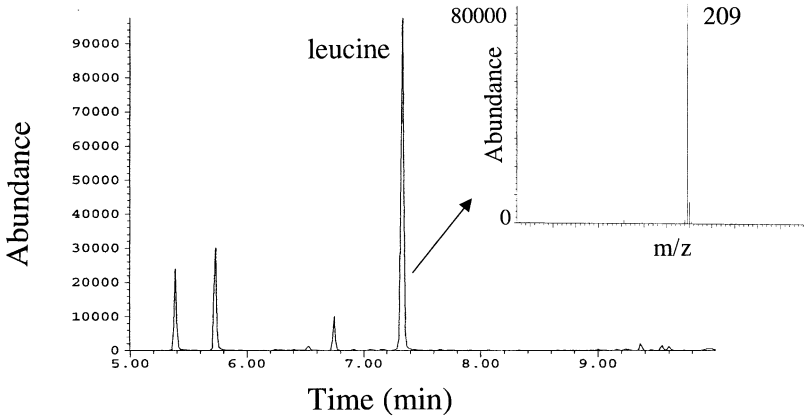

Fig. 6. Total ion chromatogram of the oxazolinone derivative of leucine isolated from HDL apolipoprotein A-I (apoA-I). Insert is the NCI molecular ion for the leucine peak eluting at 7.35 min. GC-MS conditions are the same as those in Fig. 2.

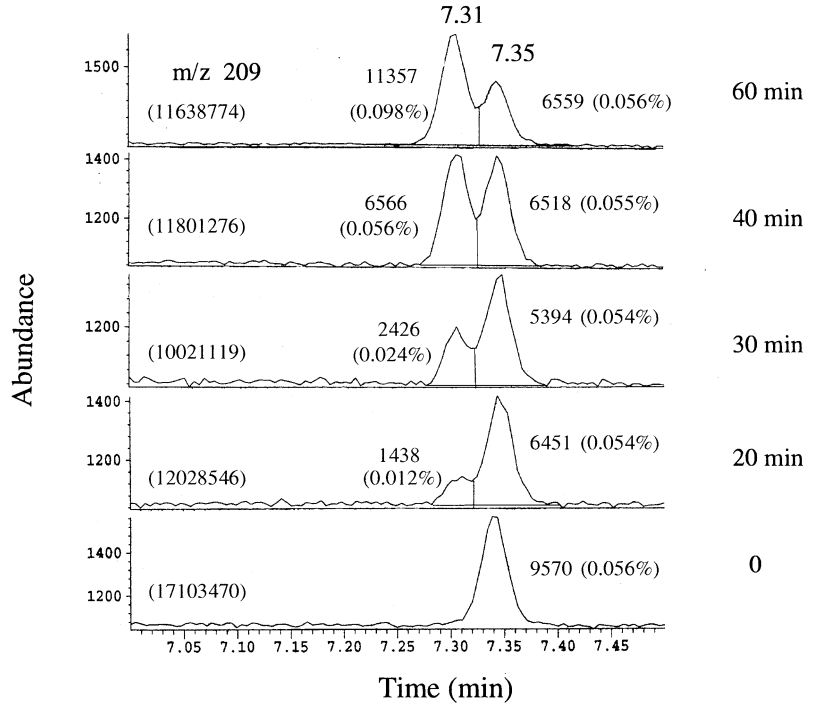

Fig. 7. Selected ion monitoring chromatograms (*m/z* 212) of labeled and unlabeled leucine derived from HDL apoA-I from 0 to 60 min following injection with d_3-leucine. The chromatograms show separation of the d_3-labeled compound from the nondeuterated 212 isotopomer. Area counts are given for each peak together with the *m/z* 209 area count for reference (number in parentheses). The size of the 212 peaks as a percentage of the 209 peak is given in parentheses next to the peak area.

Figures 5 and **6** show total ion chromatograms and NCI spectra of leucine derived from LDL apoB and HDL apoA-I, respectively. The absence of isoleucine in apoA-I is evident in Fig. 6. **Figure 7** shows the selected ion monitoring chromatogram (*m/z* 212) in the region of leucine for samples derived from HDL apoA-I at time zero and up to 60 min following injection of d_3-leucine. This figure shows the gradual enrichment with d_3-leucine as a separate peak from the endogenous leucine isotopomer. In a study of over 20 subjects, in no case did we see any peak co-eluting with d_3-leucine at time zero (before any injection of d_3-leucine); however, after 6 weeks follow-up, small traces of d_3-leucine were present in time zero samples (data not shown).

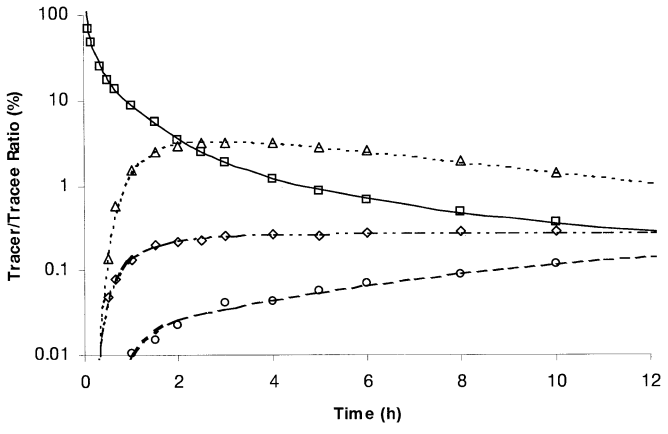

Fig. 8. Isotopic enrichment of leucine, expressed as the tracer/trace ratio following a single bolus injection of d_3-leucine (5 mg/kg). The lines through the enrichment data show the fit of the compartment model to the tracer data. Plasma leucine (squares), VLDL apoB (triangles), LDL apoB (circles), HDL apoA-I (diamonds).

The d_3-leucine enrichment data for apoB in VLDL and LDL and for apoA-I in HDL following a single bolus dose of 5 mg/kg given to a 52-year-old male volunteer (BMI = 33, total chol = 7.0 mmol/l, TG = 2.5 mmol/l, HDL chol = 1.0 mmol/l) are shown in **Fig. 8**. The lines through the observed tracer data show the fit of the compartment model to the tracer data. Fitting a model provides estimates of the FCR from which measures of production can be estimated as the product of the FCR and apoprotein pool size.

DISCUSSION

In metabolic studies using stable isotope traces, GC-MS is often used to measure the TTR (*z*) of the compound under study. It can be difficult to determine the kinetics of proteins with a slow rate of turnover such as apoA-I because of poor precision in determining the low values of *z*. Much of this difficulty arises because *z* is measured as the difference between the ion ratios of the enriched metabolite and the background of the unenriched metabolite. The isotopic background arises because of the presence of naturally occurring stable isotopes, principally carbon-13, and isotopes of silicon in silylated derivatives. In the case of leucine ($C_6H_{13}NO_2$), the isotopomer containing one carbon-13 constitutes 6% of the molecules of the amino acid. In derivatives such as *n*-propyl ester and *N*-heptafluorobutyramide, the number of carbon atoms in the molecule more than doubles, increasing the abundance of the natural isotopes and, consequently, the background against which the enrichment must be measured. The use of multiply substituted stable isotopes sharply reduces the problem of isotopic back-

ground. The oxazolinone derivative has previously been used for the gas chromatographic analysis of amino acids (6). This derivative, being of relatively small molecular weight, has the advantage of minimizing the contribution of naturally occurring isotopes. The small size of the oxazolinone derivative also gives the molecule favorable chromatographic properties such that the trideuterated leucine is almost completely resolved from the natural compound. The retention time change is due specifically to deuterium, because the naturally occurring carbon-13 isotopomer of the same mass as the trideuterated compound has unchanged mobility. The ability to integrate the enrichment peak separately from the naturally occurring leucine could further help to reduce the contribution of both isotopic and chemical background. Negative chemical ionization not only provides a very sensitive method of detection for these derivatives, but also gives a very simple mass spectrum consisting generally of the molecular ion and associated isotopomers. This derivative measured by negative chemical ionization has excellent sensitivity and precision for determining low levels of isotopic enrichment. It enables the study of small amounts of proteins excised from gels, in cell culture studies, or when only small samples are available from humans or animals. However, the critical feature of the method is its ability to accurately determine very low levels of isotopic enrichment. The derivatization reagents are cheap, and the derivatizing procedure requires only a single step with short reaction time. The sensitivity with respect to TTR and mass allows the use of single bolus injection of labeled amino acid in kinetic studies. The bolus protocol is more convenient to carry out, less invasive for patients, and gives data that is easier to interpret due to the decreased contribution of recycling of amino acid compared with primed constant infusions (12). The derivative has been shown to be suitable for the gas chromatography of a number of other amino acids (6), and it is probable that these will also be very sensitively detected by negative ion GC-MS. The method is therefore likely to be of general use for the measurement of low concentrations of alpha amino acids, both natural and synthetic.

In conclusion, the oxazolinone derivative provides a robust and sensitive technique from which excellent kinetic data can be obtained for apoB and apoA-I from subjects who have undergone injection of d_3-leucine.

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REFERENCES

1. Schaefer, J. R., D. J. Rader, and H. B. Brewer, Jr. 1992. Investigations of lipoprotein kinetics using endogenous labeling with stable isotopes. *Curr. Opin. Lipidol.* **3**: 227–232.
2. Gaw, A. C., J. Packard, and J. Shepherd. 1992. VLDL and LDL turnover: methods and clinical application. *Curr. Opin. Lipidol.* **3:** 384–388.
3. Foster, D. M., P. H. R. Barrett, G. Toffolo, W. F. Beltz, and C. Cobelli. 1993. Estimating the fractional synthetic rate of plasma apolipoproteins and lipids fom stable isotope data. *J. Lipid Res.* **34:** 2193–2205.
4. Patterson, B. W., D. L. Hachey, G. L. Cook, J. M. Amann, and P. D. Klein. 1991. Incorporation of a stable isotopically labelled amino acid into multiple human apolipoproteins. *J. Lipid Res.* **32:** 1063–1072.
5. Lichtenstein, A. H., J. S. Cohn, D. L. Hachey, J. S. Millar, J. M. Ordovas, and E. J. Schaefer. 1990. Comparison of deuterated leucine, valine and lysine in the measurement of human apolipoprotein A-I and B-100 kinetics. *J. Lipid Res.* **31:** 1693–1701.
6. Ferrito, V., R. Borg, J. Eagles, and G. R. Fenwick. 1979. Gas chromatographic and mass spectrometric characteristics of 2-trifluoromethyl-3-oxazol-5-one: a potentially useful derivative for α-amino acids. *Biomed. Mass Spectrom.* **6:** 499–501.
7. Riches, F. M., G. F. Watts, R. P. Naoumova, J. M. Kelly, K. D. Croft, and G. R. Thompson. 1998. Hepatic secretion of very-low-density lipoprotein apoB-100 studied with a stable isotope technique in men with visceral obesity. *Int. J. Obes.* **22:** 414–423.
8. Riches, F. M., G. F. Watts, J. Hua, G. R. Stewart, R. P. Naoumova, and P. H. R. Barrett. 1999. Reduction in visceral adipose tissue is associated with improvement in apolipoprotein B-100 metabolism in obese men. *J. Clin. Endocrin. Metab.* **84:** 2854–2861.
9. Egusa, G., D. W. Brady, S. M. Grundy, and B. V. Howard. 1983. Isopropanol precipitation method for the determination of apolipoprotein B specific activity and plasma concentrations during metabolic studies of very low density lipoprotein apolipoprotein B. *J. Lipid Res.* **24:** 1261–1267.
10. Parhofer, K. G., P. H. R. Barrett, D. Bier, and G. Schonfeld. 1991. Determination of kinetic parameters of apolipoprotein B metabolism using amino acids labeled with stable isotopes. *J. Lipid Res.* **8:** 1311–1323.
11. Patterson, B. W., G. Zhao, and S. Klein. 1998. Improved accuracy and precision of gas chromatography/mass spectrometry measurements for metabolic tracers. *Metabolism.* **47:** 706–712.
12. Barrett, P. H. R., and D. M. Foster. 1996. Design and analysis of lipid tracer kinetic studies. *Curr. Opin. Lipidol.* **7:** 143–148.