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ANALYTICAL CAPILLARY ISOTACHOPHORESIS: A ROUTINE TECHNIQUE FOR THE ANALYSIS OF LIPOPROTEINS AND LIPOPROTEIN SUBFRACTIONS IN WHOLE SERUM

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SUMMARY

A capillary isotachophoretic separation technique was developed for lipoproteins in native serum which, compared with previous electrophoretic techniques, has negligible molecular sieve effects, does not need gel casting, is suitable for whole serum and has a high discriminative power for lipoprotein subfractions. The technique is based on pre-staining whole serum lipoproteins for 30 min at 4°C before separation of 0.5 μl of the sample in a free-flow capillary system (0.5 mm I.D.) with a discontinuous buffer system.

In normolipidaemic sera, high-density (HDL) and low-density lipoproteins (LDL) are separated into two major subpopulations according to their net electric mobility. The identification of these fractions was confirmed by substitution with ultracentrifugally isolated lipoproteins and by their complete absence from Tangier- and abetalipoproteinaemic serum. Triglyceride-rich very low-density lipoproteins (VLDL) revealed a defined zone between the HDL and LDL subpopulations. Our preliminary results indicate that the separation of human whole serum lipoproteins by capillary isotachophoresis is a promising method for the determination of lipoprotein subfractions.

INTRODUCTION

Human plasma lipoproteins are heterogeneous in size, density and lipid composition[1,2]. Numerous methods have been described for the separation of human serum lipoproteins, including ultracentrifugation[3–6], polyanion precipitation[1,7–8] and electrophoretic techniques[10–17]. However, only polyanion precipitation and some of the electrophoretic techniques allow the analysis of large numbers of samples. Polyanion precipitation yields quantitative data, as demonstrated for the determination of high-density lipoprotein (HDL) cholesterol[1], but the available precipitation methods are still restricted to the direct determination of the HDL fraction[1].

The available electrophoretic methods are agarose electrophoresis[12,17,18], discontinuous polyacrylamide gel electrophoresis[10,11], gradient gel electrophoresis[13] and isoelectric focusing[14–16]. These methods involve either pre-staining of native lipoproteins with lipophilic dyes prior to electrophoresis[19–21] or post-electrophoretic

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detection by polyanion precipitation[17], staining with lipid or protein dyes[20] or treatment with enzymatic cholesterol reagent[22], followed by densitometric analysis.

We have developed a method for the separation of lipoproteins and lipoprotein subfractions using capillary isotachophoresis[23,24]. This method is very sensitive (picomole range), has negligible molecular sieve effects, is relatively fast (10 min for whole serum lipoprotein analysis) and has a high discriminative power.

EXPERIMENTAL

The isotachophoretic separations were performed on a modified LKB 2127 Tachophor system (LKB, Bromma, Sweden). The instrument was equipped with 20–25 cm PTFE capillaries of 0.5 mm I.D., which were cooled by an air stream (adequate cooling of the capillary system is very important). Detection was effected at 570 or 280 nm. The optical signals were recorded on a LKB 2210 two-channel recorder or directed to a Hewlett-Packard 9826A desk-top computer, which stores the data on a 5.25-in. floppy disc, has a graphic display and produces hard copy on a Hewlett-Packard 2671 graphic printer.

Serum preparation

Serum was prepared from venous blood of patients and from normolipidaemic volunteers after clotting and subsequent centrifugation for 10 min at 800 *g*. The samples were analysed within 24 h after taking the blood.

Staining

Two lipophilic dyes were used: Fat Red 7B (Sigma, St. Louis, MO, U.S.A. Cat. No. F-1000) and Sudan Black B (Merck, Darmstadt, F.R.G.). Fat Red 7B was dissolved at a concentration of 5 g/l in dimethylformamide (Merck) and 25 μl of this stock solution were activated immediately before use by adding 5 μl of 0.1 *M* sodium hydroxide, and one drop of Triton X-100 (Merck). A 1-μl volume of this dye solution is sufficient to pre-stain the lipoproteins in 175 μl of serum[19]. Sudan Black B was dissolved to give a 1% solution in ethylene glycol (Merck), stirred for 3 min at 65°C and filtered. The dye solution was mixed with the serum in a ratio of 1:2 and incubated for 30 min at 4°C.

Preparation of leading and terminating electrolytes

All chemicals used were of analytical-reagent grade. The water was doubly distilled in an all-quartz apparatus. Histidine, β-alanine and hydroxypropylmethylcellulose (HPMC) were supplied by Sigma. HPMC was dissolved to a concentration of 1% in glass-distilled water with stirring for 3 days at 4°C, then dialysed against glass-distilled water. The stock solution can be stored in glass bottles at 4°C.

The standard leading buffer contained 5 m*M* hydrochloric acid and 0.2% of HPMC, which was added in order to reduce electroendosmosis. The solution was titrated to pH 6.5 with histidine.

The standard terminating electrolyte contained 10 m*M* β-alanine and 10 m*M* histidine, adjusted to pH 9.0 with saturated barium hydroxide. Other buffer systems evaluated for suitability are described in the Results section.

Separation conditions

The equipment was operated at constant current, starting at 100 μA and *ca.* 4 kV. After 6 min (equivalent to about 18 kV) the current was reduced to 50 μA. The analysis of one sample required *ca.* 12 min while the voltage increased to 20 kV.

Zone identification

Plasma proteins. For the identification of the separated zones at 280 nm, purified human plasma proteins, purified albumin, transferrin β-globulin (Cohn fraction IV 1) and γ-globulins (Cohn fraction II) purchased from Sigma were used.

Lipoprotein fractions. Lipoprotein fractions [very low-density lipoprotein (VLDL), $d < 1.006$, low-density lipoprotein (LDL), $d = 1.019$–1.063, HDL_2 $d = 1.063$–1.125, and HDL_3, $d = 1.125$–1.21 g/ml KBr) were isolated from sera of normolipidaemic volunteers and patients with hypertriglyceridaemia by sequential ultracentrifugation at 4°C using a 50.3 Ti rotor at 49.000 rpm in a Beckman L8-70 ultracentrifuge[25]. All separated lipoprotein fractions were dialysed exhaustively against 0.9% sodium chloride.

Other methods

Lipid analysis of sera was performed on a Cobas-Bio centrifugal analyser (Hoffmann La Roche, Basle, Switzerland). Triglycerides were determined by the "Rapid Test"(Hoffmann La Roche). For total and free cholesterol, the CHOD–PAP method (Boehringer, Mannheim, F.R.G.) was used. Esterified cholesterol was calculated from the difference between the total and free cholesterol values.

Patients

Normal sera were obtained from normolipidaemic male and female students. The patients affected with Tangier disease (I, 51 years old, female, total cholesterol 64 mg/dl, triglycerides 356 mg/dl; II, 49 years old, male, total cholesterol 36 mg/dl, triglycerides 359 mg/dl) or affected with abetalipoproteinaemia (III, 29 years old, male, total cholesterol 27 mg/dl, triglycerides 2 mg/dl; IV, 22 years old, male, total cholesterol 27 mg/dl, triglycerides 7 mg/dl) have been described previously[26,27]. The other patients mentioned in this study were hospital patients whose sera were sent to our laboratory for routine clinical chemical analysis.

RESULTS

At present, four different types of detection systems are used in isotachophoretic analyses: UV, conductometric, potential gradient and thermometric detectors. However, none of these systems permits a specific distinction between lipoproteins and other serum protein fractions. In our studies, we evaluated the specific detection of lipoproteins in native serum. We tested a number of lipophilic dyes[19,21] that have previously been shown to selectively stain lipoproteins without significantly changing the electrophoretic properties of the various serum lipoprotein fractions[28]. Two of the available lipophilic dyes are suitable for use in capillary isotachophoresis. Both Sudan Black B and Fat Red 7B are diazo dyes with absorption maxima at 600 and 527 nm, respectively. Fig. 1 shows that Sudan Black B pre-staining (A) yields far more pronounced photometric signals than Fat Red 7B (B) with the same sera at a

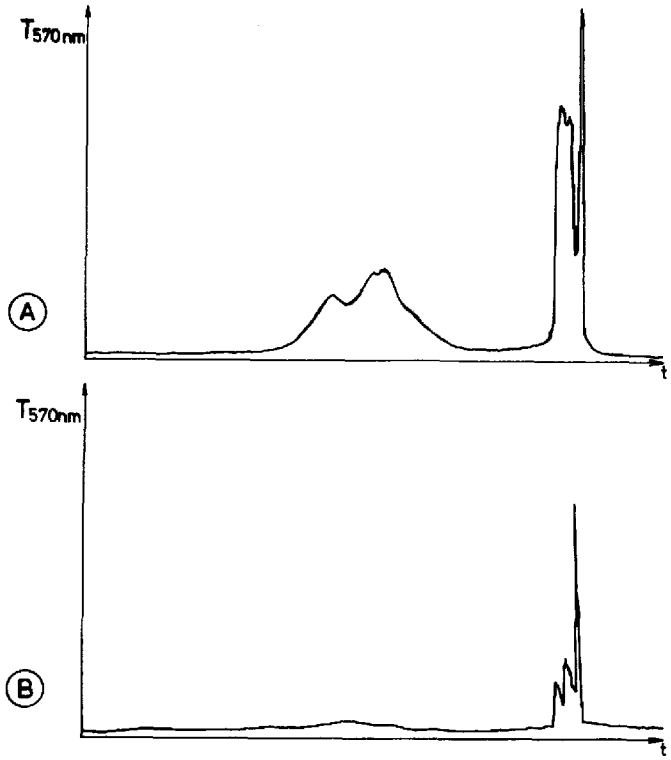

Fig. 1. Isotachophoretic patterns of sera pre-stained with lipophilic dyes. (A) Separation pattern at 570 nm of 1 μl of serum after incubation with Sudan Black B for 40 min at 4°C. (B) Separation pattern at 570 nm of 1 μl of serum after incubation with Fat Red 7B for 40 min at 4°C. Unstained sera exhibited no absorbing zones at this wavelength.

wavelength of 570 nm and identical settings of the photometric system. Hence we used the Sudan Black B pre-staining technique for all of the subsequent analyses. The staining properties of Sudan Black B dye reveal high sensitivity for cholesteryl esters, followed by triglycerides. Phospholipids and free cholesterol, however, have a lesser affinity towards Sudan Black B[21].

In order to establish the optimal staining time and stability of the lipoprotein–dye complexes, sera were incubated for different time intervals at 4°C with a defined concentration of the dye solution prior to isotachophoretic analysis. As demonstrated in Fig. 2, the optimal association of the dye is reached within 30 min of incubation at 4°C (A), and there is a relatively stable peak pattern up to 6 h (B and C); longer incubation times, however, revealed a change in the lipoprotein pattern (D).

In order to find the optimal leading and terminating buffer systems, various combinations were tested. The leading buffer system employed contained 5 m*M* hydrochloric acid and 0.2% HPMC and the pH was varied between 6.0 and 9.2 by the addition of histidine (pH 6.0–6.8), Tris (pH 7.2–8.4), or ammediol (pH 8.8–9.2) counter ions. A leading buffer pH of 6.5 with histidine as the counter ion yielded the best

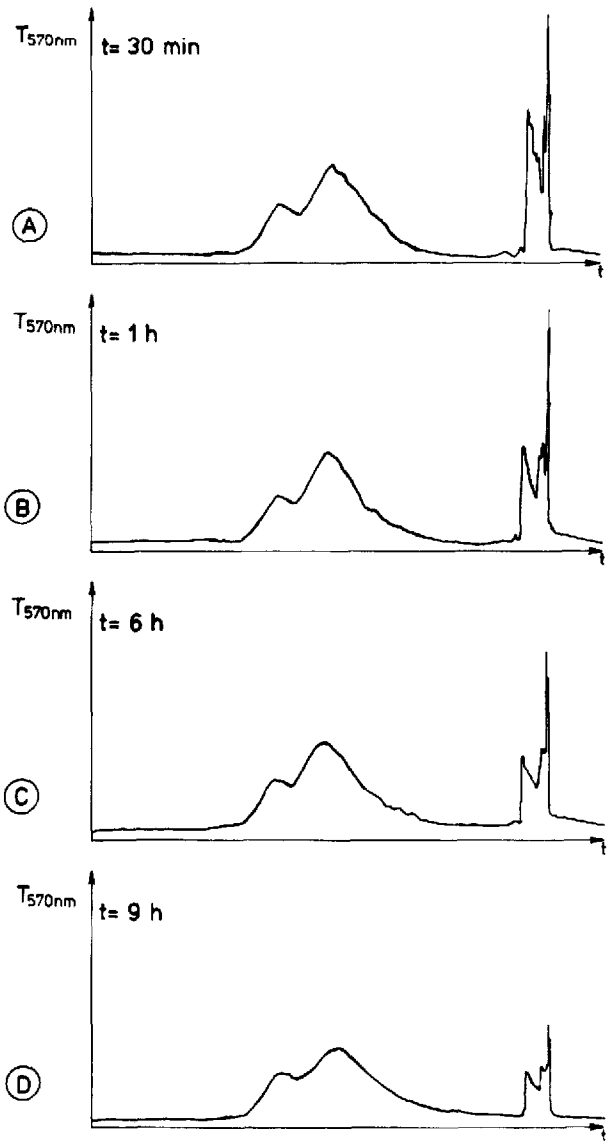

Fig. 2. Isotachophoretic pattern of sera incubated for different time periods with Sudan Black B (570 nm). (A) Isotachophoretic scan of 1 μl of serum after incubation with Sudan Black B for 30 min. at 4°C. (B) Patttern of 1 μl of the same serum after incubation with Sudan Black B for 1 h at 4°C. (C) Same as in A and B after incubation with Sudan Black B for 6 h at 4°C. (D) Same as A, B and C after incubation with Sudan Black B for 9 h at 4°C.

resolution of the individual zone groups. The terminating buffer system employed contained 10 m*M* histidine and 10 m*M* β-alanine and its pH was varied between 8.0 and 10.0 by the addition of barium hydroxide; a terminating pH value of 9.0 gave the best results. In addition to the variation in pH, the terminating ion concentration was varied between 2.5 and 12.5 m*M* β-alanine; 10 m*M* β-alanine was optimal in the

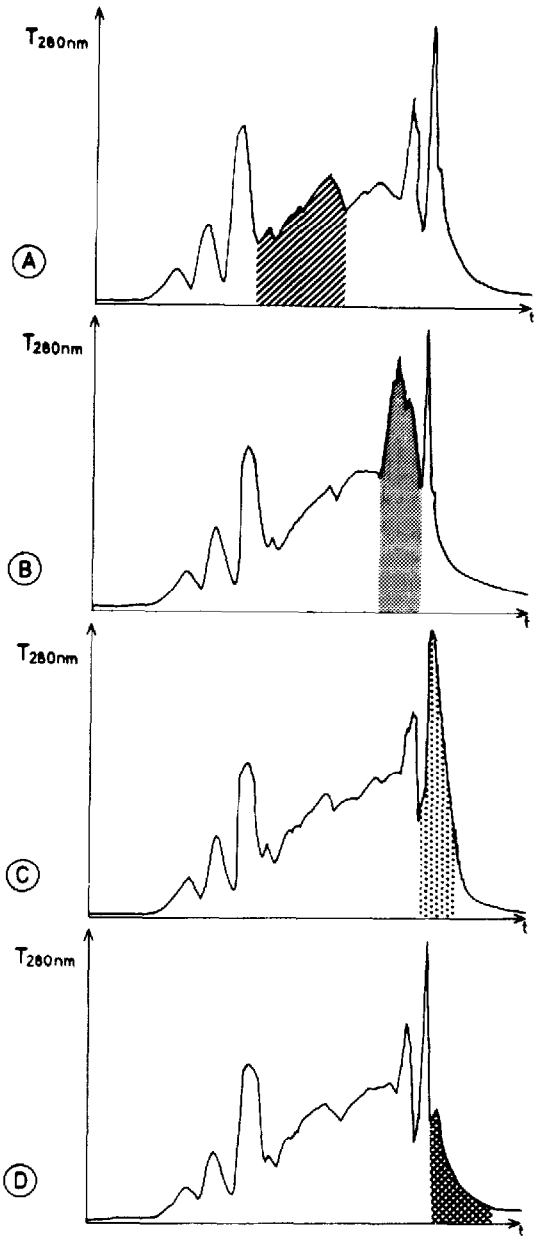

Fig. 3. Isotachophoretic pattern of unstained serum measured at 280 nm after addition of various enriched plasma protein fractions. (A) Separation pattern of 1 μl of unstained serum with addition of 8 μg of albumin. (B) Same serum as in A, with addition of 4 μg of transferrin. (C) Same as in A and B, with addition of 10 μg of β-globulins. (D) Same as in A, B and C, with addition of 10 μg of γ-globulins.

selected buffer system. The separation patterns shown in Figs. 1 and 2 (and all other separations presented in this study) were achieved with this optimized buffer system.

To compare the separation patterns of the major plasma proteins in the se-

lected buffer system with data from other buffer systems described for plasma protein separations, experiments with purified human albumin, transferrin, β-globulins and γ-globulins added to normal serum were performed and the absorption patterns measured at 280 nm. It is obvious from Fig. 3 that albumin has the highest mobility (A) followed by transferrin (B), β-globulins (C) and γ-globulins (D), which demonstrates

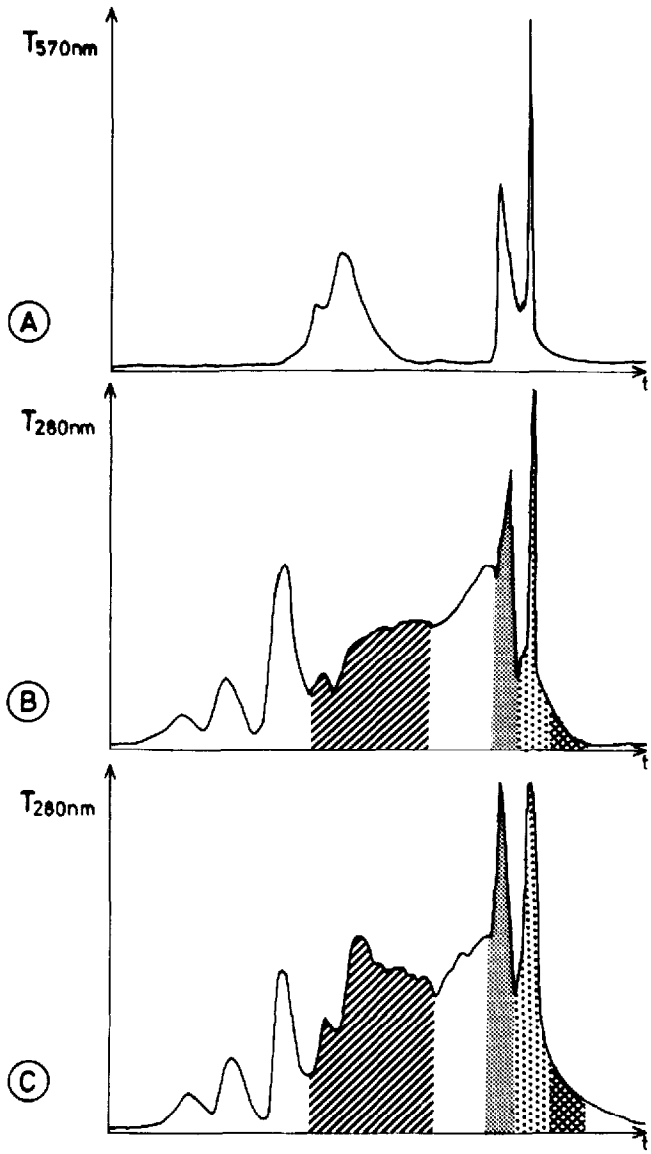

Fig. 4. Isotachophoretic pattern of pre-stained serum at 280 and 570 nm compared with the pattern of unstained serum at 280 nm. (A) Separation pattern at 570 nm of 1 μl of serum pre-stained with Sudan Black B. (B) Separation pattern at 280 nm of 1 μl of the same unstained serum. (C) Separation pattern at 280 nm of 1 μl of the same serum pre-stained with Sudan Black B.

that the mobilities of the tested plasma proteins are comparable to published values[29].

Fig. 4 demonstrates the separation pattern of identical sera at 280 nm, without (B) and with Sudan Black B staining (C). The lipoprotein zones, plotted in Fig. 4A, reveal mobilities in the albumin, transferrin and β-globulin regions, which result in an increase in absorption at 280 nm (C). However, unstained sera, measured at 570 nm, revealed no measurable photometric signals (not shown). These results indicate that the isotachophoretic separation patterns derived from Sudan Black B-prestained sera represent serum lipoprotein fractions exclusively. The major lipoproteins (VLDL, LDL, HDL) can be identified by comparing the isotachophoretic lipoprotein patterns of the different mutants. The pattern of normal whole serum lipoproteins (Fig 5A) was compared with sera derived from patients suffering from Tangier disease (Fig. 5B, patients I and II) and abetalipoproteinaemia (Fig. 5C, patients III and IV). As expected, in analphalipoproteinaemia no HDL peaks were detectable and in abetalipoproteinaemia no LDL peaks were present. However, there are additional subfractions detectable with different net mobilities and absorption profiles within the HDL and LDL fractions. Chylomicrons and their remnants were identified in

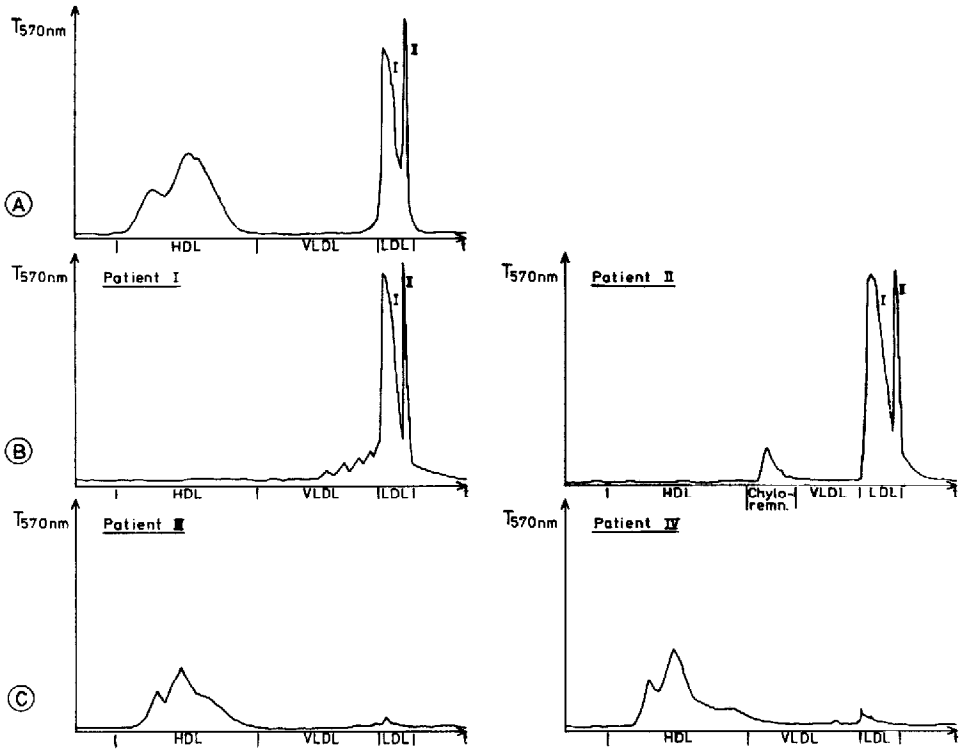

Fig. 5. Isotachophoretic pattern at 570 nm of normal serum in comparison with the pattern of sera from patients with dyslipoproteinaemia (A) Separation pattern of 0.5 μl of normal serum (TG 77 mg/dl, Chol 176 mg/dl). (B) Pattern of 0.5 μl of serum of patients I and II with Tangier disease (patient I, TG 356 mg/dl, Chol 64 mg/dl; patient II, TG 359 mg/dl, Chol 36 mg/dl). (C) Pattern of 0.5 μl of serum of patients III and IV with abetalipoproteinaemia (patient III, TG 2 mg/dl, Chol 27 mg/dl; patient IV, TG 7 mg/dl, Chol 27 mg/dl).

fasting serum from Tangier patient II (Fig. 5B) in the position between the HDL and VLDL zones. Three HDL subfractions instead of two (compared with normal controls) could be identified in both patients with abetalipoproteinaemia (Fig. 5C).

DISCUSSION

The proposed capillary isotachophoretic method for the separation of lipoproteins involves separation by carrier-free electrophoresis and the specific detection of lipoproteins in native serum using lipophilic dyes. It has been shown previously[30,31] that the specific binding of ligands to particular proteins in complex mixtures resulted in altered UV signals of the zones (an increase or decrease depending on the intrinsic absorption of the ligand). With lipoproteins, a further refinement was possible because the "affinity marker" employed, the lipophilic diazo dye Sudan Black B, absorbs in the visible region. Detection at 570 nm thus results in the elimination of all non-lipoprotein components of serum from the photometric trace. By means of analytical capillary isotachophoresis, an analysis of native lipoprotein subfractions from whole serum was possible within 12 min. Only 0.5–1.0 μl of serum is necessary, and the only pre-treatment required is pre-staining with Sudan Black B in appropriate proportions. The analysis of sera of patients affected by analphalipoproteinaemia (Tangier disease) and abetalipoproteinaemia (Bassen–Kornzweig disease) clearly demonstrates that the isotachophoretic method can distinguish between the major lipoprotein fractions, VLDL, LDL and HDL.

The decisive advantage of the capillary isotachophoretic method over more conventional gel techniques[10,15] is that the free-flow nature of the system eliminates molecular sieve effects, thus yielding a higher resolution for small charge differences within the lipoprotein subclasses. Further, the isotachophoretic configuration results in concentration of zones, with the obvious accompanying advantage for diluted samples and, in contrast to zonal electrophoresis, the resolution is improved by the continuous counteraction of diffusion (zone sharpening effect).

The evidence from our current investigations indicates that the isotachophoretic method is useful for screening in order to detect abnormal lipoprotein subfractions that may play a role in atherogenesis. However, further studies are necessary to characterize in detail the composition of the abnormal particles separated by this technique and to develop automated equipment to allow data calculations for large-scale sample analysis.

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