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Optimization of *N*-methyl-*N*-[*tert*-butyldimethylsilyl]-trifluoroacetamide as a derivatization agent for determining isotopic enrichment of glycerol in very-low density lipoproteins

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Stable isotope kinetic studies play an important role in the study of very-low density lipoprotein (VLDL) metabolism, including basic and clinical research. Today, [1,1,2,3,3-$^2H_5$]glycerol is the most cost-effective alternative to measure glycerol and triglyceride kinetics. Recycling of glycerol from glycolysis and gluconeogenesis may lead to incompletely labelled tracer molecules. Many existing methods for the measurement of glycerol isotopic enrichment involve the production of glycerol derivatives that result in fragmentation of the glycerol molecule after ionization. It would be favourable to measure the intact tracer molecule since incompletely labelled tracer molecules may be measured as fully labelled. The number of methods available to measure the intact tracer in biological samples is limited. The aim of this project was to develop a gas chromatography/mass spectrometry (GC/MS) method for glycerol enrichment that measures the intact glycerol backbone and is suitable for electron ionization (EI), which is widely available. A previously published method for *N*-methyl-*N*-[*tert*-butyldimethylsilyl]trifluoroacetamide (MTBSTFA) derivatization was significantly improved; we produced a stable derivative and increased recovery 27-fold in standards. We used the optimized MTBSTFA method in VLDL-triglyceride and found that further modification was required to take matrix effects into account. We now have a robust method to measure glycerol isotopic enrichment by GC/EI-MS that can be used to rule out the known problem of tracer recycling in studies of VLDL kinetics. Copyright © 2010 John Wiley & Sons, Ltd.

Abnormal concentrations of lipids and apolipoproteins are markers of several lipid disorders and can result from changes in the production, conversion or catabolism of lipoprotein particles. Thus, although static measurements are important, they do not reveal the underlying mechanisms involved in the dysregulation of lipid disorders. To infer this information, it is necessary to perform *in vivo* tracer/tracee studies in which the rates of synthesis or catabolism of a particular lipoprotein or apolipoprotein can be determined. The metabolism of lipoprotein particles can be followed by injecting amino acids labelled with stable isotopes which are then incorporated into proteins such as apoB100.[1] The triglyceride content can be followed by infusion of labelled glycerol or free fatty acids.[2] Early studies used radioactive [^{14}C or ^{3}H]glycerol[3,4] but all *in vivo* lipoprotein kinetic studies are now performed using infusion of stable isotope labelled compounds such as [1,2,3-$^{13}C_3$]glycerol or [1,1,2,3,3-$^2H_5$]glycerol.[2] Kinetic studies of very-low density lipoproteins (VLDL) have been informative especially when sub-divided according to density ($VLDL_1$ and $VLDL_2$).[5,6] Several approaches to determine the enrichment of glycerol $^2H_5$ have been developed, using gas chromatography/mass spectrometry (GC/MS) and electron ionization (EI),[2,7–9] or chemical ionization (CI).[10,11] These include derivatization to triacetate glycerol,[7,8] or *tris*-trimethylsilyl glycerol,[9] and EI-MS. A potential weakness using these methods is that fragmentation of the molecule occurs between the carbon 1 and 2, or 2 and 3 of the glycerol backbone during electron ionization. Methods that lead to fragmentation of the glycerol backbone are inherently less sensitive.[12] In addition, it has been found that a considerable amount of the glycerol in VLDL-triglyceride can be derived from glycolytic cycling that result in the formation of partially de-deuterated glycerol.[2] It is important to exclude this recycling from the kinetic modelling. Any such recycling would not be detectable unless the entire glycerol backbone remained intact during GC/MS analysis, enabling the M+5 ion to be detected. Particular derivatization techniques that sub-

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sequently tend to leave the glycerol backbone intact while in the mass spectrometer include reaction with heptafluorobutyric anhydride (HFBA, both EI and CI)[2,11] and reaction with acetic anhydride in combination with CI.[10] In these cases, the fragmentation preferentially occurs in the glycerol side chains created by the derivatization reagent.

The number of methods that use EI while retaining the structure of glycerol is limited. An EI method is desirable for GC/MS compared with positive or negative CI as it keeps the source cleaner. In addition, although CI is becoming more common, many low-cost bench-top mass spectrometers do not posses this facility as standard. Although EI has been used to analyze glycerol derivatized with HFBA, the relevant ion is of low intensity.[11] The reagent *N*-methyl-*N*-[*tert*-butyldimethylsilyl]trifluoroacetamide (MTBSTFA) is commonly used to create TBDMS (*tert*-butyldimethylsilyl) derivatives of metabolites[13] in order to make them more suitable for analysis by MS. MTBSTFA reacts with groups such as hydroxyl, carboxyl, thiol, and primary and secondary amines and has been used to derivatize glycerol before its analysis by GC/EI-MS.[12] However, here we report significant problems using a method that uses MTBSTFA to derivatize glycerol when analyzed by EI.[12] We describe improvements in the protocol which have resulted in a clean, robust method. We have also compared results using derivatization with MTBSTFA (with EI) and acetic anhydride (with EI) on the kinetic parameters calculated using a multicompartmental model of $VLDL_1$ and $VLDL_2$. We also tested the improved MTBSTFA method on samples prepared by solvent extraction, which allows simultaneous fatty acid analysis.

EXPERIMENTAL

Subjects

Eight subjects with a varied degree of liver fat (4–25%), body mass index (BMI) (27–40 $kg \cdot m^{-2}$) and waist measurement (82–133 cm) were recruited for this study. The purpose, nature, and potential risks of the study were explained to all subjects before their written consent was obtained. The study protocols were approved by the Ethical Committee of the Helsinki University Central Hospital.

Equipment

The GC/MS system (Agilent, Santa Rosa, CA, USA) consisted of a gas chromatograph (model 6890N) equipped with an autosampler injector (model 7683B) and a mass spectrometer (model 5975B) with an electron ionization (EI) source. The column was a DB5MS (30 m, 0.25 mm, film thickness 0.25 μm; 122-5532GL; J&W, Folsom, CA, USA) equipped with a guard column of approximately 10 m. The carrier gas was helium.

Materials

[1,1,2,3,3-$^2H_5$]Glycerol was obtained from Sigma/Isotec (Miamisburg, OH, USA). Cation resin (No. 142-1441), AG-50W-X8 (100–200 mesh), hydrogen form and anion resin (No. 140-1444), AG-1-X8 (100–200 mesh), formate form, were obtained from Bio-Rad (Sundbyberg, Sweden). Acetonitrile (No. 20062) from Pierce (Rockford, IL, USA) and pyridine (No. 27530) from Thermo (Rockford, IL, USA) were derivatization grade. MTBSTFA (No. 19915-5) and glycerol (No. 7757) were from Sigma (Stockholm, Sweden). Zeolite (No. 96096) was from Fluka Biochemika (Buchs, Switzerland).

Methods

Standards and samples

Stock solutions (10 mmol/L) of unlabelled and [1,1,2,3,3-$^2H_5$]glycerol were prepared in deionized H_2O. For isotopic standard curve samples, the stock solutions were mixed 1:1 for a 50 atom percent excess (APE) solution and sequentially diluted 1:1 with the unlabelled solution for solutions with 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.098, 0.049 APE. These standard curve samples were evaporated to dryness in a Speed-Vac® sample concentrator (Savant Instruments, Farmingdale, NY, USA) from volumes of 100 μL and were kept frozen at −20°C. Plasma from eight subjects was collected after injection of [$^2H_3$]leucine and [1,1,2,3,3-$^2H_5$]glycerol.[7] The samples were analyzed for enrichment of leucine in plasma, $VLDL_1$ and $VLDL_2$-apoB as described[7] and for glycerol in plasma and $VLDL_1$- and $VLDL_2$-triglyceride as described below.

Preparation of VLDL

$VLDL_1$ and $VLDL_2$ were isolated in a total volume of 8.4 mL EDTA-plasma.[14] For further analysis, 1 mL of $VLDL_1$ or 500 μL of $VLDL_2$ was used. The samples were deproteinized with isopropanol and delipidated twice with ethanol/diethyl ether.[15] The supernatants were combined, and the volume was increased to 20 mL with isopropanol. The phospholipids were removed by adding 2 g of activated zeolite to each tube and mixing for 20 min.[7] After centrifugation, the supernatants were evaporated under N_2 at 80°C, dissolved in isopropanol (1 mL), transferred into a 1.5 mL vial, and dried on a heating block at 80°C.

At this point, samples could be treated in one of two ways (in order to release glycerol from the triglyceride molecule) depending on which further analyses were to be performed.

1. Saponification of triglycerides with potassium hydroxide

Potassium hydroxide stock solution was prepared by dissolving 33 g KOH in 100 mL deionized H_2O. On the day of analysis, 3 mL of the stock solution were dissolved in 50 mL 95% ethanol. After removal of the phospholipids with zeolite, the sample was saponified by adding 250 μL saponification solution. After incubation at 60°C for 2 h, the sample was cooled to room temperature. We modified the protocol by including a purification step; 200 μL chloroform was added, followed by 200 μL H_2O. After mixing and centrifuging, 200 μL of the aqueous phase (top layer) were transferred to a new vial and dried under nitrogen. This was necessary to achieve effective derivatization with MTBSTFA. The samples were then derivatized with either MTBSTFA or acetic anhydride as described in the relevant sections below.

2. Transesterification and ion-exchange chromatography

We wished to test the MTBSTFA method using an alternative preparative procedure that would allow for the simultaneous

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isolation of VLDL-fatty acids after removal of phospholipids. Therefore, the dried samples from the 'VLDL preparation' were subjected to a transesterification/methylation procedure. In this procedure,[2,16] the fatty acid methyl esters were extracted into a solvent layer, leaving the glycerol backbone from the triglyceride molecule in aqueous solution. The samples were dissolved in 400 μL toluene, mixed and transferred to 15 mL Pyrex® tubes. Fatty acid methyl esters were prepared from triglycerides (plus, in this case, any small amount of cholesteryl ester present in the VLDL) by adding 800 μL of newly prepared 1.5% H_2SO_4 in methanol to the glass tubes. The tubes were vortexed briefly, incubated at 80°C for 1 h, and allowed to cool down to room temperature. The samples were neutralized using 2 mL neutralizing solution (25 g $KHCO_3$ (0.125 M) + 34.6 g K_2CO_3 (0.125 M)) made up to 500 mL with distilled water and diluted 1:5 for use, and then phase-separated using 2 mL cyclohexane and mixing for 15 min on a rotary mixer before centrifuging at 1000 *g* at room temperature for 10 min. The supernatant was removed (and could be used for further fatty acid analyses) and any remaining solvent was evaporated using nitrogen (35–50°C, 5–10 min). The aqueous phase (which contained the glycerol released from the triglyceride) was purified by a standard ion-exchange chromatography protocol as follows.

Anion and cation resins were mixed separately with deionized H_2O (1:1 v/v) in a beaker and stirred at room temperature for 5 min. A polypropylene frit was placed into a 3 mL column and, after stirring the resins, 1 mL cation resin was added with a pipette and the wall and resin were washed with 1 mL deionized H_2O. Then 1 mL anion resin was added on top of the cation with a pipette and the walls and resin were washed with 3 × 1 mL deionized H_2O. The columns could be stored at room temperature for 3 days before use. The samples were carefully added to the prepared ion-exchange columns and carefully eluted into 15 mL tubes with 3 × 1 mL deionized H_2O. The samples were then frozen at −80°C for at least 2 h before being placed in a freeze-drier over night (approximately 15 h). Since glycerol is volatile care must be taken not to dry samples excessively. Thus, the samples were removed when a few hundred μL of the samples remained and they were then put under nitrogen at 35°C to evaporate until dry.

Optimized derivatization with MTBSTFA

The samples that had been through the transesterification procedure were derivatized by adding 100 μL pyridine and 100 μL MTBSTFA, and samples that had been passed through saponification were derivatized in 50 μL pyridine and 50 μL MTBSTFA. The samples were incubated for 4 h at 70°C, cooled down, and transferred to autosampler vials before being evaporated under nitrogen at 35°C and re-dissolved in 50 μL pyridine. The vials were tightly closed with crimp lids.

*GC/MS analysis of tri-[*tert*-butyldimethylsilyl]glycerol*

For GC/MS analysis, the injector temperature on the gas chromatograph was 320°C and column flow rate was 1.1 mL/min. The initial oven temperature was 110°C and it was ramped at 10°C/min to 210°C and then at 20°C/min to 310°C, and was held at this temperature for 3 min (total run time 18 min). The MSD interface temperature was 320°C. A volume of 1 μL of the sample was injected at a split ratio 50:1. Tri-[*tert*-butyldimethylsilyl]glycerol has a molecular mass of 434 and the major EI peak corresponds to a fragmentation where a butyl group is removed and results in a fragment ion with a monoisotopic mass-to-charge ratio (*m/z*) of 377.3. The peaks of *m/z* 377.3 and 382.3 were then measured using selective ion monitoring. If necessary, samples were rerun with a variable split ratio to ensure that the 377.3 peak areas were of similar size (±20%).

Derivatization with acetic anhydride

The triacetate protocol was performed as previously described.[7] Glycerol was derivatized to its 1,2,3-triacetate ester by adding equal volumes (20 μL) of pyridine and acetic anhydride and was left at room temperature for 30 min. An additional purification step was included; 100 μL chloroform and 50 μL water were added and, after mixing and centrifuging, 95 μL of the chloroform phase were transferred to the autosampler vials, dried under nitrogen and re-dissolved in 30 μL pyridine.

GC/MS analysis of glycerol triacetate

The initial oven temperature was 170°C and this was held constant for 5 min before being ramped at 10°C/min to 210°C and held constant for 6 min. The injector and interface temperatures were both 320°C and the column flow rate was 1.2 mL/min. A volume of 1 μL of the sample was injected at a split ratio of 50:1. The peaks areas corresponding to *m/z* 145, 147 and 148 were measured. The tracer/tracee ratio (TTR) was calculated either as the ratio of *m/z* 148 to 145 using a fixed *m/z* 147-to-145 ratio determined from the baseline samples, which were run in duplicates,[7] or by a direct ratio of the *m/z* 145 and 148 peaks.

Preparation of plasma samples (free glycerol)

The plasma samples were de-proteinized by adding 2 mL ice-cold acetone to 0.5 mL of plasma. The samples were mixed by vortex and incubated for 1 h on ice. After centrifuging at 3200 *g* at room temperature for 10 min, the supernatant was transferred to a new tube. The acetone was separated from the water phase by adding 2 mL chloroform, centrifuging at 3200*g* at 4°C for 10 min and transferring the aqueous phase (upper 0.5 mL) to a new tube. This procedure was repeated once more after the addition of 0.5 mL deionized H_2O. To remove any remaining acetone, the samples were incubated under nitrogen for 10–15 min at 45–50°C. The aqueous phase was then passed through an ion-exchange column as described above, and samples derivatized with MTBSTFA.

Data analysis

Isotopic standard curve samples that had been derivatized with the same reagent (MTBSTFA or acetic anhydride) and were run before and after each sample sequence. The mean ratio of the measured to expected APE, expressed as a percentage, was calculated for the standard curves and sample enrichments were corrected using a linear interpolation between the 'before' and 'after' standard curves. The atom percent excess (APE) was calculated for each sample by calculating the tracer/tracee ratio (*TTR*), followed by

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subtracting the background TTR from a baseline sample and then normalizing by total count (e.g. 1 + TTR) and converting into a percentage.[17]

Kinetic modelling

We performed a kinetic study in eight subjects (2 groups of 4) with infusions as previously described,[7] and compared the results obtained using the optimized MTBSTFA and acetic anhydride methods. In four subjects we compared the optimized MTBSTFA method with glycerol isolated during transesterification/methylation of fatty acids (group G1M) with the acetic anhydride protocol after saponification (G1A). In the other four subjects we compared the MTBSTFA (G2M) with acetic anhydride (G2A) derivatization after saponification (Fig. 1).

RESULTS

Incomplete derivatization after 30 min at room temperature

Un-enriched glycerol standards were first derivatized for 30 min with MTBSTFA at room temperature according to the method of Flakoll *et al*.[12] We realized that derivatization was continuing in the GC vial, so we sought to investigate under what conditions the reaction became complete.

Optimization of MTBSTFA derivatization conditions

Initial experiments at room temperature showed that the peak area increased by approximately six-fold from 30 min to 70 h (data not shown). Thus, standard samples were derivatized under different conditions and we found the

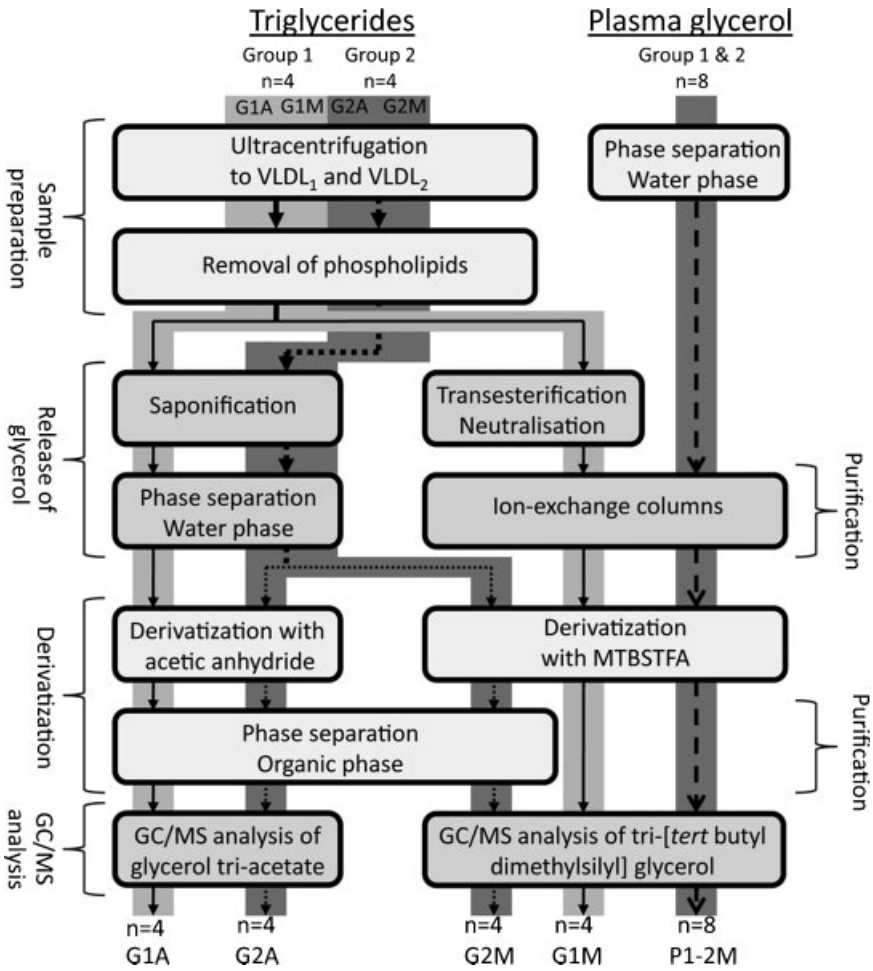

Figure 1. Schematic overview of methods. Samples from eight subjects were analyzed. After ultracentrifugation and removal of phospholipids, samples were split in two groups of four. Four samples (group 1) were split into two equal volumes and either treated with the modified saponification protocol and derivatized with acetic anhydride and analyzed by GC/EI-MS (G1A), or transesterified using the method of Flakoll *et al*.[12] and derivatized using MTBSTFA and analyzed by GC/EI-MS (G1M). The other four samples (group 2) were treated with the modified saponification protocol and split into two equal volumes after phase separation and then derivatized with either acetic anhydride (G2A) or MTBSTFA (G2M). Plasma samples from all eight subjects were analyzed with the same protocol (P1-2M).

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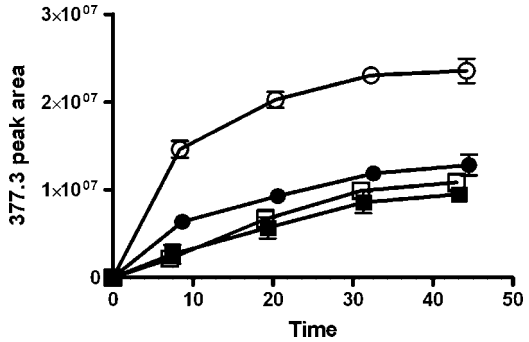

Figure 2. Peak areas after reaction at room temperature or 70°C and the effect of TBDMCS. Glycerol was derivatized with MTBSTFA with or without TBDMCS at either room temperature or at 70°C for 4 h and then left at room temperature. At room temperature there was little difference between the peak areas with or without TBDMCS at 8 h. Performing the reaction at 70°C for 4 h increased the peak area 7-fold compared with that at room temperature; however, when TBDMCS was included the increase was only 3-fold. Open boxes, room temperature; closed boxes, room temperature with TBDMCS; open circles, 70°C 4 h; closed circles, 70°C 4 h with TBDMCS.

peak area to increase with the derivatization time and temperature (Fig. 2).

Including the accelerator *tert*-butyldimethylchlorosilane (TBDMCS) in the derivatization reaction mixture yielded no improvement in recovery, either at room temperature or at 70°C. In fact, the peak areas were lower when TBDMCS was included when incubating at 70°C (Fig. 2).

To establish a final derivatization protocol, the standards were then derivatized at 70°C for 30 min, 1, 2, 4, 6 and 8 h. With increasing derivatization time, the product peak area increased 27-fold from 30 min to 8 h (Fig. 3). The *m/z* 382.3 peak area could be measured accurately in un-deuterated samples as the *m/z* 377.3 peak area reached 20×10^6, and the product was found to be stable for at least 5 days.

Recovery of glycerol enrichment is linear between 0 and 50 APE

Running sequential dilution series between 0 and 50 APE showed that the method made it possible to detect

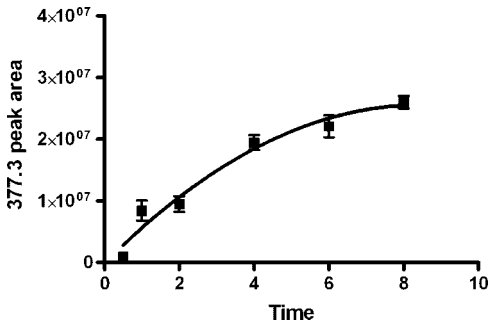

Figure 3. Reaction speed versus time. We derivatized glycerol for 30 min, 1, 2, 4, 6 and 8 h and found that the derivatization continued for at least 8 h. We conclude that 4 h at 70°C was sufficient for the reaction to be sufficiently completed. Closed boxes, *m/z* 377.3 peak area.

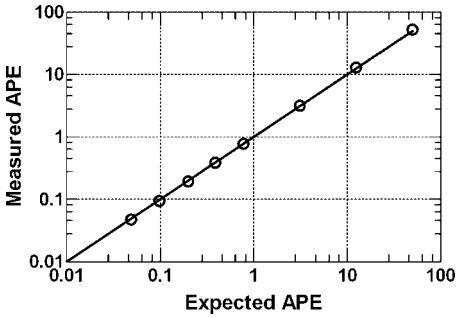

Figure 4. Measured and expected APE from sequential dilution series. The recovery was linear in the range 0–50 APE. Solid line, theoretical 100% recovery; Open circles, measured APE (n = 22). Coefficient of variation was 1–8%.

enrichment of tri-[*tert*-butyldimethylsilyl]glycerol in a linear manner in this range (Fig. 4). The ratio of observed to expected APE was 1.01 ± 0.01 (mean ± standard error of the mean (SEM), n = 22) and ranged from 0.94 to 1.10 (n = 22). In addition, the measured TTRs in the standard curve samples analyzed immediately after the samples were within 1% of the TTRs measured in the standard curve samples run prior to sample measurements.

Comparison of isotopic enrichments

The ratio between the observed and theoretical APE in the standard curves was close to 1.0 for tri-[*tert*-butyldimethylsilyl]glycerol (see results above) but only 0.74 ± 0.005(mean ± SEM, n = 8) for glycerol triacetate, and the sample enrichments were adjusted accordingly. The isotopic enrichments in glycerol from VLDL-triglycerides were higher (on average 9–34% for all sample points in $VLDL_1$ and $VLDL_2$) with the MTBSTFA protocol than with the triacetate method[7] (Fig. 5).

Data presented by Patterson *et. al.*[2] in which the relative proportions of different isotopomers in VLDL glycerol (non-fragmented) were presented show that only 25% of the glycerol tracer was m+5 (the form administered), some 6%

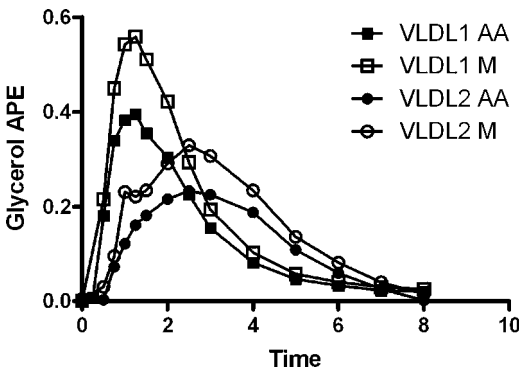

Figure 5. Enrichment curves in glycerol from $VLDL_1$- and $VLDL_2$-triglycerides after a bolus injection of [1,1,2,3,3-$^2H_5$] glycerol. Glycerol enrichment (APE) in $VLDL_1$ and $VLDL_2$ using two different derivatization methods. Open symbols, optimized MTBSTFA (M) method; closed symbols, acetic anhydride (AA) method.

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Table 1. Kinetic parameters determined using the different methods

	Group 1			Group 2			Groups 1 & 2		
	G1A	G1M	p[a]	G2A	G2M	p[a]	G1-2A	G1-2M	p[b]
Triglycerides									
$VLDL_1$ FCR	11.23	10.28	0.625	11.00	11.96	0.375	11.12	11.12	0.996
$VLDL_1$ Sec	34350	32036	0.375	21032	22189	0.625	27691	27113	0.710
$VLDL_2$ FCR	10.64	10.26	0.875	11.87	8.87	0.250	11.26	9.56	0.124
$VLDL_2$ DSec	4444	4525	0.875	2915	2301	0.250	3679	3413	0.290
ApoB									
$VLDL_1$ FCR	8.40	8.42	0.875	9.44	10.03	0.250	8.92	9.22	0.383
$VLDL_1$ Sec	1170	1167	1.000	842	902	0.250	1006	1035	0.476
$VLDL_2$ FCR	4.54	4.33	0.625	4.25	3.89	0.625	4.39	4.11	0.286
$VLDL_2$ DSec	363	364	0.875	222	226	1.000	293	295	0.806

FCR, fractional catabolic rate [pools/day]; Sec, Secretion [mg/day]; DSec, Direct Secretion [mg/day]; G1A, group 1 saponification + acetic anhydride; G1M group 1 transesterification + MTBSTFA; G2A, group 2 saponification + acetic anhydride; G2M group 2 transesterification + MTBSTFA.

[a] *p*-values attained from paired Wilcoxon paired rank test.

[b] *p*-values attained from paired t-test.

was m+4 and 13% was m+3. In our samples derivatized to triacetate glycerol, five-labelled glycerol will occur as m+3 in the fragment ion monitored. Four-labelled glycerol will always occur as m+2 since it is the product of conversion of glycerol-3-phospate into dihydroxyacetone phosphate and back, which replaces the deuterium on the second carbon.[18] Further processing of dihydroxyacetone produces glyceraldehyde-3-phosphate in which one hydrogen atom on the first carbon is replaced. This produces a three-labelled glycerol molecule that can either result in an m+2 or an m+1 fragment ion, depending on where the molecule is disrupted during ionization. Thus, the m+2 peak may very well be overestimated and therefore the calculated m+3/m+2 ratio results in an underestimation of the true TTR.

Impact of choice of method for glycerol enrichment measurements on kinetic modelling

The $VLDL_1$ and $VLDL_2$ kinetic parameters calculated using a multi-compartmental model[7] are summarized in Table 1. There were no significant differences when comparing results from the same subjects using different methods, as explained in Fig. 4 (G1A vs. G1M and G2A vs. G2M; NS Wilcoxon paired rank test), nor was there any significant difference when grouping subjects based on derivatization method (G1-2A vs. G1-2M).

To test if the amplitude of the APE curve affected the model outcome we scaled the $VLDL_1$ and $VLDL_2$ enrichments curves by factors of 0.5, 0.75, 1.5 and 2.0 and re-analyzed the results using the mathematical model. The change in kinetic parameters after scaling was less than 1%.

DISCUSSION

In this paper we present a method for preparing glycerol from triglycerides in $VLDL_1$ and $VLDL_2$ and measuring the enrichment of the intact glycerol backbone using MTBSTFA and EI-MS. The starting point for this analysis was to compare the protocol described by Flakoll *et al.*[12] with our previously published protocol. However, when using MTBSTFA to derivatize glycerol,[12] it was found that the reaction was far from complete. We therefore investigated the effect of temperature and time on the reaction, and have developed a more robust and sensitive method.

Several changes were made to the protocol in order to maximize the amount of material derivatized, increase the peak area of the selected ions, and reduce the amount of underivatized material injected onto the column. Using our revised protocol, we increased the amount of derivatized material 27-fold. The original protocol stated that 30 min at room temperature was enough for complete derivatization,[12] but we found that the reaction continued for many hours. Moreover, we found that increasing the temperature to 70°C further increased the amount of derivatized material several-fold. For practical reasons, 4 h at 70°C was chosen as the reaction time and temperature. Under these conditions, the reaction was nearly complete. However, it was possible to incubate for up to a further 24 h at room temperature. We included an evaporation step and re-dissolved the derivatized sample in pyridine to ensure a stable solution and to avoid continuation of the reaction in the autosampler vials.

TBDMCS is often used as an accelerator for MTBSTFA in derivatization protocols. However, we found that adding 1% TBDMCS to the MTBSTFA neither enhanced the reaction speed nor stabilized the product, and therefore the accelerator was not necessary. We found that the standard curves were linear from 0 to 50 APE, with a linear detection range using our GC/MS instrumentation. A wide linear range is necessary since plasma glycerol enrichment may reach 50 APE immediately after tracer injection, whereas the typical enrichment of $VLDL_1$ and $VLDL_2$ samples is below 1.0 APE.

We measured higher isotopic enrichment in the described MTBSTFA methods than in the triacetate method. This can be attributed to the fact that the triacetate method measures the m+2 and m+3 peaks instead of the m+0 and m+3 peaks of the fragment ion. The reason that this method was originally employed is that the ratio of the peak areas of m+3 and m+0 is only 0.58×10^{-3} in the natural molecule and it is thus difficult to measure low abundances and the background abundance with good accuracy. As it has been shown that

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partially deuterated products are formed during tracer recycling,[2] it is a potential drawback of the method to have to measure the m+2 peak. However, this does not seem to impact on the results of the modelling since the differences in estimated parameters were small and non-systematic (Table 1). We also tested the impact on the amplitude itself of the enrichment curves by scaling the amplitude (between 0.5- and 2-fold) and found a difference less than 1% in all estimated parameters. Thus, we suspect that even if the two methods give different amplitudes, the small observed difference in results is not due to the difference in amplitude (which was 9–34% in the analyzed time series). Amplitude variation (within the given range) does not influence the results of the model. This is an inherent effect of the design and implementation of the model and reflects the fact that the production rate of VLDL is invariant of the contribution of different glycerol sources. On the other hand, the relative contribution from different glycerol sources cannot be determined using this experimental setup.

Even if there was no significant difference in the outcome of the kinetic model, applications may still exist where it is important to be able to measure the intact glycerol molecule. Furthermore, the findings in this study also strengthen the conclusions in earlier studies using the old protocol for the measurement of glycerol isotopic enrichment since it was not known how much these results were influenced by the fragmentation of the glycerol molecule after ionization.[7]

Previously, EI-MS has been used with HFBA derivatization, but the analytical response is of low intensity.[11] Since fluorine, and other halogens, are highly electronegative, compounds containing these halogens preferentially form negative ions and they are often analyzed by negative chemical ionization. The MTBSTFA method presented here gives a high signal, suggesting that lower initial amounts of material could be used and thus reduce the amount of blood drawn from the subject. HFBA, on the other hand, has the advantage that it seems to be able to derivatize glycerol in the presence of salts, such as directly after transesterification of VLDL-triglycerides or directly in the glycerol-containing aqueous phase of plasma,[2] resulting in less time-consuming procedures.

CONCLUSIONS

We have compared three different protocols for the derivatization of glycerol from triglycerides derived from lipoproteins. We optimized the derivatization reaction with MTBSTFA with respect to time, temperature and the inclusion of TBDMCS. We then compared the results from kinetic modelling using data derived from our original protocol using acetic anhydride, the optimized protocol (optimized from Flakoll *et al.*[12]), and a new simpler protocol for preparing VLDL-triglyceride glycerol, also using MTBSTFA. We conclude that the results from kinetic modelling using glycerol enrichment curves derived with the different methods did not differ significantly, under the current circumstances, and this allows for comparison between results derived using these methods. However, the possibility that there are circumstances where it is important to measure the intact molecule cannot be ruled out, and here we present such a method suitable for use with EI-MS. In practice, the saponification protocol with derivatization with MTBSTFA is the recommended protocol whereas the transesterification protocol is applicable when the free fatty acids are also to be measured.

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REFERENCES

1. Parhofer KG, Barrett PH. *J. Lipid Res.* 2006; **47**: 1620.
2. Patterson BW, Mittendorfer B, Elias N, Satyanarayana R, Klein S. *J. Lipid Res.* 2002; **43**: 223.
3. Harris WS, Connor WE, Illingworth DR, Rothrock DW, Foster DM. *J. Lipid Res.* 1990; **31**: 1549.
4. Zech LA, Grundy SM, Steinberg D, Berman M. *J. Clin Invest.* 1979; **63**: 1262.
5. Malmstrom R, Packard CJ, Caslake M, Bedford D, Stewart P, Yki-Jarvinen H, Shepherd J, Taskinen MR. *Diabetologia* 1997; **40**: 454.
6. Adiels M, Taskinen MR, Packard C, Caslake MJ, Soro-Paavonen A, Westerbacka J, Vehkavaara S, Hakkinen A, Olofsson SO, Yki-Jarvinen H, Boren J. *Diabetologia* 2006; **49**: 755.
7. Adiels M, Packard C, Caslake MJ, Stewart P, Soro A, Westerbacka J, Wennberg B, Olofsson SO, Taskinen MR, Boren J. *J. Lipid Res.* 2005; **46**: 58.
8. Beylot M, Martin C, Beaufrere B, Riou JP, Mornex R. *J. Lipid Res.* 1987; **28**: 414.
9. Wolfe RR. *Radioactive and Stable Isotope Tracers in Biomedicine*. Wiley-Liss: New York, 1992; 424.
10. Ackermans MT, Ruiter AF, Endert E. *Anal. Biochem.* 1998; **258**: 80.
11. Gilker CD, Pesola GR, Matthews DE. *Anal. Biochem.* 1992; **205**: 172.
12. Flakoll PJ, Zheng M, Vaughan S, Borel MJ. *J. Chromatogr. B Biomed. Sci. Appl.* 2000; **744**: 47.
13. Schwenk WF, Berg PJ, Beaufrere B, Miles JM, Haymond MW. *Anal. Biochem.* 1984; **141**: 101.
14. Lindgren FT, Jensen LC, Hatch FT. In *Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism*, Nelson GJ (ed). Wiley-Interscience: New York, 1972; 181–274.
15. Egusa G, Brady DW, Grundy SM, Howard BV. *J. Lipid Res.* 1983; **24**: 1261.
16. Chong MF, Fielding BA, Frayn KN. *Am. J. Clin. Nutr.* 2007; **85**: 1511.
17. Wolfe RR, Chinkes DL. *Isotope Tracers in Metabolic Research: Principles and Practice of Kinetic Analysis*. Wiley-Liss: New Jersey, 2005.
18. McMurry J. *Organic Chemistry*. Brooks/Cole Publishing Company: Pacific Grove, CA, 1996.

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