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Simultaneous gas chromatographic determination of concentration and isotopic enrichment of fatty acids in human plasma using flame ionization and mass spectrometric detection

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Abstract

Free fatty acids (FFAs) are important not only because they provide substrate for oxidation but also because they have the potential to regulate several metabolic and hormonal processes. Using stable isotope tracers, these processes can be studied. Here we present a gas chromatographic method to measure FFA concentrations and enrichments after extraction from plasma and subsequent derivatization in one analytical run, using both flame ionization and mass-selective detection. For concentration determinations intra-assay variation ranged from 1.5 to 4.9%, inter-assay variation ranged from 3 to 11%. Intra- and inter-assay variations of the enrichment determination of palmitic acid were 1.4 and 0.9%, respectively.

Keywords: Free fatty acids

1. Introduction

Adipose tissue contains the major energy reserve. After lipolysis of triglycerides free fatty acids enter the plasma compartment. Besides providing substrate for oxidation, free fatty acids have the potential to regulate several metabolic and hormonal processes, not only by substrate competition [1], but also by activation of the nuclear receptor complex peroxisome proliferator-activated receptor alpha/9-*cis*-retinoic acid receptor alpha (PPAR-alpha/RXR-alpha) [2]. Because of their regulatory potential, free fatty acids are involved in the pathophysiology of several metabolic disorders. For instance in obesity, excess body fat results in increased release of free fatty acids, which has been clearly associated with insulin resistance of both liver [3] and muscle [4], hypertriglyceridemia [5] and impaired β-cell insulin secretion [6]. In addition, there is a growing line of evidence, that different types of fatty acids exert distinct effects [7–10]. Stable isotope tracer methods provide a useful tool to study in vivo metabolism of fatty acids under various conditions.

In our hospital a study was done on the effects of variations in carbohydrate/fat ratio in eucaloric diets on post absorptive lipolysis [11]. For this study we

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needed a method to determine concentration and isotopic enrichment of palmitic acid in the free fatty acid fraction of the plasma.

Many methods have been reported to measure fatty acids [12–14], but concentrations and isotopic enrichments are always determined in separate runs. Here we describe a method where both are measured in one run. The fatty acids are extracted, isolated, and derivatized as described by Ingalls and co-workers [15,16], with some small modifications. The separation of the free fatty acids (FFAs) from other lipid fractions is done by column chromatography on silica gel using mixtures of organic solvents with increasing polarity to elute the different fractions. Concentrations are measured by flame ionization detection (FID) and enrichments by mass selective detection. To achieve reliable results for the tracer/tracee ratios (TTRs), calibration curves are measured as described by Patterson et al. [17].

2. Experimental

2.1. Equipment

All gas chromatographic (GC) analyses were carried out on a Hewlett-Packard Model 6890 instrument equipped with a split/splitless injection port, a Model 6890 automated liquid sampler, an FID system and a Model 5973 mass-selective detector. Data collection and calculations were performed using the HP Chemstation software (Hewlett-Packard, Palo Alto, CA, USA). The analytical column was an EC-WAX column (polyethylene glycol stationary phase, 30 m×0.25 mm I.D., 0.25 μm film thickness; Alltech, Deerfield, IL, USA). The column was split at the end using a vitreous silica outlet splitter (SGE, Austin, TX, USA). One part of deactivated silica tubing was inserted into the FID system, another part was connected to the mass-selective detector. Both parts were the same length, this resulted in good responses for both FID and mass spectrometry (MS). Through variation of the lengths of silica tubing, different splitting ratios between FID and MS can be achieved. Helium was used as the carrier gas (flow-rate 2.5 ml/min) and as make-up gas for FID (flow-rate 45 ml/min).

All evaporation steps were performed using nitrogen.

2.2. Chemicals

Isooctane, ethyl acetate, heptane, glacial acetic acid, methanol, chloroform and silicagel 60 (230–400 mesh) were obtained from Merck (Darmstadt, Germany). KOH in 95% ethanol was obtained from Riedel-de Haan (St. Louis, MO, USA), borontrichloride was obtained from Supelco (St. Louis, MO, USA). Tripentadecanoin and all fatty acids were obtained from Sigma (St. Louis, MO, USA). A mixture of methylated fatty acids (AOCS mix) was obtained from Alltech, hexadecanoic-2,2-d_2 acid (d_2-palmitic acid) was obtained from Isotec (Miamisburg, OH, USA)

Water was purified by passage through a Milli-Q apparatus (Millipore, Bedford, MA, USA).

2.3. Sample treatment

Human blood was collected in vacutained tubes containing potassium ethylenediaminetetraacetic acid (K_3EDTA) as anticoagulant. After centrifuging the plasma was transferred to plastic tubes and stored at −70°C until assay.

2.4. Standards and reagent solutions

Separate stock solutions of 10 m*M* of the following fatty acids were made in chloroform: myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), nonadecanoic acid (C19:0), arachidonic acid (C20:4), henecosanoic acid (C21:0), erucic acid (C22:1) and d_2-palmitic acid (d_2-C16:0). A stock solution of 3 m*M* trinonadecanoin (tri-C19:0) was made in chloroform.

A standard working solution was prepared by pipetting 200 μl of the stock solutions of C14:0, C16:1, C18:0, C18:3, C20:4 and C22:1 (final concentrations 100 μ*M*) and 1 ml of the stock solutions of C16:0, C18:1, C18:2 and C19:0 (final concentrations 500 μ*M*) into a volumetric flask and adding chloroform to a total volume of 20 ml. An internal

standard solution was made by pipetting 2 ml of the stock standards of C15:0, C17:0 and C21:0 into a volumetric flask and adding chloroform to a total volume of 10 ml. Concentrations were 2 m*M*. A triglyceride recovery standard was prepared by diluting 1.5 ml of the trinonadecanoin stock standard to a total volume of 10 ml with chloroform. Final concentration was 450 μ*M*. Standards for measurement of the enrichment of palmitic acid were made by combining solutions of C16:0 and d_2-C16:0 in various proportions. TTRs were 0.0107, 0.0267, 0.534 and 0.1067.

All standard solutions were stored at −20°C.

2.5. Extraction of plasma lipids

To 100×13 mm glass test tubes 25 μl of the internal standard solution and 100 μl of the triglyceride recovery standard solution was added and the solvent was evaporated under a stream of nitrogen. A 200-μl volume of plasma sample was added and the extraction procedure was done according to the protocol of Ingalls et al. [16].

2.6. Isolation of triglycerides and free fatty acids fractions

The fractions of triglycerides and free fatty acids were isolated as described by Ingalls et al. [16], except that to the triglyceride fraction 75 μl of internal standard was added. All fractions were evaporated.

2.7. Derivatization of triglycerate fatty acid (TGFA) and FFA

To the residues of the saponified triglyceride fractions and the free fatty acid fractions 50 μl BCl_3 (3 *M* in methanol) was added, the vials were closed with PTFE lined caps and incubated for 15 min at 45°C. After cooling down to room temperature, 0.5 ml hexane and 0.5 ml purified water were added and the vials were vortex-mixed and centrifuged. The hexane layer was transferred into GC vials and evaporated under a stream of nitrogen. The residues were dissolved in 100 μl of hexane and transferred to limited volume inserts for the GC vials. The vials were capped with aluminium TFE/butyl rubber lined caps.

2.8. Chromatography

A 1-μl volume of the dissolved samples was injected into the GC system (split ratio 1:10). The column oven temperature was 100°C at injection and after 2 min programmed to 260°C at a rate of 40°C/min. The oven was maintained at 260°C for 5 min. The injector temperature was 250°C and FID temperature was 270°C. The MS interface temperature was 260°C, ion source temperature was 230°C. Mass spectra were recorded using electron ionization with an electron energy of 70 eV. Selected ion monitoring was done at m/z 270 and 272 for C16:0.

2.9. Quantification

All standards were isolated, derivatized and prepared for GC analysis as described before.

For determination of the concentrations of the fatty acids, three different volumes (50, 100 and 200 μl) of working standard solution were pipetted into 100×13 mm glass tubes and 25 μl of internal standard solution was added. The FID signal of the GC system was used to calculate the concentrations. Three internal standards were used: C15:0 for quantifying C14:0, C17:0 for C16:0, C16:1, C18:0, C18:1 and C18:2, C21:0 for C18:3, C19:0, C20:4 and C22:1 [18].

For the calculation of the recovery of the triglycerides, 100 μl triglyceride recovery standard was mixed with 75 μl of internal standard solution and saponified and derivatized. The amount of C19:0 measured as FFA in this sample is set at 100% and the amount of C19:0 measured in the plasma samples is used to calculate the extraction recovery. The calculated concentrations of the other fatty acids in the triglyceride (TG) fraction is divided by the extraction recovery.

To calculate the enrichment of the palmitic acid, the processed enrichment standards were injected four times with different split ratios, respectively, 1:10, 1:20, 1:40 and 1:80 to obtain a range of peak areas. The MS signal of the C16:0 peaks was used for the calculations [17].

2.10. Validation

The fatty acid standards were calibrated against a commercially available mixture of methylated fatty acids containing methylated C14:0, C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3 (AOCS mix, Alltech). To make a solution with known concentrations of the methylated fatty acids an exact mass of the AOCS mixture was dissolved in hexane in a volumetric flask (20 mg AOCS mix in 20 ml hexane). This solution was added to derivatized internal standard solution and extracted as the standards. The concentration of the fatty acids in the working solution were calculated against this AOCS solution in 15 separate runs.

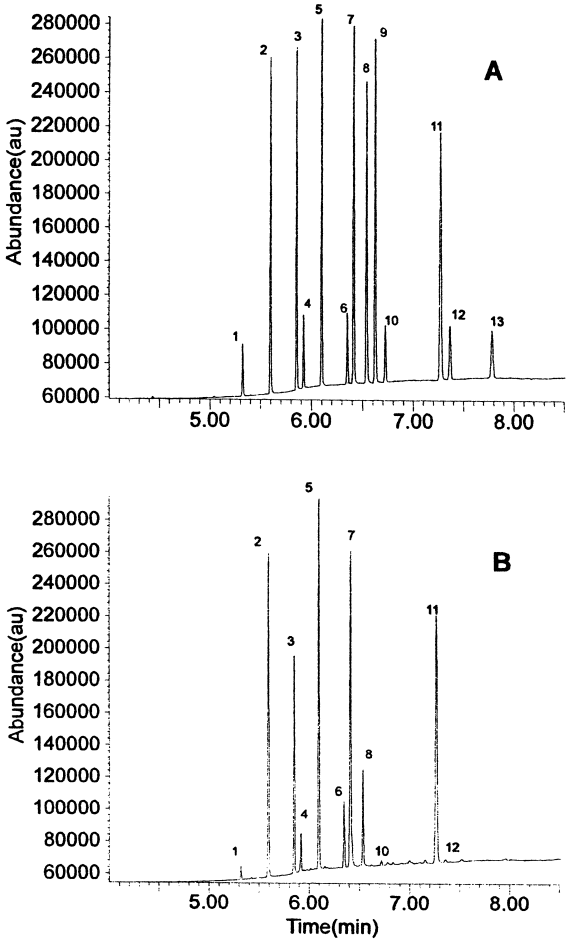

Fig. 1. FID chromatograms of a standard (A) and a plasma sample (B). 1=C14:0, 2=C15:0, 3=C16:0, 4=C16:1, 5=C17:0, 6=C18:0, 7=C18:1, 8=C18:2, 9=C19:0, 10=C18:3, 11=C21:0, 12=C20:4, 13=C22:1.

2.11. Precision

One plasma sample was spiked with C16:0 and d_2-C16:0 for calculation of the intra- and inter-assay variations of the enrichment of palmitic acid. Another plasma sample was spiked with all measured fatty acids for the calculation of the intra- and inter-assay variations of the concentration.

2.12. Recovery

The recovery of the method was established by spiking a plasma sample with standard working solution at two concentrations (200 μl plasma spiked with 50, 100 μl standard), and assaying it in triplicate.

The concentrations of the fatty acids in the non-spiked plasma were subtracted from the concentrations in the spiked plasma and the recovery percentages were calculated by dividing the calculated concentrations by the expected concentrations.

2.13. Limit of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were determined by measuring the blank signal (y) and the standard deviation of the

Table 1
Recovery, limit of detection (LOD) and limit of quantification (LOQ) of free fatty acids (LOD and LOQ quantified for a 200 μl sample)

	Recovery (%)	LOD (μM)	LOQ (μM)
C14:0	91	0.9	1.6
C16:0	97	1.1	1.6
C16:1	99	0.7	1.3
C18:0	97	0.7	1.2
C18:1	100	0.7	1.3
C18:2	101	0.8	1.4
C18:3	100	0.8	1.6
C20:4	103	1.0	2.0
C22:1	99	1.2	2.2

Table 2
Intra- and inter-assay variation of the determination of FFA concentration and enrichment of palmitic acid in plasma

	Intra-assay variation (n=10)		Inter-assay variation (n=27)	
	Mean concentration (μM)	RSD (%)	Mean concentration (μM)	RSD (%)
C14:0	39.0	4.9	42.2	6.5
C16:0	212.8	2.1	218.4	3.7
C16:1	31.0	2.5	43.4	7.2
C18:0	53.4	1.7	53.6	7.8
C18:1	206.6	2.1	238.3	3.1
C18:2	214.2	2.0	215.7	3.3
C18:3	42.3	2.3	41.1	5.4
C20:4	38.3	2.6	40.2	11.1
C22:1	41.1	1.5	41.2	3.7
	Intra-assay variation (n=10)		Inter-assay variation (n=15)	
	Mean TTR	RSD (%)	Mean TTR	RSD (%)
d_2-C16:0	0.0283	1.4	0.0280	0.9

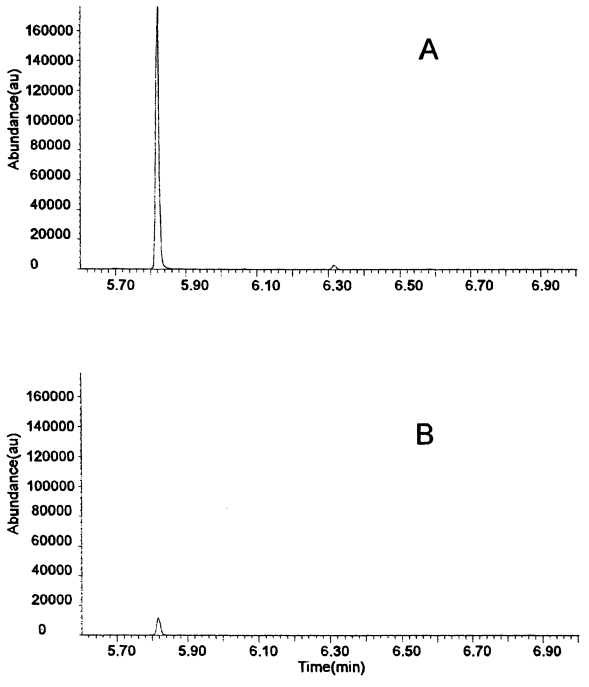

Fig. 2. Mass-selective detection mass spectra of a plasma sample using selected ion monitoring of m/z 270 (A, C16:0) and m/z 272 (B, d_2-labeled C16:0).

noise (s) and calculating the concentrations of $y+3s$ for LOD, according to the IUPAC rules [19] and $y+10s$ for LOQ as suggested by Miller and Miller [20]. This was calculated from the results of three separate runs.

3. Results and discussion

Fig. 1 shows a typical FID chromatogram of a standard and a plasma sample. Clearly can be seen that baseline separation is achieved for all components within 8 min. Fig. 2 shows mass spectra made by MS recording masses m/z 270 and m/z 272.

The calibration of our fatty acid standard against the commercially AOCS mix proved to be good (expected total concentration was 2.84 mM, measured 2.75 mM, recovery is 97%).

The recovery of the analysis is between 91 and 103% (shown in Table 1).

LOD is between 0.7 and 1.2 μM, LOQ between 1.2 and 2.2 μM (see Table 1).

Intra- and inter-assay variations of the free fatty acids are shown in Table 2. The intra-assay variation

for the concentration ranged from 1.5 to 4.9%, the inter-assay variation from 3.1 to 11.1%.

The intra- and inter-assay variations of the enrichment determination are 1.4% and 0.9%, respectively (Table 2).

4. Conclusion

Using the presented method one can measure concentration of distinct fatty acids and enrichments in one single analytical run using two detection methods, namely FID and MS, with good precision and reproducibility. The advantage of this method is that analysis time is minimized and results for concentrations and enrichment are obtained from the same injection ruling out bias from different sample clean-ups or injections.

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