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Determination of Human Apolipoproteins A-I, B, and E by Laser Nephelometry

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Summary: A laser immunonephelometric procedure for the determination of human apolipoproteins A-I, B, and E in serum and lipoproteins was developed. Coefficients of variation were 2.3%–3.4% within-run, 4.7%–4.9% between-run. Serum apolipoprotein A-I values were similar in normal and hyperlipoproteinaemic subjects with a correlation coefficient of r = 0.585 to HDL-cholesterol levels. LDL-apolipoprotein B values were highly (r = 0.935), VLDL-apolipoprotein B values less strongly (r = 0.472) correlated to the corresponding cholesterol levels. Serum apolipoprotein E concentrations were distinctly elevated in Type III and not correlated to cholesterol values.

Bestimmung der Humanapolipoproteine A-I, B und E durch Lasernephelometrie

Zusammenfassung: Eine lasernephelometrische Methode zur Bestimmung der menschlichen Apolipoproteine A-I, B und E in Serum und Lipoproteinen wurde entwickelt. Die Variationskoeffizienten waren 2,3%–3,4% (Intraassay) und 4,7%–4,9% (Interassay). Die Serumapolipoprotein A-I-Konzentrationen waren bei Gesunden und hyperlipoproteinämischen Patienten ähnlich mit einem Korrelationskoeffizienten von r = 0,585 zu den HDL-Cholesterinwerten. Die LDL-Apolipoprotein B-Konzentrationen waren hoch (r = 0.935), die VLDL-Apolipoprotein B-Konzentrationen weniger ausgeprägt (r = 0,472) mit den entsprechenden Cholesterinwerten korreliert. Die Serumapolipoprotein E-Konzentrationen waren eindeutig bei der Typ III-Hyperlipoproteinämie erhöht und korrelierten nicht mit den Cholesterinwerten.

Introduction

The close relationship of atherosclerotic vessel diseases to disorders of serum lipoprotein levels has stimulated research on the protein moieties of lipoproteins. Apolipoprotein A-I, the main constituent of high-density lipoproteins (HDL), plays an important role in the transport of peripheral cholesterol to the liver (1). Serum lipoproteins in the very low- and low-density range (VLDL and LDL) which contain apolipoprotein B and/or apolipoprotein E have attracted considerable interest because of their ability to interact with cell surface receptors, thereby delivering cholesterol to cells (2, 3). Various methods have been utilized for the quantitative determination of serum apolipoproteins, including radial immunodiffusion, electroimmunoassay, immunonephelometry, and radioimmunoassay (4, 5, 6).

We have recently developed a rapid and sensitive laser immunonephelometric assay for the determination of apolipoprotein E (7). This method was extended to apolipoproteins A-I and B with the aim of studying normal and hyperlipoproteinaemic states. Major emphasis was placed on the relations between the concentrations of apolipoproteins A-I, B, and E and of cholesterol in serum and lipoproteins.

Materials and Methods

General procedures

Serum samples from normal male volunteers and hyperlipoproteinaemic patients (aged from 23 to 65 years), taking no medication and having fasted overnight, were analysed. The samples were stored at 4 °C until analysis, which was performed within 2 days.

VLDL were isolated from serum by ultracentrifugation at 1.006 kg/l at 40,000 min^{-1} for 22 hours at 4 °C in Beckman 40.3 rotors, and the lipoprotein fractions were recovered by tube slicing (8). In the 1.006 kg/l infranate HDL were separated from LDL by precipitation of LDL using a commercially available combination of sodium phosphotungstate and magnesium chloride according to the described method (Boehringer-kit, Mannheim, FRG). A lipoprotein-free serum was obtained after ultracentrifugal flotation of lipoproteins at 1.21 kg/l. The mass of cholesterol and triglycerides in whole serum and separated lipoproteins was determined in an automated analyser by enzymatic techniques (Boehringer-kits, Mannheim, FRG). LDL-cholesterol was calculated as the difference in the mass of cholesterol in the 1.006 kg/l infranate and in HDL. Before assay of apolipoproteins, serum and ultracentrifuged fractions were stored at −30 °C for up to 1 month. Hyperlipoproteinaemic subjects were obtained from a group of individuals originally referred to the outpatient clinic. The distribution of lipid levels was determined in the families of the propositi in order to obtain a genetic diagnosis (9). Propositi with either (heterozygous) familial hypercholesterolaemia (Type IIa) or familial combined hyperlipidaemia (Type IIb) or familial hypertriglyceridaemia (Type IV) were then selected for evaluation of their lipoproteins. Diagnosis of Type III hyperlipoproteinaemia was confirmed by analytical isoelectric focusing of the apolipoprotein E isoforms (10). Statistical methods included the unpaired *Wilcoxon* two-sample test and simple regression analysis using *Pearson* correlation coefficient r (11).

Preparation of the antigens

Apolipoproteins A-I and E and LDL (1.030 kg/l < d < 1.050 kg/l) were used as antigens. Apolipoproteins were isolated from ultracentrifuged HDL (apolipoprotein A-I) and VLDL (apolipoprotein E) by Sephacryl S 200 gel filtration as described (12). Apolipoprotein E was further purified by chromatofocusing (13). LDL were prepared by 2 sequent ultracentrifugations. The homogeneity of apolipoproteins was assayed by polyacrylamide gel electrophoresis in urea and sodium dodecyl sulphate (SDS) with molecular weight determination (14, 15), and amino acid analysis was performed on an automated analyser (Liquimat III, Kontron, Munich, FRG). LDL yielded a single band on 3.5% polyacrylamide gel electrophoresis in SDS with the mobility of apolipoprotein B-100 (16). The mass of protein in isolated apolipoproteins and LDL was determined according to *Lowry* et al. (17) using human albumin as standard.

Preparation of the antisera

Antisera to apolipoproteins A-I and E and LDL were produced by injecting rabbits subcutaneously on 3 occasions 14 days apart and bleeding them 7 days after the last injection. The total amount of antigen given per inoculation was 0.3 mg protein (apolipoproteins A-I and E dissolved in 0.05 ml 0.01 mol/l NH_4HCO_3 buffer, pH 8.6) and emulsified in an equal volume of complete *Freund*'s adjuvant (Difco, Detroit, USA). The purity and specifity of the antisera were checked by double immunodiffusion and immunoelectrophoresis using whole serum and purified apolipoprotein fractions.

Light-scattering measurements

Light-scattering was measured with a commercially available laser nephelometer (Behringwerke, Marburg, FRG). The amount of light-scattering was estimated from the potential difference, expressed in volts, on the digital indicator of the instrument. Antisera were diluted in 0.01 mol/l sodium phosphate buffer, pH 7.4. 10-fold dilutions for apolipoproteins A-I and E, and a 40-fold dilution for apolipoprotein B of filtered (Minisart® P, Sartorius, Göttingen, FRG, pore size 0.45 µm) antisera were found to be optimal for routine analysis. Sample dilutions were made with phosphate buffer containing 0.33 g/l hydroxypolyethoxydodecane (Thesit®, Desitinwerke Karl Klinke, Hamburg, FRG, (18)) to remove the turbidity increase artificially induced by triglycerides. Dilutions were 600-fold (apolipoprotein A-I) and 100-fold (apolipoproteins B and E), respectively. The nephelometry was carried out either unenhanced (apolipoproteins A-I and B) or enhanced with 40 g/l polyethylene glycol 6000 (Serva, Heidelberg, FRG, apolipoprotein E). The reaction mixture contained 0.2 ml of diluted antiserum and 0.1 ml of the diluted sample, and was allowed to stand for 2 hours. Apolipoprotein concentrations of serum and of the 1.006 kg/l infranate (only apolipoprotein B assay) were obtained from a frozen serum pool which was calibrated by laser nephelometry against serial dilutions of isolated apolipoproteins and LDL in the Thesit®-phosphate buffer. A lipoprotein-free serum was added to the LDL-standard (19). VLDL-apolipoprotein B was calculated as the difference between the mass of apolipoprotein B in serum and in the infranate.

Apolipoprotein quantitation by electroimmunoassay

Rocket immunoelectrophoresis was done in 12.5 g/l agarose plates using a 0.02 mol/l barbital buffer, pH 8.6, after adding antisera (0.015, 0.007, and 0.03 l/l of apolipoprotein A-I, B, and E antisera, respectively) at 52 °C (20). 5 mm diameter holes for 15 samples were punched at the cathodal end. Serum samples were diluted 100-fold (apolipoprotein A-I) and 20-fold (apolipoproteins B and E) in barbital buffer (with additional 8 mol/l urea for apolipoprotein A-I (21), and 0.33 g/l Thesit® for apolipoproteins B and E). Apolipoprotein concentrations were obtained from standard curves of isolated apolipoproteins and LDL. For staining, Coomassie Blue R 250 (Serva, Heidelberg, FRG) was used. The height of the rockets was measured with a ruler to the nearest 0.5 mm. The coefficients of variation (CV) of these assays ranged from 2% to 5%.

Results

Analytical variables

After dilution in the Thesit®-phosphate buffer, isolated apolipoproteins A-I and E and LDL reacted well with their specific antisera showing a linear relationship between light-scattering and protein concentration. An additional step to expose antigenic sites in serum and infranate samples was not necessary. The within and between assay precision was determined using the calibrated serum standard. Within-run variation was assayed 20 times in one assay. The mean concentrations were 1.31 ± 0.03 g/l, with CV = 2.4%, for apolipoprotein A-I, 0.89 ± 0.03 g/l, with CV = 3.4%, for apolipoprotein B, and 0.098 ± 0.002 g/l, with CV = 2.3%, for apolipoprotein E. Run-to-run variation was assayed 15 times during 3 weeks. The mean concentrations were 1.31 ± 0.06 g/l, with CV = 4.9, for apolipoprotein A-I; 0.89 ± 0.04 g/l, with CV = 4.7%, for apolipoprotein B; and 0.098 ± 0.004 g/l, with CV = 4.7%, for apolipoprotein E. We determined the limit of detection of apolipoproteins by assaying dilutions of calibration standards. Under standard operating conditions the lowest limits of detection were 0.10 g/l for apolipoprotein A-I, 0.05 g/l for apolipoprotein B, and 0.005 g/l

Tab. 1. Serum and lipoprotein lipid and apolipoprotein levels (g/l ± SD) in normal and hyperlipoproteinaemic subjects.

Subjects	Cholesterol			Triglycerides		Apolipoproteins A-I	Apolipoproteins B			E
	Serum	HDL	LDL	VLDL	Serum	VLDL	Serum	Serum	LDL	VLDL	Serum
Normal (n = 20)	1.93 ± 0.30	0.48 ± 0.12	1.31 ± 0.28	0.14 ± 0.09	0.87 ± 0.34	0.42 ± 0.19	1.34 ± 0.09	0.93 ± 0.08	0.83 ± 0.08	0.10 ± 0.03	0.098 ± 0.008
Type IIa (n = 10)	3.27 ± 0.41	0.47 ± 0.14	2.59 ± 0.38	0.12 ± 0.07	0.78 ± 0.19	0.40 ± 0.15	1.36 ± 0.10	1.49 ± 0.12	1.37 ± 0.09	0.12 ± 0.03	0.111 ± 0.010
Type IIb (n = 10)	3.31 ± 0.45	0.36 ± 0.10	2.48 ± 0.38	0.44 ± 0.29	2.77 ± 1.04	1.20 ± 0.70	1.31 ± 0.08	1.51 ± 0.16	1.32 ± 0.14	0.19 ± 0.12	0.108 ± 0.010
Type III (n = 3)	3.94 ± 0.67	0.39 ± 0.11	1.30 ± 0.59	1.98 ± 0.50	4.68 ± 1.31	2.04 ± 1.01	1.32 ± 0.09	1.04 ± 0.17	0.81 ± 0.15	0.23 ± 0.13	0.426 ± 0.022
Type IV (n = 12)	2.32 ± 0.49	0.28 ± 0.08	1.24 ± 0.28	0.65 ± 0.18	4.64 ± 1.67	2.24 ± 0.90	1.28 ± 0.08	1.15 ± 0.11	0.86 ± 0.13	0.29 ± 0.08	0.116 ± 0.013

for apolipoprotein E. The comparison of results obtained by laser immunonephelometry and electroimmunoassay (12 normal and 12 hyperlipoproteinaemic serum samples) resulted in good correlations between the 2 assays with slopes of the regression line close by 1.00 (r = 0.986 for apolipoprotein A-I, r = 0.967 for apolipoprotein B, and r = 0.939 for apolipoprotein E).

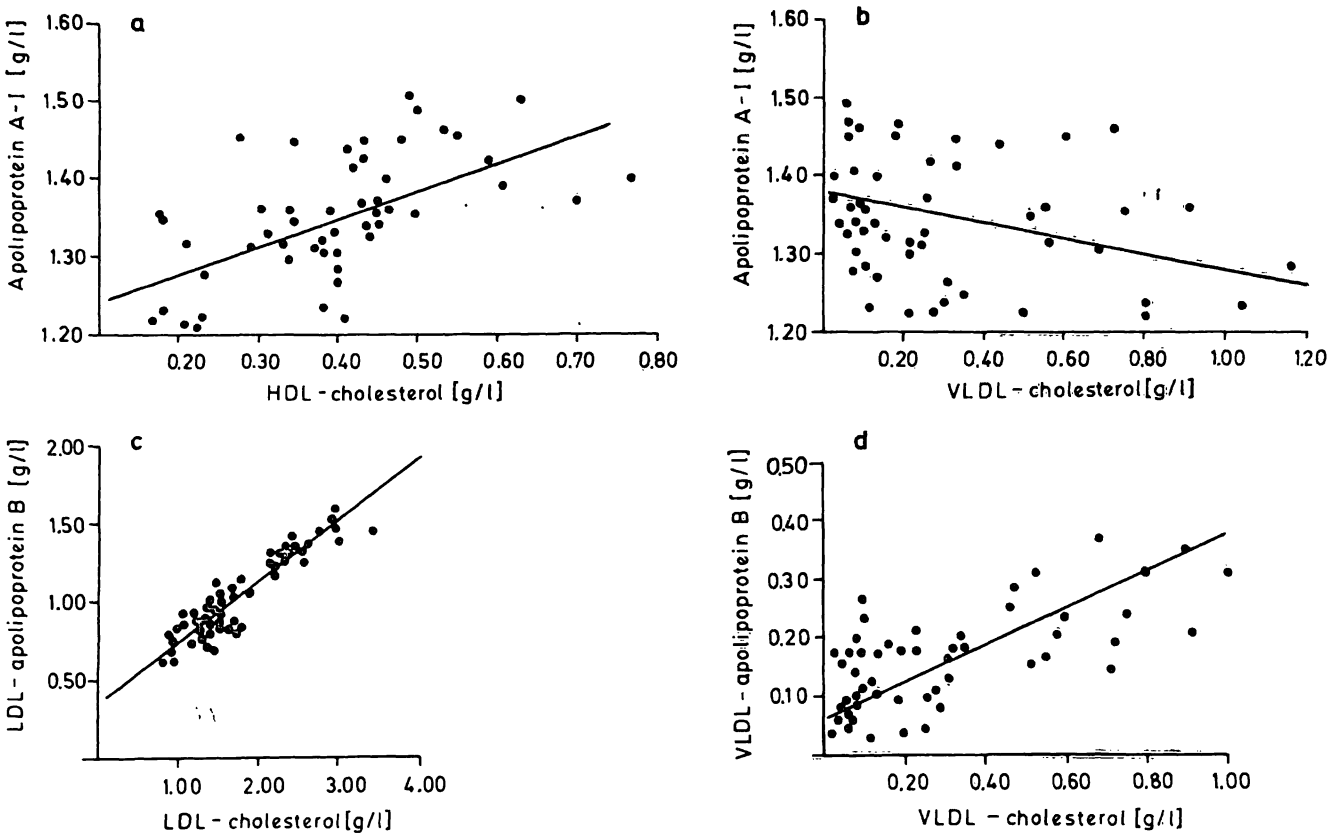
Apolipoprotein concentrations in serum and lipoproteins

Serum and lipoprotein concentrations and mass ratios of cholesterol and apolipoproteins are given in tables 1 and 2. Mean serum apolipoprotein A-I levels in normal and hyperlipoproteinaemic subjects were similar to each other. Mean ratios of HDL-cholesterol to apolipoprotein A-I were significantly lower in Type III and Type IV patients than in normal subjects. The correlation coefficients between all HDL-cholesterol and apolipoprotein A-I values and all VLDL-cholesterol and apolipoprotein A-I values were r = 0.585 and r = −0.266, respectively (correlations except Type III patients, fig. 1a and 1b).

Mean serum apolipoprotein B levels were highest in Types IIa and IIb, indicating increased LDL-apolipoprotein B concentrations. The less marked increase of serum apolipoprotein B levels in Types III and IV implied elevated concentrations of VLDL-apolipoprotein B. LDL-apolipoprotein B represented 89.3, 92.0 and 87.4% of the serum apolipoprotein B in normal, Type IIa, and Type IIb subjects, and

Tab. 2. Mass ratios of lipoprotein cholesterol to apolipoproteins ±SD, $^{+}p < 0.01$.

Subjects	Ratio HDL-cholesterol to apolipoprotein A-I	Ratio LDL-cholesterol to LDL-apolipoprotein B	Ratio VLDL-cholesterol to VLDL-apolipoprotein B
Normal (n = 20)	0.38 ± 0.09	1.59 ± 0.31	1.45 ± 0.99
Type IIa (n = 10)	0.35 ± 0.08	1.79 ± 0.29	1.27 ± 1.01
Type IIb (n = 10)	0.28 ± 0.06	1.74 ± 0.30	2.13 ± 1.60
Type III (n = 3)	0.27 ± 0.03^{+}	1.60 ± 0.21	8.01 ± 2.41^{+}
Type IV (n = 12)	0.22 ± 0.05^{+}	1.55 ± 0.27	2.28 ± 1.43



Figs. 1a–d: Significant relationships between the concentrations of apolipoproteins and cholesterol in serum and lipoproteins (n = 52, $^{+}p < 0.05$, $^{++}p < 0.001$):

	Slope	y-intercept	r
Fig. 1a	0.338	1.213	0.585^{++}
Fig. 1b	−0.089	1.374	-0.273^{+}
Fig. 1c	0.379	0.369	0.935^{++}
Fig. 1d	0.324	0.065	0.472^{++}

77.9 and 74.8% of the total apolipoprotein B in Type III and Type IV subjects. The mean ratios of LDL-cholesterol to LDL-apolipoprotein B were quite similar among groups. LDL-cholesterol was, furthermore, strongly positively correlated with LDL-apolipoprotein B (r = 0.935, fig. 1c). The mean ratio of VLDL-cholesterol to VLDL-apolipoprotein B was highest in Type III, but not significantly different from normal in Types IIa, IIb, and IV. The correlation coefficient between all VLDL-cholesterol and VLDL-apolipoprotein B values was r = 0.472 (fig. 1d).

Mean serum apolipoprotein E levels in Type IIa, Type IIb, and Type IV patients were similar to that in normal subjects. Apolipoprotein E values of patients with Type III hyperlipoproteinaemia were nearly 4-fold higher than the other ones. Apolipoprotein E showed no correlations with either serum lipids or lipoprotein cholesterol.

Discussion

One aim of these studies was to ascertain the advantages of laser nephelometry. Its rapidity, simplicity, and high precision fulfil the criteria required for standard procedures in clinical chemistry. The problem of the accuracy of apolipoprotein A-I and B immunoassays have been recently discussed (5, 6). For the measurement of these apolipoproteins as well as of apolipoprotein E (22) the use of a secondary serum standard has been proposed, because the immunoreactivity of highly purified apolipoprotein might be less stable than that of apolipoprotein in unprocessed serum (23). The presence of detergent in the concentration employed in this assay leads to a disintegration of triglyceride-rich particles (24) which is necessary to avoid nonspecific light-scattering. Preincubation of hypertriglyceridaemic serum samples with lipases can then be omitted. The good agreement between immunonephelometric assay

and electroimmunoassay provides evidence that laser immunonephelometry correctly estimates apolipoprotein values.

Another aim of the study was to obtain serum and lipoprotein apolipoprotein concentrations in normal and hyperlipoproteinaemic states and to evaluate the relations between cholesterol and apolipoproteins. Though quite different methods have been used in the determination of serum apolipoproteins A-I and B, there is a reasonable agreement between the normal values in different studies (reviews, l.c. (5), (6)). With respect to apolipoprotein E, discrepancies of normal values (7) may be related to differences in the apolipoprotein E preparation which is less standardized than the preparation of pure apolipoprotein A-I and LDL-standards.

In hyperlipoproteinaemia, apolipoprotein A-I concentrations have been reported to be similar to normal values (25, 26, 27). Changes in the relative proportions of HDL-components may be due either to different amounts of HDL_2 (28) or to alterations in the composition of HDL-particles (26). However, in hypertriglyceridaemic serum samples a significant part of apolipoprotein A-I is contributed by VLDL, which can be avoided by precipitation of apolipoprotein B-containing lipoproteins prior to immunoassay (29). In accordance with our data a weak negative relationship between VLDL-triglycerides and apolipoprotein A-I has been reported earlier (26).

With respect to apolipoprotein B we could confirm quite similar ratios of LDL-cholesterol to LDL-apolipoprotein B for normal and hyperlipoproteinaemic subjects and an excellent relationship between both parameters (30–33, 35). In spite of ratios of VLDL-cholesterol to VLDL-apolipoprotein B with considerable overlap among groups (except Type III) we found a less strong relationship between these two parameters (r = 0.472) than *Wieland* et al. (r = 0.987 (32)). Other authors found correlation coefficients from 0.198 to 0.690 (33, 34, 35). The reason for these discrepancies may lie in the heterogeneity of the VLDL-composition in different types of hyperlipoproteinaemia, as described for familial combined hyperlipoproteinaemia ("Type IIb") and familial hypertriglyceridaemia ("Type IV") by *Brunzell* et al. (36).

Distinctly elevated apolipoprotein E concentrations in type III hyperlipoproteinaemia have been demonstrated in all reports (22, 37–40). An incomplete catabolism of triglyceride-rich lipoproteins or unusual synthetic products may account for a slight increase of serum apolipoprotein E levels in other forms of hyperlipoproteinaemia (22, 37, 39). Because of the fact that the ultracentrifugation procedure results in the appearance of considerable amounts of apolipoprotein E in the lipoprotein-free fraction of serum (22, 39), a discrimination into VLDL- and HDL-associated apolipoprotein E is problematic. In agreement with *Carlson & Holmquist* (40) no significant relationships could be observed between serum or lipoprotein lipids and apolipoprotein E.

In order to appreciate the predictive ability of serum apolipoproteins in the evaluation of the risk of atherosclerotic vessel diseases, laser immunonephelometry of apolipoproteins allows simple, fast, reliable, and precise investigations. Further studies are necessary to achieve a general standardization of apolipoprotein immunoassays and to establish the advantage of combined measurement of lipids and apolipoproteins.

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