CHAPTER 5

The $^{13}$C-palmitic acid test for detection of mild fat malabsorption in healthy adults on calcium supplementation

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Abstract

**Background & Aims:** Recently we developed a [1-$^{13}$C]palmitic acid absorption test for the detection of fat malabsorption in rats with chronic bile diversion. In the present study in healthy human adults we investigated whether this test was sensitive enough to detect mild fat malabsorption induced by dietary supplementation of calcium carbonate. **Methods:** After oral supplementation of [1-$^{13}$C]palmitic acid (10 mg kg$^{-1}$) to 10 healthy adults, breath and plasma samples were obtained for 8 h and feces was collected for 72 h. Dietary fat intake was assessed on the basis of a 4-day dietary record. After collection of feces, volunteers were supplemented with 1000 mg calcium twice daily for 1 week, after which the [1-$^{13}$C]palmitic acid experiment was repeated. **Results:** Percentage of total fat absorption in healthy volunteers on their habitual diets was (mean ± SEM) 96.6 ± 0.6%. Daily calcium supplementation led to a slight but significant decrease in total fat absorption (94.9 ± 0.9%, $P<0.05$). The 8-h cumulative percentage of $^{13}$CO$_2$ expiration decreased from 11.4 ± 1.2% under control conditions to 10.3 ± 1.1% upon calcium supplementation ($P<0.05$). Yet, plasma $^{13}$C-palmitic acid concentrations were significantly higher after calcium supplementation when compared to the control experiment. **Conclusion:** Dietary calcium supplementation to healthy adults leads to a slight impairment of fat absorption. Although calcium supplementation clearly affects the outcomes of the [1-$^{13}$C]palmitic acid test, present data do not indicate that the test is sensitive enough to reliably quantitate this degree of fat malabsorption in human adults.
Introduction

Adequate absorption of dietary fats by the intestine is required for supply of energy, membrane constituents, and precursors for the formation of hormones or inflammatory mediators [1-4]. In Western diets, triacylglycerols composed of long-chain fatty acids constitute 92 to 96% of dietary fats [2]. The absorption of these fats involves several processes. Firstly, lipolysis, by lipolytic enzymes originating predominantly from the pancreas, leads to hydrolysis of triacylglycerols into fatty acids and 2-monoacylglycerols. Secondly, during the process of solubilization, mixed micelles are formed, consisting of bile acids, phospholipids, cholesterol and the products of lipolysis (free fatty acids and monoacylglycerol) [5]. The mixed micelles are thought to act as the physiological transport vehicles of lipolytic products from dietary fats in the intestinal lumen. Finally, the fatty acids and monoacylglycerols are translocated across the intestinal epithelium, reassembled into chylomicrons and secreted into the lymph [2,6,7].

The efficiency of intestinal fat absorption in patients is routinely determined by means of a fat balance, requiring detailed analysis of daily fat intake and the complete recovery of feces for 72 h. However, the fat balance does not provide information on the etiology of fat malabsorption, i.e. impaired lipolysis or solubilization. This feature limits its use for defining optimal treatments for patients. One approach to address specifically the causes of fat malabsorption involves the determination of (mal)absorption of stable isotopically-labeled fats. Recently, we described the results of the application of $^{13}$C-labeled linoleic acid [8] and of palmitic acid [9] for detection of fat malabsorption due to chronic bile diversion in rats. In these studies we observed that, after intraduodenal administration of the label, plasma $^{13}$C-lipid concentrations reflect the absorption efficiency of dietary lipids. The present study was designed to determine whether the $[1-^{13}$C]palmitic acid test is sensitive enough to detect a slight fat malabsorption in healthy adults.

Under physiological conditions, healthy individuals excrete approximately 4-6 g day$^{-1}$ of fat via the feces [10], which generally means that over 96% of the dietary fats entering the intestinal lumen is absorbed [10]. Even under physiological circumstances, non-lipid components in the diet, such as calcium, have been demonstrated to interfere with the efficient absorption of the lipids [11-14]. Oral calcium supplementation in healthy subjects has been reported to increase fat excretion via the feces in a dose-dependent fashion, presumably due to intestinal precipitation of bile salts and/or formation of insoluble calcium-fatty acid complexes, leading to impaired solubilization [13-17]. Thus, oral calcium supplementation in humans seems to be a reproducible method to induce a slight fat malabsorption due to impaired solubilization of long-chain fatty acids.

The aim of the present study was to investigate whether the $[1-^{13}$C]palmitic acid absorption test could detect a mild degree of fat malabsorption in humans. Fat malabsorption was achieved by supplementation of calcium. We investigated whether the absorption efficiency of dietary fats was mildly decreased upon calcium supplementation for 1 week to healthy adults and whether this possible effect could be quantified after oral ingestion of administered $[1-^{13}$C]palmitic acid. Quantification was achieved by determination of plasma $^{13}$C-palmitic acid concentrations and breath $^{13}$CO$_2$ concentrations.
Materials and methods

Human volunteers

10 healthy students (7 females, 3 males) with a mean ± SEM age of 22 ± 0.3 y and a body mass index of 20.5 ± 0.1 kg m$^{-2}$ participated in the studies. The volunteers were healthy according to medical histories and showed no symptoms of diarrhea, fat malabsorption, or gastrointestinal complaints. The study protocol was approved by the Medical Ethics Committee of the University Hospital Groningen.

Study protocol

Each subject completed two tests with [1-$^{13}$C]-labeled palmitic acid separated by an interval of one week. [1-$^{13}$C]palmitic acid was purchased from Isotec Inc. (Matheson, USA) and was 99% $^{13}$C-enriched. The subjects were asked to maintain their usual dietary habits during the total experimental period of 11 days. The subjects were instructed to avoid consuming naturally $^{13}$C-enriched foods (e.g. corn products, pineapple, cane sugar) for at least two days prior to and during each three-day study period. The same pre-selected test meal was consumed throughout both tests to limit any effect that diet may have on the metabolism of [1-$^{13}$C]palmitic acid. During each test, intake of nutrients was calculated from 4-day consecutive food diaries by a clinical dietitian using The Netherlands Nutrients Table “NEVO” 1993.

Subjects started with a control experiment during which they had their habitual dietary calcium intake. After an overnight fast, the subjects consumed [1-$^{13}$C]palmitic acid at a dose of 10 mg kg$^{-1}$ body weight as part of a controlled standard test meal consisting of 2 slices of wheat bread, 20 g butter, 1 boiled chicken egg, 25 g cheese or liver-pie according to their personal preference, 150 ml orange juice, and 150 g full fat yogurt (3500 kJ; 37 g fats, 94 g carbohydrates, 32 g proteins). Butter was used as a vehicle to administer the [1-$^{13}$C]palmitic acid. Before consuming the test meal, breath samples were collected in duplicates to provide a measure of baseline $^{13}$C-excretion in expired CO$_2$. After ingestion of the test meal, breath samples were collected periodically at 30-min intervals for a period of 8 hours. A baseline blood sample (5 ml, EDTA) was collected before consuming the test meal, and was then collected hourly during the 8-h study period. Plasma was isolated and stored frozen (-20ºC) until further analysis. A baseline feces sample was collected on the day before administration of [1-$^{13}$C]palmitic acid. Thereafter, all stools passed were collected for three days, homogenized, and frozen at -20ºC until further analysis. All the subjects were rested for the duration of the test. No additional food or liquids were permitted during the test except for non-calorie drinks such as water and tea.

From 3 days after the first study, calcium intake was increased for 7 days by oral supplementation of 2000 mg calcium per day in the form of calcium carbonate, divided over two doses (before breakfast and before dinner). At day 5 after the start of the calcium supplementation, the [1-$^{13}$C]palmitic acid test was repeated identical to the procedures described above. Before ingestion of the test meal (breakfast), calcium supplementation was administered.
The influence of the test meal on $^{13}$C-enrichment in breath $^{13}$CO$_2$ was examined by having one subject completing the study, using unlabeled palmitic acid instead of [1-$^{13}$C]palmitic acid.

**Analytical techniques**

*Breath sample analysis*. End expiratory breath was collected via a straw into a 10 mL tube (Exetainers; Labco Limited, High Wycombe, United Kingdom), from which aliquots were taken to determine $^{13}$C-enrichment by means of continuous flow isotope ratio mass spectrometry (Finnigan Breath MAT, Finnigan MAT GmbH, Bremen, Germany). The $^{13}$C-abundance of breath CO$_2$ was expressed as the difference per mil from the reference standard Pee Dee Belemnite limestone ($\delta^{13}$C$_{PDB}$, ‰). The proportion of $^{13}$C-label excreted in breath CO$_2$ was expressed as the percentage of administered $^{13}$C-label recovered per hour (% $^{13}$C dose h$^{-1}$), and as the cumulative percentage of administered $^{13}$C-label recovered over the study period (cum % $^{13}$C).

Mean values of whole body CO$_2$ excretion were measured by indirect calorimetry (Oxycon, model ox-4, Dräger, Breda, The Netherlands) at 2 separate periods of 5 minutes during both test days. As a control, this sampling method was compared to sampling every 30 min (results not shown). These results indicated that, under the test conditions chosen, the mean values of the CO$_2$ production obtained from 2 randomly chosen periods were within the 95% confidence interval of the mean values obtained when sampling occurred every 30 min.

*Plasma fats*. Plasma fats were extracted, hydrolyzed and methylated according to Lepage and Roy [18]. Resulting fatty acid methyl esters were analyzed both by gas chromatography to measure the total amount of palmitic acid and by gas chromatography combustion isotope ratio mass spectrometry to measure the enrichment of palmitic acid. The concentration of $^{13}$C-palmitate in plasma was expressed as the molar percentage of the dose per liter plasma (% dose L$^{-1}$).

*Fecal fats*. Total fecal fat excretion in human subjects was measured according to the method of Van de Kamer et al. [19]. Feces was partly freeze-dried and mechanically homogenized. Aliquots of freeze-dried feces were extracted according to the method of Bligh and Dyer [20], and subsequently hydrolyzed and methylated [18]. Resulting fatty acid methyl esters were analyzed by gas chromatography to calculate both total fecal fat excretion and total palmitic acid concentration. Fatty acid methyl esters were analyzed by gas chromatography combustion isotope ratio mass spectrometry to calculate the isotopic enrichment of palmitic acid. Total fecal fat excretion was expressed as g fat day$^{-1}$ and the percentage of total fat absorption was calculated from the daily dietary intake and the daily fecal fat output and expressed as a percentage of the daily fat intake.

$$\text{Total fat absorption} = \frac{\text{Fat intake (g day}^{-1}) - \text{Fat output (g day}^{-1})}{\text{Fat intake (g day}^{-1})} \times 100\%$$

A similar calculation was performed to measure the absorption of [1-$^{13}$C]palmitic acid.

*Gas liquid chromatography*. Fatty acid methyl esters were separated and quantified by gas liquid chromatography on a Hewlett Packard gas chromatograph Model 5880 equipped
with a CP-SIL 88 capillary column (50 m x 0.32 mm) and an FID detector [21,22]. The gas chromatograph oven was programmed from an initial temperature of 150°C to 240°C in 2 temperature steps (150°C held 5 min; 150-200°C, ramp 3°C min\(^{-1}\), held 1 min; 200-240°C, ramp 20°C min\(^{-1}\), held 10 min). Quantification of the fatty acid methyl esters was achieved by adding heptadecanoic acid (C17:0) as internal standard.

Gas chromatography combustion isotope ratio mass spectrometry. \(^{13}\)C-enrichment of the palmitic acid methyl esters was determined by a gas chromatography combustion isotope ratio mass spectrometer (Delta S/GC Finnigan MAT, Bremen, Germany) [23]. Separation of the methyl esters was achieved on a CP-SIL 88 capillary column (50 m x 0.32 mm). The gas chromatograph oven was programmed from an initial temperature of 80°C to 225°C in 3 temperature steps (80°C held 1 min; 80-150°C, ramp 30°C min\(^{-1}\); 150-190°C, ramp 5°C min\(^{-1}\); 190-225°C, ramp 10°C min\(^{-1}\), held 5 min).

Calculations and statistics

The experimental data are reported as means ± SEM. Differences between sample means were calculated using the two-tailed Student’s t-test for paired data. For correlating two variables, linear regression lines were fitted by the method of least squares and expressed as the Pearson correlation coefficient \(r\). Differences between means were considered statistically significant at the level of \(P<0.05\).

Results

Total fat absorption

In Table 5.1 the nutritional data of the control and the calcium supplementation experiments are shown. After 1 week calcium supplementation, the percentage of total fat absorption showed a small but significant decrease: 94.9 ± 0.9% compared to 96.6 ± 0.6% in the control situation (\(P<0.01\)). Habitual calcium intake of the subjects was approximately 1000 mg per day. Upon calcium supplementation, the calcium intake increased 3-fold. Yet, no correlation was found between total calcium intake and percentage of total fat absorption (\(r=0.44, P=0.06\)).

<table>
<thead>
<tr>
<th>Subject</th>
<th>Calcium intake (mg day(^{-1}))</th>
<th>Total fat absorption (% intake)</th>
<th>Breath 8-h (^{13})CO(_2) recovery (% dose)</th>
<th>[1-(^{13})C]palmitic acid absorption (% dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>951 ± 133</td>
<td>96.6 ± 0.6</td>
<td>11.4 ± 3.8</td>
<td>77.4 ± 4.9</td>
</tr>
<tr>
<td>Calcium</td>
<td>2909 ± 103</td>
<td>94.9 ± 0.9(^*)</td>
<td>10.3 ± 1.1(^*)</td>
<td>82.6 ± 4.3</td>
</tr>
</tbody>
</table>

A symbol indicates a significant difference compared to the control situation: * \(P<0.05\), # \(P<0.01\).

Excretion of \(^{13}\)C-palmitic acid into feces

Table 5.1 shows the percentage absorption of [1-\(^{13}\)C]palmitic acid, assessed by fecal \(^{13}\)C-palmitate concentration. The amount of \(^{13}\)C-palmitic acid excreted into the feces was calculated for the 72-h period following administration of [1-\(^{13}\)C]palmitic acid. In the control

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experiment and in the calcium experiment the absorption of $[1^{-13}C]$palmitic acid was similar: 77.4 ± 4.9% and 82.6 ± 4.3% dose, respectively ($P=0.39$). No correlation was found between calcium intake and percentage absorption of $[1^{-13}C]$palmitic acid ($r=0.17$). A significant relationship was observed between percentage of total fat absorption and absorption of $[1^{-13}C]$palmitic acid (Figure 5.1; $r=0.47$, $P<0.05$), indicating that $[1^{-13}C]$palmitic acid was handled by the intestine in a similar fashion as the mass of unlabeled dietary fats.

Figure 5.1 Correlation between total fat absorption and the absorption of $[1^{-13}C]$palmitic acid (10 mg kg$^{-1}$ body weight) of 10 healthy adults in the control situation (■) and upon calcium supplementation (□) (2000 mg day$^{-1}$; CaCO$_3$). $r=0.47$, $P<0.05$.

**Plasma $^{13}$C-palmitate concentration**

In the control experiment, analysis of the $^{13}$C-palmitic acid concentration in plasma samples showed a slow increase over the time and a maximum of 0.44 ± 0.10% dose L$^{-1}$ plasma was obtained after 5 h in the control experiment (Figure 5.2). Upon calcium supplementation the $^{13}$C-palmitate concentrations in plasma were initially similar, yet after 4 h significantly increased when compared with the controls ($P<0.05$, Figure 5.2), with a maximum of 0.81 ± 0.21% dose L$^{-1}$ plasma obtained after 6 h, respectively.

Figure 5.2 Time courses of $^{13}$C-palmitate concentration in plasma of 10 healthy adults in the control situation (■) and upon calcium supplementation (□) (2000 mg day$^{-1}$; CaCO$_3$) after a single oral dose of $[1^{-13}C]$palmitic acid (10 mg kg$^{-1}$ body weight) (* $P<0.05$).
Breath $^{13}$CO$_2$ excretion measurements

The $^{13}$C excretion rate in breath in the control experiment increased slowly and reached a maximum value of $2.6 \pm 0.3\%$ $^{13}$C dose h$^{-1}$ between 7 and 8 h after administration of the label (Figure 5.3A). In most subjects no decay of $^{13}$C was observed. The $^{13}$C expiration rate during calcium supplementation rose more slowly, and reached a similar level after 8 h ($3.2 \pm 0.3\%$ $^{13}$C dose h$^{-1}$) when compared with the control experiment (Figure 5.3A).

![Graph](image1)

**Figure 5.3** Time courses for the (A) $^{13}$CO$_2$ excretion rate and (B) cumulative $^{13}$CO$_2$ excretion in breath over the 8-h study period following oral ingestion of [1-$^{13}$C]palmitic acid (10 mg kg$^{-1}$ body weight) to 10 healthy adults in the control situation (■) and upon calcium supplementation (□) (2000 mg day$^{-1}$; CaCO$_3$) (* P<0.05, # P<0.01, ** P<0.001).

The cumulative $^{13}$CO$_2$ excretion data are summarized in Figure 5.3B and Table 5.1. At 5 h after [1-$^{13}$C]palmitic acid administration, the cumulative $^{13}$CO$_2$ excretion was significantly lower upon calcium supplementation compared to the control situation and this difference persisted until the end of the experiment (Figure 5.3B). At 8 h after [1-$^{13}$C]palmitic acid administration, the cumulative $^{13}$CO$_2$ excretion amounted to 11.4 ± 1.2% in the control experiment, and to 10.3 ± 1.1% upon calcium supplementation.

![Graph](image2)

**Figure 5.4** Correlation between the $^{13}$CO$_2$ excretion rate and plasma $^{13}$C-palmitic acid at all time points following oral ingestion of [1-$^{13}$C]palmitic acid to 10 healthy adults in the control situation (■) and upon calcium supplementation (□) (2000 mg day$^{-1}$; CaCO$_3$). $r=0.63$, P<0.001.
In order to compare whether $^{13}$CO$_2$ excretion in breath may be extrapolated directly to absorption efficiency, the relationship between the breath $^{13}$CO$_2$ excretion rates and plasma $^{13}$C-palmitic acid concentrations was determined. A significant relationship was observed between breath $^{13}$CO$_2$ excretion rates and plasma $^{13}$C-palmitic acid concentrations (Figure 5.4; $r=0.63$, $P<0.001$).

**Background $^{12}$C-enrichment after an unlabeled test meal**

The background enrichment of $^{13}$C was examined in breath, plasma and feces in 1 subject by performing the control experiment and the experiment during the calcium supplementation without administration of the label. There was no detectable change in the plasma $^{13}$C-palmitate, breath $^{13}$CO$_2$ and fecal $^{13}$C-palmitate over the 8-h study period in either of the experimental settings (data not shown).

**Discussion**

Recently we characterized the [1-$^{13}$C]palmitic acid absorption test for the detection of fat malabsorption due to long-term bile diversion in rats [9]. In the present study we investigated whether this test was sensitive enough to detect mild fat malabsorption in healthy volunteers, induced by oral supplementation of 2000 mg calcium carbonate per day. It has been demonstrated that an increased calcium intake leads to modestly increased amounts of fat in the feces, leading to decreased percentages of total fat absorption [15,16]. Also in our study, percentage of total fat absorption was slightly but significantly decreased upon calcium supplementation.

The absorption of dietary fats was significantly correlated with the absorption of [1-$^{13}$C]palmitic acid, assessed by fecal $^{13}$C-palmitate concentrations (Figure 5.1), indicating that the fate of [1-$^{13}$C]palmitic acid parallels the fate of total mass of dietary fats with respect to absorption under the experimental circumstances of this study. Correlations between total fat absorption and absorption of labeled fats have been reported before with outcomes varying from a strong correlation [9,24] to absence of a correlation [25,26]. It could be that the presentation of [1-$^{13}$C]palmitic acid to the absorptive site may not always be the same, as palmitic acid is normally hydrolyzed from dietary triacylglycerols in the gastrointestinal tract, indicating that analysis of fecal $^{13}$C-palmitate concentrations is not a representative method to determine dietary fat absorption.

Theoretically, based on the positive correlation between total fat absorption and absorption of [1-$^{13}$C]palmitic acid, one would expect that, upon calcium supplementation, not only total fat absorption would be decreased but also the absorption of [1-$^{13}$C]palmitic acid, resulting in reduced amounts of $^{13}$C-palmitic acid in blood plasma and decreased amounts of $^{13}$CO$_2$ in breath. Indeed, in breath a small but significantly decreased amount of $^{13}$CO$_2$ was recovered after 8 h upon calcium supplementation when compared with controls. Our results with respect to breath $^{13}$CO$_2$ excretion during the control experiment are rather similar to what other scientists report [25,27]: peak excretion rate of $^{13}$CO$_2$ appears rather late (after approximately 6 h) and does not exceed 3% dose h$^{-1}$. Only a few studies have appeared in which the [1-$^{13}$C]palmitic acid breath test was studied in patients with disturbed fat absorption.
Watkins et al. [27] reported data on the [1-\(^{13}\)C]palmitic acid breath test in patients with known bile salt deficiency, and found a significantly lower 6-h cumulative \(^{13}\)CO\(_2\) expiration when compared with healthy controls.

In contrast to the decrease observed in breath \(^{13}\)CO\(_2\) excretion upon calcium supplementation, plasma \(^{13}\)C-palmitic acid concentrations after 4 h were significantly increased upon calcium supplementation when compared with controls. The apparent contradiction between plasma \(^{13}\)C-palmitic acid concentrations and breath \(^{13}\)CO\(_2\) recovery suggests that post-absorptive metabolic changes take place. Previously, it has been reported that plasma triacylglycerol concentrations were increased upon dietary calcium fortification of 1800 mg day\(^{-1}\) in humans [16]. However, it is not known whether this observation is related to the results we obtained.

In summary, we show in healthy humans that percentage of total fat absorption can be manipulated to a minor extent with the use of calcium administration. Calcium supplementation resulted in a small but significant decrease of percentage of total fat absorption due to impaired bile solubilization. After oral ingestion of [1-\(^{13}\)C]palmitic acid, the calcium-induced fat malabsorption was associated with a decreased cumulative expiration of \(^{13}\)CO\(_2\) in breath but with increased \(^{13}\)C-palmitic acid concentrations in plasma. Present data indicate that calcium supplementation does not only affect the overall quantity of fat absorption, but also leads to alterations in post-absorptive metabolism. Finally, the present data indicate that the [1-\(^{13}\)C]palmitic acid test is not sensitive enough to detect mild fat malabsorption induced by calcium supplementation in human adults.

References

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