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Determination of fatty acid and triacylglycerol composition of human very-low-density lipoproteins

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ABSTRACT

The fatty acid composition of human very-low-density lipoproteins (VLDL) was studied in a population from western Andalusia with a diet in which the fat content came mainly from olive oil. The lipid composition of VLDL, including the fatty acid composition of the phospholipids and triacylglycerols, was examined by capillary gas chromatography. Twenty-five peaks were resolved, ranging in chain length from 14 to 24 carbon atoms, including geometric and positional isomers. The major fatty acids present in phospholipids were 16:0, 18:0, 18:1($n-9$) and 18:2($n-6$), and in triacylglycerols were 18:1($n-9$), 16:0 and 18:2($n-6$). The major triacylglycerol was POO, followed by PLO and OOO. MLP, PPS and LLL were absent. The presence of a large amount of OOO in this fraction demonstrates that the triacylglycerol composition of the VLDL depends on the type of diet consumed.

INTRODUCTION

Because lipids are insoluble in water, they must form molecular aggregates with proteins in order to be transported in the blood. These aggregates are the lipoproteins.

The very-low-density lipoproteins (VLDL) constitute a family of lipoproteins with a significant degree of variability in terms of their size, density and chemical composition. Generally, they have a diameter between 25 and 70 nm, a density between 0.95 and 1.006 g/ml and a molecular mass between $5 \cdot 10^6$ and $10 \cdot 10^6$. VLDL are formed by a non-polar core consisting mainly of cholesterol esters and triacylglycerols. After their synthesis in the liver, triglycerides predominate in the core [1], but as catabolism progresses and their diameter decreases, the principal components of this hydrophobic nucleus are the cholesterol esters [2]. The apolipoprotein of the VLDL present in the highest proportions is apo-B100, which represents from 30% to 50% of the total protein content of the particle. Significant amounts of apo-C and apo-E are also present, although the amounts depend on the metabolic stage of the particle [3].

The VLDL are formed in the liver from the cholesterol and fatty acids that enter it, as well as from those synthesized in this organ. Once in the plasma, the particle undergoes a process of maturation, consisting of a gain of apolipoprotein C proceeding from the HDL. By this means its structure becomes adapted for interaction with lipoproteinlipase (LPL) [4], the enzyme responsible for its degradation. While some of its

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phospholipids are degraded by LPL and other lipases, apo-B undergoes conformational changes that make the VLDL particle capable of trapping apo-E. Hydrolysis of its triacylglycerols, and transfer of non-esterified cholesterol and apolipoproteins from VLDL to HDL and of cholesterol esters from HDL to VLDL [5], leads to the transformation of the VLDL first into IDL (with apo E) and then, probably with the participation of hepatic lipase [6], by the loss of apo E, into LDL.

There is a wide variation in the prevalence of coronary heart disease among different populations around the world. In particular, the Mediterranean region shows a very low incidence of cardiovascular disease. Among the different hypotheses proposed to explain this, the most appealing has been that the typical Mediterranean diet contains a high proportion of mono-unsaturated fatty acids. This is supported by data from the Seven Countries Study [7]. The most important mediators in the relationships between dietary fat and coronary heart disease are blood lipids and lipoproteins [8].

Traditionally, determinations of free and total fatty acids have been performed using gas chromatography (GC) on polar stationary phases absorbed on supporting materials (packed columns). However, packed-column GC is limited in its separating power, owing to undesired absorption properties. In addition, it usually requires the injection of a relatively large amount of analyte [9,10]. The use of capillary GC with flame ionization has allowed us to study the composition of human VLDL more systematically. Thus we have been able to study the major and minor VLDL.

EXPERIMENTAL

Subjects and diets

The study was carried out on 22 healthy normolipemic (total cholesterol under 5.7 m*M*) young men, recruited among medical students at the Córdoba University. Their mean age was 23 ± 0.4 years, and their mean body mass index (BMI) at the beginning of the study was 24.3 ± 0.5. Before the study, all subjects underwent a complete medical examination and routine laboratory tests to ensure their normal status. None of them was taking medication. Dietary information, including alcohol consumption, was collected for one week. Information about physical activity was used to calculate their caloric requirements. Students did not receive monetary compensation for their participation in the study and were encouraged to maintain their regular physical activity and lifestyle habits. Written informed consent was obtained from each subject after a full explanation of the procedure had been given. The protocol was that approved by the Institutional Committee on Investigation in Humans.

The experimental period lasted 77 days. Caloric intake was adjusted as needed to maintain the initial BMI. Body weight was measured twice a week. All meals were prepared in the kitchen of the clinical hospital and consumed in the dining room.

The composition of the diets was calculated using the USDA food composition tables or the Spanish food composition tables for certain local foodstuffs. Table I shows the food composition.

TABLE I

MEAN DAILY INTAKE ON THE DIETS

Protein (% total energy intake)	
Calculated	15.0
Analysed	14.6
Fat (% total energy intake)	
Saturated	
Calculated	10.0
Analysed	8.5
Mono-unsaturated	
Calculated	20.0
Analysed	21.6
Polyunsaturated	
Calculated	8.0
Analysed	6.2
Cholesterol (mg/day)	
Calculated	285.0
Carbohydrate (% total energy intake)	
Calculated	47.0
Analysed	49.1

Blood sampling and biochemical determinations

Blood samples from fasting subjects were taken at the end of the experimental period by venipuncture into tubes containing EDTA (1 mg/ml). Tubes were immediately placed in ice, and plasma was separated by low-speed centrifugation within 1 h of sampling. Lipid apolipoprotein analysis was performed within 24 h.

Lipoprotein fractions were isolated from fresh plasma samples by sequential ultracentrifugation [11] using a Beckman Model L5 ultracentrifuge with a Type 5 rotor (Beckman, Fullerton, CA, USA). VLDL were separated by ultracentrifugation for 20 h at 105 000 *g* at a temperature of 4°C.

Standards

Fatty acid methyl ester (FAME) standards were obtained from Larodan Fine Chemicals (Malmo, Sweden). The internal standard solution was prepared by dissolving 200 mg of tricosanoic acid methyl ester (C23:0) in 100 ml of hexane. The calibration solutions were prepared by dissolving known amounts of FAME standards in hexane containing 2,6-di-*tert.*-butyl-*p*-cresol (butylated hydroxy toluene, BHT) obtained from Sigma (Poole, UK).

Apparatus

For sequential ultracentrifugation a type 50 rotor and an L5-50 ultracentrifuge (Beckman, Fullerton, CA, USA) were used.

The FAMEs thus obtained were eluted with hexane and analysed in a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector and an Omegawax 320 fused-silica capillary column (30 m × 0.32 mm I.D., 0.25 μm film), obtained from Supelco (Bellafonte, PA, USA).

Mass spectral data were obtained with an automated gas chromatographic–mass spectrometric (GC–MS) system, composed of an HP-5890 gas chromatograph interfaced directly to an AEI MS-30 VG/70 update mass spectrometer and a VG-11/250 data system (VG Analytical, Manchester, UK).

Triacylglycerol analysis was carried out using a Chrompack CP 9000 gas chromatograph (Chrompack International, Middleburg, Netherlands) fitted with a split injector and a flame ionization detector.

Plasma biochemical determinations

Levels of glucose, total and VLDL cholesterol, total and VLDL triglycerides and total and VLDL phospholipids were determined in venous blood from fasting subjects by standard enzymatic procedures (Boehringer Mannheim, Mannheim, Germany) with an Hitachi Model 705 automatic analyser, using Precilip and Precilip EL (Boehringer Mannheim) as quality controls.

Lipid extraction

Quantitative extraction of total lipids from VLDL was carried out following the method of Folch *et al.* [12].

Separation and quantification of lipids

Neutral lipids from the VLDL were separated by thin-layer chromatography (TLC) on plates of silica gel 60 (Kieselgel 60 F_{254}, Merck) using a solvent system of hexane–diethyl ether–acetic acid (80:20:1, v/v/v). After development, the solvent was allowed to evaporate. This system separates phospholipids, cholesterol, triacylglycerols and cholesterol esters in increasing order of R_F. Individual lipid zones were scraped from the TLC plates and eluted from the silica gel with either diethyl ether or chloroform–methanol, depending on the individual lipids.

The VLDL lipid classes were quantified following their separation on thin silica-coated quartz rods (Chromarod S) using an Iatroscan (Technical Marketing Associates, Mississanga, Ont., Canada) equipped with a flame ionization detector (hydrogen flow-rate, 175 ml/min; air flow-rate, 1850 ml/min), a scanner (scanning speed, 0.47 cm/s) and an integrator and recorder (sensitivity, 10 mV; chart speed, 0.42 cm/min). The Chromarods (type S9) were successively developed using hexane–diethyl ether–formic acid (90:10:3, v/v/v).

Preparation of FAMEs

Lipids were transmethylated according to the method of Morrison and Smith [13]. The lipid bands on plates of silica gel 60 were sprayed

lightly with a solution of 0.1% (w/v) BHT in methanol prior to detection. Neutral lipids were eluted with two 15-ml portions of chloroform–methanol (2:1, v/v). The solvent was evaporated in a stream of nitrogen, and 10 μg of tricosanoic acid (23:0), the internal standard, were added immediately, together with 200 μl of boron trifluoride–methanol complex. The sample was flushed with nitrogen, sealed in a vial fitted with a PTFE-lined cap, and heated at 120°C for 1 h. After the sample had cooled, the FAMEs were extracted with 500 μl of hexane.

Analysis of FAMEs

The sample (a 1-μl injection of test material was made) was injected into the gas chromatograph. Following injection, the oven temperature was maintained at 200°C for 10 min, then programmed to rise at 2°C/min to a final temperature of 230°C. The helium flow-rate was 2 ml/min, the column head-pressure 250 kPa, the splitting ratio 1:25, the detector and injector temperatures 275°C, and the detector auxiliary flow-rate 25 ml/min. For MS analysis, a Supelcowax-10 fused-silica column (60 m × 0.25 mm I.D.; film thickness, 0.25 μm) was used with helium as carrier gas. The column temperature was programmed from 130°C at 2°C/min to 200°C (to a total of 100 min) at 4°C/min. The injector temperature was 250°C. The MS conditions were as follows: electron-impact ionization, 70 eV; accelerating voltage, 4 kV; emission current, 100 μA; ion source temperature, 200°C. The data were processed with a VG 11/250 data system. Each FAME present in the extract was identified by comparison of its retention time and mass spectrum with those of authentic compounds.

Analysis of triacylglycerols

Samples (1 μl) of triacylglycerol in hexane (0.1%) were injected into the gas chromatograph equipped with a 25 mm × 0.25 mm I.D. 400 65 HT aluminium-clad silica capillary column coated with 65% phenylmethylsilicone (Quadrex, New Haven, CT, USA) and operated under the following conditions: injector and detector temperatures, 360°C; initial column temperature, 350°C maintained for 1 min and then increased at 0.5°C to 360°C at which temperature it was held for 6 min. Helium was used as the carrier gas at a column head-pressure of 130 kPa, and the splitting ratio was 30 ml/min. The triacylglycerols were identified by their elution times, because retention is affected not only by the number of carbon atoms but also by the number of double bonds [14].

RESULTS AND DISCUSSION

The study was carried out on normal subjects with no known metabolic disorders and who showed no hyperglycaemia at the time when the sample was taken. Table II shows the general data and the blood parameters of the subjects under study. The subjects had an average age of 23 ± 0.4 years and a mean BMI of 24.3 ± 0.5. No significant changes in BMI occurred during the experiment.

Total lipid composition

The total lipid composition of human VLDL is given in Table III. It can be seen that triacylglycerols accounted for 57.3% of the total lipids, esterified cholesterol for 18.4%, phospholipids for 15.2%, and free cholesterol for 9.1%. It should be noted that in these samples we found virtually no diglycerides, monoglycerides or free fatty acids.

TABLE II

GENERAL DATA, BLOOD PARAMETERS AND PLASMA LIPID LEVELS

Parameter	Mean ± S.D.
Age (years)	23.0 ± 0.4
BMI (Quetelet index)	24.3 ± 0.5
Glucose (m*M*)	4.7 ± 0.4
Total cholesterol (m*M*)	4.0 ± 0.8
Total triglycerides (m*M*)	0.8 ± 0.2
Total phospholipids (m*M*)	3.7 ± 0.5
Apo AI (mg/dl)	117.5 ± 18.8
Apo B (mg/dl)	91.3 ± 19.3
VLDL	
Cholesterol (m*M*)	0.3 ± 0.1
Triglycerides (m*M*)	0.5 ± 0.1
Phospholipids (m*M*)	0.4 ± 0.1

TABLE III

LIPID CLASS COMPOSITION OF HUMAN VLDL

Lipids	Composition (mean ± S.D.) (%)
Neutral lipid (TG)	57.3 ± 0.8
Phospholipid	15.2 ± 0.4
Free cholesterol	9.1 ± 0.2
Esterified cholesterol	18.4 ± 1.1

Fatty acid composition

The fatty acid profile of polar and neutral lipids of human VLDL can be seen in Table IV. The major fatty acids present in the phospholipids were palmitic (16:0), stearic (18:0), oleic (18:1 $n-9$) and linoleic (18:2 $n-6$) acids. Triacylglycerols contained the largest amount of oleic acid (18:1 $n-9$), accounting for 42.4% of the triacylglycerol fatty acids, followed by palmitic (16:0) and linoleic (18:2 $n-6$) acids. The fatty acid composition of the phospholipids was more diverse than that of the triacylglycerols. It can be seen that, in the phospholipids, eicosapentanoic (20:5 $n-3$) and eicosanoic (20:1 $n-11$) acids were identified. Although these fatty acids are typical of fish, they can sometimes be isolated from the polar fraction of mammalian tissue lipids. Their presence in the phospholipids studied here was undoubtedly due to the diet of the subjects, from which fish was not excluded at any point. It is similarly beyond doubt that these fatty acids present in the phospholipids of the VLDL were destined to form part of the cell membranes.

Arachidonic acid (20:4 $n-6$) deserves special attention, because it is the precursor of the prostaglandins and of the leucotrienes. It was detected in significant amounts (*ca.* 6%) in the phospholipids, whereas in the triacylglycerols it never represented more than 1%. The presence of this acid, together with that of docasehexanoic acid (22:6 $n-3$) is especially significant. Both of these were detectable only in the phospholipid fraction of the VLDL. If we examine the overall fatty acid composition, it is clear that the saturated fatty acids constitute the most abundant type in the phospholipids, making up almost 50% of the total, while the mono-unsaturated fatty acids never represent more than 20%. In the triacylglycerols, however, the most important group are the mono-unsaturated fatty acids, which constitute almost half of the total, the remaining fatty acids being saturated and poly-unsaturated fatty acids in equal parts. In the phospholipids, the most important group of fatty acids is that formed by the $n-6$ fatty acids, whereas in the triacylglycerols the $n-9$ fatty acids account for almost half of the fatty acids,

TABLE IV

FATTY ACID COMPOSITION OF PHOSPHOLIPIDS AND NEUTRAL LIPIDS OF HUMAN VLDL

Fatty acid	Composition (mean ± S.D.) (%, w/w)	
	Phospholipids	Triacylglycerol
14:0	0.71 ± 0.46	1.07 ± 0.02
16:0	30.35 ± 4.14	24.37 ± 1.43
16:1($n-7$)	0.14 ± 0.12	1.49 ± 0.02
16:1($n-9$)	0.35 ± 0.64	1.93 ± 0.37
16:4($n-3$)	0.88 ± 0.58	0.11 ± 0.01
18:0	17.04 ± 1.17	2.52 ± 0.36
18:1($n-9$)	16.28 ± 2.3	42.30 ± 0.22
18:1($n-7$)	1.81 ± 0.27	2.53 ± 0.21
18:1 *trans*	0.18 ± 0.15	–
18:2($n-6$)	14.84 ± 1.74	20.36 ± 3.22
18:3($n-3$)	0.83 ± 0.63	0.48 ± 0.10
18:4($n-3$)	0.28 ± 0.20	–
20:0	0.26 ± 0.11	0.16 ± 0.01
20:1($n-11$)	0.11 ± 0.02	–
20:1($n-9$)	0.21 ± 0.09	0.35 ± 0.02
20:2($n-6$)	0.15 ± 0.07	–
20:3($n-6$)	2.45 ± 0.74	–
20:4($n-6$)	5.82 ± 2.17	1.04 ± 0.01
20:4($n-3$)	2.1 ± 1.87	–
20:5($n-3$)	0.44 ± 0.32	–
22:1($n-11$)	0.35 ± 0.34	–
22:5($n-6$)	1.39 ± 1.64	–
22:5($n-3$)	0.43 ± 0.17	0.21 ± 0.04
22:6($n-3$)	2.57 ± 0.79	0.67 ± 0.03
24:1($n-9$)	0.04 ± 0.02	0.11 ± 0.02
Σ Saturated	48.36	28.12
Σ Mono-unsaturated	19.48	48.71
Σ Poly-unsaturated	32.18	22.87
Σ $n-3$	7.53	1.47
Σ $n-6$	24.65	21.4
Σ $n-9$	16.88	44.69
Σ $n-6$/Σ $n-3$	3.27	14.56
Σ $n-9$/Σ $n-3$	2.24	30.4

there being a low percentage of $n-3$. For this reason the ratios $n-6/n-3$ and $n-9/n-3$ are lower in the triacylglycerols than in the phospholipids.

In the present study, the lipid composition of the VLDL, including their fatty acid composition, was determined using capillary columns. This technique has not been used previously. Studies carried out on other groups of lipoproteins demonstrate that their fatty acid composition affects their physico-chemical properties.

Polar capillary GC provides new insights into the molecular association of the fatty chains in the relatively saturated animal tissue triacylglycerols, which are not readily resolved by $AgNO_3$ TLC or by GC on non-polar columns. Although not all molecular species were fully characterized, on the basis of their relative retention times the absence of species containing C_{16} and C_{18} saturated acids is prominent, as is that of the C_{18} mono- and di-unsaturated fatty acids [15].

Triacylglycerol composition

The most novel results of this work were obtained in the study of the triacylglycerols (TGs) (Table V). On the capillary column, TGs are separated according to chain length (CN separation). Moreover, each CN number is split up owing to the polarity differences in the TGs. Polarity increases with increasing degree of unsaturation in fatty acids (L > O > S) and with the total number of double bonds in the TGs (OOO > SOO > SOS > SSS). Accordingly, retention is highest for unsaturated fatty acids. Fig. 1 shows the analysis of TG VLDL on this capillary column.

Table V shows the average TG composition of VLDL. The major TG is POO (glycerol–palmitate–oleate–oleate), followed by PLO (glycerol–palmitate–linoleate–oleate) and OOO (glycerol–trioleate). These three together make up 67% of the total. The TG composition of the VLDL lipoproteins is not comparable with that found in other studies, because no studies dealing specifically with this fraction have been published. However, the present study can be compared with others performed on whole plasma and on populations consuming drastically different diets. For example, studies performed on Canadian populations [16,17] demonstrate that these populations differ substantially in the composition of their TGs. In addition, in the Andalusian population examined in the present study, the percentages of POO and PLO are significantly higher than those in the Canadian population [16]. Furthermore, the most important aspect of the comparison between these two studies is the highly significant difference in the concentration of triolein, which in the Andalusian population constituted 12.2% and in the

TABLE V

AVERAGE TRIACYLGLYCEROL COMPOSITION OF VLDL IN HUMANS

Triacylglycerol[a]	Composition (mean ± S.D.) (%, w/w)
MMP	0.65 ± 0.04
MMPo	0.23 ± 0.06
MPP	0.25 ± 0.10
MOM	0.20 ± 0.01
MLM	0.82 ± 0.01
PPP	0.52 ± 0.09
MOP	0.23 ± 0.06
MLPo	0.19 ± 0.02
POP	5.95 ± 1.7
PLP + PPoO	5.49 ± 1.9
PLPo – MLO	1.22 ± 0.2
MLL	0.27 ± 0.01
POS	0.99 ± 0.07
POO	32.83 ± 3.29
PLO	22.30 ± 1.32
PLL	4.63 ± 0.6
SOS	0.25 ± 0.04
SOO	0.86 ± 0.08
OOO	12.20 ± 1.6
SOL	1.09 ± 0.2
OLO	7.53 ± 0.7
OLL	1.23 ± 0.21
Σn	99.93

[a] Fatty acids:
M = myristic acid, tetradecenoic acid, $C_{14:0}$
P = palmitic acid, hexadecenoic acid, $C_{16:0}$
S = stearic acid, octadecenoic acid, $C_{18:0}$
Po = palmitoleic acid, hexadecenoic acid, $C_{16:1}$
O = oleic acid, *cis*-9-octadecenoic acid, $C_{18:1}$
L = linoleic acid, *cis*, *cis*-9, 12-octadecenoic acid $C_{18:2}$
Glycerides:
PPP = glycerol–tripalmitate
MLP = glycerol–myristate–linoleate–palmitate
PLO = glycerol–palmitate–linoleate–oleate

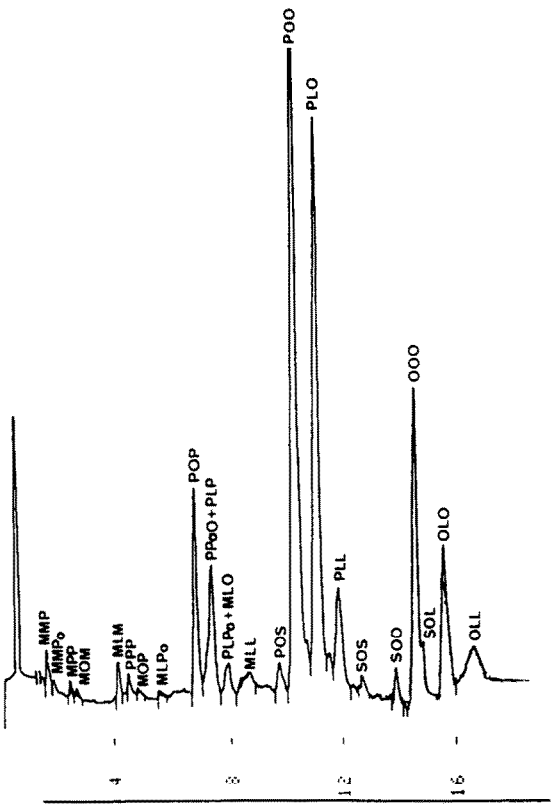

Fig. 1. Polar capillary GC of human VLDL triacylglycerols. The triacylglycerols are identified by the combination of the component fatty acids without regard to positional location. GC conditions and instrumentation as in Experimental. Peak identification as in Table V.

Canadian population 34%. This difference is undoubtedly due to the fact that in the population studied in the present work, the oil consumed in the diet is primarily olive oil, in which triolein is one of the major TGs. However, if we compare the TG composition determined in the present work with a previous study of a population from Andalusia [18], it can be seen that the TG composition of human tissue is similar, in terms of the major TGs present, to that of the VLDL lipoproteins found in the cited study. Examining the concentrations of the TGs present in lower amounts, however, and comparing them with the results from the previously-mentioned study [16] performed on adipose tissue, it can be seen that in the VLDL, MLP (glycerol–myristate–linoleate–palmitate), PPS and LLL are all absent. The absence of these triglycerides in the VLDL may be due to the fact that the VLDL primarily transport triglycerides of dietary origin, whereas the triglycerides that are absent are fundamentally of endogenous origin and thus are chiefly present in adipose tissue. Another important difference between the adipose tissue and the VLDL is that the latter contain more OLO (glycerol–oleate–linoleate–oleate) and PLL (glycerol–palmitate–dilinoleate), which reinforces the hypothesis previously made, namely that the triglyceride composition of the VLDL reflects the diet, whereas that of the adipose tissue is influenced not only by diet but also by triglycerides of endogenous origin.

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