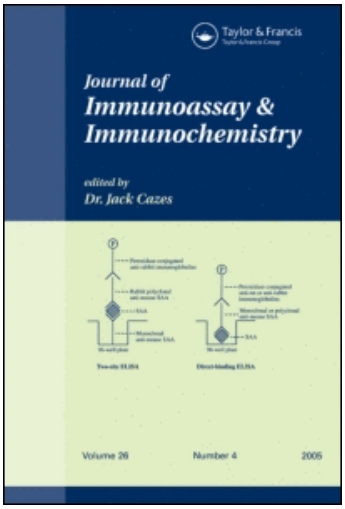

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Quantitative Determination of Different Apolipoprotein B Containing Lipoproteins by an Enzyme Linked Immunosorbent Assay: Apo B with Apo C-III and Apo B with Apo E

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QUANTITATIVE DETERMINATION OF DIFFERENT APOLIPOPROTEIN B CONTAINING LIPOPROTEINS BY AN ENZYME LINKED IMMUNOSORBENT ASSAY: APO B WITH APO C-III AND APO B WITH APO E

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ABSTRACT

A non competitive enzyme-linked immunosorbent assay (ELISA) for total apolipoprotein (apo) B, apo B with apo C-III (LpC-III:B), and apo B with apo E (LpE:B), was developed. Microtiter plates were used as solid-phase, and subdivided into three parts, coated respectively with affinity purified antibodies to apo B, to apo C-III and to apo E. After incubating the antigen with coated plates, a horseradish peroxidase-labelled antibody to apo B was added to all the plates to estimate total apo B, LpC-III:B and LpE:B.

(KEY WORDS : Lipoprotein particles ; ELISA; Apo B ; LpC-III:B ; LpE:B.)

INTRODUCTION

Studies on the protein moities have underlined the complex chemical nature of the plasma lipoprotein system by disclosing a marked protein heterogeneity of lipoprotein density classes. Results of immunological characterization and apolipoprotein quantification have shown clearly that each density class consists of several particles rather than single homogeneous lipid protein complexes (1,2). For that reason, a new classification system of lipoproteins has been proposed where lipoprotein particles are defined by their apolipoprotein composition (3).

Apo B has been investigated as a potential risk factor of heart disease (4). Several immunological methods for measuring this apolipoprotein have been developed, but these methods do not distinguish between the different apo B containing particles such as lipoprotein particles which contain B and C-III (LpC-III:B), B and E (LpE:B).

By using a two site differential immunoenzymatic assay we were able to quantify in whole plasma different associations of apo B. In this paper, we describe a procedure for the quantification of two populations of apo B containing particles : particles containing B and C-III called LpC-III:B and particles containing B and E called LpE:B.

MATERIALS AND METHODS

Blood Samples

Samples of human plasma were obtained from overnight fasted normolipidemic donors. Hypertriglyceridemic; TG > 1.5 g/l, (TG > 1.70 mmol/l) hypercholesterolemic subjects; cholesterol > 2.5 g/l (> 6.45 mmol/l) and type III hyperlipoproteinemic subjects were included in this study.

Isolation of Lipoprotein Density Classes

Major lipoprotein density classes including very low density, VLDL (d < 1.006 g/ml) intermediate density, IDL (d = 1.006 - 1.019 g/ml), low density LDL (d = 1.019 - 1.063 g/ml), and high density lipoproteins HDL (d = 1.063 - 1.21 g/ml) were isolated from plasma samples of fasting subjects by sequential preparative ultracentrifugation (5). Using a TLA 100.2 rotor in a Beckman TL 100 ultracentrifuge , each centrifugation was carried out for 2 hours at 10°C and 100 000 rpm.

Preparation and Purification of Antibodies to Apo B, Apo C-III and Apo E

Antisera to the individual apoproteins were produced in rabbits (6). Preliminary purification of antibodies was made by Na_2SO_4 precipitation and then purified by affinity chromatography using the corresponding pure antigen coupled to CNBr-activated Sepharose 4B as previously described (7).

Labelling of Antibodies to Apo B

Horseradish peroxidase (EC 1.11.1.7) labelled-affinity purified antibodies were made following the procedure described by Nakane and Kawaoi (8). The conjugate solution was stored at -20°C in small aliquots after addition of glycerol to a final dilution of 50 % (V/V) (7).

Isolation of Lipoproteins containing Apo B and Apo C-III (LpC-III:B) and Lipoproteins containing Apo B and Apo E (LpE:B)

Purified antibodies to apo C-III or to apo E were coupled covalently to CNBr activated Sepharose 4B.

In order to remove all apo C-III, 2 ml of whole fresh plasma were passed through 30 ml of immunoaffinity column specific for apo C-III ; with total capacity of 0.5 mg. The total capacity of immunoaffinity column specific to apo E was 0.3 mg per 15 ml of the gel. To minimize the disruptive effects of the dissociating agent (3M, NaSCN), 3 volumes of Sephadex G25 were used for 1 volume of antibody Sepharose as described by McConathy et al (9). The retained and unretained fractions were concentrated under vaccum to a volume equal to that of the starting plasma, corresponding respectively to LpC-III:B and apo B without C-III. The same procedure was applied for isolation of LpE:B and apo B without apo E.

Immunoprecipitation procedure

Immunoprecipitation was performed in bovine serum albumin (BSA) precoated eppendorf tubes. Antibodies to apo C-III or to apo E were added to duplicate dilutions of the plasma. The mixtures were incubated at 4°C overnight. The precipitates were separated by centrifugation at 9000 rpm for 15 minutes in TH2 1 Bioblock Scientific centrifuge, at room temperature. The supernatants obtained were analysed for apo B, apo C-III when anti apo C-III was used and for apo B and apo E when anti apo E was used. The amount of apo B found in supernatants in which apo-CIII or apo E was not detectable corresponds to apo B without C-III or apo B without apo E. Total apo B, apo C-III, apo E was measured by using an enzyme linked immunoassay on microtiter plates as earlier described by our laboratory (7, 10, 11).

Immunoassay procedure for measurement of LpC-III:B and LpE:B

Solid-phase: Ninety six wells polystyrene microtiter plates (Costar, Sercolustar USA) were washed three times with 0.1 M phosphate buffer saline (PBS) before use. Coating, washing, addition of conjugate, substrate, HCl and finally spectrophotometer reading were performed by use of an automatic ELISA Processor(Behring Institute, Marburg West Germany). In order to minimize non specific binding to microtiter wells, the assay buffer (dilution of antigen and conjugate contained 1 % bovine serum albumin.

Samples and standard were diluted with an electronic dilutor (Diluterend, Boehringer Mannheim).

Peroxidase substrate solution: 3g of o-phenylenediamine dihydrochloride (Sigma, Chemical Co, St Louis, MO) were dissolved per litre of 0.1 M phosphate citrate buffer, pH 5.5 containing hydrogen peroxide (3.5 mM).

In order to measure total apo B, LpC-III:B and LpE:B , the plate was subdivided into three parts and coated respectively with purified antibodies to apo B, to apo C-III and to apo E. Each well was coated with 0.1 ml of appropriate purified antibody solution and incubated overnight at room temperature.

After washing the coated plates four times with 0.1 M PBS, 0.1 ml of appropriate dilution of standard or plasma were added to each part of the microtiter plate, and the reaction was allowed to incubate for 2 hours at 37°C. The plate was washed again, and 0.1 ml per well of peroxidase labelled antibody to apo B was added to the entire plate and incubated for 2 hours at 37°C. The plate was washed and aspirated dry. Then, 0.1 ml of fresh substrate solution was added to each well and the enzymatic reaction was allowed to proceed for 30 mn in the dark at room temperature. The reaction was stopped by the addition of 0.1 ml of 1 M HCl and the absorbances were read at 492 nm. Relating absorbances to apo B, LpC-III:B and LpE:B concentrations and plotting on a semi log scale paper, standard curves were obtained for each respective assay.

Standardization procedure

Primary standards were prepared from human fasting plasma and consisted in lipoproteins isolated by immunoaffinity chromatography. For Lp C-III:B assay, the retained fraction of an anti apo C-III column was used as a primary standard while the standardization of the Lp E:B assay was done with the retained fraction of an anti apo E column. These fractions were measured for apo B by ELISA . This gave us the amount of apo B associated with apo C-III (Lp C-III:B) or apo B associated with apo E (Lp E:B) and was used to plot the standard curve of the corresponding assays.

This primary standard was used to determine the concentration of LpC-III : B and LpE : B of a pool of plasma which was used as a secondary standard.

RESULTS

Standardization

The amount of apo B in the immunoaffinity isolated LpC-III:B and LpE:B was used as the primary standard value. Adequate dilutions of immunoaffinity

isolated LpC-III:B and LpE:B, in parallel of those of whole plasma (secondary standard) were added respectively to the anti apo C-III and anti apo E coated wells. The slope of the curve of the immunoaffinity isolated LpC-III:B was 1.008 and 1.002 for the secondary standard. Those of LpE:B were respectively 1.024 and 1.025 ; indicating that both LpC-III:B and LpE:B were measured with satisfactory accuracy irrespective of their initial source.

On the other hand the secondary standard was analysed by the immunoprecipitation procedure. When anti apo C-III was used in this method, no further decrease of apo B, and no detectable apo C-III was observed in the supernatant after the addition of 0.6 mg/ml of affinity purified antibodies to apo C-III (Fig. 1). The value of LpC-III:B evaluated by subtracting the concentration of apo B remaining in the supernate from the total apo B concentration was 0.33 g/l ± 0.07, n = 3 ; and was comparable with that obtained from immunoaffinity isolated LpC-III:B which was 0.36 ± 0.06 g/l, n = 3.

When a secondary standard was treated with antibodies to apo E, the curve reached a plateau after the addition of 0.5 mg/ml of anti apo E (Fig 2). The LpE:B value of the secondary standard deduced from immunoaffinity isolated LpE:B was compared to that obtained by immunoprecipitation procedure. (0.51 ±0.07 g/l and 0.46 ± 0.10, n = 3, respectively).

Optimizing Assay conditions

Optimal coating of microtiter plates with the affinity purified antibodies to apo C-III or to apo E was examined by coating different immunoglobulin concentrations (0 to 100 µg/ml), followed by incubation with a fixed dilution (1/1000 fold) of the working standard. The optimal concentration of antibody for coating was 15 µg/ml of anti apo C-III and 20 µg/ml of anti apo E. These values correspond to those obtained when total apo C-III or total apo E were also measured.

The optimal dilution of anti apo B conjugate (the highest that gives a high range of absorbance value, and low zero blank) was about 1/4000.

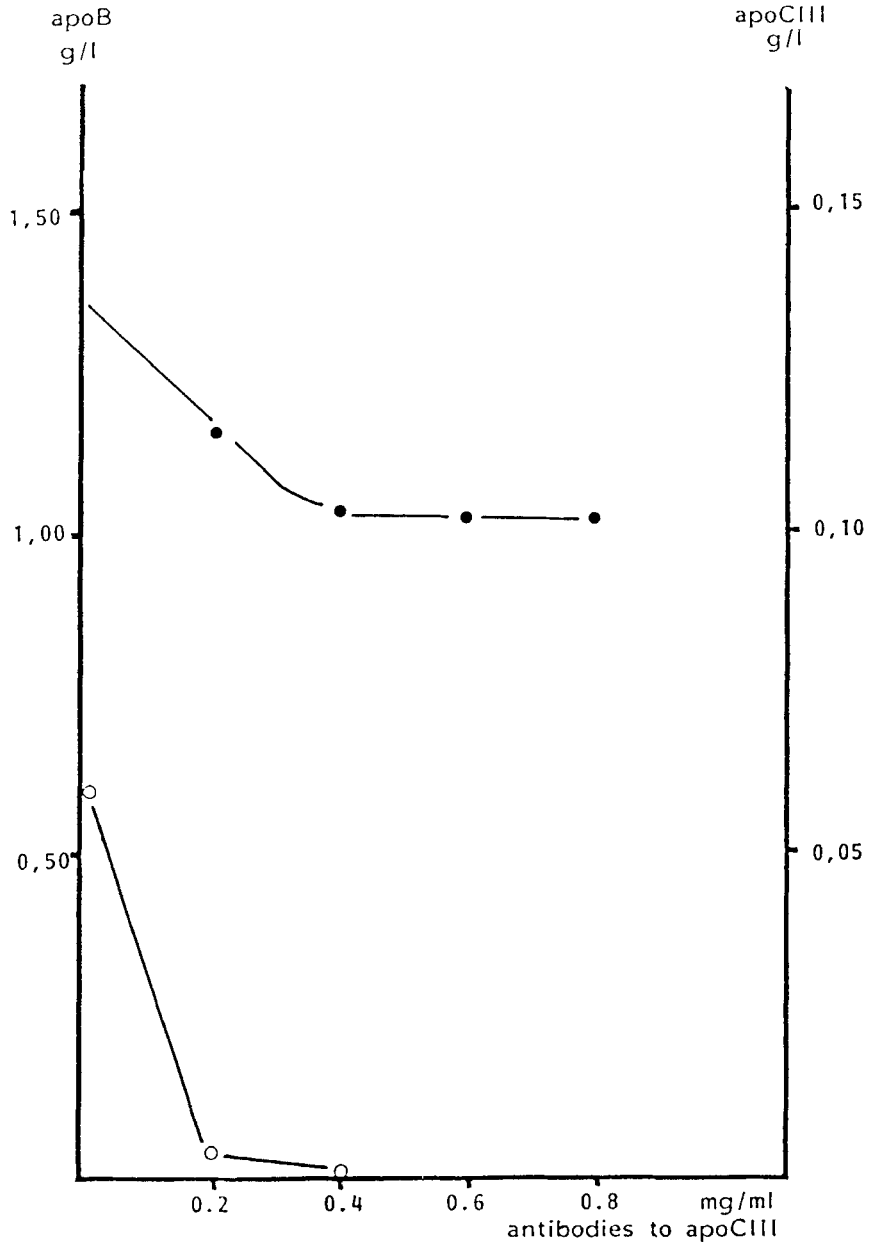

FIGURE 1: Immunoprecipitation procedure of LpC-III:B. 1V of fixed dilution of the standard (1/100 fold) incubated overnight at 4°C with 1V of different dilutions of purified antibodies to apo C-III. Apo B (●) and apo C-III (o) were assayed in all supernatants.

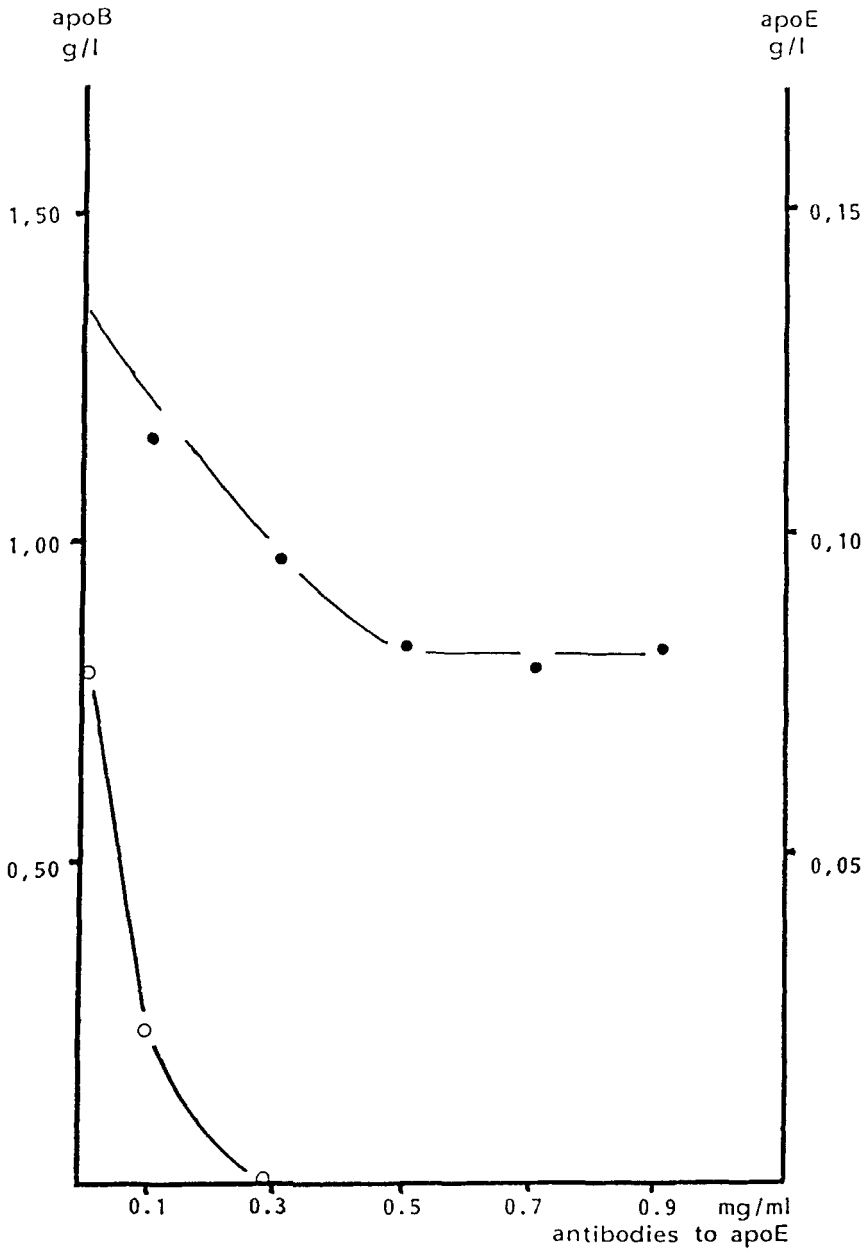

FIGURE 2: Immunoprecipitation procedure of Lp E:B. 1V of fixed dilution of the standard (1/50 fold) incubated overnight at 4°C with 1V of different dilutions of purified antibodies to apo E. Apo B (●) and apo E (o) were assayed in all supernatants.

Sensitivity, Reproductibility, Accuracy, Specificity and Recovery of the assays

The conditions of the assay have been optimized for measuring the concentration of Lp C-III:B and LpE:B in plasma. The working range of the assay was 10 ng/ml to 200 ng/ml for Lp C-III:B and 20 ng/ml to 500 ng/ml for Lp E:B. The lowest quantity that could be measured in 0.1 ml of sample applied per well was 1 ng of LpC-III:B and 2 ng for LpE:B. Figure 3 shows the typical curves of both assays.

The within-run assay precision CV, estimated by 20 replicate analyses in two samples of human plasma with low and high LpC-III:B concentrations (0.12 g/l and 0.52 g/l) were respectively 3.2 % and 3.0 %. The intra assay coefficient of variation were 3.3 % and 2.7 % for LpE:B levels of 0.06 g/l and 0.30 g/l. The between-run assay precision, assessed by running the two specimens for 5 consecutive working days was 4.9 % and 5.6 % for LpC-III:B; 7.5 % and 9.2 % for LpE:B.

The accuracy of the assays was verified by the addition of five known amounts of the immunoaffintity isolated LpC-III:B or LpE:B, to the plasma previously measured. The mean percentage recovery was 105 ± 8 for LpC-III:B and 107 ± 12 for LpE:B.

To investigate the specificity of the assays for LpC-III:B and LpE:B, low dilutions of the plasma (1/10 to 1/1000) of a patient with abetalipoproteinemia were assayed. This patient was characterized by a lack of apo B and low levels of apo A-I, apo A-II, apo C-III and apo E. The concentrations of these apolipoproteins were respectively 0.24 g/l, 0.16 g/l, 0.03 g/l and 0.02 g/l. Neither apo B nor LpC-III:B nor LpE:B were detectable.

Results obtained by the proposed method were compared with those of immunoprecipitation. Table 1 shows a good correlation between these two

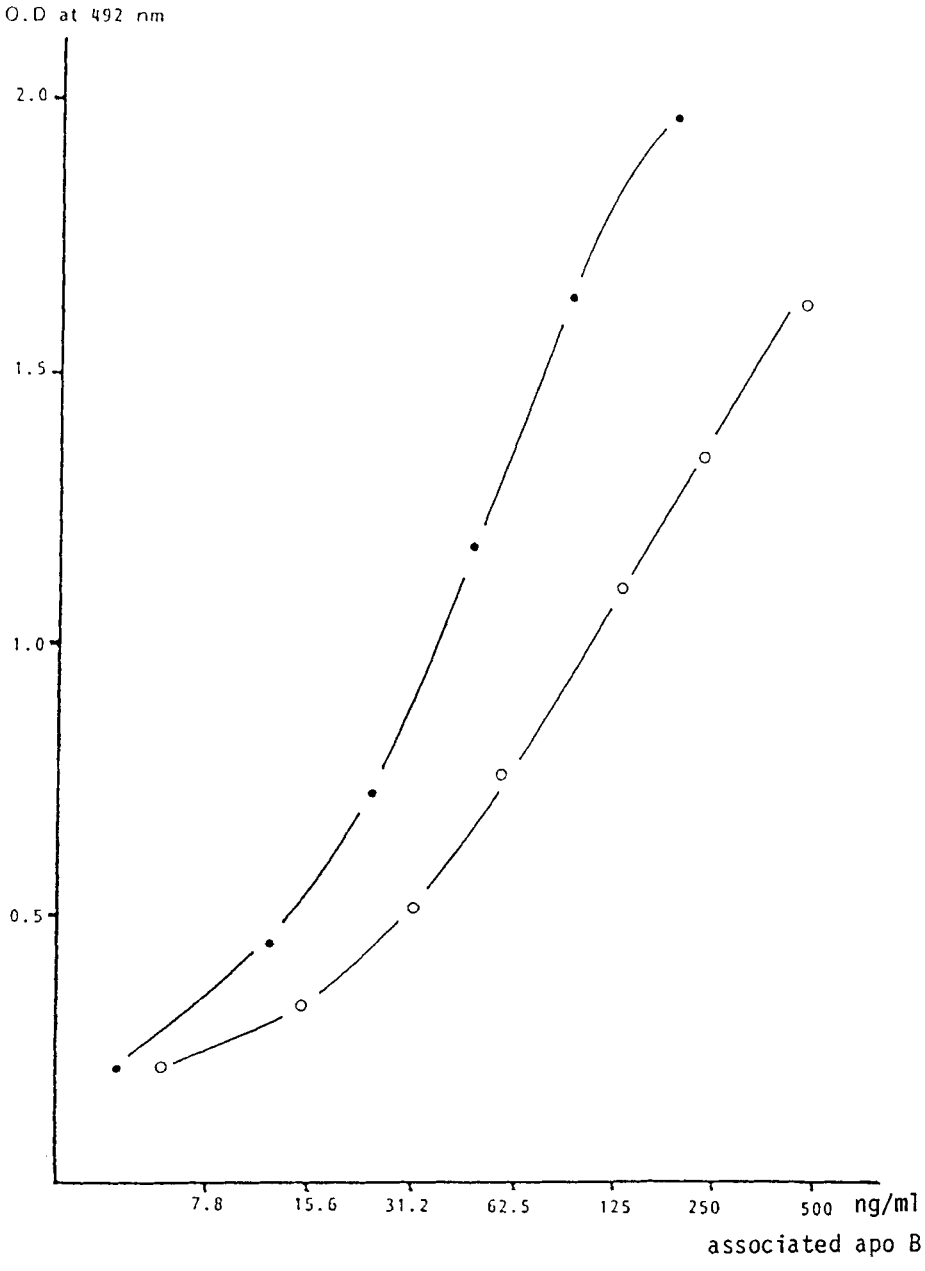

FIGURE 3: Typical standard curves of both LpC-III:B (●) and LpE:B (o).

TABLE 1

Comparison of the proposed method (y) with the immunoprecipitation procedure (x).

	LpC-III:B	LpE:B
Mean of y ± SD g/L (n = 19)	0.163 ± 0.119	0.255 ± 0.173
Mean of x ± SD g/L (n = 19)	0.197 ± 0.179	0.229 ± 0.170
Equation of Regression Line	y = 0.595x + 0.046	y = 0.912x + 0.048
Correlation coefficient	R = 0.90	R = 0.89

methods. The coefficient of the correlation were 0.90 and 0.87 for LpC-III:B and LpE:B respectively.

Distribution of LpC-III:B and LpE:B among Lipoprotein Subfractions (TABLE 2)

LpC-III:B and LpE:B were found in all major lipoprotein density classes. The distribution of LpC-III:B seems to be uniform across density classes. However this particle mainly accumulated in VLDL of hypertriglyceridemic subjects and represents 92 % of apo B.

The highest concentration of LpE:B for the three analysed groups, was found in VLDL, and this concentration decreased with increasing density. Most apo B, if not all, in normolipidemic VLDL was bound to apo E and 45 % to apo

TABLE 2

Distribution of LpC-III:B and LpE:B in major Lipoprotein Density Classes.

		TG g/L	Chol. g/L	Apo B g/L	LpCIII:B g/L	LpE:B g/L	LpCIII-B % of apo B	LpE:B % of apo B
WP	1	1.00 ± 0.19	2.18 ± 0.25	1.22 ± 0.12	0.20 ± 0.05	0.22 ± 0.07	16.3 ± 3.5	18.7 ± 7.6
	2	1.52 ± 0.83	3.07 ± 0.34	2.23 ± 0.32	0.44 ± 0.13	0.31 ± 0.16	19.6 ± 5.2	13.3 ± 6.2
	3	3.78 ± 1.96	2.57 ± 0.26	1.79 ± 0.44	0.66 ± 0.03	0.43 ± 0.23	39.7 ± 11.0	28.0 ± 22.0
VLDL	1	0.19 ± 0.11	0.12 ± 0.04	0.10 ± 0.01	0.04 ± 0.006	0.08 ± 0.03	45.2 ± 12.0	89.2 ± 15
	2	0.62 ± 0.48	0.25 ± 0.15	0.17 ± 0.09	0.12 ± 0.09	0.12 ± 0.07	66.0 ± 11.0	76.0 ± 9
	3	2.23 ± 1.19	0.56 ± 0.21	0.27 ± 0.02	0.25 ± 0.04	0.24 ± 0.11	92.0 ± 0.9	89.0 ± 19
IDL	1	0.20 ± 0.05	0.24 ± 0.08	0.15 ± 0.04	0.03 ± 0.004	0.06 ± 0.01	20.2 ± 6.0	44.3 ± 18.0
	2	0.33 ± 0.17	0.37 ± 0.13	0.25 ± 0.10	0.07 ± 0.05	0.10 ± 0.06	27.0 ± 7.9	39.0 ± 15.2
	3	0.33 ± 0.14	0.25 ± 0.07	0.14 ± 0.03	0.07 ± 0.03	0.08 ± 0.02	50.0 ± 7.0	53.0 ± 7.4
LDL	1	0.26 ± 0.04	0.91 ± 0.17	0.74 ± 0.17	0.05 ± 0.02	0.03 ± 0.04	7.1 ± 2.9	3.7 ± 1.0
	2	0.31 ± 0.06	1.51 ± 0.16	1.20 ± 0.16	0.09 ± 0.03	0.06 ± 0.01	7.1 ± 1.8	5.2 ± 0.8
	3	0.31 ± 0.03	0.94 ± 0.26	0.95 ± 0.18	0.10 ± 0.02	0.04 ± 0.02	10.2 ± 1.0	4.8 ± 2.9
HDL	1	0.21 ± 0.04	0.63 ± 0.11	0.12 ± 0.04	0.02 ± 0.009	0.004 ± 0.002	19.8 ± 4.4	4.1 ± 2.2
	2	0.21 ± 0.05	0.56 ± 0.15	0.13 ± 0.04	0.03 ± 0.01	0.007 ± 0.002	20.0 ± 4.6	5.7 ± 1.1
	3	0.25 ± 0.03	0.32 ± 0.03	0.12 ± 0.04	0.03 ± 0.007	0.003 ± 0.001	28.9 ± 6.3	3.5 ± 2.1

1: Normolipidemia (n=10); 2: Hypercholesterolemia (n=5); 3: Hypertriglyceridemia (n=4); WP: Whole Plasma

C-III, suggesting the existence of at least two apo B containing particles : LpE:C-III:B and LpE:B. In the latter, apo B represents 55 % of VLDL apo B. In hypertriglyceridemic VLDL, most apo B was bound to both apo E and apo C-III.

Although, the distribution of LpC-III:B and LpE:B across the density classes was different, the percentage of apo B bound to apo C-III and apo B bound to apo E decreases with increasing density (with the exception in the HDL), generating the simple form of apo B (LpB).

The recovery of total apo B, LpE:B and LpC-III:B using a TL100 ultracentrifuge, averaged respectively 85 %, 94 % and 68 % for nineteen individual preparations, indicating the damaging effect of ultracentrifugation conditions on LpC-III:B.

Concentration of LpC-III:B and LpE:B in Normolipidemic and Hyperlipidemic plasmas (TABLE 3)

Comparisons of apo B containing particles, between normolipidemic and hyperlipidemic subjects show a significantly elevated plasma concentrations of both Lp C-III:B and LpE:B in the three hyperlipidemic groups.

Hypercholesterolemic patients have higher levels of apo B, LpC-III:B and LpE:B, and slightly elevated levels of apo C-III and apo E. However, it was interesting to note the elevated amount of apo B without apo C-III and without apo E. Thus the significant increase of apo B levels in hypercholesterolemic patients could reflect the increase of LpB, a simple lipoprotein characterized by the presence of apo B as a sole apolipoprotein constituent.

Patients with hypertriglyceridemia have an elevated level of apo B, apo C-III, apo E, Lp-CIII:B and LpE:B, but apo B without apo E and without apo CIII concentrations remain normal, indicating that the increased concentration of total apo B was due to the accumulation in the plasma of LpC-III:B and LpE:B.

Type III hyperlipoproteinemia is characterized by significantly higher levels of LpE:B. Apo B for these patients was mainly associated with apo E ; about 59 % of total apo B (range between 30 % and 92 %).

TABLE 3

LpC-III:B and LpE:B profiles of subjects with Hyperlipidemia compared to Normolipidemic subjects

	T.G	Chol.	ApoB	ApoCIII	ApoE	LpCIII:B	LpE:B	Apo B without Apo CIII	Apo B without Apo E
	g/L								
Normolipidemia (n = 35)	0.72 (a) (0.30)	1.97 (0.33)	1.16 (0.27)	0.07 (0.02)	0.04 (0.02)	0.17 (0.05)	0.29 (0.08)	0.99 (0.25)	0.87 (0.21)
Hypertriglyceridemia (n = 31)	2.29 (0.80)	2.25 (0.31)	1.32** (0.24)	0.09*** (0.03)	0.06*** (0.03)	0.34*** (0.12)	0.44*** (0.20)	NS 0.98 (0.19)	NS 0.88 (0.26)
Hypercholesterolemia (n = 20)	1.07 (0.29)	2.95 (0.15)	1.47*** (0.28)	0.10*** (0.03)	0.05* (0.02)	0.30* (0.08)	0.39** (0.12)	1.17** (0.24)	1.08** (0.22)
Familial Type III (n=6)	3.49 (1.01)	3.57 (0.97)	NS 1.15 (0.22)	0.18*** (0.05)	0.47*** (0.13)	0.33*** (0.05)	1.01*** (0.36)	NS 0.83 (0.22)	0.13*** (0.11)

TG : Triglyceride ; Chol : Total Cholesterol
(a) : Means g/L
(S.D)
* : p <0.05 ; ** : p <0.01 ; *** : p <0.001 ; NS : not significant

DISCUSSION

Characterization of apo B containing particles shown earlier, complexes association of apo B with apo E and/or apo C (12,13,14). Discrete particles isolated from lipoprotein density classes differed not only in chemical and apoprotein composition but also in in vitro catabolism (15,16,17,18,19). Methods used to isolate these particles include immunoprecipitation (14), affinity chromatography using concanavalin A (20), heparin Sepharose (21,22) and immunosorbers (12). By using a two site differential immunoenzymatic assay to quantify some apo B containing particles, we were able to measure in the same microtiter plate total apo B, apo B associated with apo C-III and apo B associated with apo E. The amount of apo B without apo CIII and apo B without apo E can be deduced from total apo B. The method is fast, specific, sensitive and well correlated with the immunoprecipitation procedure. It offers a specific method for measuring lipoprotein complexes in plasma without previous isolation.

It may be possible that apo B containing particles may have various numbers of apo E or apo C-III depending the size and density of the particle. To check that increasing apo E and/or apo C-III content of the particle does not alter the result, we measured Lp E : B and Lp C-III : B in VLDL, IDL and LDL isolated from normolipidemic plasma at different dilutions. The regression lines did not differ between density classes. This indicate that the results obtained with our assays do not depend on the number of apo E and/or apo C-III per particle.

The distribution of LpC-III:B and LpE:B across the density spectrum, showed that these particles may extend in HDL, about 7 % of total plasma Lp C-III:B and 2 % of total plasma LpE:B was found in HDL. The fact that the percentage of apo B bound to apo C-III or to apo E decreases with increasing density is in agreement with the known data; where VLDL is remodelled in vivo to form IDL and LDL, the dynamic apo E and C peptides are progressively removed with a concomitant enrichment of the particle with apo B (16, 19).

By studying lipolytic degradation of VLDL by human lipoprotein lipase, Alaupovic's group (23) has shown that LDL contains Lp B as the main

lipoprotein form of apo B, while LpB:E:C-I and LpB:CI:CII:CIII account respectively for 5-10 % and 5 % of total of apo B in native LDL. These results are in agreement with our findings (Table 2). Measurement of VLDL LpC-III:B and LpE:B, showed that apo B was mainly associated with apo E (about 90 % of VLDL apo B), but only 45 % of apo B was bound to apo C-III. These results showed that VLDL from normolipidemic donors consists probably of two types of apo B containing particles ; LpC-III:B:E and LpE:B. While VLDL from hypertriglyceridemic patients contains LpC-III:E:B as the main complex particle.

Studies on hyperlipidemic subjects showed that in the case of hypercholesterolemia apo B without apo C-III and without apo E is significantly higher than in normolipidemic donors. Hypertriglyceridemic subjects were characterized by significant increases of apo B containing apo C-III and apo E, accumulated in VLDL, In familial type III hyperlipoproteinemia, apoB without apo E was dramatically diminished (Table3).

Our basic studies have clearly shown that apo B containing lipoproteins do not behave in the same way regarding the receptor specific pathway when apo E and/or apo C-III is present (24). Basically, apo E increases the affinity but decreases the number of particles bound to the receptor. Apo C-III decreases the affinity in such a way that particles containing apo B and apo C-III but free of apo E do not bind to the receptor. Measuring total ap B does not distinguish between these particles with different behavior. The results presented in this paper indicate clearly that in some pathological situations, such as type III dyslipoproteinemia, LpE : B increases dramatically, while total apo B does not change.

These results indicate that the assays described in this paper may potentially add new information or the lipoprotein profile of different individuals suffering of dyslipoproteinemia. Further investigation is necessary to determine the clinical significance of these new parameters, particularly in regard to the risk of atherosclerosis.

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