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High-performance liquid chromatographic determination of plasma triglyceride type composition in a normal population of Barcelona

Relationship with age, sex and other plasma lipid parameters

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ABSTRACT

A coupled TLC–HPLC procedure is proposed for the separation and determination of plasma triglycerides. The method was tested by application to plasma samples corresponding to a normal population of Barcelona (Spain). Eighteen different triglyceride types were identified and their relative proportions were established, in order to give a "normal profile" for men and women. Sex-related differences ($p < 0.05$) were only found for dioleostearin and palmitodilinolein + linoleooleopalmitolein (LLP + LOPa). A correlation study showed that palmitodiolein and total cholesterol levels increase with age, whereas LLP–LOPa decreases in men and palmitolinoleoolein + palmitooleopalmitolein in women.

INTRODUCTION

Plasma lipid fractions are currently receiving a great deal of attention owing to their involvement in the development of several atherogenic diseases with a wide incidence. Some of the parameters studied, such as total plasma cholesterol levels and lipoprotein distribution, have been shown to be risk factors for coronary heart disease [1–5]. However, the involvement of plasma triglycerides (TGs) in these diseases seem to be less clear [6–8], although it would be interesting to determine the relationship between total plasma TG levels and other factors. Other studies have attempted to establish a relationship between total plasma TG levels and/or plasma fatty acid composition and some metabolic upsets [9–11] and diet [12–21]. The increasing popularity of the Mediterranean diet, which is associated with lower morbidity and mortality caused by these diseases, has also led to further studies [22–26]. Although there have been numerous studies dealing with the chromatographic (GC and HPLC) separation, identification and determination of triglycerides in food and biological lipids [27–30], hardly any detailed research has been carried out into the proportion of each individual TG type in plasma [31]. For this reason, the purpose of this study was to determine the profile of plasma TG type composition in normal subjects who live in Barcelona and who follow the Mediterranean dietary patterns. We also propose a chromato-

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graphic procedure for the separation and determination of individual plasma TG types suitable for routine analytical application.

EXPERIMENTAL

Samples

The study subjects were 27 adult men and 32 adult women from Barcelona with the following characteristics: (a) aged 18–61 years, mean (±S.D.) age 34 ± 10 years; (b) not overweight, with mean (±S.D.) mass and height 73 ± 5 kg and 175 ± 5 cm in men and 59 ± 8 kg and 162 ± 6 cm in women; (c) no subject was taking known medication (such as diuretics for hypertension, drug treatment for hyperlipaemia or insulin); (d) total plasma TG levels ranged from 40 to 170 mg/dl and cholesterol levels from 150 to 258 mg/dl; total plasma TGs and cholesterol were determined with an AutoAnalyzer (Technicon, Tarrytown, NY, USA); (e) other biochemical measurements (such as GOT, GPT and uric acid) were also normal.

Blood samples were obtained in 0.1 ml of EDTA (15%) from subjects in a fasting state (12 h overnight fast) by clean venipuncture, and plasma was separated at 4°C by centrifugation. The plasma was stored at −20°C until analysis.

Lipid extraction

A 1-ml volume of plasma was added to 9.5 ml of chloroform–methanol (2:1, v/v) following a modification of the method of Folch *et al.* [32]. The mixture was shaken for 30 min and centrifuged at 2000 *g* for 5 min. The organic phase was washed with 5 ml of distilled water and separated by centrifugation under the same conditions. It was then washed with 8 ml of saturated NaCl solution and centrifuged again. After filtration through anhydrous sodium sulphate, the lipid extract (clean chloroform fraction) was evaporated to dryness under nitrogen and the residue was dissolved in 100 μl of chloroform.

TLC isolation of triglyceride fraction

TGs were separated from other lipids by TLC on silica gel G60 (Merck, Darmstadt, Germany) with a hexane–diethyl ether–acetic acid (85:15:1, v/v/v) as solvent [33] and detected by spraying the plate with 2,7-dichlorofluorescein (0.2% solution in ethanol). The band corresponding to TGs was scraped from the plate, eluted with 10 ml of chloroform and stored at −20°C (in a conical tube) after drying under nitrogen. All chemicals used for sample preparation were of analytical-reagent grade (Merck).

HPLC determination of TG types

The TG analysis was performed on a Perkin-Elmer Series-10 high-performance liquid chromatograph coupled to a Perkin-Elmer (Norwalk, CT, USA) Model LC 25 refractive index (RI) detector and a Hewlett-Packard (Avondale, PA, USA) Model 3390A integrator. The HPLC column was 5-μm Spherisob ODS-2 (250 × 4 mm I.D.) (Tracer, Barcelona, Spain). The analysis was carried out at room temperature, using acetone–acetonitrile (64:36, v/v) as the mobile phase at a flow-rate of 1 ml/min [34]. Prior to analysis, the samples were dissolved in 50 μl of acetone and 10 μl of this solution were injected into the HPLC system. Acetone and acetonitrile were of HPLC grade (SDS, Peypin, France).

RESULTS AND DISCUSSION

Characteristics of the method

For identification of TG peaks we used a standard mixture of simple saturated TGs and vegetable oils whose TGs were well known, according to Goiffon and co-workers [35,36].

The fatty acid composition was determined in a pooled plasma in order to evaluate the average composition of plasma lipids from the subjects studied.

Quantification was carried out by normalization assuming that the detector response for all TG types was the same [37–39]. TGs were further quantified by grouping them by their equivalent carbon number (ECN).

A TG type is defined as a TG in which the three fatty acids are known, but not their position. The following code is used for fatty acids: L = linoleic (18:2), Ln = linolenic (18:3), O = oleic (18:1), P = palmitic (16:0), Pa = palmitoleic (16:1), M = myristic (14:0), S = stearic (18:0), La = lauric (12:0), where the number before the

colon indicates the acyl carbon number and that after the colon the number of double bonds.

The identification of the plasma TG types is shown in Table I, together with their respective experimental log α values. Some peaks could not be identified (NI), although they may correspond to one of the following groups of triglycerides: peak NI + NI with log $\alpha = -0.273 \pm 0.007$ might be MPaL–LaOL (48:3), MLM–LaPL (46:2), MOLa (44:1), MMM–LaMP (42:0); all included in the ECN-42. Other unidentified peaks in ECN-44 were log $\alpha = -0.173 \pm 0.002$, -0.156 ± 0.002 and -0.143 ± 0.002, which might be MOL–PLPa (50:2), MPL–MPaO–LaOO (48:2), MMO–LaPO (46:1) and MMP–LaPM (44:0) respectively.

TABLE I

IDENTIFICATION OF PLASMA TG TYPES IN THE SUBJECTS ANALYSED

Equivalent carbon number (ECN)	CN:n[a]	TG type	Log α[b]
42	54:6	LLL	−0.360 ± 0.004
42	54:6	LnLO	−0.337 ± 0.008
42	52:5	LnLP	−0.320 ± 0.015
42	–	NI + NI[c]	−0.273 ± 0.007
44	54:5	LLO	−0.241 ± 0.003
44	52:4	LLP	−0.208 ± 0.002
44	52:4	LOPa	−0.195 ± 0.002
44	–	NI[c]	−0.173 ± 0.002
44	–	NI[c]	−0.159 ± 0.002
44	–	NI[c]	−0.143 ± 0.002
46	54:4	LOO	−0.121 ± 0.002
46	54:3/50:2	PLO + POPa	−0.089 ± 0.002
46	50:2	PLP	−0.049 ± 0.002
46	50:2	MOO	−0.038 ± 0.002
46	–	NI[c]	−0.019 ± 0.002
48	54:3	OOO	0.000
48	52:2	POO	0.032 ± 0.002
48	50:1	POP	0.075 ± 0.003
48	48:0	PPP	0.121 ± 0.003
50	–	NI[c]	0.136 ± 0.003
50	54:2	SOO	0.146 ± 0.003
50	52:1	SOP	0.186 ± 0.004

[a] CN:n = Acyl carbon number : double bond number.
[b] α = Reduced retention time of triglycerides relatives to triolein. Values of log α are means ±S.D. ($n = 59$).
[c] NI = not identified.

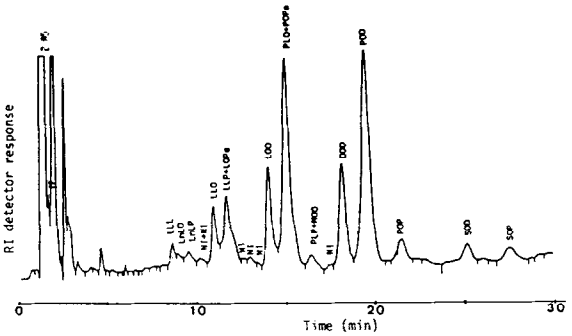

Fig. 1. HPLC of plasma triglycerides in a normal man.

Finally, the chromatographic peak with log $\alpha = -0.019$ may be assigned to MOS (50:1), MOP–PPPa (48:1) or MPP (46:0). Fig. 1 shows an HPLC profile of plasma triglycerides in a normal man.

In order to establish the sensitivity of the method, the detection limits were calculated by using a signal-to-noise ratio of 3:1 for the detection limit (DL) and of 10:1 for the quantification limit (QL). Results for triolein were DL = 0.82 μg and QL = 1.31 μg.

TG type composition of plasma

Details analysis of the TG type composition of plasma corresponding to this normolipaemic population is given in Fig. 2. Among the eighteen TG types identified, POO shows the highest proportion (26.5%). Other relevant groups were PLO + POPa with 24%, OOO with 11% and LLP + LOPa with 9.5%. This composition reveals a clear influence of the diet followed by the

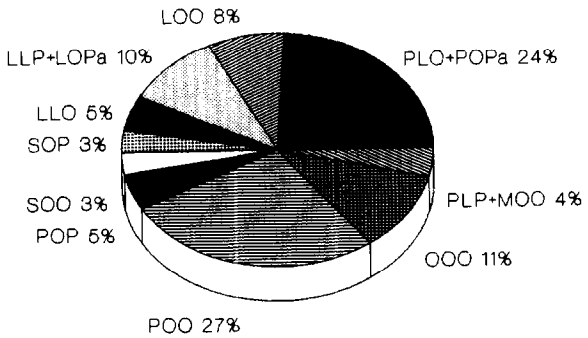

Fig. 2. Plasma TG type composition in the studied population ($n = 59$). Data given as relative percentage (mean ±S.D.).

population studied, which is characterized by high monounsaturated fatty acid levels (olive oil) and a lower intake of animal fats.

Influence of sex in plasma TG types, total TGs and cholesterol levels

The differences in the TG type proportions, ECN proportions and total triglyceride and cholesterol levels between men and women are shown in Table II. Applying Student's *t*-test we found statistically significant differences for ECN-50 ($p < 0.05$) and SOO ($p < 0.05$), which is included in this ECN. Moreover, the differences between men and women were statistically significant for the critical pair LLP + LOPa ($p < 0.05$). In both instances the values are higher for men. However, despite the tendency for OOO and PLO + POPa to show differences between men and women, these were not statistically significant ($p = 0.050$ and 0.057, respectively). It should be noted that the women had a significantly lower mean total TG level than the men ($p < 0.001$), which agrees with data reported by others [9].

We also studied the correlations between the different TG types in each sex group (Tables III and IV). In these instances, we only consider correlations for which $p < 0.001$.

Correlations with age and total cholesterol

We also studied correlations between total cholesterol, total TGs, TG types and ECN

TABLE II

COMPARATIVE STUDY OF THE TG TYPE PROPORTIONS, ECN PROPORTIONS AND TOTAL TGs AND CHOLESTEROL LEVELS BETWEEN MEN AND WOMEN

Data given as a percentage of total TG types. Values are means ±S.D.

Variable	Men ($n = 27$)	Women ($n = 32$)
ECN-42	5.74 ± 2.13	5.12 ± 1.81
ECN-44	15.69 ± 4.58	14.64 ± 4.07
ECN-46	33.95 ± 3.45	33.15 ± 4.02
ECN-48	38.91 ± 7.39	40.42 ± 6.22
ECN-50	5.65 ± 1.78	6.64 ± 2.43[a]
LLO	4.53 ± 1.87	4.70 ± 1.85
LLP + LOPa	10.32 ± 3.11	8.95 ± 2.63[a]
LOO	8.16 ± 2.30	8.63 ± 2.11
PLO + POPa	24.72 ± 3.01	23.34 ± 3.47
PLP + MOO	4.07 ± 2.00	4.15 ± 1.86
OOO	10.00 ± 3.86	11.74 ± 4.09
POO	27.04 ± 5.19	26.15 ± 4.25
POP	5.14 ± 2.14	5.32 ± 1.91
SOO	3.13 ± 1.06	3.78 ± 1.19[a]
SOP	2.84 ± 1.19	3.16 ± 2.05
Total TGs (mg/dl)	95.22 ± 33.62	65.87 ± 21.02[b]
Total cholesterol (mg/dl)	199.29 ± 26.01	190.06 ± 29.54

[a] Significantly different between men and women, $p < 0.05$ by Student's *t*-test.
[b] Significantly different between men and women, $p < 0.001$ by Student's *t*-test.

TABLE III

CORRELATIONS BETWEEN TG TYPES IN MEN ($n = 27$)

Variable	r[a], $p < 0.001$ (d.f. = 25 $r_t = 0.5974$)[b]
LLO/LLP + LOPa	0.62121
LLO/POO	−0.63628
LLP + LOPa/PLO + POPa	0.62286
LLP + LOPa/POO	−0.88157
LLP + LOPa/SOO	−0.71763
PLO + POPa/OOO	−0.80107
PLP + MOO/OOO	−0.69767
PLP + MOO/POP	0.63858
POO/SOO	0.63292

[a] r = Experimental correlation coefficient.
[b] d.f. = Degrees of freedom, r_t = theoretical correlation coefficient.

TABLE IV

CORRELATIONS BETWEEN TG TYPES IN WOMEN ($n = 32$)

Variable	r[a], $p < 0.001$ (d.f. = 30 $r_t = 0.5541$)[b]
LLO/LLP + LOPa	0.68683
LLO/POO	−0.56684
LLP + LOPa/POO	−0.58328
LOO/POP	−0.55438
PLO + POPa/OOO	−0.79697
PLP + MOO/POP	0.68875

[a,b] See Table III.

TABLE V

CORRELATIONS WITH AGE IN MEN

Variable	r (d.f. = 25)[a]
ECN-44	−0.52508[b]
LLP + LOPa	−0.58892[b]
ECN-48	0.59268[b]
POO	0.6022[c]
Total cholesterol	0.40479[d]

[a] See Table III.
[b] Statistical significance: $p < 0.01$, theoretical correlation coefficient $r_t = 0.4868$.
[c] Statistical significance: $p < 0.001$, $r_t = 0.5974$.
[d] Statistical significance: $p < 0.05$, $r_t = 0.3809$.

fractions with age for men and women separately, for those that showed statistical significance.

In men (Table V), there is a increase in POO percentage with age ($p < 0.001$), and also ECN-48 ($p < 0.01$), which includes POO, and a decrease in LLP + LOPa ($p < 0.01$) and ECN-44 ($p < 0.01$), which includes LLP + LOPa. The results also show an increase in total plasma cholesterol level with age ($p < 0.05$).

In women (Table VI) there is an increase in POO and ECN-48 with age ($p < 0.05$), which is less significant than in men. A decrease is also observed in PLO + POPa ($p < 0.05$), which is not observed in men. The total cholesterol level also increases with age ($p < 0.05$) in women. It seems that levels of more unsaturated TG types fall slightly with age, while the total plasma cholesterol increases.

TABLE VI

CORRELATIONS WITH AGE IN WOMEN

Variable	r (d.f. = 30)[a]
PLO + POPa	−0.37083[b]
ECN-48	0.35058[b]
POO	0.43363[b]
Total cholesterol	0.48288[c]

[a] See Table III.
[b] Statistical significance: $p < 0.05$, theoretical correlation coefficient $r_t = 0.3493$.
[c] Statistical significance: $p < 0.01$, $r_t = 0.4487$.

TABLE VII

CORRELATIONS OF TG TYPES AND TOTAL TGs WITH TOTAL CHOLESTEROL IN PLASMA

Variable	r[a]
Men (d.f. = 25)[a]	
ECN-44	−0.43879[b]
LLO	0.46311[b]
LLP + LOPa	0.42124[b]
Total TGs	0.40350[b]
Women (d.f. = 30)[a]	
POO	0.37719[c]
Total TGs	0.49610[d]

[a] See Table III.
[b] Statistical significance for men: $p < 0.05$, theoretical correlation coefficient $r_t = 0.3809$.
[c] Statistical significance for women: $p < 0.05$, $r_t = 0.3493$.
[d] Statistical significance for women: $p < 0.01$, $r_t = 0.4487$.

Table VII shows the correlations found for ECN fractions, TG types and total TGs with cholesterol levels for both sexes. This shows that the percentage of POO increases in women, as does total plasma TG in both men and women, and LLO and LLP + LOPa decrease in men with increase in the total cholesterol level. All this suggests that in plasma there is a replacement of unsaturated TGs by more saturated TGs with increase in cholesterol.

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