HORMONE MEASUREMENT GUIDELINES

Tracing lipid metabolism: the value of stable isotopes

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Abstract

Labelling molecules with stable isotopes to create tracers has become a gold-standard method to study the metabolism of lipids and lipoproteins in humans. There are a range of techniques which use stable isotopes to measure fatty acid flux and oxidation, hepatic fatty synthesis, cholesterol absorption and synthesis and lipoprotein metabolism in humans. Stable isotope tracers are safe to use, enabling repeated studies to be undertaken and allowing studies to be undertaken in children and pregnant women. This review provides details of the most appropriate tracers to use, the techniques which have been developed and validated for measuring different aspects of lipid metabolism and some of the limitations of the methodology.

Key Words

- ▶ stable isotopes
- ▶ tracers
- ▶ fatty acids
- ▶ cholesterol
- ▶ lipoproteins

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Introduction

The global increase in obesity has led to an increased focus on understanding the regulation of lipid metabolism. Obesity is associated with insulin resistance, hyperlipidaemia, non-alcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus and atherosclerotic cardiovascular disease (Hubert *et al.* 1983, de Marco *et al.* 1999). Fatty acids are one of the mediators of insulin resistance (Boden 1998), and an imbalance in fatty acid metabolism results in the accumulation of ectopic fat, which can lead to NAFLD, fatty infiltration of the pancreas and epicardial fat (Morelli *et al.* 2013). Hyperlipidaemia, i.e. increased levels of triacylglycerol (TAG) and cholesterol, is a major risk factor for atherosclerosis, the major cause of cardiovascular disease (Duffield *et al.* 1983). This review will provide an overview of the techniques that can be used to measure lipid metabolism using stable isotopes in humans.

The concentration of lipids in a blood sample can provide information about whether levels are abnormal, but it cannot provide information about the underlying

mechanisms for any abnormality. The concentration of any lipid in a blood sample is a product of its local synthesis and influx into the circulation, absorption from dietary sources and efflux (and local degradation) over time. Therefore, a high concentration of plasma cholesterol, for example, could be a result of its increased synthesis and/or absorption or decreased removal from the circulation. To gain an understanding of lipid metabolism and how it is regulated, we need to be able to measure these fluxes. To do this, the lipid of interest needs to be labelled so that it is indistinguishable from the naturally occurring lipid in the body but is distinct in some way that enables it to be detected and measured accurately. Since the labelled lipid needs to behave metabolically like the unlabelled lipid, the labelling must not change its metabolic characteristics. One way to achieve this is to use stable or radioactive isotopes. Stable isotopes are not a source of ionising radiation, unlike radioactive isotopes. Labelling with stable isotopes

has become a gold-standard method to study the metabolism of lipids and lipoproteins in humans. The quantities of stable isotopes that are administered are non-toxic, and studies can be safely undertaken in humans, including pregnant women and children.

Theory

Some elements are composed of atoms that are chemically identical but have a different mass due to different numbers of neutrons. These are termed isotopes. In the biological environment, the most abundant isotopes of the major elements, i.e. hydrogen (H), carbon (C), nitrogen (N) and oxygen (O), are ^1H , ^{12}C , ^{14}N and ^{16}O . These are stable, but there are also other stable isotopes of these elements which are much less common (ranging from 0.02 to 1.1%). These are ^2H (deuterium), ^{13}C , ^{15}N , ^{17}O and ^{18}O . In this review the term 'stable isotope' will refer to these less common isotopes. When one of these less abundant isotopes is substituted for the common form in a molecule, this is known as 'labelling' and creates a 'tracer'. The original molecule is known as the tracee. The tracer and tracee have the same chemical properties. The amount of tracer relative to the amount of tracee is expressed as the tracer:tracee ratio (TTR). TTR corrected for the background TTR is referred to as enrichment. An alternative way of expressing enrichment is as mole percent excess or atom percent excess (APE). This is the amount of tracer (in moles or atoms) relative to the sum of tracer+tracee multiplied by 100. The different mass of stable isotopes enables them to be detected and accurately measured by mass spectrometry (MS).

One of the first studies where stable isotopes were used to study lipid metabolism was published in 1937 (Schoenheimer 1937). In this study, deuterium-labelled linseed oil was fed to mice, and it was shown that 33% of the labelled fatty acids could be detected in adipose tissue (Schoenheimer 1937). However stable isotope-labelled tracers were not easily obtainable and the mass spectrometers needed for their measurement were costly, so this methodology was of limited use for most researchers. However there was a plentiful supply of radioisotopes, which could be used for creating radioactive lipid tracers. These could be measured by scintillation counters which were readily available. The health risk associated with radioactive tracers in humans and the development of affordable mass spectrometers led to the greater use of stable isotope tracers in the late 1960s and early 1970s. Stable isotopes are now widely used for lipid tracer studies,

although many of the techniques that are used were developed using radioactive tracers.

Measurement

Isotopic enrichment of biological samples can be measured by nuclear magnetic resonance or MS; the latter is often interfaced with an instrument which first separates the molecule of interest, e.g. gas chromatography (GC), liquid chromatography etc. In GCMS and LCMS the labelled molecule, e.g. $[1-^{13}\text{C}]$ palmitate (the tracer), is measured relative to the unlabelled palmitate (i.e. the tracee). A major advantage of stable isotopes over radioactive isotopes is that both tracer and tracee are measured simultaneously with very high precision. An isotope ratio mass spectrometer (IRMS) measures the isotopic enrichment of a gas. This technique is very sensitive and so can be used when enrichment is low. Samples are converted to gases through combustion or pyrolysis, which are then separated. Samples containing ^{13}C -labelled molecules can be converted to CO_2 by combustion, while deuterium-containing molecules can be converted to hydrogen by pyrolysis. An IRMS is also used for measuring expired $^{13}\text{CO}_2$ generated from the oxidation of a ^{13}C -labelled molecule and for measuring the enrichment of labelled plasma water ($^2\text{H}_2\text{O}$) which is used in the measurement of fatty acid synthesis. For more details on measurement techniques see Schierbeek *et al.* (2012).

Lipid metabolism can be measured by the administration of a tracer either orally or intravenously. There is a natural abundance of stable isotopes, so any isotopic tracer administered will add to the existing background. It is therefore essential that the background levels are measured before administering the tracer. Because stable isotope tracers have mass, the quantities used should be low so that the pool size of the tracee is not affected, as this would perturb the kinetics of the system being studied. This requirement is usually fulfilled, since measurement techniques are very sensitive.

Tracer supply and choice

Stable isotope tracers can be purchased from a number of suppliers. For human studies they are relatively expensive because of the quantity required. The cost varies depending on the number of labels and the site of labelling in the molecule. If they are to be administered intravenously in humans, they must be either purchased sterilised and prepared for human use or prepared in this way in a hospital pharmacy. This adds another layer of cost.

Figure 1

Sites of utilisation of palmitate, glycerol, water and acetate tracers used for the measurement of lipid metabolism.

The standard notation for a stable isotope tracer indicates the mass number as a superscript to the left of the atom and the number of the atoms substituted with the isotope as a subscript to the right; for example, $[\text{}^2\text{H}_4]$ palmitic acid has four atoms of ^2H . Uniformly labelled, represented by 'U', means all the atoms are substituted with the isotope. Thus $[\text{U-}^{13}\text{C}]$ palmitic acid indicates that every carbon has been replaced by ^{13}C . The choice of tracer is determined by the level of sensitivity required, whether oxidation rate is to be measured, whether other tracers are to be administered in the same study, whether tracer recycling is likely to occur and other factors such as cost. More details on the most appropriate tracers to use are given when describing each method below.

Stable isotope techniques for measuring lipid kinetics

There are four techniques which are used for measuring lipid kinetics: i) dynamic isotope dilution; ii) the product precursor method; iii) tracer conversion; and iv) the dual isotope method. The choice of technique is dependent on the metabolic pathway to be measured. Some of the tracers used to study lipid metabolism, and the points where they are utilised, are shown in Fig. 1.

Adipose tissue lipolysis

The technique of dynamic isotope dilution can be used to measure whole body production rates of metabolites secreted into the circulation. Since the whole body

production rate of non-esterified fatty acids (NEFA) into the blood from tissues is mainly from peripheral adipose tissue in the fasting state, this is used as an index of whole body adipose tissue lipolysis of stored TAG. Although i.v. lipolysis of plasma lipids can contribute, in a fasting state the former is considered to make only a minor contribution (Wolfe *et al.* 1985). In a postprandial state this becomes more significant (Evans *et al.* 1999). In a fasting state the rate of production of fatty acids is equal to the rate of whole body uptake, which can also be of interest.

A palmitate tracer is most commonly used for measuring NEFA production rate because it is the cheapest tracer. It is assumed that palmitate is representative of all fatty acids. The tracer can be administered as an i.v. bolus or as a constant i.v. infusion. The latter is the most frequently used, since a bolus requires very rapid blood sampling, as the clearance of NEFA from the circulation is very fast (Mittendorfer *et al.* 2003). With the constant infusion method, a known amount of the fatty acid tracer, e.g. $[\text{}^1\text{-}^{13}\text{C}]$ palmitate, is infused intravenously at a constant rate. Samples are collected ideally from arterialed venous blood or from an artery. In a metabolic steady state, e.g. during fasting, the TTR or APE increases with time until a plateau is reached indicating that an isotopic steady state is achieved (Fig. 2). At this point the rate of removal of the tracer from the system is equal to the rate that it appears, i.e. the infusion rate. In the plasma pool the tracer is mixed with tracee entering the system from adipose tissue, and measurement of its dilution provides a

Figure 2

Schematic example of a constant infusion of tracer to achieve an isotopic steady state.

Figure 3

Calculating rate of appearance (Ra) at isotopic steady state. Rd, rate of disappearance; black circles represent the tracer; white circles represent the tracee. Reproduced, with permission, from Wolfe RR & Chinkes DL (2004) *Isotope Tracers in Metabolic Research: Principles and Practice of Kinetic Analysis*, 2nd Edition. Copyright 2004 Wiley-Blackwell.

measure of the rate at which palmitate is appearing in the body pool. This technique requires the unlabelled palmitate to be constant during the study, i.e. in a steady state. It is assumed that the tracer is diluted in a single body pool, e.g. plasma. The dilution of the tracer by the tracee at isotopic steady state is a measure of the rate of appearance (Ra) of palmitate into the pool, which in a steady state is equal to the rate of loss from the pool (also known as the flux) (Fig. 3).

$$Ra \text{ (mg/min)} = \text{Tracer infusion rate (mg/min)} / (TTR_{SS} - TTR_{(t=0)})$$

where SS = isotopic steady state

To convert palmitate Ra to NEFA Ra, the concentration of palmitate relative to total NEFA needs to be measured. Other fatty acids can be used as tracers; palmitate, oleate and linoleate have all been shown to provide good estimates of NEFA Ra, whereas myristate and stearate do not (Mittendorfer *et al.* 2003). Since long-chain fatty acids are insoluble in aqueous solution, the stable isotope tracer of a fatty acid salt, e.g. potassium palmitate, is complexed with human albumin to aid solubility. Deuterated tracers are cheaper, but if $[U-^{13}C]$ palmitate is used, the infusion rate can be reduced if GC-combustion IRMS is used to measure enrichment. This reduces the amount of albumin which needs to be infused. If $[U-^{13}C]$ palmitate is used, palmitate oxidation rate can also be measured.

NEFA production rate can underestimate adipose tissue lipolysis, because some NEFA released from lipolysis is re-esterified within adipose tissue. The production rate of glycerol measured with a glycerol tracer provides a more accurate measure of lipolysis, since glycerol kinase levels are very low in adipose tissue, so there is very little recycling (Miles *et al.* 2004). As with using NEFA

production rate as a measure of lipolysis, i.v. lipolysis of plasma lipids can also contribute some glycerol to the plasma pool, although in a fasting state this would be a minor contribution (Robinson & Newsholme 1967). The technique used is identical to the one described above for palmitate. There are several options in the choice of tracer: $[^2H_5]$ glycerol, $2-^{13}C_1$ and $U-^{13}C$ glycerol have all been shown to give similar measurements of whole body glycerol production rate (Wolfe 1992).

The isotope dilution technique can also be used in the non-steady state. With the tracee in a steady state, e.g. during fasting, an isotopic steady state is achieved first with the tracer infusion. The steady state can then be disturbed by, for example, the subject exercising or with the infusion of a hormone. The changes in TTR and concentration with time are used to calculate the change in the flux of the tracee using non-steady-state equations or mathematical modelling (Steele *et al.* 1965). Using this technique, the insulin sensitivity of lipolysis can be measured by combining a constant i.v. infusion of labelled glycerol or labelled palmitate with a low-dose insulin infusion (Shojaee-Moradie *et al.* 2007) (Fig. 4).

NEFA Ra can also be measured during the postprandial period, using non-steady-state equations. However, in the postprandial period, some fatty acids hydrolysed from dietary fat (chylomicrons) can directly enter the plasma pool, a process known as spillover, so NEFA Ra is a measure of both lipolysis and spillover (Bickerton *et al.* 2007).

The isotope dilution technique can be combined with the measurement of arterio-venous balance measurements in specific tissues, e.g. subcutaneous adipose tissue.

Figure 4

Glycerol production rate in a healthy subject measured with a constant infusion of $[^2H_5]$ glycerol during fasting and following an infusion of insulin. The decrease in glycerol production rate is a measure of the insulin sensitivity of lipolysis.

Figure 5

Plasma NEFA oxidation calculated using the acetate correction factor when infusing [U- ^{13}C]palmitate. The correction factor is the fractional ^{13}C -label recovery in breath CO_2 , observed after an infusion of [1,2- ^{13}C]acetate. Reproduced, with permission, from Schrauwen P, van Aggel-Leijssen DP, van Marken Lichtenbelt WD, van Baak MA, Gijsen AP, Wagenmakers AJ (1998) Validation of the [1,2- ^{13}C]acetate recovery factor for correction of [U- ^{13}C]palmitate oxidation rates in humans. *Journal of Physiology* **513** 215–223. Copyright 2004, John Wiley and Sons.

This can provide measurements of tissue-specific rates of production and uptake (Bickerton *et al.* 2007). This method requires the measurement of blood flow through the tissue.

Fatty acid oxidation

Whole body NEFA oxidation can be measured using the technique of tracer conversion. If ^{13}C -labelled palmitate is administered, the appearance of ^{13}C in CO_2 can provide a measure of palmitate oxidation rate. Small bags or tubes are used to collect expired air. The $^{13}\text{CO}_2$ content can then be measured by IRMS. To calculate the $^{13}\text{CO}_2$ expiry rate, CO_2 production rate (VCO_2) must also be measured. This can be obtained using a gas analyser when collecting the breath samples. A quantitative measure of palmitate oxidation rate can be determined if a steady state of $^{13}\text{CO}_2$ is achieved. An isotopic steady state of $^{13}\text{CO}_2$ can take up to 8 h due to a slow rate of exchange of $^{13}\text{CO}_2$ with the bicarbonate pool. This can be shortened by priming the bicarbonate pool by administering an i.v. bolus of ^{13}C sodium bicarbonate when the tracer infusion is initiated. The other consideration with this method is correction for any fixation of $^{13}\text{CO}_2$ in the body bicarbonate pool and

loss of ^{13}C during the TCA cycle. A method for correcting for this has been developed using ^{13}C -labelled acetate (Schrauwen *et al.* 1998). In this method ^{13}C -labelled acetate is infused in a separate study, which mimics the conditions employed for the determination of palmitate oxidation (Schrauwen *et al.* 1998). Acetate enters the TCA cycle as acetyl CoA, and the difference between the amount of ^{13}C acetate infused and the amount recovered in $^{13}\text{CO}_2$ is a measure of the amount of fixation which needs to be corrected for (Fig. 5). The need for an additional study adds an extra burden to this measurement.

Lipoprotein metabolism

Lipoprotein particle kinetics can be measured by labelling one of the apolipoprotein components with an isotopically labelled amino acid, e.g. ^{13}C leucine. Lipoprotein TAG kinetics can be measured by labelling TAG in the TAG-rich lipoproteins, i.e. VLDL and/or chylomicrons. To measure the synthesis rates of macromolecules such as proteins and triglycerides, the precursor-product method is used. A stable isotope-labelled precursor is administered either orally or intravenously, and the incorporation of the precursor into the macromolecule is measured over a period of time. If the TTR of the precursor is known or can be measured, the fractional rate of synthesis (FSR) of the product can be determined. In a steady state, i.e. when the protein mass or TG pool is constant, the FSR is equal to the fractional catabolic rate (FCR), a measure of metabolic clearance. FSR and FCR have per unit of time. An absolute secretion rate (ASR) (e.g. mmol/h) is calculated by multiplying the FSR by the product pool size.

Apolipoprotein kinetics

ApoB100 is a large structural protein in VLDL, IDL and LDL. There is one apoB100 molecule/lipoprotein particle. VLDL, IDL and LDL apolipoprotein (apo) B100 FCR and ASR can be measured with either an i.v. bolus or constant infusion of a labelled amino acid (Millar *et al.* 1995, Demant *et al.* 1996). Most researchers use [1- ^{13}C]leucine or [$^2\text{H}_3$]leucine, although labelled valine and lysine have also been used.

With the infusion method the rate of increase in apoB enrichment measured relative to the precursor pool is a measure of apoB fractional secretion rate (FSR). With the bolus method, the decrease in apoB enrichment over time is measured. With both methods the change in enrichment with time is not linear, so fitting the data with

Figure 6

Typical curve fit for leucine tracer:tracee ratio in VLDL, IDL and LDL apoB100 using mathematical modelling following an infusion of [$1\text{-}^{13}\text{C}$]leucine. VLDL shown as large squares. IDL shown as triangles. LDL shown as circles. The solid lines indicate the curve fit. The modelling provides a measure of VLDL, IDL and LDL apoB100 fractional catabolic rate and production rate.

a linear regression is inappropriate. A monoexponential function can be fitted to the enrichment data (Cummings *et al.* 1995) to calculate FSR, but a mathematical model can provide a better curve fit and more accurate measurements of FSR (Parhofer *et al.* 1991). Because VLDL is metabolised to IDL and then LDL in the circulation, and during this process apoB100 is retained within the lipoprotein, if apoB100 enrichment is measured in each fraction, it is possible to describe this whole metabolic pathway using a multicompartmental mathematical model (Fig. 6). A number of different model structures have been proposed (Millar *et al.* 1995, Duvillard *et al.* 2000). If VLDL apoB100 enrichment achieves an isotopic steady state during a constant-infusion study, this plateau of enrichment is a measure of the enrichment of the precursor pool. If this is not the case, an alternative measure of the precursor pool is used. Plasma leucine does not provide an appropriate measure of the precursor pool, since hepatic intracellular leucine (the true precursor pool) is diluted by intracellular proteolysis. Within hepatic cells, leucine is deaminated to α ketoisocaproate (α KIC), and the enrichment of α KIC, which can be found in the circulation, has been shown to provide a good estimate of hepatic intracellular leucine enrichment (Barazzoni *et al.* 1999).

VLDL and IDL apoB100 enrichment can be measured by GCMS, but there is considerable dilution of the tracer in the large pool of LDL apoB100. This necessitates the measurement of LDL apoB100 enrichment by GC-combustion IRMS, which has a much higher sensitivity

than GCMS. In several studies VLDL has been subfractionated and the enrichment and kinetics of two forms of VLDL measured: VLDL1, a large triglyceride-rich lipoprotein; and VLDL2, a less triglyceride-rich lipoprotein (Adiels *et al.* 2005). VLDL2 can be formed by direct secretion from the liver or from catabolism of VLDL1, and both these pathways can be determined by mathematical modelling. Unlike VLDL, LDL has a very slow turnover, so a constant infusion of tracer over a single day provides a limited description of the LDL apoB enrichment curve. An i.v. bolus of tracer provides an alternative approach, which enables LDL apoB enrichment to be measured over several days if volunteers are willing to return to the clinical research centre for a single blood sample over the following 12–14 days. This provides a more accurate measurement of LDL kinetics (Demant *et al.* 1996).

In studies where constant meal feeding is used to achieve a postprandial steady state, labelling of apoB48, the structural protein of chylomicrons, with a labelled amino acid such as [$1\text{-}^{13}\text{C}$]leucine can provide a measure of chylomicron particle kinetics. The methodology is identical to that described for VLDL apoB100. Both apoB48 and apoB100 are isolated by SDS polyacrylamide gel electrophoresis (Lichtenstein *et al.* 1992).

By labelling the main protein component, apoA1, HDL particle kinetics can be measured (Cohn *et al.* 1990). This has been measured with a constant i.v. infusion of a labelled amino acid over a single day. However HDL has a half-life of 5 days, which means a very limited description of the apoA1 enrichment curve is gained in 1 day. As with measuring LDL kinetics, the i.v. bolus technique with measurement of enrichment over several days combined with mathematical modelling should provide a more robust measurement of HDL apoA1 kinetics. There are, however, no published studies which have investigated this using stable isotopes.

VLDL and chylomicron TAG kinetics

VLDL TAG kinetics can be measured with an i.v. bolus or constant infusion of labelled glycerol (Sarac *et al.* 2012) or a fatty acid, usually palmitate (Patterson *et al.* 2002). Glycerol is the preferred tracer, since recycling of a fatty acid tracer can occur, which can result in an underestimation of VLDL TAG synthesis rate (Patterson *et al.* 2002). Administration of an i.v. bolus of $^2\text{H}_5$ glycerol with measurement of the rise and decline in glycerol enrichment in triglyceride over 8–12 h is the most widely used method (Sarac *et al.* 2012). The FSR (and FCR) can be determined by mathematical modelling as described

Figure 7

Typical curve fit of VLDL₁ and VLDL₂ glycerol tracer:tracee ratio in TAG, following an i.v. bolus of [²H₅]glycerol, using mathematical modelling. VLDL₁ shown as large squares. VLDL₂ shown as small circles. The solid lines indicate the curve fit. The modelling provides a measure of VLDL₁ and VLDL₂ TAG fractional catabolic rate and production rate. Reproduced, with permission, from Sarac I, Backhouse K, Shojaaee-Moradie F, Stolinski M, Robertson MD, Bell JD, Thomas EL, Hovorka R, Wright J & Umpleby AM (2012) Gender differences in VLDL1 and VLDL2 triglyceride kinetics and fatty acid kinetics in obese postmenopausal women and obese men. *Journal of Clinical Endocrinology and Metabolism* **97** 2475–2481. Copyright 2012 The Endocrine Society.

above for apoB100 kinetics (Adiels *et al.* 2005, Parhofer *et al.* 1991). Depending on the ultracentrifugation methods used to isolate VLDL, either total VLDL TAG kinetics or VLDL1 and VLDL2 TAG kinetics can be measured (Fig. 7).

An i.v. bolus of ²H₅ glycerol has been shown to be incorporated into chylomicron TAG, enabling chylomicron TAG synthesis to be measured in a constant-feeding study (Fig. 8) (Shojaaee-Moradie *et al.* 2013). This is a laborious method because chylomicron particles must be separated from VLDL particles. This cannot be achieved by ultracentrifugation. In the above study an immunoaffinity technique using three antibodies to apoB100 was used to isolate VLDL from chylomicrons (Shojaaee-Moradie *et al.* 2013).

Hepatic fatty acid synthesis

The precursor-product method is also used for measuring hepatic fatty acid synthesis. Since fatty acids are synthesised from multiple units of acetyl CoA, an i.v. infusion of ¹³C acetate (the precursor) should enable the determination of hepatic fatty acid synthesis (frequently referred to as *de novo* lipogenesis; DNL) if the TTR of the precursor (acetate) is known and the TTR of the product, e.g. palmitate, the major product of hepatic synthesis, is

measured in VLDL TAG (which is produced by the liver). However, because there are different pools of acetyl CoA in the liver used for different metabolic pathways, the plasma acetate TTR cannot be assumed to be the true precursor. One way to calculate the enrichment of the true precursor uses mass isotopomer distribution analysis (MIDA). This method determines the precursor enrichment from the pattern of labelling in the product (Hellerstein & Neese 1999). A constant i.v. infusion of the acetate tracer is administered for 6–8 h, with measurement of the enrichment of palmitate in VLDL TAG at the end of the infusion period. The frequency of double-labelled relative to single-labelled palmitate is measured and the TTR of the precursor is calculated using an algorithm. This method has been used in both fasting and feeding studies. In feeding studies repeated mixed meal feeding is used to achieve a postprandial steady state in VLDL TAG (Stanhope *et al.* 2009). With an infusion period of 8 h, the fractional synthesis of palmitate is determined as

$$\text{FSR } (\%/h) = \frac{\text{TTR}_{\text{palmitate}}(t_8) - \text{TTR}_{\text{palmitate}}(t_0)}{\text{TTR}_{\text{precursor}} \times (t_8 - t_0)} \times 100$$

where TTR_{palmitate} is the enrichment of palmitate in VLDL TAG at time 0 (before the tracer infusion) and 8 h, and TTR_{precursor} is the enrichment of the precursor pool (acetate) at 8 h.

Figure 8

Glycerol tracer:tracee ratio in chylomicron TAG in healthy subjects (white circles) and subjects with MetS (black circles) following an i.v. bolus of [²H₅]glycerol (mean ± s.e.m.). Mathematical modelling of this data provides a measure of chylomicron TAG fractional catabolic rate and production rate. Reproduced, with permission, from Shojaaee-Moradie *et al.* (2013). *Diabetes*, American Diabetes Association, 2013. Copyright and all rights reserved. Material from this publication has been used with the permission of American Diabetes Association.

An alternative method for measuring hepatic fatty acid synthesis uses labelled water. During palmitate synthesis, H from NADPH and H₂O is added, and this can be utilised to measure palmitate synthesis rate (Leitch & Jones 1993). When ²H₂O is given orally, it rapidly equilibrates with total body water and ²H is incorporated into palmitate as it is synthesised. A plateau of deuterium enrichment is reached within 12 h. In the most widely used method an oral dose (3 g/kg body weight) of ²H₂O which aims to produce 0.45% enrichment of body water is given in the evening, half with the evening meal and half at 22 h. Subjects drink only water (0.45% enriched) to prevent dilution of the labelled body water with ingested water, until after a blood sample is taken the following morning for the measurement of VLDL TAG-palmitate enrichment and plasma water enrichment. There are 31 hydrogens in palmitate which could potentially be labelled. However, not all hydrogens are equivalent. Seven H are directly from H₂O, 14 are from NADPH and ten are from acetyl CoA. Complete equilibration with the labelled body water pool does not occur with the latter two pools. It has been shown, using MIDA, that the maximum number of labelled H that can be incorporated into palmitate is 21 (Diraison *et al.* 1996).

If all VLDL TAG-palmitate are derived from DNL, the enrichment in TAG-palmitate will be equal to the enrichment of the plasma water multiplied by the maximum number of labelled H that can be incorporated.

Maximum theoretical palmitate TTR = $TTR_{H_2O} \times 21$

The maximum theoretical proportion of VLDL TAG-palmitate which can be derived from DNL is 100%. The actual proportion is calculated from the ratio of the observed VLDL TAG-palmitate enrichment (TTR) to the maximum theoretical palmitate enrichment (the theoretical enrichment if all VLDL TAG-palmitate is derived from DNL).

$$\%DNL = \frac{\text{VLDL TAG-palmitate TTR}}{\text{maximum palmitate TTR}} \times 100$$

The absolute rate of VLDL TAG synthesis from DNL can be calculated by multiplying %DNL by total VLDL TAG synthesis rate (e.g. measured using a glycerol or palmitate tracer as described in the section on VLDL TAG kinetics).

De novo cholesterol synthesis and absorption from the diet A high cholesterol level is a strong independent risk factor for cardiovascular disease. Understanding the regulation of cholesterol metabolism is therefore of major importance. Plasma cholesterol is derived from

endogenous synthesis and exogenous sources (diet and bile acids). Stable isotope tracers can be used to measure cholesterol absorption from the diet and from *de novo* synthesis.

Cholesterol absorption The dual-isotope method is used for measuring oral absorption of cholesterol (Bosner *et al.* 1993). It is based on a method previously developed by Zilversmit & Hughes (1974) using radioisotopes. An oral dose of ²H₅ cholesterol (or ²H₆ or ²H₇ cholesterol) in a meal and an i.v. injection of ¹³C₅ cholesterol (or ¹³C₆ or ¹³C₇ cholesterol) is administered on day 0, and on day 3 the plasma enrichment of the two tracers is measured. At this point it has been shown that the ratio of enrichment of the oral/i.v. tracer in the plasma is stable (Bosner *et al.* 1993). A limitation of the method is that the labelled cholesterol absorption is not necessarily the same as cholesterol absorption from food. Percentage cholesterol absorption is calculated from the ratio of plasma ²H₅ cholesterol TTR/¹³C₅ cholesterol TTR $\times 100$ on day 3. This method has been shown to have good precision and repeatability (Bosner *et al.* 1993). An alternative method has used ²H₅ cholesterol as the tracer and ²H₄ sitostanol as a non-absorbable marker given orally for 7 days. Cholesterol absorption was calculated from faecal samples collected over days 4–7 and measured by GCMS (Lutjohann *et al.* 1993).

Cholesterol synthesis The precursor-product method is used for measuring free cholesterol synthesis. There are two principal methods which are very similar to methods used for measuring fatty acid synthesis as described above. An i.v. infusion of ¹³C acetate combined with the MIDA method to determine the true precursor pool has been used to measure the FSR of cholesterol (Neese *et al.* 1993). To measure the absolute synthesis rate, blood samples can be collected over the next few days to measure the decay of the labelled cholesterol with time. The rate constant for this removal rate provides an estimate of the cholesterol pool size. The absolute synthesis rate of cholesterol can then be calculated as:

$$FSR (\%/day) \times \text{cholesterol pool size}$$

The pool size is assumed to be free cholesterol in liver, plasma and red cells, as these free cholesterol pools are in rapid equilibrium in humans. What is measured by this method is the synthesis of cholesterol (which is predominantly in the intestine and liver) which is secreted into the plasma cholesterol pool. Total body cholesterol synthesis is not measured by this method, because tissue cholesterol

synthesis that does not leave the cell or equilibrate with the plasma pool is not measured. While some researchers have used an 8-h i.v. infusion of ^{13}C acetate, a 24-h infusion is recommended.

An alternative method to measure the fractional synthesis rate (FSR) of cholesterol uses the oral administration of $^2\text{H}_2\text{O}$ and measurement of the incorporation of the deuterium tracer into plasma cholesterol or erythrocyte cholesterol (Jones *et al.* 1988). The deuterated water equilibrates across the body water pool and intracellular NADPH pools which are precursors for 22 of the 46 hydrogens in cholesterol. Measurements are usually made over 24 h to account for the diurnal rhythm in cholesterol synthesis. Cholesterol enrichment can be measured by GCMS and plasma water by IRMS or by GC-MS, via acetone exchange (Shah *et al.* 2010). FSR is calculated as

$$\text{FSR (\%/d)} = \frac{\text{cholesterol TTR}(t_{24} - t_0)}{(\text{plasma water TTR} \times 0.478)}$$

The factor 0.478 is the fraction of hydrogen atoms/cholesterol molecule that can become enriched with deuterium during cholesterol synthesis. This can be converted to an absolute synthesis rate if assumptions are made about the size of the free cholesterol pool.

The FSR measured by an i.v. infusion of ^{13}C acetate and the MIDA method agrees well with FSR measured with oral $^2\text{H}_2\text{O}$, showing a significant correlation ($r=0.84$, $P=0.0007$) in 12 subjects (Di Buono *et al.* 2000).

Conclusion

Measuring lipid concentrations in blood samples provides only a static measurement of lipid metabolism. This provides inadequate information for understanding lipid metabolism in health and disease in humans. Using stable isotope tracers, the fluxes of molecules through lipid metabolic pathways can be measured *in vivo*. There are many different methods which can be used, and the choice of method and tracer depends on the lipid metabolite to be studied. The techniques and tracers can be used individually or several techniques and tracers can be combined in a single study enabling multiple metabolic pathways to be measured simultaneously.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this guideline.

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