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Fast gas chromatographic–mass spectrometric method for the evaluation of plasma fatty acid turnover using [1-^{13}C]palmitate

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Abstract

Turnover of plasma free fatty acids (FFAs) can be determined from the palmitate enrichment of plasma after administration of analogues labeled with stable isotopes. We studied the conditions to measure both the concentration and the ^{13}C enrichment of plasma palmitate by gas chromatography–mass spectrometry (GC–MS) using crude extracts. The method used plasma extraction after addition of heptadecanoic acid as internal standard and methylation with diazomethane. Subsequently the samples were analyzed by GC–MS. Plasma palmitate levels determined with this simplified method did not differ statistically from those obtained by a more "classical" procedure using FFA separation from other plasma lipids. Palmitic acid turnover rates (R_a) were evaluated in the steady-state period, in two normal subjects after 90 min infusion with [1-^{13}C]palmitate bound to human albumin. The rate of appearance (R_a) was found to be 0.92 and 1.08 mmol kg^{-1} min^{-1}, which is in good agreement with the turnover rate previously reported for normal subjects. Sample preparation and GC–MS analysis by the proposed procedure are simple and rapid and thus the method appears to be particularly useful in clinical studies where numerous samples have to be analyzed.

1. Introduction

Turnover of plasma free fatty acids (FFAs) has been extensively studied in animals and humans using radiolabeled compounds, particularly [^{14}C]palmitic acid [1,2]. Over the last decade the radioactive compounds used for this type of metabolic studies have been more and more replaced by compounds labeled with stable isotopes, *e.g.* ^{2}H or ^{13}C. Technical and ethical problems usually encountered with the use of radioactive tracers are thus avoided. So far, two methods have been reported for the determination of plasma palmitic acid enrichment with the [1-^{13}C] labeled acid [3,4]. The first one involves a rather laborious sample preparation with thin-layer chromatographic separation of the free fatty acids from the others lipids [3] after the extraction step. In the second method, purification of the free fatty acid fraction is obtained by multiple extraction at different pHs [4]. Since it was reported that plasma free fatty acids can be determined by gas chromatography of the crude extracts [5–8] we have studied the con-

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ditions necessary to measure both ^{13}C enrichment and concentration of plasma palmitate in these extracts by gas chromatography–mass spectrometry (GC–MS). The procedure described here gives good reproducibility and accuracy while sample preparation and GC–MS analysis are simple and rapid. Thus numerous samples can be analyzed daily.

2. Experimental

2.1. Materials

Palmitic acid and heptadecanoic acid were purchased from Sigma Chemical Co. (Bellefonte, CA, USA). Biosil-A (100–200 mesh) was from BioRad (Richmond, CA, USA). Sep-Pak cartridges C_{18} were from Millipore (Milford, MA, USA). [1-^{13}C]Palmitic acid (99 atom% excess) was obtained from Tracer Technologies (Somerville, MA, USA). Fatty-acid-free human albumin was purchased from ORHA (Behring, Germany) and human albumin (20% solution) was from AVIS (Milan, Italy).

2.2. Binding of palmitate to human albumin

Palmitic acid was converted into its sodium salt by treatment with 1 *M* NaOH solution (0.3% molar excess) and dilution with distilled water at 60°C to obtain a 5.2–43 m*M* concentration range. In order to determine the maximal amount of palmitic acid that can be bound to albumin, 1-ml aliquots of palmitate solutions at the different concentrations were mixed with 1 ml of fatty-acid-free human albumin, previously dissolved in distilled water at 45°C (10% solution), or with 1 ml of commercial human albumin (20% solution).

2.3. Preparation of the infusate containing [1-^{13}C]palmitate

Weighed amounts of [1-^{13}C]palmitic acid were bound to fatty-acid-free human albumin by mixing 200 ml of the albumin solution (75 mg/ml) at 45°C and 430 mg of labeled palmitate solution (3.3 mg/ml) kept at 60°C. The solution was then filtered through a 0.8-μm and a 0.22-μm sterile filter. After collecting three aliquots (10 μl each) for the analysis of palmitate concentration, the infusion was immediately started.

2.4. Human study

Two healthy volunteers (men, age 25–35 years) of normal weight (65–72 kg) and height (160–180 cm) were infused with fatty-acid-free albumin solution (45%) to which 29 mg [1-^{13}C]palmitate/g protein had been added, and which had been sequentially filtrated on 0.80-μm and 0.22-μm filters. Palmitate in the filtered infusate was analyzed by GC–MS under the conditions described below, in order to evaluate its concentration and isotope enrichment before infusion. Infusion was carried out at a flow-rate of 40–65 ml/h for 90 min as described elsewhere. Crude fatty acid extracts prepared from blood withdrawn at 10 and 5 min before infusion and at 75, 80, 85, 90 min after infusion were analyzed by GC–MS after esterification with CH_2N_2. The turnover rate of the FFAs was calculated as previously described [9] using the equation for steady-state data.

2.5. Sample preparation

To aliquots of 0.5–1 ml of plasma 20 μl of heptadecanoic acid (25 μg) in 1-propanol as internal standard were added. The samples were then processed according to either one of the following methods.

2.6. Purification of FFA extracts

(A) Lipids were extracted from plasma using the procedure described by Bligh and Dyer [10]. Briefly, 2.5 ml of methanol and 1.25 ml of chloroform were added to the 1-ml sample. The obtained monophasic solution was then mixed with 1.25 ml of chloroform and 1.25 ml of water and vortex-mixed. The organic fraction was separated and dried under nitrogen. Part of the extract, corresponding to *ca.* 0.5 ml of plasma was then esterified for GC and GC–MS analysis

as described below. The remaining part was dissolved in 1 ml of hexane and applied to a pre-washed silicic acid open chromatographic column (0.5 g of silicic acid, Biosil-A). The column was first eluted with 10 ml of hexane–diethyl ether (96:4, v/v). The FFAs were then eluted from the column with 10 ml of diethyl ether. After solvent evaporation the FFAs were converted to their methyl esters with a freshly prepared solution of diazomethane.

(B) The plasma samples were acidified with 0.1 *M* HCl, 1 ml of acetonitrile was added and the obtained suspension was applied to a pre-conditioned Sep-Pak C_{18} cartridge. After washing with 10 ml of H_2O–CH_3CN (1:1, v/v) the FFAs were eluted with 10 ml of CH_3CN–MeOH (99:1, v/v). The solvent was evaporated under vacuum with a Savant centrifuge and residues were treated with diazomethane as described above.

(C) Crude extracts prepared as in A were purified by TLC (Kieselgel 60F254 coated plates, 0.25-mm thickness; Merck, Darmstadt, Germany) using hexane–diethyl ether–acetic acid (70:30:1, v/v) as the eluting solvent and iodine for the detection. The free fatty acids recovered from silica with methanol were dried and then esterified as in A.

2.7. Calibration curves

Fixed amounts of palmitic acid (25 μg) were mixed with [1-^{13}C]palmitic acid to obtain a calibration curve with a molar percent excess (MPE) of ^{13}C ranging from 0 to 20%. For one curve samples were processed as described in A (crude extract and silicic acid), for the second curve samples were treated as reported in B (Sep-Pak), and for the third curve samples were methylated without any previous manipulation (pure standards).

Another set of calibration curves to be used for the quantification of palmitate plasma levels was prepared by mixing 25 μg of heptadecanoic acid with various amounts of palmitic acid (0, 1, 10, 20, 50 μg) and then processed as described above. Plasma palmitic acid concentration was calculated on the basis of the regression line computed from the GC data obtained for the calibration curve.

2.8. GC analysis

The dried samples were dissolved in 50 μl of ethyl acetate and 1-μl aliquots were injected onto a Fractovap Model 4160 HRGC gas chromatograph (Carlo Erba, Milan, Italy) equipped with a splitless injector and a FID detector. Chromatographic separation of palmitic acid from heptadecanoic acid and from other acids was carried out on an SPB-1 fused-silica capillary column (25 m × 0.53 mm I.D., film layer thickness 1 μm; Supelco, Bellefonte, CA, USA). The injector and detector temperatures were 270°C and 280°C, respectively. The oven temperature was held for 1 min at 200°C and then increased to 260°C at 20°C/min. Palmitic acid and heptadecanoic acid methyl esters had retention times of 5.30 and 6.30 min, respectively. The concentration of the plasma palmitic acid was then calculated on the basis of the palmitate/heptadecanoate peak-area ratios measured with an integrator (Waters Model 740) and of the calibration curve described above.

2.9. GC–MS analysis

Aliquots of plasma extracts were injected onto a Finnigan 4021 or an LKB 2091 instrument to determine the plasma palmitic acid enrichment. The mass spectrometric conditions of the two instruments were set as previously described [11]. Analyses were carried out in the selected-ion monitoring (SIM) mode, monitoring ions at *m/z* 270 and *m/z* 271 for the natural and the labeled palmitic acid and at *m/z* 284 for the heptadecanoic acid. Dwell times were 70 ms for ions 270, 271 and 284 when the Finnigan instrument was used to obtain at least 35 points per peak. With the LKB instrument peak width was set at 8 s so that dwell times were 20 ms for the 270 and 284 ions and 6 ms for 271, respectively. Chromatographic separation of the fatty acid methyl esters was achieved with an SPB-1 column (30 m × 0.32 mm I.D. with a film layer thickness of 1 μm; Supelco) in a Fractovap

Model 4160 HRC gas chromatograph equipped with a splitless injector. The oven temperature was set at 100°C for 1 min and then programmed to 260°C at 25°C/min. Peak heights were used for the following calculation. Plasma palmitic acid enrichment was determined as described by Magni *et al.* [11] based on the equation of the regression line of the enriched calibration curve. Plasma palmitic acid concentration in FFA was calculated using the peak-height ratio of ions 270 and 284 and converting this value in mg/ml using the equation obtained from the respective standard curve.

2.10. Statistics

Data were analyzed by Student-t test with a commercial software for IBM computers. The statistical level of significance was set at 95%.

3. Results

3.1. Binding of palmitate to human albumin

When palmitate was added (10 mg per g of protein, 2.6 mol/mol) to commercial solutions of human albumin not deprived of fatty acids, the binding capacity of the albumin was found to be already saturated. When palmitate was added to fatty-acid-free albumin (30 mg palmitate per g albumin), 95 ± 3.9% (mean ± S.D., $n = 6$) was found to be bound (7.6 ± 0.3 mol/mol). Any attempt to improve the palmitate/albumin ratio in the perfusate resulted in precipitation which hampered the subsequent sterilization of the perfusate by filtration on 0.22-μm sterile filters. Moreover, binding did not exceed the above reported maximal ratio, as determined from the concentration of the bound acid measured in the filtered perfusate by GC, using heptadecanoic acid as internal standard.

3.2. Validation of the evaluation of plasma palmitate concentration using crude extracts

Calibration curves for the evaluation of plasma palmitate concentration by GC and by GC–MS using heptadecanoic acid as internal standard are reported in Table 1. Linearity was ensured by the high value of the correlation coefficient under all tested conditions, including analysis of the crude extracts. Results shown in Table 2 on the plasma palmitate concentration measured in crude and purified extracts confirm the validity of the assay using crude extracts.

3.3. Evaluation of plasma palmitate ^{13}C-enrichment using crude plasma extracts

Calibration curves prepared as described in the Experimental section for the evaluation of palmitate MPE showed high correlation coefficients under all the three tested conditions (Table 3). The slopes values did not differ significantly from 1, which is expected for MPE lines calculated as described previously [11]. There was no difference between the "pure standard" curve, for which the only manipulation was the esterification with diazomethane,

Table 1
Calibration curves for the evaluation of plasma palmitate, using heptadecanoic acid as internal standard

	GC		*GC–MS*	
	$y = a + bx$	r^2	$y = a + bx$	r^2
Pure standards	$y = 1.09x$	0.9990	$y = 1.04x$	0.9970
Crude extract	$y = 0.98x$	0.9994	$y = -0.06 + 1.06x$	0.9781
Silicic acid	$y = 0.09 + 1.08x$	0.9965	$y = 0.10 + 0.92x$	0.9988
C_{18} Sep-Pak	$y = 0.31 + 1.28x$	0.9754	$y = 0.26 + 1.06x$	0.9904

Curves were prepared and analyzed as described in the text. Lines were calculated by regression analysis from the ratios of peak areas of palmitate/heptadecanoate (y) and the palmitate/heptadecanoate mass ratio (x).

Table 2
Basal concentration of palmitic acid in plasma[a]

Plasma	Concentration of palmitic acid (μg/ml)		
	TLC	Crude extract	Silicic acid
A	29.3	28.7	–
B	30.3	31.7	–
C	31.3	31	–
D	–	25.0 + 3.1	27.8 + 1.9
E	–	16.8 + 2.2	17.3 + 2.6
F	–	26.6 + 2.7	24.4 + 3.2

Plasma samples were prepared with blood collected from healthy subjects. Aliquots (1–0.5 ml) of each plasma were extracted and were then either directly analyzed or purified by silicic acid chromatography or by TLC.
[a] Values are either mean of two plasma aliquots (A–C) or mean ± S.D. of four plasma aliquots (D–F).

and the other curves for which analysis was preceded by sample processing with solvents.

The value of the m/z 271/270 ratio in the analysis of authentic palmitate was 0.221 ± 0.003 (mean ± S.D.; $n = 5$, 0.8 nmol palmitate per injection). In the analysis of pure [1-^{13}C]palmitate the peak height at the methyl palmitate retention time for the m/z 270 ion was 1% of that at m/z 271.

This 271/270 ratio was unchanged (0.217 ± 0.001, $n = 5$) when 8 nmol injections of palmitate were performed. Therefore using the LKB 2091 instrument imprecision (C.V.%) of the ratio determined at 0 MPE was 1.4% and 0.46% for the lower and higher injected amounts, respectively. When similar amounts of an enriched sample at *ca.* 1.5 MPE were analysed the imprecision of the 271/270 ratio was 1.3% (ratio = 0.234 ± 0.003) and 0.43% (ratio = 0.232 ± 0.001) for the lower and higher injected amounts, respectively. With the Finnigan mass spectrometer the ratio was 0.244 ± 0.008 and 0.352 ± 0.005 (for 0.8 and 8 nmol injected) indicating a higher variability with this instrument and a significant increase of the ratio on increasing the injected amount ten-fold. The latter result is in good agreement with the recent observations by Patterson and Wolfe [12] who found the (M + 1)/(M + 0) ratio of methyl palmitate to increase with a ten-fold increase of the injected amount of palmitate (0.2 nmol/2 nmol) and with a 2.8% C.V. for the mean value. Based on the above described results we used only the magnetic instrument for the analysis.

Table 3
Calibration curves for MPE determination

	$y = a + bx$	r^2
Pure standard	$y = -0.05 + 1.00x$	0.998
Crude extract	$y = 0.08 + 0.97x$	0.999
Silicic acid	$y = -0.01 + 1.00x$	0.995

Standard mixtures of natural and [1-^{13}C]palmitic acid were methylated with diazomethane either without previous manipulation (pure standards) or as described for method A. Lines were calculated by regression analysis as reported previously [11].

Correlation between the MPE determined on crude extracts and on purified FFA extracts of plasma samples enriched with palmitate bound to albumin is reported in Fig. 1. The enrichment determined on the crude extract and on the purified palmitic acid fraction of these enriched

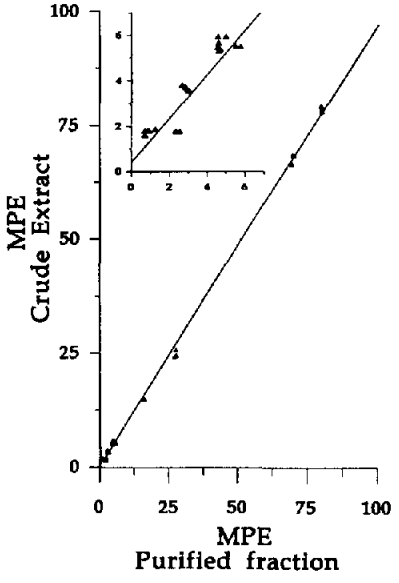

Fig. 1. Each point represents the MPE values obtained in the evaluation of palmitate in the crude extract and in the fraction purified by silicic acid column prepared from the same plasma sample. Enrichment of the samples was obtained by spiking control plasma specimens with [1-^{13}C]palmitate bound to human albumin. MPE was calculated as described in the text.

Table 4
Palmitic acid turnover determination after [1-^{13}C]palmitic acid infusion

	GC	GC–MS		R_a
	(μg/ml)	(μg/ml)	(MPE)	(μmol/kg/min)
Subject 1	17.3 ± 2.6	15.3 ± 3.9	3.6 ± 0.8	0.92
Subject 2	24.4 ± 3.1	19.2 ± 0.8	5.1 ± 0.5	1.08

Each value is the mean of the plasma palmitic acid concentration and enrichment determined 75, 80, 85 and 90 min after start of the infusion.

plasma samples were compared. Statistical analysis showed a high correlation between the two procedures ($r^2 = 0.999$) and a slope value not significantly different from 1 ($y = 0.4 + 0.967x$).

Results obtained from the evaluation of the plasma palmitate concentration and enrichment allowed to calculate the plasma turnover rate in two healthy subjects given [1-^{13}C]palmitate by infusion (Table 4).

4. Discussion

A number of investigations have shown the utility of stable isotopes in the evaluation of *in vivo* fatty acid turnover [13]. In recent studies of this type, labeled palmitic acid bound to human albumin was infused and isotopic enrichment of palmitate in plasma was determined by GC–MS, in order to determine the plasma free fatty acid turnover [3,4]. Few information is available on the maximal amount of palmitic acid that can bind to albumin, which is particularly important when long-lasting experiments are carried out. Therefore, the rate of albumin infusion must be maintained as low as possible while keeping the rate of palmitic acid infusion high enough for the isotopic enrichment to be measurable. With the aim to find useful analytical conditions for clinical studies involving a rather large number of subjects, we first quantified the protein binding of palmitate to human albumin solutions. Results indicating that *ca.* 8 mol palmitate was bound to one mole of albumin under the conditions used were in good agreement with the number of high-affinity binding sites of first and second class previously reported [14]. It is worth noting that the use of commercial albumin solution necessarily reduces the enrichment of the fatty acid with the labelled isotopomer due to the dilution of the added acid with that already present in the solution. Despite this drawback, the use of this type of solutions in clinical studies may be useful due to both their low cost and to the standardization of the albumin concentration of these preparations. However, due to the variability of the FFA concentration among the various batches, the level and the enrichment of palmitic acid in the infusion medium must be always checked before its administration.

Conditions that would allow high simplicity and rapidity, in addition to high specificity, reproducibility and accuracy of the procedure for the determination of the isotopic enrichment of plasma palmitate, were then considered in this study. For this purpose, three types of purification procedures, silicic acid column, Sep-Pak C_{18}, and TLC were tested and the results obtained using purified extracts were compared with those obtained with crude extracts. With regard to the linearity of the GC evaluation of the palmitate concentration, comparable results were obtained for the silicic acid purified samples and for the non-chromatographed samples (Table 1); with Sep-Pak purification instead a steeper slope was observed for the regression line, likely due to an unknown contaminant that co-eluted from the gas chromatographic column with methyl palmitate. In fact the difference between the lines was not observed when the analyses were carried out by GC–MS which allows a more specific determination of palmi-

tate. In all cases the fitted lines showed a small positive intercept on the y-axis, which was more pronounced when Sep-Pak C_{18} was used for the purification. As demonstrated from the analysis of blank samples, to which palmitate and/or heptadecanoate were not added, the intercept appears to be due to small amounts of palmitic acid ($<0.001\%$) present in the solvents, even if the highest commercially available purity was used. Due to these problems the Sep-Pak C_{18} purification procedure was not considered further.

When the different procedures were applied to various plasma samples to determine the palmitate concentration in the free-fatty-acid fraction, no statistical difference was found between the value obtained by GC for the crude extract and that found in the palmitate fraction purified by silicic acid chromatography or by TLC (Table 2). It was reported that FFA levels could be determined by direct methylation of plasma [15]. However, more recent studies have shown that under these conditions the fatty acid levels are abnormally high, due to hydrolysis of plasma phospholipids [16]. Our data ensure that the conditions used for methylation in the present study do not cause hydrolysis of phospholipids. Comparison of the values obtained for crude extracts with those of the fatty acid TLC band obtained for the same extracts also exclude the formation of significant amounts of methyl palmitate by transmethylation of fatty acid esters present in the crude extracts (Table 2).

The highly linear correlation of the MPE evaluation between crude extracts and purified FFA fractions (Fig. 1) also ensures the validity of using the former. Moreover, the results on palmitate enrichment in volunteers after infusion of labeled palmitate (Table 4) show that the overall procedure described here fulfills the requirements for studies in humans since the FFA turnover (R_a) was in good agreement with previously reported values [17,18].

In conclusion, the results of the present study confirm that plasma enrichment of [1-^{13}C]palmitate can be determined as described here, without previous separation of FFAs and the other plasma lipids. This simplified procedure allows 70–80 plasma specimens to be processed completely within three working days. The reduction in processing time is of great importance in clinical studies where large numbers of samples have to be handled.

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