ELSEVIER

Journal of Chromatography B, 658 (1994) 369–374

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Determination of plasma fatty acid composition in neonates by gas chromatography☆

E.M. Bondia[a], A.I. Castellote[a], M.C. Lopez*[,a], M. Rivero[b]

[a]*Unitat de Nutrició i Bromatologia, Departament de Ciències Fisiològiques, Humanes i de la Nutrició, Facultat de Farmacia, Universitat de Barcelona, 08028 Barcelona, Spain*

[b]*Departament Cientific. ORDESA, S.L. Sant Boi de Llobregat, Barcelona, Spain*

First received 6 December 1993; revised manuscript received 13 June 1994

Abstract

Total fatty acids in plasma of neonates have been analysed as their methyl esters by gas chromatography. They were separated on a capillary column coated with a SP-2380 stationary phase. As little as 100 μl of plasma is used for the analysis. The extraction procedure was performed with dichloromethane–methanol (2:1) and fatty acids were methylated with boron trifluoride–methanol. The quantification of fatty acids is based on an internal standard method. Absolute values (μg fatty acid per 100 μl plasma) are given together with relative values (%). At a signal-to-noise ratio of 3, the detection limits for flame ionisation detection are between 0.08 to 0.51 ng. The high sensitivity and precision permits the effective determination of the fatty acids in neonate plasma.

1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA) are components of structural lipids in all cell membranes, and are particularly important for the development and the function of the central nervous system and retina [1]. The essential fatty acids ($C_{18:2\omega-6}$, $C_{18:3\omega-3}$) are metabolized to form longer-chain more highly unsaturated fatty acids as arachidonic acid ($C_{20:4\omega-6}$, AA); 5, 8, 11, 14, 17-eicosapentaenoic acid ($C_{20:5\omega-3}$, EPA) and 4, 7, 10, 13, 16, 19-docosahexaenoic ($C_{22:6\omega-3}$, DHA). It has been suggested that elongation of polyunsaturated fatty acids from dietary $C_{18:2\omega-6}$ and $C_{18:3\omega-3}$ precursors may be deficient in the newborn [2]. Therefore, it is necessary to monitor the level of these LC-PUFA in plasma of neonates, in order to determine possible deficiencies, and, if necessary, incorporate AA and DHA into the infant formula. Plasma and erythrocytes are highly feasible markers of fatty acid metabolism in pediatric clinical investigation [3,4].

In principle, the qualitative and quantitative measurement of fatty acids is relatively easy. In our case, however, sufficient sample was not available since patients were neonates. Several authors have determined fatty acids in plasma [2,5–15]. The classical method for the extraction of the lipids in most cases, was the method of Folch et al. [16]. Farrel and Gutcher [7], Bjerve and Fischer [6], follow the Bligh and Dyer method [17] and Liebich and Wirth [9] and Welz et al. [13] follow the method of Lepage and Roy [18].

* Corresponding author.

☆ Presented at the *22nd Annual Meeting of the Spanish Chromatography Group, Barcelona, October 20–22, 1993.*

0378-4347/94/$07.00
SSDI 0378-4347(94)00287-F

We have used the method of Folch et al. with slight modifications; we have replaced the binary mixture chloroform–methanol (2:1) by dichloromethane–methanol (2:1) since it is less toxic [19,20] and we have added butylated hydroxytoluene (BHT) to the mixture as an antioxidant [6,11,15]. The next step was the methylation of the fatty acids. The method of Morrison and Smith [21] has been used by various authors with only slight variations: addition of methanolic NaOH and BF_3–MeOH [6–8] or methanolic HCl [10,11]. On the other hand, direct transesterification with acetyl chloride in methanol has been reported [9,13] following the procedure of Lepage and Roy [18]. Pace-Asciak [22] also described a one-step method for the derivatization and extraction of non-esterified fatty acids in plasma with methanol and rapid methylation with diazomethane in diethyl ether. Clark et al. [2] and Carter et al. [23] methylated with 1% H_2SO_4 in methanol. Fatty acid methyl esters (FAME) were extracted into *n*-hexane [13] more efficiently than petroleum ether [7] or *n*-heptane [2], dried over anhydrous Na_2SO_4. We have quantified each fatty acid using heptadecanoic acid ($C_{17:0}$) as an internal standard, in order to give absolute values together with relative values. Previous studies [2,4,7,11] give only relative amounts (percentage) of the fatty acids. Only few papers show absolute values [6,10]. As the concentration of total lipids in plasma increases after birth [24], the percentage values may not quantitatively reflect the real status of essential and polyunsaturated fatty acids. The purpose of this study is to develop a micromethod, as little as 100 μl plasma sample is used for the analysis, from an existing macromethod [7,8,11]. This permits the determination of total fatty acids in neonate plasma whereby special attention is paid to the analysis of AA and DHA, because both are essential for normal neural development.

2. Experimental

2.1. Reagents

Dichloromethane, methanol, anhydrous sodium sulfate (99% minimum purity) and sodium chloride were purchased from Probus (Badalona, Spain). Boron trifluoride–methanol and *n*-hexane (analytical-reagent grade) were purchased from Merck (Darmstadt, Germany). Butylated hydroxytoluene (BHT) and EDTA were from Fluka (Buchs, Switzerland).

2.2. Standard solutions

Stock solutions of all compounds (1 mg/ml) and the internal standard (0.5 mg/ml), were prepared by dissolving pure standards in *n*-hexane and were stored at 4°C. Working standard solutions were prepared weekly: 200 μg/ml for $C_{16:0}$, $C_{18:0}$, $C_{18:1\omega-9}$, $C_{18:2\omega-6}$, $C_{18:3\omega-3}$ (hexadecanoic, octadecanoic, octadecenoic, octadecadienoic, octadecatrienoic acids) in a kit purchased from Supelco (Bellefonte, PA, USA); 20 μg/ml for $C_{20:4\omega-6}$ (arachidonic acid, AA) purchased from Larodan Fine Chemicals (Malmö, Sweden); 20 μg/ml for $C_{22:6\omega-3}$ (docosahexaenoic acid, DHA) purchased from Sigma (St. Louis, MO, USA). A series of working standards were prepared by pippeting aliquots of the working standard solutions, evaporating to dryness under nitrogen and redissolving in 50 μl of *n*-hexane. Three levels for every fatty acid were made: 0.2, 0.4, 0.8 μg/μl of $C_{16:0}$, $C_{18:0}$, $C_{18:1\omega-9}$, $C_{18:2\omega-6}$, $C_{18:3\omega-3}$; 0.1, 0.2, 0.3 μg/μl of AA; 0.01, 0.04, 0.08 μg/μl of DHA and a constant solution of 0.5 μg/μl of $C_{17:0}$ (heptadecanoic acid) as internal standard (IS), obtained from Fluka (Buchs, Switzerland). Fatty acid reference mixtures (RM-3, PUFA 1, and PUFA 2) were from Supelco. Their purities were tested and, in most instances, were greater than 98%.

2.3. Sample collection and storage

Cord samples were collected from the umbilical vein within a few minutes of birth. One mililiter blood sample from a newborn infant was collected in brown polypropylene tubes containing EDTA K_3 anticoagulant (15% in saline solution). The blood was centrifuged at 3000 *g* for 5 min (Model Hermle ZK 380) and the

plasma and buffy layer were removed by aspiration. Plasma was stored in conical bottoms with amber polypropylene microcentrifuge tubes (Elkay Prod., Shrewsbury, MA, USA) in the dark at −70°C.

2.4. Sample preparation

Lipid extraction

At the beginning of the extraction, 50 μl of a hexane solution (0.5 mg/ml) of internal standard $C_{17:0}$, were pippeted into a Pyrex tube with a PTFE lined screw cap and evaporated to dryness under nitrogen, then, 100 μl of plasma was added. Total lipids were extracted from plasma with 5 ml of dichloromethane–methanol (2:1, v/v) according to a variation of the method of Folch et al. [16], butylated hydroxytoluene (BHT), 5 μg/ml, was added to the solvents as antioxidant. The plasma sample (100 μl) was extracted twice. Plasma lipid extracts were evaporated to dryness under nitrogen and stored at −20°C.

Fatty acid methylation

Lipid extracts were methylated with methanolic NaOH and boron trifluoride–methanol according to the method of Morrison and Smith [21]. The reaction time in a boiling water bath at 100°C was 10 min and the reaction was performed in Pyrex tubes fitted with PTFE-lined screw caps. After cooling, the resulting methyl esters were extracted by *n*-hexane and transferred to vials containing anhydrous Na_2SO_4 as the dehydrating agent and analyzed by gas chromatography (GC).

2.5. Gas chromatography

Separation was performed on a Model 5890 A gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame ionization detector and split-splitless injector. A 60 m × 0.25 mm I.D. fused-silica capillary column SP-2380 (biscyanopropylphenylpolysiloxane) (Supelco) with a 0.20-μm film thickness was used under the following conditions: the initial oven temperature was 160°C with a hold for 2 min and then a rise of 4°C/min until reaching 240°C with a final hold for 3 min. The run time was 25 min. Helium was used as a carrier gas at 1 ml/min and nitrogen as a make-up gas at 32 ml/min. The flame ionization detector temperature was at 300°C and air and hydrogen flows were adjusted to give maximum detector response. The injector temperature was at 270°C. The split ratio was 1:30. The sample was concentrated to 50 μl in *n*-hexane prior to injection, an aliquot of 1 μl was injected.

Methyl esters were identified by comparison with retention times of standards and also by their log of relative retention time (log RRT) [25].

2.6. Quantification

The concentrations of fatty acids (FA) were calculated using the internal standard method as follows: FA concentration (μg/100 μl) = (peak area of FA/peak area of 17:0) × amount of 17:0 (μg) × RRF × (1/100 μl plasma). The RRFs (relative response factors) were determined using a series of working standards of each fatty acid and the amount of IS remained constant at 25 μg (see Standards solutions section): RRF = (amount of FA/amount of 17:0) × (peak area of 17:0/peak area of FA).

3. Results and discussion

Fig. 1 shows a typical chromatogram of the fatty acids methyl esters of a 100-μl plasma sample of newborn. Only 25 min were necessary for the simultaneous determination of all peaks studied. The fatty acids were identified by comparison of log RRT with reference mixtures (RM-3, PUFA 1 and PUFA 2) (Table 1).

The relative response factors (RRF) are shown in Table 2; calculation of the RRF on the basis of standard FAME was used to test linearity of the detector response, the repeatability of the apparatus and that of the integrator. PUFA 20 and 22 carbon atoms show an appreciable increase in the RRF. Thus, if these RRF values are not determined and applied, the errors in

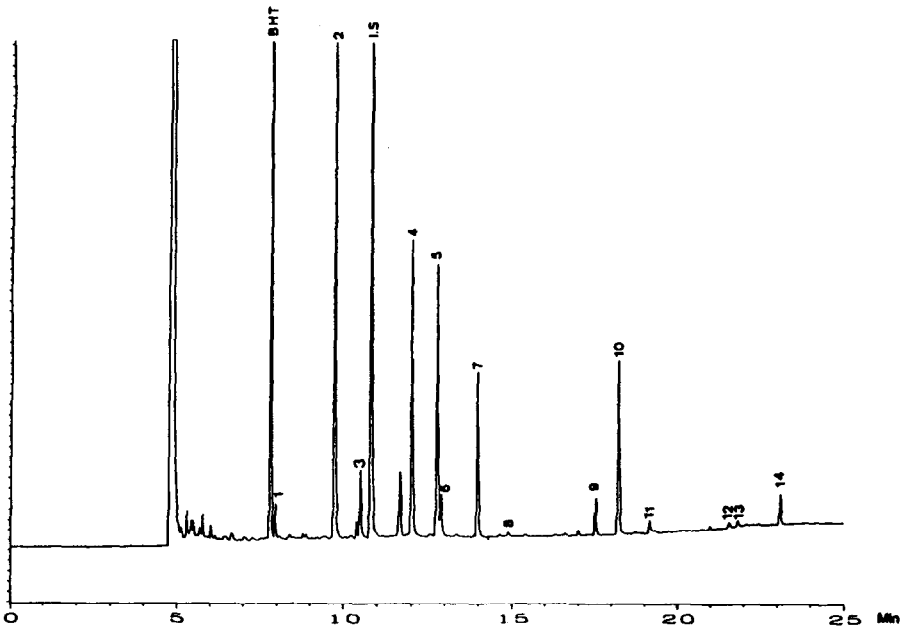

Fig. 1. GC profile of FAMEs from plasma of neonates including the antioxidant BHT, and 17:0 as internal standard (IS). Peaks: 1 = 14:0, 2 = 16:0, 3 = 16:1$\omega-7$, 4 = 18:0, 5 = 18:1$\omega-9$, 6 = 18:1$\omega-7$, 7 = 18:2$\omega-6$, 8 = 18:3$\omega-3$, 9 = 20:3$\omega-6$, 10 = 20:4$\omega-6$, 11 = 20:5$\omega-3$, 12 = 22:4$\omega-6$, 13 = 22:5$\omega-3$, 14 = 22:6$\omega-3$. At trace levels were: 18:3$\omega-3$, 20:5$\omega-3$, 22:4$\omega-6$, 22:5$\omega-3$.

Table 1
Logarithm of relative retention times of standards (PUFA 1, PUFA 2) and plasma sample.

Fatty acid	log RRT		
	PUFA 2	PUFA 1	Plasma sample
$C_{14:0}$	−0.4610	−0.4616	−0.4632
$C_{16:0}$	−0.2684	−0.2670	−0.2699
$C_{16:1\omega-7}$	−0.2038	−0.2038	−0.2047
$C_{18:0}$	−0.1053		−0.1036
$C_{18:1\omega-9}$	−0.0590	−0.0616	−0.0604
$C_{18:1\omega-7}$	−0.0547	−0.0550	−0.0534
$C_{18:2\omega-6}$	reference peak	reference peak	reference peak
$C_{18:3\omega-6}$	0.0404	–	–
$C_{18:3\omega-3}$	0.0620	–	–
$C_{20:3\omega-6}$	0.1393	–	0.1399
$C_{20:4\omega-6}$	0.1639	–	0.1647
$C_{20:5\omega-3}$	0.2087	0.2099	–
$C_{22:4\omega-6}$	0.2427	–	–
$C_{22:5\omega-3}$	0.2806	0.2802	–
$C_{22:6\omega-3}$	0.2966	0.2979	0.2996
$C_{20:1\omega-9}$	–	0.0640	–
$C_{18:4\omega-3}$	–	0.1009	–
$C_{22:1\omega-11}$	–	0.1502	–
$C_{22:1\omega-9}$	–	0.1542	–

Table 2
Relative response factors of FAMEs

Fatty acid	RRF (mean ± S.D.)	Coefficient of variation (%)
$C_{14:0}$	1.0095 ± 0.04	3.96
$C_{16:0}$	0.9916 ± 0.04	4.03
$C_{16:1\omega-7}$	1.1235 ± 0.04	3.56
$C_{18:0}$	0.9860 ± 0.03	3.04
$C_{18:1\omega-9}$	1.0040 ± 0.03	2.98
$C_{18:2\omega-6}$	1.0283 ± 0.06	5.83
$C_{20:3\omega-6}$	1.0939 ± 0.05	4.57
$C_{20:4\omega-6}$	1.1895 ± 0.06	5.04
$C_{22:6\omega-3}$	1.2115 ± 0.07	5.77

RRF values are given in relation to FA 17:0 (IS), and were calculated using the equation given in the text. ($n = 9$).

fatty acid analyses are considerable (over 15–20% for AA and DHA).

A measure of precision was obtained calculating standard deviation and coefficient of variation of ten repeated analyses, (Table 3). The inter-day precision is within the limits of acceptable variability in analytical methods proposed by Horwitz [26] for analyte concentrations in the order of μg/100 μl.

The standard addition method was followed to calculate the recovery rates of $C_{22:6\omega-3}$, $C_{20:4\omega-6}$ and $C_{18:1\omega-9}$ by spiking the samples with three different levels of standards. The mean recovery ($n = 9$) for DHA was 91.3%, for AA 93% and 94.3% for oleic acid.

BHT was added in small portion to the solvents, to prevent autooxidation during the analytical procedure. After BF_3–methanol reaction, BHT might interfere with the FAME at two sites [15]. The first peak is BHT (Fig. 1), which might completely overlap the peak of methyl myristate, however, in our case the column gives sufficient resolution to separate distinctly the peaks of BHT and methyl myristate. The second peak, is the derivative of BHT produced by permethylation during the BF_3–methanol reaction, which was described by Heckers et al. [27] as having a retention time identical to that of $C_{16:1\omega-7}$, moreover, the BHT was partly lost in the injector.

Table 3
Inter-day precision

Compound	Concentration (μg/100 μl)	Coefficient of variation (%)
$C_{16:0}$	22.47	4.60
$C_{18:0}$	15.73	3.02
$C_{18:1\omega-9}$	16.70	2.99
$C_{18:2\omega-6}$	11.03	5.17
$C_{20:4\omega-6}$	10.38	7.20
$C_{22:6\omega-3}$	1.78	7.48

Detection and quantification limits for flame ionization detection (FID) are shown in Table 4. These limits demonstrate the excellent sensitivity of the method proposed, according to the criteria of Kaiser [28], Kateman and Pijpers [29], Long and Winefordner [30] and Bonate [31]. For the determination of the detection and quantification limits, the signal-to-noise ratio was measured at 3 and 10, respectively. We have developed a sensitive, precise method for the determination of total fatty acids in plasma samples.

Table 5 shows the mean values of total fatty acids in plasma of neonates. Values are given in μg/100 μl plasma and in percentages. It is observed in the bibliography [7,11,32,33], that the % content of EFA ($C_{18:2\omega-6}$, $C_{18:3\omega-3}$) is higher in samples of human adult plasma than in plasma of neonates, however, the content of LC-PUFA (AA and DHA) is higher in neonate plasma; Nordoy and Dyerberg [33], give the first following percentages for plasma total fatty acids in adults in comparison to Farrel and Gutcher [7] who give the second percentages for plasma total fatty acids in neonates, that are in accordance

Table 4
Detection and quantification limits for FID

Fatty acid ($n = 9$)	Detection limit (ng)	Quantification limit (ng)
$C_{14:0}$	0.42	1.10
$C_{16:0}$	0.51	1.41
$C_{18:0}$	0.50	1.35
$C_{18:1\omega-9}$	0.50	1.38
$C_{18:2\omega-6}$	0.35	0.95
$C_{20:3\omega-6}$	0.11	0.30
$C_{20:4\omega-6}$	0.16	0.42
$C_{22:6\omega-3}$	0.08	0.22

Table 5
Fatty acid composition of neonate plasma

Fatty acid	Composition (mean ± S.D., $n = 10$)	
	%	$\mu g/100\ \mu l$
$C_{14:0}$	1.77 ± 0.15	1.01 ± 0.08
$C_{16:0}$	31.28 ± 0.90	24.03 ± 0.91
$C_{16:1\omega-7}$	4.30 ± 0.29	4.30 ± 0.26
$C_{18:0}$	16.90 ± 0.90	15.94 ± 0.95
$C_{18:1\omega-9}$	15.10 ± 0.83	16.91 ± 0.90
$C_{18:1\omega-7}$	3.26 ± 0.20	3.46 ± 0.21
$C_{18:2\omega-6}$	10.60 ± 0.66	11.35 ± 0.65
$C_{20:3\omega-6}$	2.50 ± 0.25	2.75 ± 0.30
$C_{20:4\omega-6}$	11.90 ± 1.07	10.77 ± 0.97
$C_{22:6\omega-3}$	2.01 ± 0.21	1.89 ± 0.20

with our values in neonates: EFA $18:2\omega-6$ (32.1% vs. 10.5%), $18:3\omega-3$ (0.5% vs. not detected); long-chain polyunsaturated fatty acids, AA (6.9% vs. 15.6%), DHA(0.8% vs. 1.01%).

Acknowledgements

The financial support from ORDESA, S.L. and Centro para el Desarrollo Tecnológico e Industrial (CDTI) is gratefully acknowledged. We also thank the Department of Pediatrics, Hospital of Granada for their helpful assistance in collecting the samples.

References

[1] R. Uauy, E. Birch and P. Peirano, *J. Pediatr.*, 120 (1992) s168.
[2] K.J. Clark, M. Makrides, M.A. Neumann and R.A. Gibson. *J. Pediatr.*, 120 (1992) s151.
[3] D.L. Ponder, S.M. Innis, J.D. Benson and J.S. Siegman, *Pediatr. Res.*, 32 (1992) 683.
[4] S.M. Innis, *J. Pediatr.*, 120 (1992) s78.
[5] N. Iritani and R. Narita, *Biochim. Biophys. Acta*, 793 (1984) 441.
[6] K.S. Bjerve and S. Fisher, *Am. J. Clin. Nutr.*, 46 (1987) 570.
[7] P.M. Farrel and G.R. Gutcher, *Am. J. Clin. Nutr.*, 48 (1988) 220.
[8] T.A.B. Sanders, A. Hinds and C.C. Pereira, *J. Int. Med.*, 225 (1989) 99.
[9] H.M. Liebich and C. Wirth, *J. Chromatogr.*, 572 (1991) 1.
[10] K.D. Foote, H.J. MacKinnon and S.M. Innis, *Am. J. Clin. Nutr.*, 54 (1991) 93.
[11] C. Galli, C. Agostini and C. Mosconi, *J. Pediatr.*, 119 (1991) 562.
[12] A. Lohninger, P. Preis and L. Linhart, *Anal. Biochem.*, 186 (1990) 243.
[13] W. Welz, W. Sattler, H-J. Leis and E. Halle, *J. Chromatogr.*, 526 (1990) 319.
[14] B.D. Beaumelle and H.J. Vial, *J. Chromatogr.*, 356 (1986) 187.
[15] S. Makdessi, J.L. Andrieu, A. Bacconin, J-C. Fugier, H. Herilier and G.J. Faucon, *J. Chromatogr.*, 339 (1985) 25.
[16] J. Folch, M. Lees and G.H.S. Stanley, *J. Biol. Chem.*, 226 (1957) 497.
[17] E.G. Bligh and W.J. Dyer, *Can. J. Biochem. Physiol.*, 37 (1959) 911.
[18] G. Lepage and C.C. Roy, *J. Lipid res.*, 29 (1988) 227.
[19] I.S. Chen, C.-S.J. Shen, A.J. Sheppard, *J. Assoc. Off. Anal. Chem.*, 58 (1981) 599.
[20] W.W. Christie (Editor), *Gas Chromatography and Lipids*, The Oily Press, Aye, 1989, p. 31.
[21] W.R. Morrison and L.M. Smith, *J. Lipid Res.*, 5 (1964) 600.
[22] C.R. Pace-Asciak, *J. Lipid Res.*, 30 (1989) 451.
[23] H.E. Carter, W.P. Norris, F.J. Glick, G.E. Phillips and R. Harris, *J. Biol. Chem.*, 170 (1947) 269.
[24] R.J. Holman, L. Smythe and S. Johnson, *Am. J. Clin. Nutr.*, 32 (1979) 2390.
[25] L.S. Ettre, *J. High Resolut. Chromatogr.*, 16 (1993) 258.
[26] W. Horwitz. *Anal. Chem.*, 58 (1986) 67A.
[27] H. Heckers, F.W. Melcher and V.J. Shloeder. *J. Chromatogr.*, 136 (1977) 311.
[28] H. Kaiser, *Anal. Chem.*, 42, (1970) 26A.
[29] G. Kateman and F.W. Pijpers, *Quality Control in Analytical Chemistry in Chemical Analysis, Vol. 60*, Wiley, New York, NY, 1981, pp. 73–83.
[30] G.L. Long and G.L. Winefordner, *Anal. Chem.*, 55 (1983) 712A.
[31] P.L. Bonate. *J. Chromatogr. Sci.*, 28 (1990) 559.
[32] K.S. Bjerve, S. Fisher, F. Wammer and T. Egeland, *Am. J. Clin. Nutr.*, 49 (1989) 290.
[33] A. Nordoy and J. Dyerberg, *J. Int. Med.*, 225 (1989) 1.