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# GAS CHROMATOGRAPHIC EVALUATION OF PLASMA TRIGLYCERIDE COMPOSITION IN HYPERLIPIDEMIA

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## SUMMARY

The molecular distribution of human plasma triglycerides was studied by gas—liquid chromatography. Triglycerides of the total plasma lipids are separated into fractions according to molecular weight. Individual fractions consist of molecules containing constant numbers of carbon atoms in the acyl chains. The carbon number serves to designate the fraction. In normal plasma it is possible to separate plasma triglycerides into four main fractions: $C_{48}$, $C_{50}$, $C_{52}$, and $C_{54}$.

The reference group consists of 199 apparently healthy subjects (93 men, 106 women) and is stratified according to age. Triglyceride values were adjusted to age and sex. Parameters of triglyceride fractions $C_{48}$—$C_{56}$ were calculated after arcsin transformation of percentage values.

The group of hypertriglyceridemic subjects totals 113 (56 men, 57 women). Their adjusted triglyceride values exceed the 95 percentile of the reference group values. Analysis of the triglyceride fractions gives the following results:

(1) In more than a quarter of the hypertriglyceridemic patients a different molecular distribution was demonstrated. Mostly it was fraction $C_{56}$ that was increased in proportion.

(2) There seems not to be an anomaly of triglyceride molecular distribution associated with any type of hyperlipoproteinemia. Nevertheless, in a family with type III hyperlipoproteinemia we observed a constant increase of $C_{56}$ and higher fractions.

Fraction $C_{56}$ and higher molecular weight fractions contain one or more twenty-carbon or longer fatty acids chains. We conclude that hypertriglyceridemia in about one quarter of cases is associated with a disorder of the metabolism of triglyceride molecules containing $C_{20}$ or longer fatty acid chains.

## INTRODUCTION

Hypertriglyceridemia is known as an important risk factor of coronary heart disease even if the causal relationship has not yet been proved [1,2]. In all

previous studies on hyperlipidemia only total plasma triglyceride levels have been assessed. This is the consequence of methodological difficulties in analysis and quantitation of triglyceride molecular species in large-scale clinical studies.

In 1967 Kuksis and collaborators proposed gas chromatographic (GC) analysis of the plasma lipid profile [3]. This was a good step forward because the method affords a better view of plasma triglyceride composition with a relatively simple technique. In principle Kuksis' method separates triglycerides according to molecular weight. Individual triglyceride fractions are identified by their "carbon number". The carbon number is the sum of all the carbon atoms of the fatty acyl chains attached to glycerol in the individual molecule.

In our laboratory we have increased possibilities for the quantitation of triglyceride fractions [4]. At present we have several thousands of analyses for evaluating the technique for clinical purposes. This present paper reports on the comparison of hypertriglyceridemia and the reference group.

## METHODS

### *Reference group*

The reference group is composed of 199 persons (93 men, 106 women) selected according to the following criteria. Exclusion of all subjects with (1) weight index exceeding the value of 1.1; (2) arterial hypertension; (3) hyperglycemia; (4) hyperuricemia; (5) functional disorder of the thyroid; (6) xantomatus manifestations on the skin; (7) corneal arcus senilis.

We excluded all subjects with a history of manifest signs of illness, all subjects with a history of (a) myocardial infarction; (b) hysterectomy; (c) cholecystectomy; (d) thyroid surgery; (e) familial hyperlipoproteinemia; (f) viral hepatitis within the last five years.

Further criteria for exclusion were (1) hormonal contraception in women; (2) regular alcohol consumption; (3) heavy smoking habits (more than 20 cigarettes a day); (4) special dietary habits.

### *Group of hyperlipidemic patients*

The group of hyperlipidemic patients consisted of 113 persons (56 men, 57 women) with a plasma triglyceride value, adjusted for age and sex, of more than 144 mg/dl. This critical value is significant on a 5% level. No difference was made for lipoprotein type hyperlipidemia according to Fredrickson's classification, but the majority of patients were classified as combined hyperlipidemia. No exclusion was made for patients with secondary hyperlipidemia associated with obesity, alcoholism and chemical diabetes, but in the majority of patients the underlying primary disease factor of hyperlipidemia was not evident.

### *Chemicals*

Acetone, methanol and chloroform, all of analytical grade, were obtained from Lachema (Brno, Czechoslovakia). Isooctane analytical grade was provided by International Enzymes (Windsor, Great Britain). Cholesteryl butyrate was obtained from Applied Science Labs. (State College, Pa., U.S.A.). Florisil 60—100 mesh was supplied by Fluka (Buchs, Switzerland). The purity of all solvents was checked by GC.

*Chemical analysis*

Blood samples were collected from fasting subjects into test-tubes containing 1 mg EDTA per ml of blood and centrifuged within 1 h at 4° (10 min at 1000 *g*). Extraction and isolation of neutral lipids from plasma was made in a one-step procedure. The principle of this method, which will be described in another paper, is the sorption of polar lipids on Florisil simultaneously with extraction. Aliquots of the extracts were taken into 10-ml flasks, dried under nitrogen and stored at -20°. Samples were stable for 3 months. Just before GC analysis the samples were dissolved in the internal standard solution of cholesteryl butyrate (200 ng/$\mu$l) in the mixture isooctane—chloroform (80:20, v/v). Analyses were performed on a Perkin-Elmer F 30 gas chromatograph equipped with autosampler PS 4950 or on a Perkin-Elmer F 17 gas chromatograph with manual injection of the samples. Both gas chromatographs were equipped with a dual column system with FID detection. Glass columns (0.5 m × 2.0 mm I.D.) were packed with 1% OV-1 on Gas-Chrom Q 100—120 mesh. The injector was thermostatted at 300°; the oven temperature was programmed as follows: initial temperature 180°, programme rate 5°/min, final temperature 350°. Helium flow-rate was 100 ml/min. The gas chromatographs were combined with a Perkin-Elmer M-2 Calculating Integrator or a Perkin-Elmer Sigma 10 Laboratory Data system. Data were processed by a Computer MDS-2400 using a special programme [4].

*Precision and accuracy control*

Precision of the results in time was controlled by means of quality control (K card). Coefficients of variation for individual fractions of the lipid profile were as follows: 1.21 for free cholesterol; 1.13 for cholesterol esters; 5.39, 1.23, 1.14 and 2.14 for fractions of cholesteryl esters of carbon number 41, 43, 45 and 47, respectively; 1.11 for total cholesterol; 1.99 for triglycerides; 4.26, 1.96, 2.13, 3.23 and 4.41 for fractions of triglycerides of carbon number 48, 50, 52, 54 and 56, respectively.

For the quality control we used a model mixture of pure compounds, the composition of which was similar to that of plasma. Results of the total cholesterol and triglyceride determination in plasma were compared with those measured enzymatically using commercial sets of chemicals from Boehringer (Mannheim, G.F.R.) Cat. Nos. 15732 and 138355. The two methods were in good agreement: the correlation coefficients of 30 duplicate measurements were 0.986 and 0.998 for total cholesterol and triglycerides, respectively. Detailed results of the quality control will be published separately.

*Processing and analysis of data*

Regression analysis was used to calculate adjusted log triglyceride confidence limits. For classification of triglyceridemia the antilog critical value was calculated as 144 mg/dl, which represents the 95 percentile of the reference group.

Percentage values of triglyceride fractions were transformed by arcsin function before computing means and standard deviations. The distribution of transformed values was approximately normal.

Data processing was performed on a programmable calculator HP 97 (Hewlett-Packard) using commercial and our own programmes.

## RESULTS

The values for total plasma triglyceride in the reference group study are summarized in Fig. 1. The curves represent the relationship of triglyceride to age in men and women. Regression functions are the exponentials and show the change of triglyceride level with increasing age as well as the difference between the sexes.

Contrary results are evident concerning the molecular composition of plasma triglyceride. The percentage molecular composition of triglycerides in the same group of subjects is shown in Table I. The uniformity of the molecular distribution is evident for all age groups in men as well as in women and no difference between the sexes could be observed.

The fraction $C_{56}$, which contains two eighteen-carbon chains and one twenty-carbon fatty acyl chain has a content of about 1% on average. The value of 6.28% is considered as elevated at the 5% level of significance. Table II summarizes the comparison of the molecular composition of plasma triglycerides in the hypertriglyceridemia and reference groups. The increase of triglyceride fraction $C_{56}$ was found in 28 of the total number of 111 patients. In the 199 subjects of the reference group the same fraction was found in 9 individuals only. The fourfold table test is significant for the difference. The increased frequency of the augmented cholesteryl fraction with carbon number 47 can also be seen.

We were interested to see if there was some relationship between the anomaly and lipoprotein type according to Fredrickson's classification [5]. Table III shows lipoprotein type in 28 patients with heterogeneous hypertriglyceridemia; all lipoprotein types of hyperlipidemia are involved. The difference in frequencies reflects more the incidence of individual types in the population than the preference of a particular lipoprotein type for heterogeneous hyperglyceridemia.

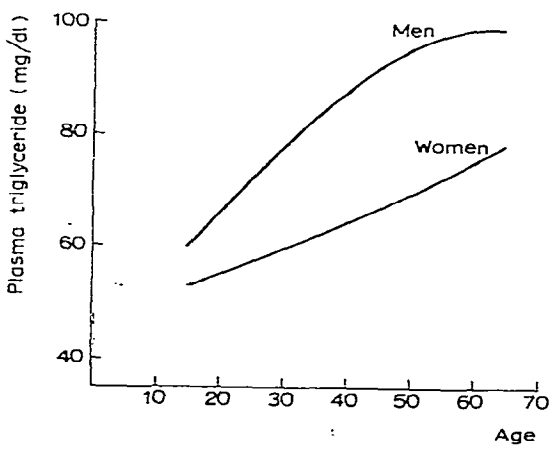


Fig. 1. Fitted regression lines for plasma total glyceride with age in men and women. Regression equations: for men $Y = 40.6062 \exp(0.027882x - 0.000219x^2)$; for women $Y = 47.4108 \exp(0.007576x)$. Coefficients are calculated from log values and re-transformed to arithmetic values.

TABLE I

PLASMA TRIGLYCERIDE COMPOSITION IN RELATION TO AGE IN MEN AND WOMEN

| Age (years) | *n* | Triglyceride composition (weight percent) | | | | | |
|---|---|---|---|---|---|---|---|
| | | $C_{48}$ | $C_{50}$ | $C_{52}$ | $C_{54}$ | $C_{56}$ | $C_{58}$ |
| *Men* | | | | | | | |
| 15—19 | 17 | 4.8* | 17.6 | 48.2 | 28.3 | 1.1 | 0 |
| 20—29 | 16 | 6.3 | 20.3 | 48.5 | 24.7 | 0.2 | 0 |
| 30—39 | 16 | 5.4 | 19.1 | 49.6 | 24.6 | 1.2 | 0 |
| 40—49 | 17 | 5.5 | 19.5 | 48.8 | 25.1 | 1.1 | 0 |
| 50—59 | 15 | 5.9 | 19.5 | 48.6 | 25.8 | 0.2 | 0 |
| 60 and over | 12 | 5.9 | 19.1 | 49.2 | 23.8 | 1.9 | 0 |
| *Women* | | | | | | | |
| 15—19 | 20 | 5.3 | 18.2 | 47.0 | 29.5 | 0 | 0 |
| 20—29 | 20 | 6.6 | 20.4 | 49.6 | 23.4 | 0 | 0 |
| 30—39 | 19 | 5.9 | 19.6 | 47.3 | 24.3 | 2.9 | 0 |
| 40—49 | 18 | 5.1 | 17.8 | 49.1 | 26.8 | 1.2 | 0 |
| 50—59 | 19 | 6.5 | 19.3 | 50.6 | 23.3 | 0.4 | 0 |
| 60 and over | 10 | 6.4 | 17.7 | 48.3 | 24.9 | 1.9 | 0.8 |

*The values are the mean for each age group.

TABLE II

FREQUENCY OF PLASMA TRIGLYCERIDE FRACTION $C_{56}$ AND CHOLESTERYL ESTER FRACTION $C_{47}$ IN HYPERLIPIDEMIA AND IN REFERENCE GROUP

| Group | *n* | Frequency of augmented fraction | |
|---|---|---|---|
| | | Triglyceride $C_{56}$ | Cholesteryl ester $C_{47}$ |
| Hyperlipidemia | 113 | 28 | 23 |
| Reference | 199 | 9 | 5 |
| Fourfold table test (*P*) | | <0.001 | <0.001 |

TABLE III

LIPOPROTEIN TYPE IN HETEROGENEOUS HYPERTRIGLYCERIDEMIA

| Group | *n* | Lipoprotein type | | |
|---|---|---|---|---|
| | | IIB | III | IV |
| Heterogeneous hypertriglyceridemia | 28 | 4 | 3 | 21 |

In Table IV the incidence of the molecular composition anomaly in a family in which genetic factors for hyperlipidemia predominate is demonstrated. The index patient, aged 48, female, reveals a classical picture of type III hyperlipoproteinemia with all clinical and laboratory signs present. The total plasma triglyceride is 1068 mg/dl and its molecular composition is quite abnormal. The same is true for her mother, who is 78 years and has a plasma triglyceride level of 650 mg/dl. In this patient the fractions with two twenty-carbon acyl chains (carbon number 58) and with three twenty-carbon acyl chains (carbon number 60) are augmented. This finding was constant in both patients over long-term observation with repeated triglyceride analyses. One sister of the index patient is normal; another sister as well as the children show a mild form of type IV hypertriglyceridemia with normal molecular composition.

TABLE IV

PLASMA TRIGLYCERIDE COMPOSITION IN A FAMILY RUNNING TYPE III HYPERLIPOPROTEINEMIA

| Patient | Position in family | Age (years) | Triglyceride (mg/dl) | Lipoprotein type | Weight percent $C_{56}$ | $C_{58}$ | $C_{60}$ |
|---|---|---|---|---|---|---|---|
| J.H. 609 | Father | 74 | 163 | N | 0 | 0 | 0 |
| M.H. 765 | Mother | 78 | 650 | III | 5.7 | 3.7 | 1.4 |
| M.P. 658 | Index patient | 48 | 1068 | III | 9.8 | 7.3 | 0 |
| J.P. 766 | Husband | 47 | 228 | IV | 3.5 | 0 | 0 |
| V.P. 607 | Sister | 38 | 172 | IV | 3.5 | 0 | 0 |
| J.B. 606 | Sister | 50 | 140 | N | 5.0 | 0 | 0 |
| J.S. 611 | Daughter | 24 | 179 | IV | 0 | 0 | 0 |
| M.P. 604 | Daughter | 22 | 160 | IV | 0 | 0 | 0 |
| J.P. 605 | Son | 25 | 287 | IV | 0 | 0 | 0 |

Interesting is the effect of treatment of the index patient which is shown in Table V. Before the treatment started the patient was highly hypertriglyceridemic with abnormal molecular composition of triglyceride. The treatment was introduced with diet and Clofibrate. After 5 months' therapy a dramatic fall of total plasma triglyceride was registered. The content of triglyceride fraction $C_{56}$ was on the border of significance.

TABLE V

PLASMA TRIGLYCERIDE COMPOSITION IN TYPE III HYPERLIPOPROTEINEMIA: EFFECT OF TREATMENT

| Conditions | Triglyceride (mg/dl) | Weight percent $C_{56}$ | $C_{58}$ |
|---|---|---|---|
| Before treatment | 1079 | 9.8 | 7.3 |
| After treatment (diet, Clofibrate) for 5 months | 92 | 5.4 | 0 |

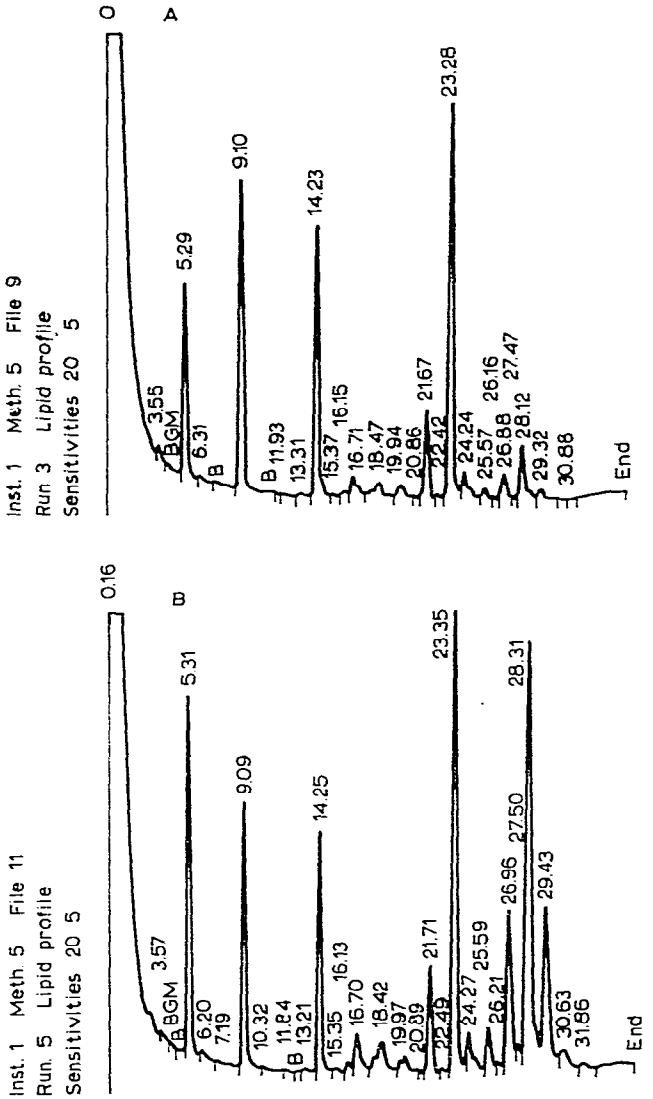


Fig. 2. Gas chromatograms of normal (A) and hyperlipidemic (B) plasma neutral lipid. Individual compounds are identified according to their elution times (min): (A): 5.29 = cholesterol; 9.10 = cholesteryl butyrate (internal standard); 14.23 = cholesteryl benzoate (standard for laboratory control); 15.37, 16.15, 16.71, 18.47 = diglycerides and decomposition products of phospholipids; 19.94, 20.86, 21.67, 22.42, 23.28, 24.24 = cholesteryl esters with carbon numbers 41, 42, 43, 44, 45, 47, respectively; 25.57, 26.16, 26.88, 27.47, 28.12, 29.32 = triglycerides with carbon numbers 48, 49, 50, 51, 52, 54 respectively. (B): 5.31 = cholesterol; 9.09 = cholesteryl butyrate (internal standard); 14.25 = cholesteryl benzoate (standard for laboratory control); 15.35, 16.13, 16.70, 18.42 = diglycerides and decomposition products of phospholipids; 19.97, 20.89, 21.71, 22.49, 23.35, 24.27 = cholesteryl esters with carbon numbers 41, 42, 43, 44, 45, 47, respectively; 25.59, 26.21, 26.96, 27.50, 28.31, 29.43, 30.63, 31.86 = triglycerides with carbon numbers 48, 49, 50, 51, 52, 54, 56, 58, respectively. Gas chromatograms were recorded by means of a Sigma 10 Data System. Vertical lines under the baseline are the marks of start and end of the integration. Sample volume 2 $\mu$l; chart speed, 5 mm/min; other analytical conditions are given in the text.

## DISCUSSION

Our results demonstrate that in hypertriglyceridemia there is an anomaly of plasma triglyceride composition in about 25% of cases. This heterogeneous hypertriglyceridemia can be proved by GC analysis of intact plasma lipids (Fig. 2).

The increased triglyceride fraction consists of molecules with carbon number 56. In some cases an augmented content of fractions $C_{58}$ and $C_{60}$ was found. It is evident that in such cases there must be some metabolic disturbance of fatty acids with twenty-carbon and twenty-two-carbon chains. Not only triglyceride but also the cholesteryl ester fraction loaded with twenty-carbon fatty acyl chains is augmented.

It is not clear if the anomaly in triglyceride composition is the result of hypertriglyceridemia or if it is its background cause. Further studies that will try to answer this question and to help towards a better understanding of the underlying pathogenetic factors of hyperlipidemia are in progress.

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