Determination of Plasma-Free Fatty Acid Kinetics with Tracers: Methodologic Considerations

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ABSTRACT. Plasma-free fatty acids (FFA) are an important source of energy for a variety of tissues. Recently, there has been an increased interest in the measurement of FFA kinetics *in vivo*, using radiolabeled or stable isotopic tracers.

Standard techniques for measurement of FFA-specific activity are relatively imprecise and have limited sensitivity. We have developed a method for determination of the concentration and specific activity of individual plasma FFA that is precise (coefficient of variation <2%) and sensitive (detection limit in the high femptomolar to low picomolar range). Using this method, one can measure the kinetics of three or more long-chain fatty acids simultaneously. Its sensitivity is a particular advantage if one wishes to measure low rates of FFA turnover such as are encountered during hyperinsulinemia.

It has been suggested that, for optimal accuracy in the determination of substrate kinetics, the tracer should be administered in the left ventricle and mixed venous blood samples should be obtained from the right heart. We have conducted experiments in dogs which demonstrate that peripheral tracer infusion and more conventional arterial (or arterialized venous) sampling actually provide more accurate estimates of FFA turnover; this is fortunate, since intracardiac infusion and sampling are not practical for human studies. (*Journal of Parenteral and Enteral Nutrition* **15:**90S–92S, 1991)

Free fatty acids (FFA) circulate bound to plasma albumin and are a major source of fuel for diverse tissues, supplying roughly half of systemic energy needs in man after an overnight fast.[1] FFA are of particular importance as a fuel in liver,[2] myocardium,[3] kidney,[4] and working,[5] as well as resting[6] skeletal muscle. Because of this prominent role, numerous studies of FFA metabolism in humans and animals have been conducted over the past three decades. In recent years, interest has heightened in the metabolism of free fatty acids for a number of reasons, including a potential role for FFA, via the glucose-fatty acid cycle,[7] in the pathogenesis of diabetes.[8] In this report, we shall address analytical issues relevant to the study of FFA metabolism *in vivo*. In addition, we will consider the infusion-sampling mode question for FFA and other substrates.

ANALYTICAL ISSUES

One of the obstacles that confronts the investigator wishing to study FFA metabolism is the measurement itself. Unlike glucose, FFA are technically difficult to measure and are particularly difficult to measure with accuracy and precision. This is in part because FFA are highly protein bound, and quite insoluble in an aqueous medium.[9] Ordinarily, thus, it is necessary to extract the FFA into an organic solvent. Over the years, most of the methods for FFA quantitation have been based on the original technique of Dole.[10] For kinetic studies, radioactivity has been determined by a simple extraction,[11] which is nonspecific, or by thin-layer chromatography[3,6,12] or back extraction into dilute base,[4,13] both of which are somewhat tedious. Unfortunately, these methods suffer somewhat from imprecision and inaccuracy because extraction of FFA from plasma with organic solvents is often not quantitative.[14]

A major advance in FFA kinetic measurements has been the development of stable isotope techniques. With this approach, a tracer (labeled either with deuterium or ^{13}C) is infused into the circulation and the isotopic enrichment in the specific fatty acid of interest is measured in plasma after extraction, thin-layer chromatography, and derivatization.[14] This technique has the advantage of high specificity and precision, since the isotopic enrichment in a specific fatty acid is quantified. Its major disadvantage is that it is necessary to infuse large quantities of albumin-bound tracer to achieve adequate enrichment; several hundred milliliters of 5% albumin is required for a human study of 3 or more hours duration.[15,16] Such a perturbation is undesirable, since the administration of gram quantities of albumin may have independent effects on the processes that one wishes to characterize. The advent of new technologies such as continuous flow isotope ratio mass spectrometry[17] may minimize this problem in the future by reducing the amount of tracer needed for such studies.

In order to eliminate problems with precision and accuracy associated with older methods for determining FFA specific activity in plasma, we have developed a technique utilizing high-performance liquid chromatography.[18] This technique measures radioactivity in individual fatty acids, rather than in total fatty acids[3,4,6,12,13] or total plasma lipids.[11] In this method [$^{2}H_{31}$]palmitate is used as an internal standard.[19] This compound elutes separately from natural palmitate and has the advantage of allowing automatic calculation of individual plasma

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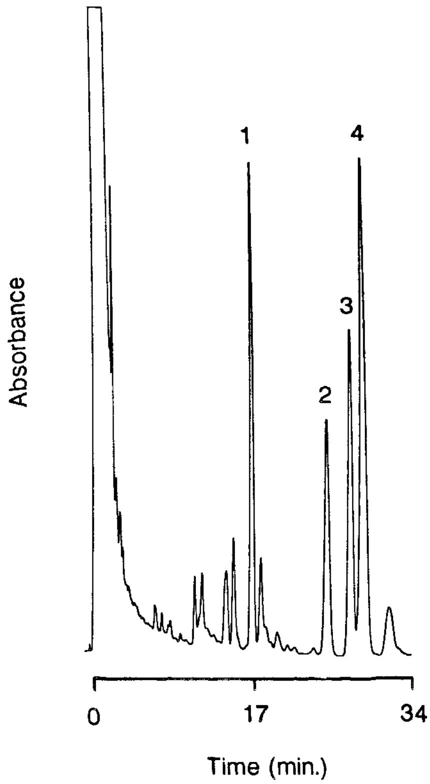

FIG. 1. A chromatogram of plasma FFA, analyzed by high-performance liquid chromatography. 1 = linoleate; 2 = [$^2H_{31}$]palmitate; 3 = natural palmitate; 4 = oleate.

FFA concentrations, using a computer-based data system (SpectroPhysics WINner/386). A representative chromatogram is shown in Figure 1. With this approach, the coefficients of variation for measurement of FFA concentration and specific activity are both <2%. Because the phenacyl esters of FFA are highly absorbent at 242 nm,[20] the method has extreme sensitivity with a detection limit in the high femptomolar to low picomolar range. Thus, it would be suitable for multiple blood sampling in rodents. Specifically, if sufficient high specific activity tracer is administered, the analysis could be performed on as little as 20 to 50 μL of plasma. The sensitivity of this method is also an advantage when one wishes to measure low rates of FFA turnover, such as are encountered during even moderate hyperinsulinemia.[21] Moreover, it is possible to simultaneously determine the kinetics of three or more long-chain fatty acids at once, since interconversion among FFA is known to be minimal.[22]

Unfortunately, it is necessary to select one of the numerous circulating FFA as a paradigm for the others. Although the metabolism of the various FFA may be sufficiently similar to justify extrapolation from measurements of the metabolism of one of the FFA to the aggregate,[13,23] there are significant differences in regional metabolism of the different classes of FFA.[24–26] Thus, caution is warranted in this regard. It is best to select a tracer of a fatty acid that is well-represented in plasma, such as palmitate, oleate, or linoleate, rather than a compound that is less well represented among the FFA such as stearate or myristate. With this advance in methodology, the concentration and specific activity of individual plasma FFA can be determined with precision and accuracy equivalent to gas chromatography mass spectrometry. In addition, one is able to avoid the infusion of large quantities of albumin with its attendant problems. Although each sample requires a fairly long analytical time (40–45 min), the availability of automation allows acceptable sample throughput.

INFUSION-SAMPLING MODE

Although FFA kinetics may be determined with a tracer bolus technique,[27,28] constant tracer infusion has been the method of choice for most investigators. This is usually accomplished by administering the infusion in a peripheral vein and sampling either arterial[2,5,18,27,28] or arterialized venous[29,30] blood. Several years ago, however, Katz[31] and Norwich,[32] in separate reports, pointed out that results of substrate kinetic studies may depend on the choice of sampling site and infusion site. It was noted that downstream sampling (i.e., infusion of tracer in a peripheral vein or central vein and sampling from an artery, or the V-A mode) gave different results than upstream sampling (e.g., infusion of the tracer in the left ventricle and sampling in the right heart, or A-V mode). It was proposed that the A-V mode may be superior for the measurement of the kinetics of lactate.[31] On the other hand, the V-A mode has been shown to be superior for measuring glucose kinetics.[33]

In order to test the relative merits of the A-V and V-A modes for FFA metabolism, we infused a [1-^{14}C]palmitate tracer in the left ventricle of anesthetized dogs.[19] [9,10-^{3}H]palmitate was infused as tracee in equal quantities in three peripheral veins, in order to simulate as much as possible the appearance of FFA from diffuse adipose tissue beds. The use of a tracer as tracee has been undertaken by others for the study of glucose metabolism,[34] and has the advantage of not perturbing the plasma-FFA pool because of the relatively high specific activity of the tracee. In the case of FFA, it is the only acceptable alternative, since emulsions of unlabeled FFA induce hemolysis when infused peripherally.[18] During steadystate infusion of tracer and tracee, palmitate ^{14}C specific activity was significant (~20%) lower in the pulmonary artery than in the femoral artery. In contrast, there was no difference in [^{3}H]palmitate specific activity between the two sampling sites. These data demonstrate that the site of downstream sampling, in the case of tritiated palmitate, does not influence the specific activity, as long as the sample is taken from a well-mixed pool. It also is concordant with the data of Katz,[31] in that there was a clear distinction in plasma [^{14}C]palmitate specific activity between the upstream and the downstream sampling sites.

In order to determine which sampling site is appropriate for the most accurate estimate of tracee inflow, the rate of appearance (Ra) of tritiated palmitate ([^{3}H]palmitate) was calculated using [^{14}C]palmitate as tracer. In this calculation, the [^{14}C] palmitate infusion rate is divided by the [^{14}C/^{3}H]palmitate ratio in plasma; this ratio is analogous to the conventional ^{14}C specific activity, in which [^{12}C]palmitate is the tracee, except that [^3H]palmitate is the tracee. As can be seen in Figure 2 (*lower panel*), the use of mixed venous specific activity resulted in an overestimate of [^3H]palmitate Ra, whereas the use of arterial (downstream) ^{14}C specific activity gave an accurate estimate of actual [^3H]palmitate inflow. Moreover, when arterial blood was analyzed, the estimate of endogenous palmitate Ra using [^3H]palmitate as tracer was identical to that when [^{14}C]palmitate was used (Fig. 2, *upper panel*). This latter observation is particularly important, since it provides indirect evidence that entry of tracee from multiple sites versus a single site of entry does not influence the choice of infusion sampling mode.

The implications of these observations deserve discussion. Numerous investigators have argued that an ideal tracer method generates a specific activity (or stable isotopic enrichment) that is similar or identical to intracellular values.[31,32,35] This is clearly not the case, for example, when one infuses a tracer of lactate or leucine and samples the "primary pool" (lactate and leucine, respectively) using the V-A mode.[35,36] This problem can be overcome by sampling the "reciprocal pool" (pyruvate and α-ketoisocaproate, respectively) using either sampling mode.[36,37] Thus, the solution which provides optimal estimates of intracellular enrichment or specific activity in two complex cases of reversibly interconverting substrates is unrelated to the choice of infusion-sampling mode, in spite of the fact that the use of the A-V mode with a primary-pool analysis will give results that more closely approximate intracellular values than when the V-A mode is used. In other words, the use of the A-V mode with primary analysis may generate an error that tends to correct a separate error associated with primary-pool analysis itself. In a separate example of reversibly interconverting substrates, the ketone bodies acetoacetate and β-hydroxybutyrate, neither reciprocal pool analysis nor infusion-sampling mode considerations appear to provide accurate kinetic data (Miles et al., unpublished results). Instead, a double tracer, open two-pool model is required.[38]

The issues are somewhat less complicated in the use of FFA, since one is dealing with a single pool from which tracer and tracee leave irreversibly. However, if local FFA reesterification occurs in adipocytes, as has been suggested by some *in vitro* studies,[39] then the V-A mode will generate kinetic data that underestimate *total* systemic lipolysis. However, the V-A mode will accurately quantify the parameter that is relevant from a physiologic point of view: the transport of FFA via the circulation to distant sites for either oxidation or reesterification. In contrast, the A-V mode will overestimate plasma FFA transport and give a value that may or may not reflect "total" lipolysis. For these reasons, we believe that the V-A mode is preferable for studies of FFA metabolism. This is fortunate, since the A-V mode is not a practical consideration for human studies because of its invasiveness.

In summary, it is now possible to study FFA metabolism using radiolabeled tracers with a high degree of accuracy and precision, both in humans and small animals. Moreover, accurate results can be obtained with the less invasive V-A sampling mode. Our understanding of FFA metabolism, which is somewhat limited when compared with our understanding of glucose metabolism, will be advanced by application of these techniques.

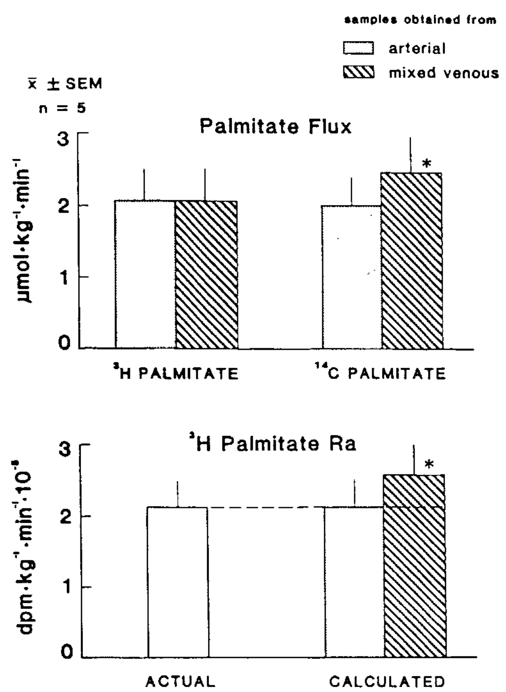

FIG. 2. *Upper panel*, endogenous palmitate flux calculated from both arterial and mixed venous specific activities, using both ^3H- and ^{14}C-palmitate tracers. *Lower panel*, ^3H-palmitate rate of appearance, actual vs values calculated from arterial and mixed venous analysis, using ^{14}C-palmitate as tracer.

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Methods of Investigating Intestinal Transport

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ABSTRACT. This article provides a brief overview of well-established *in vivo* and *in vitro* methods that have contributed the most to the understanding of transport processes across the gastrointestinal epithelium. *In vivo* perfusion techniques in humans revolve around double- and triple-lumen per oral tubes. In animals, *in vivo* techniques include the single and recirculation perfusion techniques and the double-isotope technique for measurement of net absorption. *In vitro* methods of studying intestinal transport include the everted gut sac technique, the Üssing chamber, the use of isolated epithelial cells, and the use of brush border and basolateral membranes isolated from enterocytes. The use of fluorescent probes for the measurement of intracellular ionic concentrations is a new and powerful *in vivo* technique that is now being applied to the gastrointestinal tract. (*Journal of Parenteral and Enteral Nutrition* **15:**93S–98S, 1991)

Solute transport through membranes is a highly complex process, as evident by the intricate interactions that occur between transported solutes, various ions, intracellular organelles and cellular membranes.[1–2] The transport mechanisms involved may be as simple as diffusion down electrochemical gradients or as complex as active cotransport.

In general, *in vivo* intestinal transport studies help elucidate transport phenomena. However, they treat ep-

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