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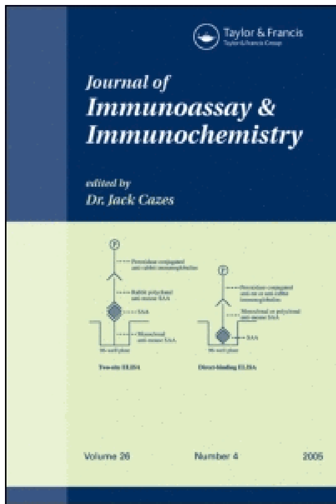

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Quantitative Determination of Human Plasma Apolipoprotein A-II by a Non Competitive Enzyme-Linked Immunosorbent Assay

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JOURNAL OF IMMUNOASSAY, 7(4), 285-307 (1986)

QUANTITATIVE DETERMINATION OF HUMAN PLASMA APOLIPOPROTEIN A-II BY A NON COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

A noncompetitive enzyme-linked immunosorbent assay (ELISA) for apolipoprotein A-II (ApoA-II) was developed. Microtiter plates were coated with affinity purified antibodies to ApoA-II. After incubation with human plasma, the amount of ApoA-II bound to the coated plate was determined with peroxidase-labeled antibodies to ApoA-II. When pure ApoA-II or delipidated reference plasma was used as standard, a single step delipidation was required in order to unmask some antigenic sites of ApoA-II. However, the underestimated ApoA-II values in untreated samples were shown to be corrected by using intact reference plasma as secondary standard. The average concentration of ApoA-II in normolipidemic plasma was 0,376 g/l.

(KEYWORDS: Noncompetitive enzyme-linked immunosorbent assay - *Apolipoprotein A-II - Human plasma)*

INTRODUCTION

Measurements of the apolipoprotein A-II (ApoA-II) concentration in human plasma are performed by various types of immunoas-

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says, including radial immunodiffusion (1), electroimmunoassay (2, 3), immunonephelometry (4, 5) and radioimmunoassay (6, 7). Since the advent of the enzyme-linked immunosorbent assay (ELISA) (8), this method has been used for quantitative determination of a variety of biologically important compounds. This has also included competitive enzyme-linked immunoassays for apolipoproteins C-I, C-II, C-III, E and B (9, 10) as well as ApoA-II (11). however, the alternative noncompetitive "sandwich" ELISA seems to be a more attractive version (12), since in this method, the antigen to be measured does not have to be labeled, which excludes the possibility of its alteration. In addition, ELISA uses small quantities of antibody, does not require disposal of radioisotopes, and displays good sensitivity.

We have recently described a noncompetitive "sandwich" ELISA for measuring the concentration of ApoA-I, the major protein component of human high density lipoproteins (13). This study describes the development of a "sandwich" ELISA for measurement of the other major protein of HDL, ApoA-II.

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MATERIALS AND METHODS

Microtiter plates (96-2311 Serocluster "U" vinyl) were purchased from COSTAR, Cambridge, MA, USA ; horseradish peroxidase, protein-A-Sepharose, bovine serum albumin and gelatin, from Sigma Chemical Company, St. Louis, MO, USA. Peroxidase color

reagent (2.2'-azino-di-3-ethylbenzothiazoline-sulfonate) was purchased from Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, USA. Rabbit serum was purchased from K.C. Biological, Lenexa, KS, USA.

Plasma

Human blood samples were obtained by venipuncture from asymptomatic normolipidemic male and female donors who had fasted overnight. For comparative purposes, a group of hypertriglyceridemic male and female patients (triglyceride values above 3 g/l) was also included in this study. The blood was collected into 10 ml vacutainer tubes containing 0.5 ml of 5 mmol/L EDTA and centrifuged for 15 min at 2,500 rpm at 4°C to separate cells from plasma. Plasma samples were kept at 4°C and always tested within 48 hours. Plasma used for the preparation of density fractions was collected by plasmapheresis from normolipidemic and hyperlipidemic donors. Lipoproteins fractions were isolated by sequential ultracentrifugation (14).

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Isolation of ApoA-II

ApoA-II was isolated from HDL by a combination of DEAE-cellulose and gel permeation chromatography as described previously (15, 16). The isolated ApoA-II was homogeneous by basic and SDS

polyacrylamide gel electrophoresis and double diffusion analysis. It showed the expected molecular weight and amino acid composition of ApoA-II. The content of ApoA-II in these preparations was determined by amino acid analyses (16).

Preparation and Purification of Antibodies to ApoA-II

Antisera to ApoA-II were produced in rabbits by intraperitoneal administration of antigen as previously described (14). After assessment of the monospecificity of antisera by double diffusion and by electroimmunoassay (2, 15), affinity purified antibodies were isolated by immunoaffinity chromatography using delipidized HDL proteins (Apo HDL) coupled to CNBr activated Sepharose 4 BCL, as described previously (13). Affinity purified antibodies were stored as 0.5 ml aliquots at -75° C.

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Preparation and Purification of Anti-ApoA-II Peroxidase Conjugate

The antibody enzyme conjugate was prepared according to Avraemas (17) using glutaraldehyde as coupling reagent. Labeled antibodies were separated from the non-conjugated peroxidase using protein A Sepharose column chromatography. After purification by protein A, peroxidase-labeled anti-ApoA-II was concentrated with dry sucrose, and stored as 0.5 ml aliquots at - 75°C.

Principle of the Assay

The two site "sandwich" ELISA was performed on microtiter plates. The assay consisted of the following six steps : a) coating of the plate with antibodies; b) blocking of unreacted binding sites on the plate; c) incubation of antigen with the antibody-coated plate; d) incubation of the peroxidase-labeled antibodies with antigen bound to the coated plate; e) incubation with the peroxidase substrate and development of color; and f) reading of the optical density.

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Optimizing Assay Conditions

Optimal coating of microtiter plates with the affinity purified antibodies to ApoA-II was first examined. Differents dilution of antibodies were made with phosphate-buffered saline 0.01M, pH 7.4 or sodium carbonate buffer 0.1M, pH 9.6 or deionized water, and incubated on plates (50 µl/well) for 18 hours at 25°C in a humidified chamber. After washing, the plates were incubated with swine peroxydase labelled antibodies to rabbit immunoglobulins diluted in Na_2HPO_4 0,15 M pH 7.2 containing 1% of bovine serum ablumine for 2 hours at 25°C. After addition of the peroxydase substrate, the measurement of the optical density allowed to determine the most efficient buffer to get maximal coating of first antibodies.

Once this above experiment was performed, these such defined optimal conditions were retained to ascertain wether they were also optimal for the complete enzyme immunoassay procedure of ApoA-II evaluation. For that purpose, serial dilutions of pure ApoA-II were prepared with 0.15M Na_2HPO_4 buffer containing 1% of the blocking agent and incubated on the precoated plate (50 µl/well) for 18 hours at 4°C. Then, the plates were washed three times and incubated for 5 hours at 25°C with peroxydase labelled antibodies to ApoA-II (50 µl/well) diluted with the same buffer as above, (1:1000 or 1:2000 or 1:4000 or 1:8000 or 1:16000).

Following incubation, plates were washed five times with 0.15M Na_2HPO_4, pH 6.5 and peroxydase substrate was added (50 µl/well). After 1 hour at 37°C, color development was monitored by measuring optical density at 405 nm using an automated ELISA reader.

The effect of pH was established by using different pH of the buffer ranging from 6 to 8.5. The blocking efficiency of bovine serum albumine and non immune rabbit pool serum were also compared by judging the intensity of background optical density. Through all the enzyme immunoassay procedure, washing and incubation buffers were always identical : same pH and same blocking agent.

Standard Curves

For the construction of standard curves different ApoA-II containing preparations were used :

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- pure ApoA-II : its apoprotein content was calculated from the amino acid composition.

- two different HDL preparations : one preparation from a normolipidemic patient and one other from a hyperlipidemic patient (total cholesterol : 3.92 g/l, triglycerides : 3.2 g/l). By quantitative polyacrylamide gel electrophoresis (1) their ApoA-II content was estimated to be 19.2% and 21.3% respectively of their total apoprotein content (7, 11).

- pool of fasting plasmas from normolipidemic donors : its ApoA-II content has been determined as above, after isolation of HDL by ultracentrifugation (11).

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Treatment of Samples

HDL or the pool plasma used for the construction of the secondary standard curves as well as samples to be tested were delipidated to compare immunoreactivity with their undelipidated state. To expose eventual masked antigenic determinants of ApoA-II, a convenient procedure was used : a delipidation in a single step extraction by a mixture of butanol and diisopropylether (18). The ratio of 20:80 (v/v) was choosen for the routine extraction procedure after a comparative study using different proportions of butanol and diisopropylether. This method was also compared with total delipidation, whereby plasma was lyophylized and extracted repeatedly by a chloroform methanol mixture as described earlier (16).

Recovery

To determine the recovery, HDL with known contents of Apo A-II, were added to plasma samples of known ApoA-II concentrations. These were then assayed.

Reproducibility

Three plasmas containing high, medium and low amounts of ApoA-II were used. Intra-assay reproducibility was determined by measuring the concentration of ApoA-II in theses samples of thirty times on the same plate. Inter-assay precision was assessed by measuring the concentration of ApoA-II in the same plasmas for 6 consecutive working days.

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Specificity

In addition to the initial assessment of the antibodies to ApoA-II by electroimmunoassay and double immunodiffusion, the specificity of the assay was also checked by measuring the concentration of ApoA-II in the presence of ten-fold excess (w/w) of purified ApoA-I (2), ApoE (19), ApoB (20), ApoC-II (21), ApoC-III (15) and human albumin (Sigma, St. Louis, MO).

Lipid Determination

Triglycerides and free and esterified cholesterol were measured according to the Lipid Research Clinic methodology (22). High density lipoprotein cholesterol was determined as described by Warnick and Albers (23).

Electroimmunoassay (EIA)

The concentration of ApoA-II in normolipidemic and hyperlipidemic plasma samples was also measured by EIA (2) in order to compare both methods.

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RESULTS

Optimal Assay Conditions

Optimal coating of first antibodies on the plates was obtained with concentration above 0,5 µg/well and when dilutions were made in desionized water (Fig. 1).

With dilution 1/4000 of anti ApoA-II peroxidase labeled antibody, response curves for pure ApoA-II, as Ag, yielded a good slope from 10 to 100 ng/ml. The effect of pH on the binding of antigen to the antibodies coated plate is shown in figure 2. The

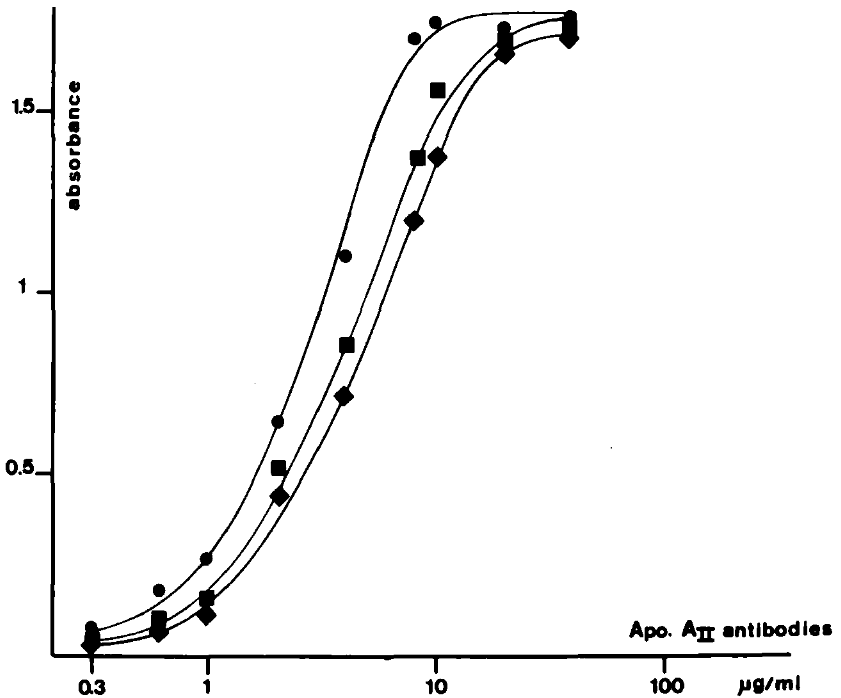

FIGURE 1. Effect of different buffer on the antibody coating to the plate : ●–● desionized buffer, ◆–◆ phosphate buffered saline 0.01M pH 7.4, ■–■ sodium carbonate buffer 0.1M pH 9.6.

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optimal binding occured between pH 6.0 and 7.0. In contrast to ApoA-I enzyme immunoassay procedure described earlier (13), no difference was found for the blocking efficiency between bovine serum albumine and non immune rabbit serum. For economy, precision and in an effort to use same conditions than for ApoA-I assay (13), the following conditions were retained for the typical enzyme immunoassay of ApoA-II : pH 6.5 for the buffer, non immune

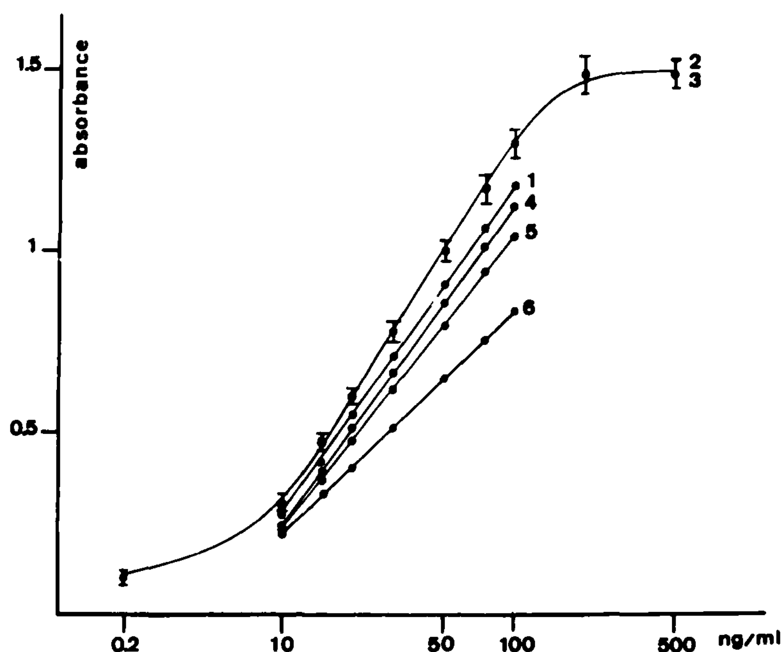

FIGURE 2. The effect of pH on the slope of a primary standard curve. ApoA-II was used as a standard at pH 6.0 (1), 6.5 (2), 7.0 (3), 7.5 (4), 8.0 (5) and 8.5 (6). Identical effect of pH was observed when reference plasma was used as standard.
Peroxidase labelled antibodies ApoA-II were diluted 1:4000. The typical standard curve was constructed with 5 determinations for each point on the same plate (bars indicate standard deviation).

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rabbit serum as blocking agent. For the measurement of ApoA-II content in unknown sample, two different dilutions (1:5000 and 1:15000) of the sample we done and tested in duplicate on the same plate.

Standard Curves

Using 1-500 ng/ml of pure ApoA-II, the binding curve showed a sigmoid form characteristic of this type of immunoassay. The

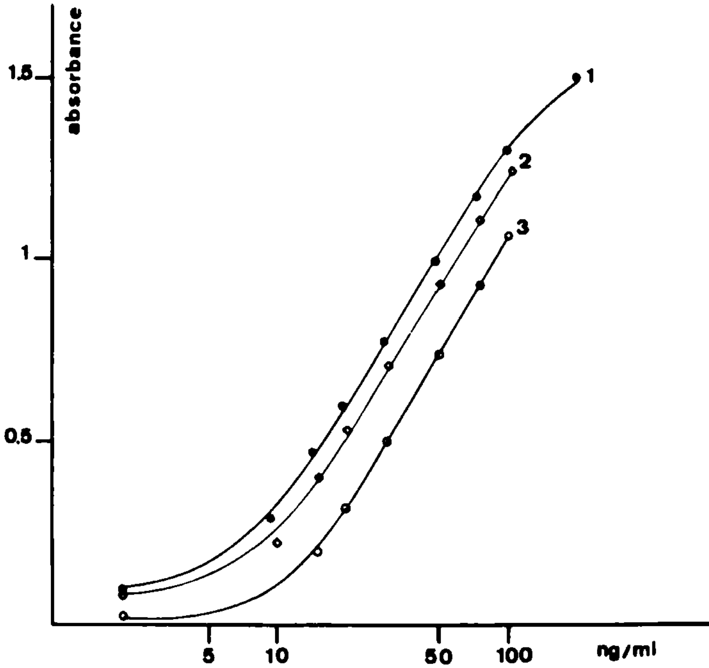

FIGURE 3. Standard curves obtained with pure ApoA-II (1), delipidated HDL preparations (1), reference plasma delipidated with butanol-diisopropylether (1), undelipidated HDL preparations (2), undelipidated reference plasma (2), reference plasma delipidated with chloroform-methanol (3).

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region of the curve closely fitting a straight line (from 10 to 100 ng/ml) was selected as the primary standard curve (Fig. 2). A normolipidemic fasting plasma served as secondary standard. As shown in Figure 3, plasma delipidated with either butanol-diisopropylether or chloroform-methanol mixtures displayed slopes identical to that of the ApoA-II, which allowed its use as a secondary standard.

Dilutions of undelipidated and delipidated reference plasma or HDL preparations produced curves closely parallel to the

primary standard throughout must of the range of the assay, but delipidation shifted the curves significantly to the left (Fig. 3).

Effect of Treatment of Samples on ApoA-II Concentrations

To expose epitopes of ApoA-II, plasma samples from normolipidemic and hypertriglyceridemic subjects were delipidated by a modification of the procedure described by Cham and Knowles (18). Using the delipidated reference plasma to construct the secondary standard curve, delipidation of unknown samples with the mixture butanol-diisopropylether (20:80) yielded significantly higher values (Table 1A) (0.410 g/l instead of 0.351 g/l for normolipidemic subjects, 0.380 g/l instead of 0.328 g/l for hypertriglyceridemic subjects), leading to an underestimation of ApoA-II concentrations in untreated plasmas. However the ApoA-II values for unknown plasma samples were corrected when the reference plasma was used untreated for the construction of the secondary standard curve (Table 1B). This finding that some antigenic sites of ApoA-II were masked in undelipidated plasmas were conflicting with a previously described enzymeimmunoassay (11). Also we tested the effect of the non ionic detergent Tween 20 on the immunoreactivity of ApoA-II in plasmas, which is usually included in this last assay buffer. Including Tween 20 at a final concentration of 0.05% (11) in all the buffers, the secondary standard curve obtained with undelipidated and delipidated reference plasma

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TABLE 1

The Effect of Delipidation on the Concentrations of ApoA-II in Normolipidemic and Hypertriglyceridemic Plasmas, using Different Proportions of Butanol and Diisopropylether.

	Butanol/Diisopropylether ratio (v/v)	Normolipidemic Subjects (n=9) (Triglycerides : 0.93 ± 0.293 g/L) (Cholesterol HDL : 0.531 ± 0.093 g/L)	Hypertriglyceridemic Subjects (n=9) (Triglycerides : 3.68 ± 1.07 g/L) (Cholesterol HDL : 0.513 ± 0.078 g/L)
A	0/0*	0.351 ± 0.075**	0.328 ± 0.044
	10/90	0.321 ± 0.048	0.318 ± 0.059
	20/80	0.410 ± 0.090	0.380 ± 0.052
	40/60	0.323 ± 0.042	0.325 ± 0.052
	60/40	0.319 ± 0.036	0.317 ± 0.045
	80/20	0.281 ± 0.032	0.312 ± 0.041
B	0/0*	0.413 ± 0.078	0.378 ± 0.055

* undelipidated plasma
** mean ± SD of concentrations of ApoA-II (g/L)

A : The standard curve was constructed using the delipidated reference plasma
B : The standard curve was constructed using the delipidated reference plasma

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were identical and superimposable. In these conditions no effect of delipidation with the mixture butanol:diisopropylether (20/80) on ApoA-II values was found (Table 2).

TABLE 2

The Effect of Tween 20 (0.05%) on the Concentrations of ApoA-II in Delipidated and Undelipidated plasma.

		no Tween 20	Tween 20
Normolipidemic subjects (n = 9)	undelipidated	0.351 ± 0.075*	0.411 ± 0.098
	delipidated	0.410 ± 0.090	0.413 ± 0.097
Hypertriglyceridemic subjects (n = 9)	undelipidated	0.328 ± 0.044	0.379 ± 0.057
	delipidated	0.380 ± 0.052	0.380 ± 0.055

* mean ± SD of concentration of ApoA-II (g/L)

The standard curve was constructed using the delipidated reference plasma

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TABLE 3

Intra- and Inter-Assay Precision

	Intra assay precision			Inter assay precision		
	Mean (g/L)	SD	CV (%)	Mean (g/L)	SD	CV (%)
High concentration of ApoA-II	0.441	0.017	3.9	0.436	0.027	6.2
Medium concentration of ApoA-II	0.382	0.012	3.1	0.386	0.020	5.1
Low concentration of ApoA-II	0.247	0.008	3.2	0.259	0.011	4.2

Recovery, Reproducibility, Specificity and Sensitivity of the Assay

ApoA-II was fully recovered (96.9% - 101.05%) when added in the form of HDL to several plasmas. The intra-and inter-assay coefficients of variation obtained for the three plasmas with high, medium and low amounts of ApoA-II were acceptable (Table 3). The cross reactivity with purified ApoA-I, ApoE, ApoC-III, ApoB or human serum albumin was, in all cases, less than 0.5%. The lowest amount of ApoA-II that could be detected by this assay was 0.5 ng.

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Concentrations of ApoA-II in HDL subfraction

To assess whether the higher lipid content of HDL_2 than of HDL_3 could affect the measurement of A-II, quantitation of A-II in HDL subfractions, HDL_2 (d = 1.10-1.21) and HDL_3 (d = 1.063-1.10), from three normolipidemic subjects and hypertriglyceridemic subjects was compared to an independent estimation of the apoprotein by quantitative polyacrylamide gel electrophoresis (PAGE) (1).

The concentration of ApoA-II by our assay was comparable to that obtained by the PAGE procedure for HDL_3 fractions as well as HDL_2 fractions (Table 4). Thus the accuracy of the measurements of ApoA-II in HDL subfractions is appeared to be adequate.

TABLE 4

ApoA-II Content in HDL Subfractions, Comparison of PAGE with ELISA.

		ApoA-II (g/l)	
		ELISA	PAGE
HDL_2	N*	0.058	0.056
	N	0.044	0.040
	N	0.048	0.048
	H	0.038	0.039
	H	0.049	0.047
	H	0.036	0.034
HDL_3	N	0.201	0.197
	N	0.181	0.182
	N	0.222	0.208
	H	0.171	0.182
	H	0.190	0.168
	H	0.223	0.225

* N : normolipidemic subjects
H : hypertriglyceridemic subjects

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Concentrations of ApoA-II in Plasma

The average level of ApoA-II in plasma of normolipidemic subjects, determined by ELISA was 0.376 g/l and is comparable to the most frequently reported concentrations of ApoA-II (5). Mean values for female and male subjects were not significantly different (Table 5). There was a significant correlation between ELISA and EIA (Table 6). The average concentration of plasma ApoA-II in a group of 20 hypertriglyceridemic patients (with average triglyceride concentration of 2.08 ± 0.38 g/l) was

TABLE 5

Concentrations of ApoA-II (g/l) in the Plasmas of Normolipidemic and Hypertriglyceridemic Subjects.

Normolipidemic Subjects Males (n = 50)	Normolipidemic Subjects Females (n = 50)	Hypertriglyceridemic Subjects Males and Females (n = 20)
0.375 ± 0.092* (0.514 ± 0.098)*	0.377 ± 0.088 (0.531 ± 0.119)	0.359 ± 0.111 (0.508 ± 0.095)

* mean ± SD
** (cholesterol HDL : mean ± SD g/L)

The differences between the three groups of subjects were not significant.

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0.359 g/l which was not significantly different from normolipidemic patients (Table 5).

DISCUSSION

The noncompetitive "sandwich" ELISA for the quantification of ApoA-II described here is a precise, specific and relatively simple procedure which can be automated. When using pure ApoA-II or the delipidated reference plasma as secondary standard, delipidation of plasma using the mixture of butanol and diisopropylether, proved bo be a valuable step in the micro ELISA procedure for ApoA-II. However, the proper choice of organic solvent

TABLE 6

Concentration of Apolipoprotein A-II in Plasmas as Determined by ELISA and EIA

	EIA (n = 52) mean ± SD	ELISA (n = 52) mean ± SD
Apo A-II (g/l)	0.351 ± 0.111	0.378 ± 0.095

Regression line : $y = 0.915x + 0.057$
$R = 0.928$ ($p < 0.001$)

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mixture seemed to be important. While the use of intact reference plasma as secondary standard made this delipidation step of the plasma samples unnecessary. It has been reported recently that delipidation of plasma did virtually not appear to expose additional antigenic determinants of apolipoprotein A-II (11). We tried to explain these conflicting results by the use in buffers of the non ionic detergent Tween 20. Indeed Tween 20, usually included in the buffer to avoid non-specific binding of antibodies or antigen to the assay plastic surface, has been shown in our study to expose more antigenic sites. The possible mechanism by which Tween 20 exposes the antigenic sites can be trough dissocating apolipoproteins from lipoprotein particles (24). Thus including Tween 20 in assay buffer, new delipidation step did not affect the amount of immunoassayable ApoA-II in human plasma. As already demonstrated for ApoA-I determination (25), present data indicate that secondary standards and unknown samples must be in a

similar physicochemical state. Under that condition, the antibodies have equal recognition of antigenic sites on both standards and unknown samples.

Several reports have been published on the relationship of plasma apolipoprotein levels to coronary artery disease. ApoA-I and ApoB have been shown to be useful in distinguishing patients with coronary heart disease (26, 27, 28, 29) whereas discrepancies exist about clinical significance of apolipoprotein A-II (28, 29, 30, 31, 32). To establish its definitive clinical value, more experience is required and in combination with the previously described "sandwich" ELISA for ApoA-I (13) and ApoB (33), the enzyme linked immunosorbent assay of ApoA-II should be useful in large clinical and/or epidemiological studies.

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