Characterization and quantification of serum lipoprotein subfractions by capillary isotachophoresis: relationships with lipid, apolipoprotein, and lipoprotein levels

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Abstract **Human serum lipoproteins are currently defined according to their density as well as according to their electrophoretic mobility. They can be fractionated into discrete subspecies which exhibit variations in their structure and function. Capillary electrophoresis has been suggested to be a potential analytical strategy in understanding metabolic lipoprotein heterogeneity. In a sample of 35 normolipidemic subjects, we analyzed ceramide-labeled serum lipoproteins by capillary isotachophoresis linked to laser-induced fluorescent detection. Capillary isotachophoresis showed advantage to be an automated, rapid (6 min) and reproducible (CV $<$ 7%) separation mode, on-line monitoring lipoprotein subfractions according to net charge. HDL were separated into three subfractions: *i*) the fast migrating HDL correlated positively with serum apoA-I ($P < 0.05$) and negatively with triglyceride ($P < 0.01$) concentrations, *ii*) the intermediate migrating HDL involved in HDL-cholesterol delivery and inversely related to LDL particles concentration ($P < 0.001$), and *iii*) the slow migrating preβ_1HDL. Triglyceride level was significantly associated with two fractions: *i*) the VLDL fraction correlated positively with apoE serum concentration ($P < 0.01$), and *ii*) the IDL fraction closely and positively associated with apoC-III-containing lipoprotein level ($P < 0.001$). Two LDL subfractions were positively related to LDL-cholesterol ($0.05 \leq P < 0.01$) and might characterize, respectively, small dense and large buoyant LDL subfractions: *i*) the fast migrating LDL, positively linked to apoB concentration and to LpCIII:B ($P < 0.01$) reflecting altered IDL metabolism, and *ii*) the slow migrating LDL. Analytical capillary isotachophoresis of fluorescent-stained lipoprotein subfractions might represent an efficient qualitative and quantitative tool which would afford complementary information on lipoprotein metabolism to current clinical lipoprotein analysis.**—Schlenck, A., B. Herbeth, G. Siest, and S. Visvikis. **Characterization and quantification of serum lipoprotein subfractions by capillary isotachophoresis: relationships with lipid, apolipoprotein, and lipoprotein levels.** ***J. Lipid Res.*** **1999.** 40: **2125–2133.**

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Lipoprotein analysis is essential in the characterization of important indicators of susceptibility to atherosclerosis development. In the fasting state, four main lipoprotein subclasses are generally described according to density: very low, intermediate, low, and high density lipoproteins (VLDL, IDL, LDL, and HDL, respectively) (1). Lipoproteins can be separated by using ultracentrifugation (2), electrophoresis (3, 4), chromatographic (5) and precipitation (6) methods, homogeneous immunoassays (7), and more recently by nuclear magnetic resonance spectroscopy (8). They are then generally quantified according to their cholesterol content (9), or more recently using antibodies specific for their apolipoprotein (apo) content (10). However, each class seems to be a heterogeneous mixture consisting of lipoprotein particles differing in size, density, lipid, and apolipoprotein composition (11, 12). The numerous analytical strategies developed for reporting lipoprotein heterogeneity use different physical properties and consequently are not always in total agreement (13, 14). Moreover, the methods are sometimes technically difficult and tedious, highly sensitive to operating conditions, require specialized equipment and large volumes of fresh plasma or serum samples, and may alter lipoprotein composition. Consequently, further technical improvements are still necessary in order to produce ho-

Abbreviations: apo, apolipoprotein; CE, capillary electrophoresis; cITP, capillary isotachophoresis; CZE, capillary zone electrophoresis; HDL, high density lipoprotein; HDL-C, HDL-cholesterol; fHDL, fast migrating HDL; sHDL, slow migrating HDL; iHDK, intermediate migrating HDL; LDL, low density lipoprotein; LDL-C, LDL-cholesterol; fLDL, fast migrating LDL; sLDL, slow migrating LDL; LpAI, lipoprotein particles containing apoA-I without A-II; LpAI:AII, lipoprotein particles containing apoA-I with apoA-II; LpB:E, lipoprotein particles containing apoE with apoB; LpEnB, lipoprotein particles containing apoE without apoB; LpCIII:B, lipoprotein particles containing apoC-III with apoB; LpCIIInB, lipoprotein particles containing apoC-III without apoB; RPA, relative peak area.

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mogeneous lipoprotein particles with respect to their native forms, compositions, and functionalities.

Recent advances in capillary electrophoresis (CE) technology has led several investigators to examine CE as one of the key analytical methodologies for future apolipoprotein and lipoprotein analysis in very small volumes of biological samples (15–18). However, it implied generally preparative ultracentrifugation steps and did not allow the detailed distinction of lipoprotein subfractions (19).

In 1985, Schmitz, Borgmann, and Assmann (20) were the first investigators to propose a lipoprotein separation from total serum by capillary isotachophoresis (cITP) according to effective mobility of particles. Analytical isotachophoresis differs from standard zone electrophoresis by using a discontinuous electrolyte system. This system is composed of a leading and a terminating electrolyte, which exhibit, respectively, a higher and a slower electrophoretic mobility than the ionic species contained in the mixed sample. The mixed sample is introduced between these two electrolytes. The ionic species separate the original mixed sample zone into pure zones of individual substances according to differences in their electrophoretic mobilities. After complete separation, a steady state exists. This state is formed by a stack of adjacent zones between the leading and the terminating zones, each containing only one susbtance. The zones migrate in the same order of decreasing mobilities of their substance but with the same velocity. The cITP steady state has a special feature of concentrating effect which may be characterized by a constant concentration throughout each sample zone, and directly proportional to concentration of the leading substance (21).

Charge of lipoprotein particles is determined by a direct contribution of negative charge from phosphatidylinositol molecules on the surface of the lipoproteins and by the conformation of the resident apolipoproteins, which is influenced by the presence of neutral lipids and also by the particle shape (22, 23). Applied to lipoprotein analysis, the cITP procedure has the decisive advantage of eliminating molecular size sieving effects occurring in the conventional electrophoresis support media, achieving separation in free solution and producing results in minutes rather than hours. Moreover, it produces a continuous zone sharpening and self-focusing effect, which improves resolution of small charge differences within the lipoparticles and increases fluorescent detection sensitivity of the minor lipoprotein subclasses (24).

According to Schmitz, Möllers, and Richter (25), the cITP procedure might allow simultaneous quantification of HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) concentrations, and also estimate detailed lipoprotein distribution inside HDL (α-apoAI-HDL, preβ-HDL), VLDL (triglyceride-rich lipoproteins and their remnants) and LDL (rich or depleted in cholesteryl esters). Despite the growing interest in understanding lipoprotein heterogeneity and the underlined efficiency of the cITP analysis, no subsequent studies were carried out in this way. Consequently we decided to evaluate the analytical free flow capillary isotachophoresis procedure by comparing the lipoprotein distribution established by cITP to the current lipid, apolipoprotein, and lipoprotein particle measurements. We tested the hypothesis reported in the single development study of the cITP tool, and tried to determine whether it might represent an informative and quantitative approach to determining lipoprotein subfraction metabolism, which might afford more precise and complementary information than conventional analysis.

MATERIALS AND METHODS

Human subjects

Thirty-five healthy subjects (17 men and 18 women), between 23 and 57 years were recruited from a Caucasian population coming to the Center for Preventive Medicine (Vandoeuvre lès Nancy, France) for a health screening after a 12-h overnight fast. They had no history of dyslipidemia, obesity, diabetes mellitus or known diseases, were not taking drugs known to affect plasma lipids (hormone therapy, hypolipidemic drugs) and had only moderate alcohol and tobacco consumption (**Table 1**).

Serum was separated in a 10-ml Vacutainer tube of fasting blood by centrifugation (1000 *g* for 15 min at room temperature). Lipid and apolipoprotein measurements were made on fresh serum, part of which was stored at −196°C for lipoprotein particles and cITP analysis. The study protocol was accepted by the Medical Ethic Committee of the "Centre Hospitalier Régional et Universitaire" of Nancy (France).

Lipid serum analysis

Total cholesterol and triglycerides were measured using standard enzymatic techniques (Merck, Darmstadt, Germany) automated on AU5000 (Olympus, Japan). HDL-C level was quantified using enzymatic measurement in supernatant obtained after precipitation with $MgCl_2$/phosphotungstate, on a Cobas-Mira Centrifugal Analyser (Roche Diagnostic Systems, Neuilly sur Seine, France). LDL-C level was calculated according to the Friedewald equation (26).

Immunological assays of total apolipoproteins and lipoprotein particles

Serum apoA-I and apoB concentrations were determined by immunonephelometry on a Behring Nephelometer Analyser, with Behring reagents (Rueil-Malmaison, France) which were re-

TABLE 1. Age, body mass index, alcohol and tobacco consumption, and lipids, apolipoproteins, and lipoprotein particles levels in the 35 subjects

	Mean	SD
Age (years)	35.2	9.4
Body mass index (kg/m^2)	23.4	3.3
Alcohol (g/day)	11.8	18.6
Tobacco (cig/day)	4.0	9.7
Cholesterol (mmol/l)	5.64	1.24
Triglycerides (mmol/l)	1.24	0.43
HDL-C (mmol/l)	1.37	0.38
LDL-C (mmol/l)	3.62	1.17
ApoA-I (g/l)	1.66	0.27
ApoB (g/l)	1.08	0.32
LpCIII (mg/l)	29.23	8.72
LpE (mg/l)	67.83	21.17
LpAI (g/l)	0.56	0.12
LpAI:AII (g/l)	1.10	0.18
LpB:E (mg/l)	32.90	15.86
LpEnB (mg/l)	34.92	20.63
LpCIII:B (mg/l)	16.06	5.75
LpCIIInB (mg/l)	13.17	5.70

spectively calibrated versus the primary reference material SP1 (apoA-I) and SP3 (apoB) proposed by the IFCC-WHO.

Quantitative determinations of apoA-I-, apoC-III-, and apoE-containing lipoprotein particles were carried out by an electroimmunodiffusion technique in agarose gel containing monospecific antibodies (Sebia, Issy-lès-Moulineaux, France) according to the manufacturer's instructions. Lipoprotein particles containing apoA-I without A-II (LpAI), total apoC-III (LpCIII), lipoprotein particles containing apoC-III without B (LpCIIInB), total apoE (LpE), and lipoprotein particles containing apoE without B (LpEnB) were measured according to rocket heights. Concentrations of lipoprotein particles containing apoA-I with apoA-II (LpAI:AII), apoC-III with apoB (LpCIII:B) and apoE with apoB (LpB:E) were calculated by the difference between apoA-I and LpAI, LpCIII and LpCIIInB, LpE and LpEnB, respectively.

Lipoprotein subfractions analysis by capillary isotachophoresis

Samples preparation. Aliquots of serum stored at −196°C were thawed once before treatment. Briefly, 10 μl of serum was diluted in deionized water (1:3) (v/v) before incubation at room temperature with a half volume of NBD-ceramide solution (0.5 mg/ml) (Molecular Probe, Interchim, Montluçon, France) prepared in polyethylene glycol (Sigma-Aldrich Chimie sarl, Saint Quentin Fallavier, France). Prior to cITP analysis, incubated samples were subsequently mixed (3:5) (v/v) with an amino acid spacers mixture buffered and diluted in the leading buffer (1:5) (v/v). The spacers mixture was composed of ACES, glucuronic acid, octansulfonic acid, TES, tricine, glutamine, methionine, serine (0.16 mg/ml), of glycine (0.08 mg/ml) and of TAPS (0.24 mg/ml). It contained also 5-carboxyfluoresceine (0.08 mg/ml). It was used as a front migration marker in order to correct run-to-run variations. All spacers mixture reagents were obtained from Sigma-Aldrich Chimie sarl (Saint Quentin Fallavier, France).

Capillary isotachophoresis. The discontinuous buffer system consisted of 10 mm HCl as leading electrolyte, and of 20 mm Alanine and saturated $Ba(OH)_2$ as terminating electrolyte. Seventeen mm of 2-amino 1-methyl 1,2-propanediol (Ammediol) was used as a common counterion. In order to stabilize the self-focusing lipoprotein bands, hydroxypropylmethyl cellulose (0.3% HPMC) was added to the leading buffer. All reagents were obtained from Sigma-Aldrich Chimie Sarl (St Quentin Fallavier, France).

A few nanoliters of ceramide-stained serum were injected by pressure (6 sec, 0.5 psi) in a coated fused silica capillary (180 μm id, Lt 27 cm, Ld 20 cm) (DB1 J&W, Alltech France Sarl, Templeuve, France) between the leading and the terminating buffers. We used a P/ACE system 5510 equipped with an argon laser-induced fluorescence detector (λ_{Em} = 488 nm). The CE material was obtained from Beckman Coulter (Fullerton, CA). Separations were performed at 20°C and 10 kV constant voltage for 7 min. The separated zones of pure lipoprotein fractions were detected by fluorescence emission at 520 nm after excitation of ceramide at 488 nm.

CITP lipoprotein peak quantification. CITP lipoprotein subfractions were characterized by their specific relative migration time (RMT) defined by the ratio (absolute peak migration time/peak migration time of the front migration marker). They were quantified according to their peak area by automated integration of the fluorescent signal using the Gold Software (Beckman Coulter, Fullerton, CA). The proportion of each fraction was expressed in terms of percent of the total peak area or relative peak area (RPA %). Based on the formula proposed previously (27) and used by Schmitz et al. (25) to reach a quantitative electrophoretic analysis of lipoprotein subfractions, we tried to estimate cholesterol concentrations in HDL and LDL by applying this percentage to total cholesterol concentration measured by enzymatic assay, for the HDL and LDL peaks groups: $HDLC_{ITP}$ = RPA(1+23+4) × total cholesterol, and $LDLC_{ITP}$ = RPA(7+8) × total cholesterol.

Statistical analysis

Statistical analyses were performed with the Biomedical Computer Program (BMDP) statistical software. First we developed a bivariate regression analysis to calculate Pearson correlation coefficients between RPA of lipoprotein fractions and other serum parameters. In order to distinguish the independent potential predictive factors of cITP measurements and to ascertain the significance of multiple variables, stepwise regression analysis was then performed. Three different models of regression were used. In the "lipid" model, equations were developed for each cITP variable by entering total cholesterol, triglycerides, HDL- and LDL-cholesterol as covariates. In the "apolipoprotein" model only apoA-I, apoB, apoE, and apoC-III were tested. The potential explanatory variables of the "lipoprotein particles" model were LpAI, LpAI:AII, LpB:E, LpEnB, LpCIII:B, and LpCIIInB. *P* level at 0.05 was used.

RESULTS

Description of the cITP lipoproteins profile

Whole serum lipoproteins were characterized by nine individual peaks. In relative intensity, peak 1 represented the major fraction followed by peaks 7 and 8. Peaks 2 and 3 formed a broadened peak and consequently were co-integrated in a single peak 23. Peak 5 represented a minor subfraction, while peak 9 was generally not distinguished from baseline and therefore it was not taken into account (**Fig. 1**).

Between-assays precision was estimated as variation coefficient of duplicate cITP analysis for 25 different serum samples. Inter-run variations remained lower than 0.6% for RMT and ranged between 0.3 and 6.9% for relative peak area. The largest variation coefficient was observed for the minor peak but remained in an acceptable range of reproducibility.

Comparison of paired fresh and frozen samples at −196°C (48 h) of six fasting normolipidemic serum samples was performed by paired Student's *t*-test and did not show any significant freezer-associated bias in the cITP lipoprotein mobilities and distribution (data not shown).

Well defined lipoprotein fractions (HDL, LDL, and VLDL) were initially identified inside the cITP profile by spiking experiments. We overloaded a pooled plasma sample with its constitutive HDL, VLDL, and LDL fractions isolated from the fresh sample by equilibrium density gradient ultracentrifugation in a swinging-bucket rotor. We observed a differential increase of the peak groups [1+23+4], [5], or [6+7+8], respectively, after plasma spiking with ultracentrifugally isolated HDL, VLDL, and LDL fractions (data not shown). The HDL group consisted of fast (fHDL), intermediate (iHDL), and slow (sHDL) migrating HDL fractions characterized, respectively, by peaks 1, 23, and 4, and representing 55.3% of the total lipoprotein profile. Peaks 7 and 8 accounted for 31% of the lipoprotein fluorescence and were, respectively, considered as fast

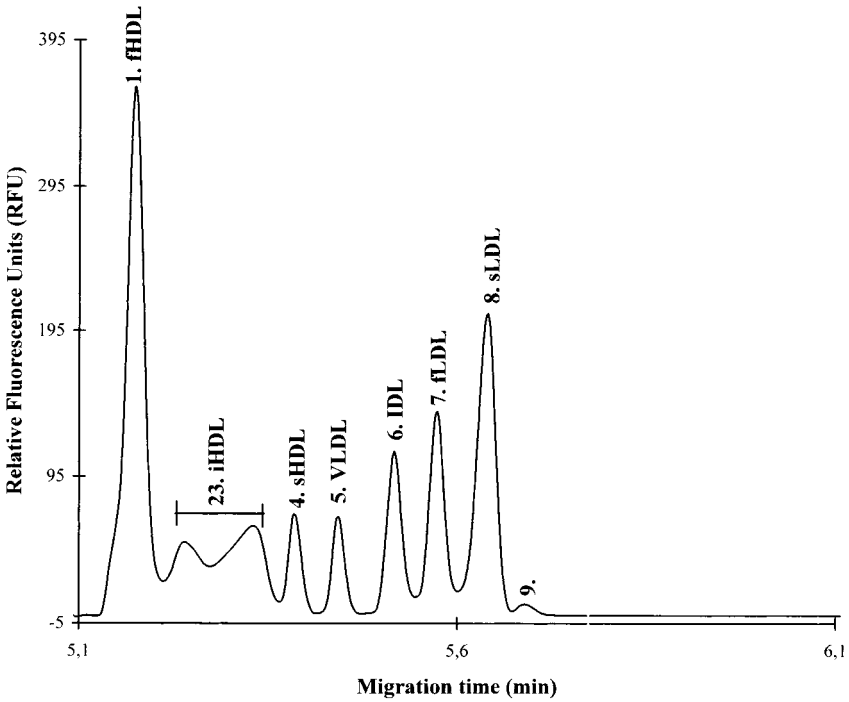

Fig. 1. Representative cITP profile of the lipoprotein subfractions separated from a normolipidemic serum.

(fLDL) and slow (sLDL) migrating LDL subfractions. The 4.3% and 9.1% remaining were attributed to two minor VLDL and IDL subfractions, respectively, separated in peaks 5 and 6 (**Table 2**). Interindividual variations of the cITP lipoprotein profile were characterized by significant differences in relative peak area of the subfractions. No change in lipoprotein peak mobilities was observed ($CV_{RMT} \leq 1\%$).

TABLE 2. Mean proportion of serum lipoprotein subfractions and calculation of cholesterol concentration in HDL and LDL

Peak Number	Relative Peak Area (RPA%) Mean	SD
1	35.7	8.7
23	13.5	4.7
4	6.2	2.3
1234	55.3	10.1
5	4.3	2.2
6	9.1	4.7
7	15.2	6.7
8	15.8	8.1
78	31.0	7.5
9	0.2	0.2
	HDL- and LDL-Cholesterol Content	
	mmol/l	
$HDL\text{-}C_{ITP}$	3.11	0.54
$LDL\text{-}C_{ITP}$	1.75	0.69

Mean proportion of serum lipoprotein subfractions was assessed by fluorescent cITP in 35 subjects, and calculation of cholesterol concentration in HDL ($HDL\text{-}C_{ITP}$) and LDL ($LDL\text{-}C_{ITP}$) by applying total cholesterol concentration to the sum of relative peak areas (1 to 4) and (7 to 8), respectively.

$HDL\text{-}C_{ITP}$ = RPA(1+23+4) × total cholesterol (mmol/l). $LDL\text{-}C_{ITP}$ = RPA(7+8) × total cholesterol (mmol/l).

Relationships between lipoprotein subfractions identified by cITP and conventional lipoprotein variables

HDL subfractions. Using bivariate analysis (**Table 3**), the sum of HDL peaks (1 to 4) was correlated positively with HDL-C level determined by precipitation ($P < 0.05$) and negatively with triglyceride, total cholesterol, and LDLC levels ($P < 0.001$). Individually, both fHDL and iHDL were negatively related to total cholesterol and LDLC concentrations ($P < 0.05$ and $P < 0.001$, respectively, for fHDL and iHDL), while only fHDL was negatively correlated with triglyceride level ($P < 0.05$). Regarding apolipoprotein and lipoprotein particle concentrations, significant negative correlation coefficients were noticed between the sum of HDL peaks and apoB, apoC-III but also LpCIII:B ($P < 0.001$) and LpB:E ($P < 0.05$) serum levels. Individually, only fHDL was significantly negatively related to apoC-III ($P < 0.01$) and LpCIII:B ($P < 0.001$) concentrations, while both fHDL and iHDL were negatively correlated with apoB level ($P < 0.01$ and $P < 0.001$, respectively). In multivariate analysis (**Table 4, Table 5**, and **Table 6**) only LDLC, apoB, and LpCIII:B levels were significant predictors of the sum of HDL peaks ($P < 0.001$), accounting, respectively, for 35.1, 38.2, and 35.7% of its global variance. Inside the HDL group, the three separated fractions had distinct behaviors. The multivariate analysis revealed that the major independent determinants of fHDL were total triglyceride concentration ($P < 0.01$) and apoA-I ($P < 0.05$) after adjustment on apoC-III serum level, reflecting the negative association obtained

TABLE 3. Correlation coefficients established by bivariate analysis between cITP lipoprotein subfractions and their potential lipid, apolipoprotein, and lipoprotein particle predictors

	cITP Lipoprotein Subfractions								
	1 fHDL	23 iHDL	4 sHDL	1234 HDL	5 VLDL	6 IDL	7 fLDL	8 sLDL	78 LDL
Lipids									
Cholesterol	−0.35[a]	−0.59[c]	—	−0.56[c]	—	+0.37[a]	+0.46[c]	—	+0.47[c]
Triglyceride	−0.40[a]	—	—	−0.50[c]	+0.35[a]	+0.68[c]		—	—
HDL-C	—	—	—	+0.34[a]	—	—	—	—	—
LDL-C	−0.37[a]	−0.66[c]	—	−0.61[c]	—	+0.37[a]	+0.47[c]	+0.32[a]	+0.54[c]
Apolipoproteins									
ApoA-I	—	—	—	—	—	—	—	—	—
ApoB	−0.42[b]	−0.60[c]	—	−0.62[c]	—	+0.48[c]	+0.52[c]	—	+0.47[c]
ApoC-III	−0.43[b]	—	—	−0.54[c]	+0.35[a]	+0.44[b]	+0.37[a]	—	+0.35[a]
ApoE	—	—	—	—	+0.46[c]	—	—	—	—
Lipoprotein particles									
LpAI	—	—	—	—	—	—	—	—	—
LpAI:AII	—	—	—	—	—	—	—	—	—
LpCIII:B	−0.57[c]	—	—	−0.60[c]	+0.36[a]	+0.59[c]	+0.40[a]	—	—
LpCIInB	—	—	—	—	—	—	—	—	—
LpB:E	—	—	—	−0.37[a]	+0.38[a]	+0.36[a]	+0.33[a]	—	—
LpEnB	—	—	—	—	—	—	—	—	—

Dash, nonsignificant; [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

with lipoprotein particles containing apoB and apoC-III ($P < 0.001$). iHDL proportions were directed by LDL-C level (44.1%) and concentration of apoB-containing lipoproteins without association with apoC-III or apoE (35.8%). sHDL proportion did not correlate significantly with any lipid or apolipoprotein levels.

VLDL subfraction. By bivariate analysis, VLDL proportion was positively correlated with triglyceride ($P < 0.05$), apoE ($P < 0.001$), apoC-III, LpCIII:B, and LpB:E ($P < 0.05$) concentrations (Table 3). In multivariate analysis, 35.3% of VLDL global variance was explained by triglyceride level. VLDL variations were positively and independently related to the apoB-containing LpE ($P < 0.001$) and to the apoB-depleted-LpE ($P < 0.05$) subfractions (Tables 4, 5, and 6).

IDL and LDL subfractions. In bivariate analysis (Table 3), total cholesterol and LDLC were positively correlated with peaks 6 ($P < 0.05$) and 7 ($P < 0.001$). Peak 8 was also related to LDL-C ($r = 0.32$) although this relationship was borderline significant ($P = 0.05$). Peak 6 showed a supplementary positive correlation with triglyceride level ($P < 0.001$). Regarding apolipoprotein measurements, peaks 6 and 7 were positively correlated with apoB and apoC-III concentrations ($P < 0.01$ and $P < 0.05$, respectively, for peaks 6 and 7). Peak 6 was more closely positively related to apoB-lipoprotein particles containing apoC-III ($P < 0.001$) and apoE ($P < 0.05$) than peak 7 (p 0.05). No significant correlation was established with peak 8. By multivariate analysis (Tables 4, 5, and 6) we accounted for 46.3% of global variance of peak 6 by triglyceride concentration while the variability of the sum of LDL peaks (7 and 8) was governed by LDL-C level (29.3%). According to apolipoprotein and lipoprotein particle concentrations, the most predictive factor of peak 6 was apoB-containing LpCIII ($P < 0.001$). ApoB-containing lipoprotein depleted of other apolipoproteins preferentially determined the sum of LDL peaks ($P < 0.01$) while lipoprotein particles containing apoB and apoC-III were more significant predictors of peak 7 ($P < 0.01$), individually.

TABLE 4. Predictors of cITP lipoprotein subfractions in multivariate analysis (lipid model)

	Independent Explanatory Variables Regression Coefficient (± Standard Error)				
Peaks	Triglycerides	HDL-C	VLDL-C	LDL-C	Intercept Total R^2
fHDL	−8.1 (3.23)[b]	—	—	—	16.2
iHDL	—	—	—	−2.68 (0.52)[c]	44.1
sHDL	—	—	—	—	—
HDL	—	—	—	−5.22 (1.19)[c]	35.1
VLDL	+1.77 (0.81)[a]	—	—	—	35.3
IDL	+7.38 (1.38)[c]	—	—	—	46.3
fLDL	—	—	—	+3.49 (1.14)[b]	21.9
sLDL	—	—	—	—	—
LDL	—	—	—	+3.45 (0.93)[c]	29.3

R^2, parts of variance of RPA explained.
[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

CITP quantification of HDL- and LDL-cholesterol content

Using spiking experiments we observed that the addition of 1 mmol/l HDL-C led to a 2.5-fold higher increase in the corresponding relative peaks area than the addition of 1 mmol/l LDL-C. When we expressed cholesterol concentration in the HDL fraction according to the sum of the relative areas of peaks 1 to 4, the linear correlation calculated between HDL-C_{ITP} (Table 2) and HDL-C measurements reached significance ($r = 0.33$, $P < 0.05$) although it revealed large differences in the range of concentrations. A similar phenomenon was observed with apoA-I levels despite a significantly better correlation ($r = 0.41$, $P < 0.01$). On the contrary, LDL cholesterol calculations according to Friedewald and cITP (Table 2) formulas were in good agreement ($r = 0.87$, $P < 0.001$). LDL-C_{ITP} estimation was also totally concordant with the apoB levels ($r = 0.80$, $P < 0.001$).

TABLE 5. Predictors of cITP lipoprotein subfractions in multivariate analysis (apolipoprotein model)

Peaks	Peaks	Independent Explanatory Variables Regression Coefficient (± Standard Error) ApoA-I	ApoB	ApoC-III	ApoE	Intercept Total R^2
fHDL	1	+10.79 (5.22)[a]	—	−0.56 (0.16)[c]	—	28.0
iHDL	23	—	−8.84 (2.06)[c]	—	—	35.8
sHDL	4	—	—	—	—	—
HDL	1234	—	−19.47 (4.31)[c]	—	—	38.2
VLDL	5	—	—	—	+0.05 (0.02)[b]	20.9
IDL	6	—	+6.97 (2.23)[b]	—	—	22.9
fLDL	7	—	+9.34 (3.26)[b]	—	—	20.0
sLDL	8	—	—	—	—	—
LDL	78	—	+11.05 (3.58)[b]	—	—	22.4

R^2, parts of variance of RPA explained.
[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

DISCUSSION

The present work describes an automated procedure for the analysis of plasma lipoproteins by using capillary isotachophoresis. In agreement with the single previous evaluation of the cITP analysis of serum lipoprotein fractions (25), we observed that this system allowed, in less than 6 min and with a good reproducibility (CV < 7%), the separation of the main lipoprotein groups with respect to the consensus lipoprotein classification: HDL, VLDL, and LDL.

As lipoprotein cholesterol content is usually considered the most accurate and informative parameter to quantify lipoprotein groups, Schmitz et al. (25) tried to estimate both HDL-C and LDL-C by applying serum cholesterol concentration, respectively, to the relative HDL- and LDL-fluorescent peaks area. They published high correlation coefficients between usual techniques for HDL-C and LDL-C quantification and cITP calculations, and concluded that ceramide staining was directly proportional to lipoprotein cholesterol content. In our study a high order and statistically significant correlation was observed between LDL-cholesterol calculations according to Friedewald and cITP formulas ($r = 0.87$, $P < 0.001$), and also with serum apoB concentration measured by immunoturbidimetric assay ($r = 0.80$, $P < 0.001$). A negative intercept and a slope lower than 1 in the linear regression curves were established for LDL-C and apoB, and suggested that the cITP method could underestimate LDL-cholesterol. Martin et al. (28) emphasized that cholesterol was implicated in the potentiation of NBD-ceramide fluorescence and suggested that this fluorescent lipophilic dye may be used to monitor cholesterol in cells. However, in normolipidemic subjects, LDL is the major cholesterol-carrying lipoprotein in serum (>50%) (29). On the contrary, in our cITP analysis, we noticed that ceramide-bound LDL represented only 31% of the lipoprotein pattern, similar to the previous study (25). NBD-ceramide is a lipophilic dye which specifically labels serum lipoproteins according to their size and surface area, the staining being not proportional to the lipoprotein cholesterol content. The spiking experiment revealed that HDL and LDL particles exhibited different chromogenicities, as previously observed for other dyes (30), and that ceramide would be bound preferentially to smaller rather than to larger lipoproteins. However, in LDL, fluorescence emission seemed to be proportional to LDL cholesterol and then to apoB contents. Nevertheless, we must keep in mind that these relationships were influenced by the cholesterol-related factor, included in the Friedewald formula and strongly correlated to apoB level ($r = 0.91$, $P < 0.001$).

The HDL-C_{ITP} calculation was significantly related to HDL-$C_{precipitation}$ ($r = 0.33$, $P < 0.05$) and to apoA-I serum concentrations ($r = 0.41$, $P < 0.01$), but low orders of cor-

TABLE 6. Predictors of cITP lipoprotein subfractions in multivariate analysis (lipoprotein particle model)

Peaks	Peaks	Independent Explanatory Variables Regression Coefficient (± Standard Error) LpAI	LpAI:AII	LpCIII:B	LpCIIInB	LpB:E	LpEnB	Intercept Total R^2
fHDL	1	—	—	−0.87 (0.22)[c]	—	—	—	32.7
iHDL	23	—	—	—	—	—	—	—
sHDL	4	—	—	—	—	—	—	—
HDL	1234	—	—	−1.05 (0.25)[c]	—	—	—	35.7
VLDL	5	—	—	—	—	+0.07 (0.02)[c]	+0.04 (0.02)[a]	25.5
IDL	6	—	—	+0.48 (0.11)[c]	—	—	—	35.1
fLDL	7	—	—	+0.55 (0.18)[b]	—	—	.—	22.1
sLDL	8	—	—	—	—	—	—	—
LDL	78	—	—	—	—	—	—	—

R^2, parts of variance of RPA explained.
[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

relation were reached. These results suggested that NBD ceramide-fluorescence did not exhibit a good proportionality with HDL cholesterol content. Discrepancies between the two methods of HDL-C evaluation might be related *i*) to a lack of analytical sensitivity of the cITP calculation, which could not distinguish small variations within a normal range of HDL-C levels, but also *ii*) to the highly variable selectivity of apoB-containing lipoprotein precipitation and of apoE-rich HDL recovery efficiency, using commercial reagents for chemical precipitation (31–33).

The lack of significant effect of freezing on cITP lipoprotein pattern tended to demonstrate that bias due to preanalytical storage did not seem to be involved in the differences observed between the two methods.

To our knowledge this work is the first which tests the cITP analysis and formulas previously developed by Schmitz et al. (25) to quantify HDL- and LDL-cholesterol content. Results are not totally in agreement and will await further confirmation, i.e., by using direct HDL-C and LDL-C measurements in larger ranges of cholesterol concentrations. At this stage, it seems that we could not consider cITP as an accurate method to determine HDL- and LDL-cholesterol concentrations in serum samples, and that lipoprotein ceramide fluorescence emission would not be directly proportional to lipoprotein cholesterol content, particularly in HDL. Nevertheless, the reproducibility of the lipoprotein peaks mobility observed within our normolipidemic population would reflect the structural and compositional stability of the cITP lipoprotein fractions properties, and led us to consider fluorescence as a valuable index of lipoprotein particles number variations. Moreover, the analytical free flow capillary isotachophoresis showed a more resolving power than current electrophoresis media (agarose, polyacrylamide) (29, 34) and would simultaneously characterize subtle lipoprotein subfractions inside the HDL, LDL, and VLDL groups. Their distributions were significantly correlated to current lipid, apolipoprotein, and lipoprotein particle concentration and might reflect informative changes in lipoprotein metabolism.

The HDL group is composed of three subfractions: fast (fHDL), intermediate (iHDL), and slow (sHDL) migrating HDL. Total RPA of the whole HDL group was directly related to HDL-$C_{precipitation}$, but we noticed that the three HDL subfractions had distinct behaviors. Only fHDL represented the bulk of apoA-I serum concentration ($P < 0.05$). Its proportion was also inversely linked to triglyceride ($P < 0.01$) and LpCIII:B levels ($P < 0.001$). These results confirmed the sequential electrophoresis of fHDL on agarose and polyacrylamide (25) and fHDL compositional analysis in analytical preparative free flow isotachophoresis (35). Previous studies have suggested that elevated triglycerides and accumulation of triglyceride-rich lipoprotein remnants (LpCIII:B) are a major independent cause of depressed HDL-C (36) but this relationship is not due to the whole HDL spectrum. HDL_3 plasma levels are rather constant among individuals whereas HDL_2 levels vary greatly and account for most of the variability of total HDL-C (37). Consequently, we might suppose that fHDL would represent αHDL_3 fraction and contain HDL_2 as a major contributor for its variance.

The second iHDL subfraction, identified according to its intermediate mobility, was essentially linked to low LDL-cholesterol ($P < 0.001$) and pure apoB serum ($P < 0.001$) concentrations. These results agree with the potential role of iHDL in receptor-mediated delivery of HDL cholesterol to the liver (35, 38) and with modulation of LDL catabolism by effective clearance of iHDL through down-regulation of hepatic LDL receptors (39).

Interestingly we were unable to characterize the third HDL population by any lipoprotein variables. This lack of correlation could be consistent with the hypothesis that the fourth cITP peak might contain $pre\beta_1HDL$. $Pre\beta_1HDL$ analysis cannot be performed by any routinely used clinical measurements and usually requires HDL fractionation in term of net charge and high sensitive detection (40).

It is now well established that HDL exist in plasma as several subfractions that differ in physicochemical and functional properties. They can be interconverted during the metabolism of other lipoproteins, which control their concentration in plasma. Triglyceride metabolism and the factors that regulate triglyceride-rich lipoprotein catabolism might be determinants of HDL distribution in serum (41). It is interesting then to assess the serum proportion of triglyceride-rich VLDL at the same time.

Using cITP we characterized a single subfraction of very low density lipoprotein, which well reflected the triglyceride level ($P < 0.05$). In agreement with the previous analysis (42), we observed that its peak area was correlated to the serum apoE level ($P < 0.01$). LpB:E particles, which are the major component of large VLDL (43), can be considered as a significant determinant of this subfraction ($P < 0.001$). However, the LpEnB particle also seems to be implicated ($P < 0.05$) and might reflect the importance of apoE transfer from HDL towards VLDL in VLDL catabolism (12, 44).

Interestingly, the most important impact of triglyceride level was observed on the cITP peak 6 ($P < 0.001$). Using cITP we can clearly distinguish IDL from VLDL and LDL particles. Nowicka et al. (42) reported that this subfraction was enriched in apoB and depressed in apoC-III. We observed in our study that LpCIII:B serum level was a more informative predictor of IDL proportion than apoB. However, these results remain consistent with the LpCIII:B increase which occurs during defective lipolysis and catabolism of triglyceride-rich lipoproteins and which leads to IDL accumulation (45). Therefore, the cITP measurement might be considered as a promising method in evaluating triglyceride-rich lipoprotein remnant enrichment of the lipoprotein pattern. Nevertheless, we observed that measurement of this fraction showed one of the most important analytical variabilities. Consequently, further studies will be necessary to demonstrate the clinical significance of this IDL measurement, despite a lack of precision.

Finally, the LDL group is subdivided into a fast- (fLDL) and a slow- (sLDL) migrating fraction. Total RPA of the LDL group was directly related to LDL-$C_{Friedewald}$ ($P < 0.001$) and apoB ($P < 0.01$) concentrations, but individu-

ally the two subfractions were involved differently in these relationships.

In 1982, Krauss and Burke (46) provided firm evidence that LDL was structurally heterogeneous. Different analytical strategies have subsequently been developed for reporting LDL heterogeneity and have led to a consensus model. It consists of a continuous spectrum of particles from the larger buoyant cholesteryl ester-rich subfraction (LDL I) to the smaller denser cholesteryl ester-depleted subfraction (LDL IV), associated with a decreasing ratio of (cholesteryl ester / apoB). The predominance of the large LDL II subfraction characterizes the "normolipidemic" pattern A, while the shift toward pattern B is associated with LDL III enrichment. Serum LDL-C level was negatively correlated to LDL I, and positively correlated to LDL II and LDL III (47). Increased apoB concentrations were associated to LDL I decrease and to LDL III increase, reflecting LDL particles accumulation as a consequence of reduced binding to LDL receptors (47–49). However, no relationship was established with LDL II. Moreover, increased levels of small, cholesteryl ester-depleted LDL particles were associated with redistribution of apoC-III from HDL to apoB-containing lipoproteins (LpCIII:B) (50).

In our study, while LDL-C concentration was a significant determinant of fLDL and, in a lesser extent, of sLDL, only fLDL was associated with apoB level. We observed also that LpCIII:B was the most predictive factor of fLDL, as IDL subfractions, suggesting that fLDL would be indicative of a high number of LDL particles and would derive from intravascular remodeling of IDL. Neither apolipoprotein nor lipoprotein particle measurements allowed us to predict significantly sLDL variations, which was the second largest peak in the cITP spectrum in our normolipidemic population. Moreover quantitative electrophoretic measurements of LDL subfractions with different densities indicated that the small dense LDL are the most negatively charged particles (51). Consequently, in agreement with a recent study (52), we suggest that sLDL might characterize the LDL II while an increased level of fLDL might be related to a shift toward dense LDL particles exhibiting a prolonged plasma residence time. Therefore, the pedigree of the two LDL subfractions separated by cITP and the relative prevalence of the faster migrating LDL or of the slower migrating LDL subfractions might be consistent with the proposed model describing LDL heterogeneity.

Conclusion

Based on our results, we suggest that the use of capillary isotachophoresis linked to laser-induced fluorescent detection for lipoprotein analysis, after ceramide labeling of whole serum, might offer the potential to measure lipoprotein subfractions simultaneously with respect to their function. We could distinguish the bulk of α-apoA-I HDL in serum from a subfraction involved in HDL-cholesterol delivery and a slow-migrating preβ_1HDL important in the efflux of cell-derived cholesterol. Two LDL subfractions would differentially characterize LDL-cholesterol and apoB serum levels, reflecting the well-described LDL heterogeneity clinically important in cardiovascular risk assessment. Triglyceride-rich lipoproteins and their remnants would be major determinants of the type and quantity of HDL and LDL subfractions present in serum. Therefore it would become useful to follow triglyceride metabolism through the VLDL fraction reflecting apoE serum concentration and the IDL fraction closely linked to apoC-III metabolism. Consequently, rather than a steady state measurement of serum lipoproteins, the cITP procedure might become an efficient analytical strategy in understanding the interrelated dynamic metabolism of lipoproteins along with their heterogeneity.

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