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Journal of Chromatography B, 716 (1998) 1–6

JOURNAL OF CHROMATOGRAPHY B

# Analysis of *tert.*-butyldimethylsilyl [1-$^{13}$C]palmitic acid in stool samples by gas chromatography–mass spectrometry with electron impact ionisation: comparison with combustion isotope-ratio mass spectrometry

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Received 3 April 1998; received in revised form 2 June 1998; accepted 2 June 1998

**Abstract**

The use of $^{13}$C-labelled compounds to study lipid metabolism is increasing. Typically less than 40% of the orally administered label is recovered in breath $CO_2$. The remainder must be either absorbed and not oxidised or not absorbed and remain in the faeces. Two methods of determining how much tracer passes through the body, and is present in the stool, were compared. Compound specific analysis of *tert.*-butyldimethylsilyl [$^{13}$C]hexadecanoic acid by gas chromatography–mass spectrometry (GC–MS) with electron impact ionisation was compared with bulk analysis of whole stool and lipid extract by continuous flow isotope ratio mass spectrometry (CF–IRMS) with a combustion interface. The mean difference between the IRMS and GC–MS methods was −0.02 mmol $^{13}$C d$^{-1}$ with a mean excretion of 14.2 mmol $^{13}$C d$^{-1}$. Combustion IRMS is both simpler and cheaper, when the objective is to determine how much administered dose appears in stool, and information about the form of the label is not required. 

*Keywords:* Hexadecanoic acid; Palmitic acid

## 1. Introduction

Stable isotopes offer a safe, repeatable, non-invasive means to measure gastrointestinal function and nutritional status in health and disease [1]. There is growing interest in the use of $^{13}$C-labelled compounds to study lipid metabolism [2–4]. However, using methods whereby $^{13}$C is recovered in breath following oral ingestion of a labelled substrate, only a small and variable proportion (0–40%) of the administered label is recovered in breath $CO_2$. To estimate the amount retained in the body it is therefore necessary to determine how much of the administered label is excreted in the stool. Classical methods of faecal fat extraction are based on the methods of Van de Kamer [5] and Folch [6]. Jeejeebhoy [7] developed a modified Van de Kamer method, employing a heptane: ether: 95% ethanol solvent system. The Jeejeebhoy method was compared with the classical Folch method.

Analysis of fatty acids by gas chromatography–mass spectrometry (GC–MS) is usually performed using methyl ester derivatives. However, under electron impact ionisation (EI) this derivative frag-

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0378-4347/98/$19.00 
PII: S0378-4347(98)00280-1

ments extensively in the ion source, resulting in loss of molecular ion information. When analysing stable-isotope labelled compounds, both tracers and internal standards, it is essential that the fragment used for analysis contains the tracer label. Furthermore, it is desirable that the same fragment contains the stable isotope of the internal standard. This goal can be achieved by using the methyl ester derivative with chemical ionisation or by using a derivative which does not fragment greatly, producing a pseudo-molecular ion under electron impact ionisation, which is most widely available in medical laboratories.

We have previously used the *tert.*-butyldimethylsilyl (TBDMS) derivative of amino acids in tracer studies with analysis by GC–MS, under electron impact ionisation [8]. Phillipou et al. [9] demonstrated the electron impact ionisation of TBDMS fatty acids. This derivative has also been used to measure organic acid profiles of tissue, saliva and urine [10–13]. Given the excellent mass spectrometric properties and ease of derivatization, it is surprising that this compound has not found wider application. We have found few publications describing TBDMS for the analysis of stable-isotope labelled long chain fatty acids [14]. Simoneau et al. [15] used this derivative for the analysis of $^{13}$C-labelled acetate by GC–MS with methane chemical ionisation.

The aim of this study was to determine whether compound specific analysis using the TBDMS derivative of 1-[$^{13}$C]hexadecanoic acid (C16:0) by GC–MS under electron impact ionisation, was superior to bulk analysis of $^{13}$C by continuous flow isotope-ratio mass spectrometry (CF–IRMS) with a combustion interface [16] for analysis of the amount of lipid tracer in human faeces.

## 2. Experimental

### *2.1. Subjects and protocol*

The study was performed at the Royal Hospital for Sick Children, Glasgow with the approval of the hospital ethics committee. After an overnight fast, eleven subjects (age 21–46 yr) consumed [1-$^{13}$C]hexadecanoic acid (99 atom% excess, Eurisotop, Saint-Aubin Cedex, France) at a dose of 5 mg kg$^{-1}$ body weight, dissolved in 75 ml single cream (18% fat). All stools passed were collected before and for 3 days after administration of the dose.

### *2.2. Compound specific analysis of [1-$^{13}$C]hexadecanoic acid in faecal lipid extract by GC–MS*

#### *2.2.1. Fatty acid standards*

Fatty acid standards were purchased from Sigma–Aldrich (Poole, Dorset, UK). Standard solutions (10 mmol l$^{-1}$) in propan-1-ol were prepared and working standards reflecting the fatty acid composition of normal faeces [17] were prepared in 2 ml glass vials (Chromacol, Welwyn Garden City, UK) with the addition of the internal standards, pentadecanoic acid (C15:0), heptadecanoic acid (C17:0) and hexadecanoic-16,16,16-d$_3$ acid ($d_3$-hexadecanoic acid; 99 atom% D). $d_3$-hexadecanoic acid was purchased from Isotech Inc., OH, USA. A gravimetric series of enriched standards were prepared from 0–10 mole% excess 1-[$^{13}$C]hexadecanoic acid (from the same source of tracer given to the volunteers).

#### *2.2.2. Sample preparation*

All solvents were of HPLC or analytical reagent grade (Sigma-Aldrich, Poole, Dorset, UK). Daily faecal collections were weighed, homogenised in a polythene bag, and an aliquot (25 g) was freeze dried to constant weight, and further homogenised with a glass pestle and mortar. An internal standard (C15:0, 0.25 mmole) was added prior to the first homogenisation. Freeze dried samples were stored at −20°C. Lipids were extracted from 0.5 g freeze-dried faeces in a 25 ml glass centrifuge tube, according to a modification of the method of Jeejeebhoy [7]. Internal standards ($d_3$-hexacanoic acid (1 mg) and C17:0 (20 μmoles)) were added prior to extraction. Samples were dried under vacuum. The samples were acidified by the addition of 1 ml 0.3 *M* HCl in 0.4% saline and lipids extracted into 10 ml Solvent 1 (heptane: diethyl ether: 95% ethanol, 1:1:1) by shaking in an ultrasonic bath for 10 min. The samples were centrifuged at 5000 g for 10 min and the supernatant transferred to a preweighed 25 ml glass centrifuge tube. Lipids were extracted twice more with 10 ml Solvent 2 (the upper phase of

heptane: diethyl ether: 95% ethanol: deionised water, 1:1:1:1), each time adding the supernatant to the preweighed tube. The lipid extracts were dried under vacuum and the yield of fat calculated. Solvent 2 (2 ml) was added and a volume equivalent to 0.5 mg lipid was transferred to a 2 ml glass vial for analysis without hydrolysis. A second aliquot containing 20 mg lipid was refluxed for 1 h at 90°C with 10 ml ethanolic NaOH (1 *M* in 90% ethanol) to saponify any triacylglycerols present. The sample was cooled, acidified by the addition of 5 ml water and 10 ml 0.5 *M* $H_2SO_4$ in saturated salt and non-esterified fatty acids extracted twice with 10 ml Jeejeebhoy Solvent 2. Replicates equivalent to 0.5 mg lipid were dried under vacuum in 2 ml glass vials.

*tert*.-Butyldimethylsilyl (TBDMS) derivatives were prepared by adding 175 μl 2,2,4-trimethylpentane (iso-octane) and 25 μl N-methyl-N-(*tert*.-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) (Regis Technologies Inc. IL, USA) to the dry samples and heating for 30 min at 80°C. In separate experiments using triheptadecanoin, we have established that this derivatisation procedure does not transesterify fatty acids.

#### 2.2.3. GC–MS

The GC–MS was a Trio-1000 system (Hewlett-Packard 5890 II gas chromatograph with Fisons Instruments A200S autosampler and VG Masslab Trio-1000 quadrupole mass spectrometer, all supplied by Fisons Instruments, Middlewich, UK).

#### 2.2.4. GC parameters

The gas chromatograph was operated in splitless mode with helium (CP grade) as carrier gas. The inlet pressure was 135 kPa. The injector temperature and the transfer line between the GC and the MS were operated at 300°C. The analytical column was a DB5MS (J & W Scientific, Folsom, CA, USA), length: 30 m, internal diameter 0.25 mm, film thickness 0.25 μm. The temperature program started at 120°C and ramped to 220°C at 10° $min^{-1}$, then from 220°C to 236°C at 4° $min^{-1}$, and finally from 236°C to 320°C at 20° $min^{-1}$, followed by a 4 min period at 320°C. The solvent delay was 4 min. The injection volume was 0.2 μl.

#### 2.2.5. MS parameters

The Trio-1000 quadrupole mass spectrometer with electron impact ionisation, was operated in positive mode with a trap current of 150 μA and electron energy of 70 eV. The source temperature was 200°C, and the detector multiplier was operated at 300 V. Selected-ion recording mode of $M-(C-(CH_3)_3)$ i.e. $M-57$ was used to measure hexadecanoic acid enrichment and concentration. The quantitation masses for hexadecanoic acid, [$^{13}$C]hexadecanoic acid, $d_3$-hexadecanoic acid and heptadecanoic acid are $m/z$ 313, 314, 316, 327 respectively. The dwell time on each mass was 0.08 s, with a span of ±0.1 amu.

#### 2.2.6. Calibration

Concentration of C16:0 acid was calculated with reference to the internal standards, and related to the daily stool output. Enrichment of 1-[$^{13}$C]hexadecanoic acid was calculated from the background corrected ratio of 314/313, with reference to a 2 point calibration from a natural abundance standard and an enriched working standard at 5 mole% excess 1-[$^{13}$C]hexadecanoic acid. The product of enrichment and concentration allowed us to express the results as mmole $^{13}$C per day to enable direct comparison between the compound specific analysis and bulk analysis.

### 2.3. Bulk analysis of $^{13}C$ in whole stool and faecal lipid extract by Continuous Flow Isotope Ratio Mass Spectrometry (CF–IRMS)

#### 2.3.1. Sample preparation

Freeze-dried, ground stool samples (2 mg) were weighed in tin boats, 8×5 mm, 150 μl capacity (Elemental Microanalysis Ltd., Okehampton, UK) on a 5-figure balance. Extracted lipid in Jeejeebhoy Solvent 2 (equivalent to 2 mg C), was pipetted into 150 μl capacity tin boats containing an inert support (acid washed Chromosorb W, mesh size 60/80, Alltech Associates, Carnforth, UK), which had been pre-combusted at 450°C for 2 h to remove residual carbon. The solvent was removed by drying in a refrigerator overnight and then freeze-drying for 2 h. This procedure prevented the sample ‘creeping’ up the sides of the combustion boat. Hexadecanoic acid standards (1 mg C per 20 μl Jeejeebhoy Solvent 1)

were prepared as above, together with blanks composed of tin boats containing pre-combusted Chromosorb W.

### 2.3.2. IRMS

The samples were combusted in a biological sample converter (Europa Scientific, Crewe, UK) as described by Preston and McMillan [16]. This was interfaced to a 20–20 isotope ratio mass spectrometer (Europa Scientific, Crewe, UK). The oxidation stage was held at 1000°C, the reduction stage was 550°C, and the GC column was isothermal at 75°C.

### 2.3.3. Calibration

The system was calibrated using sugar beet sucrose, whose $^{13}C$ enrichment had been independently calibrated against an international standard. The $^{13}C$ enrichment of the samples was calculated by comparison with working standards comprising 2.375 mg sugar beet sucrose (containing 1 mg C), after subtraction of the blank consisting of a combustion boat with Chromosorb W. Data are presented as mmole $^{13}C$ per day.

### 2.4. Statistical methods

The Bland-Altman Method [18] was used to assess agreement between the two analyses of lipid extract.

## 3. Results and discussion

The Jeejeebhoy method of faecal fat extraction [7] was compared with the classical Folch method [6]. The Jeejeebhoy method was adopted because the lipid containing phase is the upper phase, and is therefore easier to remove from solid samples processed in large numbers.

Fig. 1 shows the EI mass spectrum of TBDBS–hexadecanoic acid. The prominent peak at m/z 313 is formed by the loss of a tertiary-butyl group ($M-57$) from the molecular ion at $m/z$ 370. This fragment ion, therefore, contains all the carbon atoms originally present in both the tracer and the internal standard. Monitoring $m/z$ 313 and 314 gives isotopic information on the tracer, [1-$^{13}C$]hexadecanoic acid for enrichment analysis, and monitoring $m/z$ 316

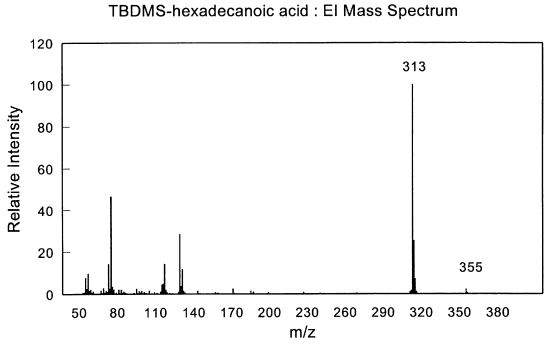


Fig. 1. Positive EI mass spectrum of TBDMS–hexadecanoic acid. The prominent peak at $m/z$ 313 is formed by the loss of a tertiary-butyl group ($M-57$) from the molecular ion at $m/z$ 370.

gives isotopic information on the internal standard, $d_3$-hexadecanoic acid for concentration analysis. Accuracy of the enrichment analysis was ensured by including a two point calibration (0 and 5 mole% excess) with each batch of samples analysed. The standard deviation of replicate sample injections was 0.16 mole% excess $^{13}C$ over a four month period including the analysis of 124 samples in triplicate ($n=372$). This included both hydrolysed and crude lipid extracts. The mean enrichment of these samples was 3.7 mole% excess $^{13}C$. The precision of the concentration analysis was 0.10 mole% excess $d_3$-hexadecanoic acid, as measured by the standard deviation of the measured 316:313 ratio, where the mean enrichment was 7.4 mole% excess $d_3$-hexadecanoic acid.

Analysis of concentration by GC–MS allows the use of a deuterated internal standard of the same compound. This ensures that any processing losses are the same for both the tracer and the internal standard, thus giving a more accurate analysis. The coefficient of variation of the measured ratio for the concentration analysis using pentadecanoic acid as internal standard was 2.1%, that of heptadecanoic acid was 2.8%, and of $d_3$-hexadecanoic acid was 0.6%. Thus the deuterated internal standard was used for quantitation of concentration.

The lipid extract was analysed as both non-esterified fatty acids in the crude extract, and after hydrolysis. The difference gives a measure of esterified hexadecanoic acid in stool. The data in Table

Table 1
Compound specific analysis of concentration and enrichment of non-esterified hexadecanoic acid from crude lipid extract compared to saponified lipid extract. Results of linear regression analysis of concentration and $^{13}C$ enrichment of non-esterified hexadecanoic acid in crude lipid extract vs saponified lipid extract are presented

| | Concentration g C16:0 $d^{-1}$ | Enrichment mmol. $^{13}C$ $d^{-1}$ |
|---|---|---|
| Gradient | 0.93 | 0.98 |
| Std. error of gradient | 0.025 | 0.051 |
| Intercept | 0.073 | 0.85 |
| Std. error of intercept | 0.107 | 5.78 |
| Number of observations | 45 | 32[a] |
| Correlation coefficient | 0.97 | 0.92 |

[a] Excluding baseline samples.

1 show that, in this study, the vast majority of extracted hexadecanoic acid was in the free form.

Both freeze dried stool and lipid extract were analysed by combustion IRMS. The analysis of both the carbon concentration and $^{13}C$ enrichment from the lipid extract was much more precise than that of whole stool due to the more homogeneous nature of the extract. Results are shown in Table 2.

The precision of analysis of whole stool reported here is poorer than that reported by Schoeller et al. [20], who used high-precision differential isotope ratio mass spectrometry, a technique that requires off-line preparation of pure $CO_2$ samples involving considerable labour, prior to analysis by IRMS. The classical technique uses larger samples and is less prone to poor precision due to sample heterogeneity. Importantly, the precision of analysis of lipid extract is similar to that of Schoeller et al. [20], reflecting the more homogenous nature of this extracted sample compared to whole stool.

Table 2
Precision of IRMS analysis of freeze dried stool and lipid extract

| | Whole stool | | Lipid extract | |
|---|---|---|---|---|
| | mg C | ape $^{13}C$ | mg C | ape $^{13}C$ |
| Mean | 1.07 | 0.041 | 0.75 | 0.14 |
| Relative S.D. (%)[a] | 13.7 | 24.8 | 5.3 | 1.0 |
| S.D. | 0.15 | 0.010 | 0.04 | 0.0014 |

[a] Relative standard deviation of duplicate analyses ($n$=50) [19].

Fig. 2 compares bulk analysis of $^{13}C$ with compound specific analysis of [1-$^{13}C$]hexadecanoic acid, in faecal lipid extract, both expressed as mmole $^{13}C$ per day. The mean difference between the bulk analysis by CF-IRMS and compound specific analysis by GC–MS was −0.02 mmole $^{13}C$ per day. The 95% confidence interval for the bias was −1.22 to 1.18 mmole $^{13}C$ per day [18]. The limits of agreement (mean±2 sd) were −6.78 and 6.74, with 95% confidence intervals of −8.86 to −4.71 and 4.66 to 8.81, respectively [18]. There is thus excellent agreement between the two methods. This observation supports the interpretation that microbial transformations such as chain elongation, hydroxylation

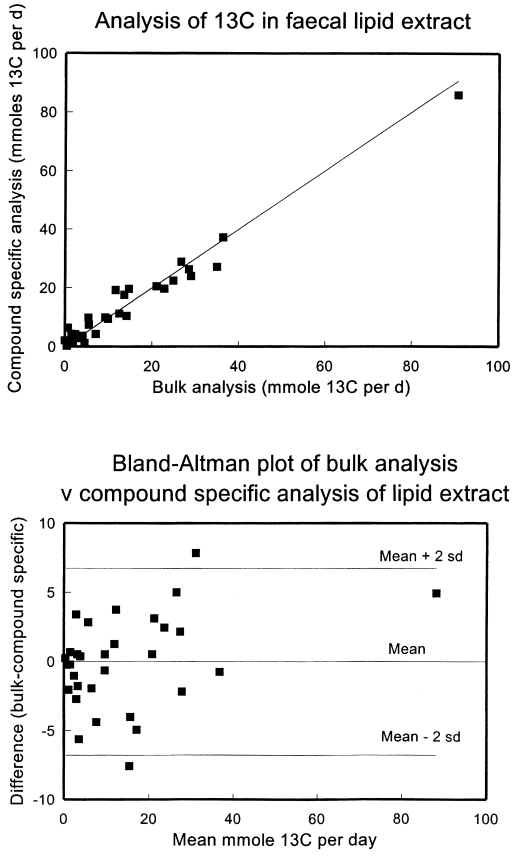


Fig. 2. Comparison of bulk analysis of $^{13}C$ by continuous flow-combustion-IRMS with compound specific analysis of [1-$^{13}C$]hexadecanoic acid by GC–MS in faecal lipid extract. The residual plot shows a mean difference of −0.02 mmole $^{13}C$ per day.

or desaturation of hexadecanoic acid are not of such quantitative significance as to lead to a systematic difference between compound specific and bulk analyses.

## 4. Conclusions

We have introduced accurate procedures for bulk and compound specific analysis of $^{13}C$-labelled lipid tracers in stool. To ensure good accuracy and precision in estimating absolute tracer quantities, both approaches analyse concentration and enrichment simultaneously.

Compound specific analysis is required when it is necessary to investigate the chemical form of the tracer, but if the objective is to determine how much $^{13}C$ label appears in the stool, then bulk analysis of the lipid extract is both simpler and cheaper and is to be recommended. The analytical cycle for IRMS is typically a quarter of the time of GC–MS analysis. In addition, data reduction of the IRMS output takes significantly less time.

The majority of fat in stool is in the form of non-esterified fatty acids, which includes soaps and free fatty acids.

There is no evidence for microbial transformations that potentially lead to underestimation of faecal tracer output by compound specific isotope analysis.

## Acknowledgements

The helpful advice of Dr Frans Stellaard and Dr Mini Kalivianakis of the Department of Paediatrics, University Hospital of Groningen, The Netherlands is gratefully acknowledged, and volunteers who agreed to provide stool samples. The project received support from the EC Biomed Programme.

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