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Note

Quantitative assessment of γ-linolenic acid in human blood and plasma with capillary gas chromatography

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γ-Linolenic acid (18:3*n*6) is an important substance for the synthesis of precursors of prostanoids [1]. Elongation of the acyl chain of γ-linolenic acid results in the formation of dihomo-γ-linolenic acid (20:3*n*6). This compound serves either as precursor of the synthesis of prostanoids of the 1 series or as substrate for the formation of arachidonic acid (20:4*n*6) by the action of Δ^5-desaturase. Arachidonic acid is the precursor of prostanoids of the 2 series.

Prompted by the question whether disturbances in human prostanoid homeostasis associated with diseases, such as the premenstrual syndrome [2–4], are related to abnormal levels of γ-linolenic acid in blood or plasma, an assay technique to monitor quantitatively γ-linolenic acid in the various circulating lipid pools has been evaluated. This paper describes a sensitive technique for the assessment of γ-linolenic acid at the nanomolar level in 0.4 ml of blood or blood plasma. Circulating lipid pools were extracted with chloroform–methanol according to Folch et al. [5]. Purification and isolation of the various lipid

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classes, such as phospholipids, triacylglycerols, cholesteryl esters and fatty acids, were accomplished with one-dimensional thin-layer chromatography (TLC) [6]. Quantitation of the content of γ-linolenic acid in the various lipid classes was performed with capillary gas chromatography (GC).

EXPERIMENTAL

Blood was obtained from healthy female ($n=10$) and male ($n=5$) volunteers between 9 and 11 a.m. The volunteers had a normal diet and lifestyle. Their age varied from 20 to 40 years. All women were in the mid-folicular phase (day 7–10) of their cycle and did not use oral contraceptives. All had normal menstrual cycles and no symptoms of dysmenorrhea, premenstrual syndrome or similar complaints.

Blood was collected on EDTA. Of the freshly obtained blood, 0.4 ml was quickly frozen in liquid nitrogen and stored at −80°C for analysis. The remainder of the blood was immediately centrifuged at 3000 *g* for 2 min at 4°C; 0.4 ml of the blood plasma was also frozen in liquid nitrogen and stored at −80°C. For the analysis, 2.0 ml of ice-cold methanol were pipetted into the test-tubes with the frozen blood or plasma, and the contents of the test-tubes were thawed by gently shaking at room temperature. Thereafter, 4.0 ml of chloroform were added to the test-tubes and, after addition of heptadecanoic acid (8 nmol), triheptadecanoin (125 nmol) and cholesteryl heptadecanoate (150 nmol) as internal recovery standards, lipids were extracted as described previously [6]. Both methanol and chloroform contained 0.01% butylated hydroxytoluene (BHT) to prevent auto-oxidation of unsaturated lipids.

The lipids extracted were separated into cholesteryl esters, triacylglycerols, fatty acids and phospholipids by one-dimensional TLC as described previously [6]. The lipid spots were made visible by spraying the plate with Rhodamine 6G followed by spraying with a solution of fluorescein in methanol (0.01%) to intensify the colouring. Thereafter the spots were scraped from the plate, transferred to test-tubes, and methylated with 7% boron trifluoride–methanol according to Morrison and Smith [7]. The fatty acid moieties of the various lipid classes were methylated at 20°C for 15 min (fatty acids), at 100°C for 30 min (triacylglycerols) and at 100°C for 45 min (cholesteryl esters and phospholipids, respectively). The methyl esters were extracted from the methylating mixture with pentane. After evaporation of the pentane under a stream of nitrogen at 30°C, the methyl esters originating from the fatty acid, triacylglycerol and phospholipid fractions were dissolved in trimethylpentane, containing appropriate amounts of the methyl ester of pentadecanoic acid (9 nmol in the case of the assay of fatty acids and 85 nmol in the case of the assay of fatty acyl moieties derived from cholesteryl esters, phospholipids or triacylglycerols). The methyl esters of fatty acid moieties originating from the cholesteryl

ester fraction were rechromatographed on silica gel plates to remove the antioxidant BHT and cholesterol derivatives. The lipid spots were extracted from the silica gel powder with diethyl ether–methanol (50:1, v/v).

A 100-μl volume of a standard solution of triacylglycerols, cholesteryl esters and fatty acids (containing ca. 280, 170 and 80 nmol of total fatty acid moieties, respectively) was analysed in parallel with the biological samples to evaluate the overall recovery of the fatty acid moieties from different lipid sources.

Quantification of the fatty acyl moieties in the various lipid classes was achieved by the use of a standard solution of fatty acid methyl esters. The composition of this standard solution was (according to their chemical notation): 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3*n*3, 18:3*n*6, 20:0, 20:1, 20:4, 22:0, 22:1, 22:4, 22:6, 24:0 and 24:1. The amount of fatty acid methyl esters present in standard solution ranged from 5 to 8 nmol when the blood or plasma fatty acid fraction was analysed, and 50 to 80 nmol of fatty acid methyl esters were present when the fatty acyl moieties originating from the esterified lipid classes in blood or plasma were assayed. An example of a chromatogram of the fatty acid methyl ester standard mixture is shown in Fig. 1A.

The methyl ester mixtures were analysed by GC using a WCOT fused-silica capillary column coated with 0.2 μm CP-Sil 88 (50 m$\times$0.22 mm I.D., Chrompack International, Middelburg, The Netherlands). Routinely 4 μl of the methyl ester mixture were injected. Using the split injection technique, 1/30 of this volume was supplied to the column. The starting temperature of the column was 180°C for 2 min, and it was increased to 190°C at 5°C/min. The temperature was kept at 190°C for 5 min, then further increased to 225°C at 10°C/min. The helium flow-rate was 20 cm/s. Detection was achieved by flame ionization.

RESULTS AND DISCUSSION

Recovery

The overall recovery of the standards of triacylglycerols, cholesteryl esters and fatty acids ranged from 90 to 105% after correction for losses during the assay procedure with the use of the internal standards triheptadecanoin, cholesteryl heptadecanoate and heptadecanoic acid, respectively. The recovery of phospholipids (not corrected for losses) was ca. 85–90%.

Blood composition of lipids

The majority of total fatty acyl moieties were present in the phospholipid fraction in blood and plasma (ca. 3.3 and 3.2 m*M*, respectively). A considerable amount is present in the cholesteryl ester fraction and less in the triacylglycerol and fatty acid fractions of blood and plasma (ca. 1.5 and 2.8, 0.8 and 1.6 and 0.2 and 0.3 m*M*, respectively). No gender differences could be observed.

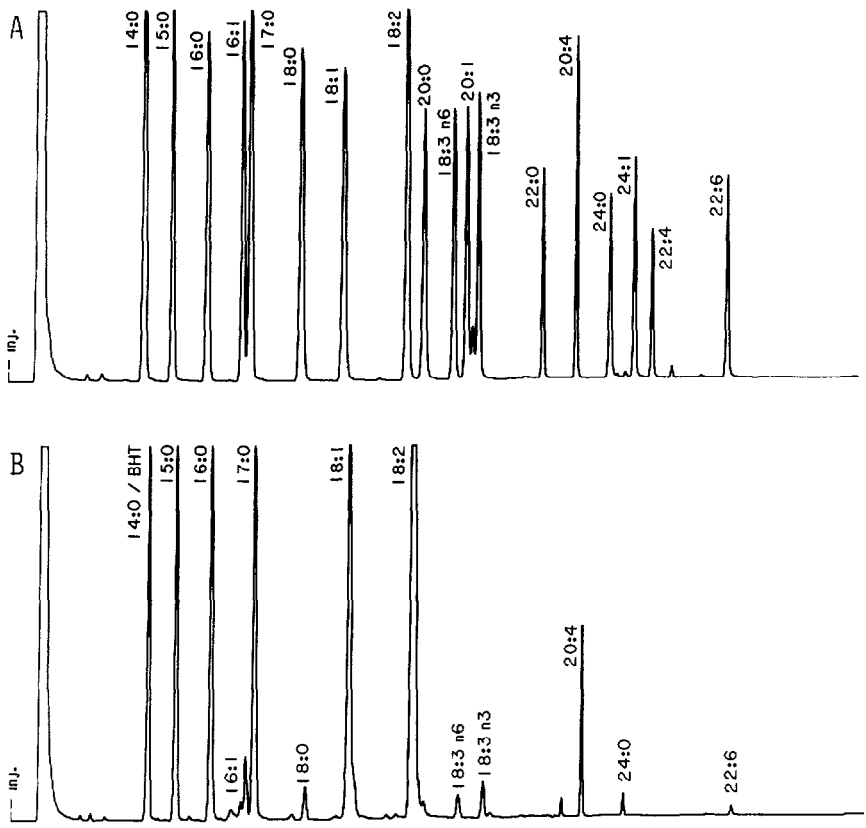

Fig. 1. (A) Typical chromatogram of methyl esters prepared from the fatty acid standard mixture. The fatty acyl moieties of the methyl esters are indicated by the usual chemical notation (see Experimental). The peak of 18.3*n*6 (γ-linolenic acid) corresponds to 25 pmol fatty acid methyl ester injected on column. The total run-time was 26 min. (B) Typical chromatogram showing the relative composition of the fatty acyl moieties in the cholesteryl ester fraction in the plasma of a male volunteer.

γ-Linolenic acid in blood lipid fractions

The total amount of γ-linolenic acid in blood and plasma is ca. 15 and 30 μM, respectively. In plasma, ca. 85% of all γ-linolenic acyl moieties are present in the cholesteryl ester fraction (Table I). A typical chromatogram showing the relative composition of fatty acyl moieties in the cholesteryl ester fraction in plasma of a male volunteer is presented in Fig. 1B. On the average ca. 1% of all fatty acyl moieties incorporated in the cholesteryl ester fraction was found to be γ-linolenic acid (Table II). This is about double the value reported earlier [8] and considerably lower than the content measured in circulating lipids of hyperlipidemic subjects [9], but in the same order of magnitude as measured in nuns fed on a normocaloric diet with 30% milk fats or peanut oil [10]. About

TABLE I

CONTENT OF γ-LINOLENIC ACID IN THE VARIOUS LIPID FRACTIONS IN BLOOD AND PLASMA OF VOLUNTEERS

N.D. indicates not detectable.

Lipid fraction	γ-Linolenic acid (mean ± S.D.) (μM)			
	Blood		Plasma	
	Female ($n=10$)	Male ($n=5$)	Female ($n=10$)	Male ($n=5$)
Fatty acids	N.D.	N.D.	N.D.	N.D.
Triacylglycerols	N.D.	N.D.	3.4 ± 3.8	4.8 ± 4.4
Cholesteryl esters	16.1 ± 9.9	12.8 ± 3.4	28.6 ± 12.2	23.8 ± 5.1
Phospholipids	N.D.	N.D.	1.5 ± 2.5	N.D.

TABLE II

RELATIVE FATTY ACID COMPOSITION OF THE VARIOUS LIPID FRACTIONS IN HUMAN PLASMA

Data obtained from female and male volunteers were combined. Values are expressed as a percentage of the total amount of fatty acids measured in the particular lipid fraction (mean and standard deviations, $n=15$). N.D. indicates not detectable.

Fatty acyl moieties	Lipid fraction			
	Fatty acids	Triacylglycerols	Cholesteryl esters	Phospholipids
14:0	3.79 ± 1.43	3.50 ± 1.92	1.01 ± 0.45	0.58 ± 0.29
16 0	28.19 ± 3.48	26.14 ± 4.18	11.85 ± 0.94	31.25 ± 2.08
16 1	2.86 ± 1.95	3.19 ± 1.12	2.49 ± 0.91	0.49 ± 0.23
18 0	12.95 ± 9.14	4.61 ± 1.26	0.91 ± 0.18	13.63 ± 1.16
18 1	33.87 ± 6.77	39.92 ± 4.55	18.47 ± 2.16	12.95 ± 4.84
18·2	16.11 ± 6.60	18.72 ± 6.20	56.50 ± 3.80	24.35 ± 7.03
18:3*n*3	0.96 ± 0.71	1.02 ± 0.46	0.60 ± 0.17	0.11 ± 0.16
18:3*n*6	N.D.	0.22 ± 0.23	0.97 ± 0.26	0.03 ± 0.06
20.0	0.24 ± 0.40	0.05 ± 0.11	0.05 ± 0.06	0.40 ± 0.14
20·1	0.47 ± 0.43	0.75 ± 0.38	0.09 ± 0.07	0.07 ± 0.12
20 4	0.33 ± 0.50	1.15 ± 0.12	6.13 ± 0.86	9.87 ± 1.30
22.0	0.04 ± 0.15	0.01 ± 0.03	0.43 ± 0.23	1.01 ± 0.17
22·4	N.D.	0.01 ± 0.05	N.D.	0.21 ± 0.29
22.5	N.D.	N.D.	N.D.	0.97 ± 0.93
22:6	0.25 ± 0.33	0.15 ± 0.21	0.49 ± 0.13	2.93 ± 0.95
24:0	0.05 ± 0.18	N.D.	0.02 ± 0.04	0.63 ± 0.13
24:1	N.D.	N.D.	N.D.	0.78 ± 0.28

12 and 3% was found in the triacylglycerol and phospholipid fractions, respectively. It should be noted that only in eight and three out of fifteen persons was γ-linolenic acid present in detectable amounts in plasma triacylglycerols and phospholipids, respectively. γ-Linolenic acid was not detectable in the fatty acid fraction (Table I). Gender differences were absent.

Since γ-linolenic acid is an intermediate in the biosynthetic pathways of prostanoids, either by serving as substrate for dihomo-γ-linolenic acid synthesis or for the formation of arachidonic acid [1], this GC technique may offer an analytical tool to elucidate disturbances in human prostanoid synthesis at the level of crucial precursors, such as γ-linolenic acid.

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