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Separation and selective detection of lipoprotein particles of patients with coronary artery disease by frit-inlet asymmetrical flow field-flow fractionation

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Abstract

An analytical method to improve the characterization of lipoprotein fractions is presented. Human plasma samples were treated with Sudan Black B to stain the lipid component in lipoproteins, then the stained lipoproteins were separated by frit inlet asymmetrical flow field-flow fractionation (FI-AFIFFF), according to the lipoprotein particle sizes, with the selective detection of eluting lipoprotein fractions, high-density lipoproteins (HDL), low-density lipoproteins (LDL) and very-low-density lipoproteins (VLDL), at 610 nm. The capability of this technique has been evaluated with plasma samples obtained from patients with coronary artery disease (CAD), and it showed that the retention profile of patients' lipoprotein samples was clearly distinct from those of healthy persons. The potential of this technique comes with the direct injection of a stained lipoprotein sample without a prior procedure such as ultracentrifugation for sample preparation, and the size calculation of lipoprotein particles from the experimental retention time by theory. Since sample relaxation was achieved hydrodynamically in an FI-AFIFFF channel, sample injection and separation processes were continuously made without stopping the separation flow. This study demonstrated the potential of the FI-AFIFFF technique to be utilized as a powerful tool for the determination of the LDL profiles of patients with CAD.

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1. Introduction

Lipoproteins are macromolecular assemblies of lipids and proteins, and their principal function is to transport lipids through vascular and extravascular

body fluids [1]. Globular shaped lipoproteins consist of hydrophobic core molecules such as neutral lipids, cholesteryl esters, and triglycerides, with amphiphilic surface molecules composed of phospholipids, free cholesterol, and apoproteins. Classically, lipoproteins are classified according to their hydrated densities into three different categories: high-density lipoproteins (HDL), low-density lipoproteins (LDL), and very-low-density lipoproteins (VLDL) [2]. Among

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them, LDL are a heterogeneous mixture of lipoproteins with various sizes, densities, and compositions [3,4]. The LDL subfraction profile, containing predominantly small, dense LDL particles, is known to be associated with a higher risk of developing coronary artery disease (CAD) [3–6]. While the characterization of LDL subfractions is one of the important steps in diagnosing CAD in clinical examinations, current analysis of LDL subclasses is not simple. Several methods have been used to determine lipoprotein profiles in human blood plasma, including analytical ultracentrifugation [7], density gradient ultracentrifugation [8], polyacrylamide gel electrophoresis [3,4,9], and chromatography [2,10]. Ultracentrifugation techniques require laborious centrifugal procedures that normally take over 24 h, and they require a certain amount of sample volume for analysis. In addition, there is a possibility of inducing structural changes to the lipoprotein complex by the shear, and the effect of ionic strength variation. While the electrophoretic method is widely used as a powerful technique to provide high-resolution separation, the technique is too laborious to be used for a routine and quick assessment of patients. Size-exclusion chromatography also requires a prior procedure, such as ultracentrifugation, for the preparation of the sample to be loaded on the column. In some cases there is a possibility of pore blockage of chromatographic packing materials.

Flow field-flow fractionation (FIFFF or flow FFF) [11–13], a separation technique for nanosized materials such as nanoparticles, proteins, etc., was utilized in this study for separating lipoproteins particles with the selective detection of lipoproteins. In a flow FFF channel, separation of particles or macromolecules is carried out by the difference in diffusion coefficients for particles of different sizes [12,14]. A small particle having a higher diffusion coefficient experiences field strengths given by the movement of crossflow across separation channel less strongly than a larger one. Thus, separation in flow FFF is made according to the order of increasing diameter of particles or increasing molecular mass. From theory, the retention ratio, R , for highly retained particles is expressed as [12,14]

$$R = \frac{t^0}{t_R} \cong \frac{2kT}{\pi\eta w^2 d_s} \cdot \frac{V^0}{\dot{V}_c} \quad (1)$$

where t^0 is the void time, t_R the retention time, V^0 the channel void volume, \dot{V}_c the cross flow-rate at the channel wall, kT thermal energy, η the viscosity of the carrier solution, w the channel thickness, and d_s the Stokes' diameter. From this relationship, it is possible to calculate the diffusion coefficient or Stokes' diameter of a particle from an experimental retention time once the experimental run conditions are provided [15–17].

In the literature, flow FFF has been utilized for the separation and size characterization of lipoprotein components [17–19]. While earlier studies demonstrated the strength and possibility of separating major components of lipoprotein particles using the flow FFF technique, detection of HDL and LDL components that were separated by flow FFF was not complete due to the co-elution of large amount of plasma protein, such as albumin [17]. In order to reduce the influences of albumin or other protein components, a membrane-selective flow FFF technique was tried by using a channel membrane having large pores, so that albumins can be swept through the channel wall during sample relaxation [18], and an asymmetrical flow FFF technique was used to separate lipoprotein components from albumin with an improved resolution [19]. However, in both cases, the relative peak intensities of the LDL components from plasma samples were too small compared to the huge intensity of the HDL peak signal to characterize the LDL subfraction profile.

In this study, a method to selectively detect the resolved lipoprotein particles was applied. Since blood plasma is a complex macromolecular colloidal solution containing many components, principally numerous proteins and various classes of lipoproteins, flow FFF separation of a plasma sample will result in the elution of all components with an increasing hydrodynamic diameter but without specific detection of lipoprotein particles. Typical UV detection at 254 or 280 nm may not differentiate lipoprotein components since they elute simultaneously with proteins of similar size. For a selective detection of lipoprotein components, plasma samples were treated with Sudan Black B, which is utilized in gel electrophoresis for the specific staining of lipid components in lipoproteins, prior to the flow FFF separation [3,9], and they were detected at a wavelength of 610 nm. In this study, separation of stained

lipoprotein particles was carried out by utilizing a modified form of flow FFF channel, the frit inlet asymmetrical flow FFF (FI-AFIFFF). Since sample injection in a FI-AFIFFF channel is made onto the flowing streamline directly while the frit flow enters the channel through the inlet frit [20–24] as shown in Fig. 1, sample relaxation in the FI-AFIFFF channel, an essential procedure in most FFF techniques for providing equilibrium conditions for sample components before separation, can be hydrodynamically obtained by the compression action of frit flow without stopping migration flow for a certain period of time [20]. Though the relaxational band broadening [21] is induced by the stopless flow operation, and it is somewhat larger than can be expected by the conventional asymmetrical flow FFF channel using the focusing/relaxation process, system operation in a FI-AFIFFF channel can be greatly simplified with a reduced risk of sample adhesion to channel membranes that can occur during stoppage of sample migration in conventional channel systems. In addition, utilizing the staining process followed by flow FFF separation is advantageous for lipoprotein characterization, since there is no pre-treatments required except for the staining process itself.

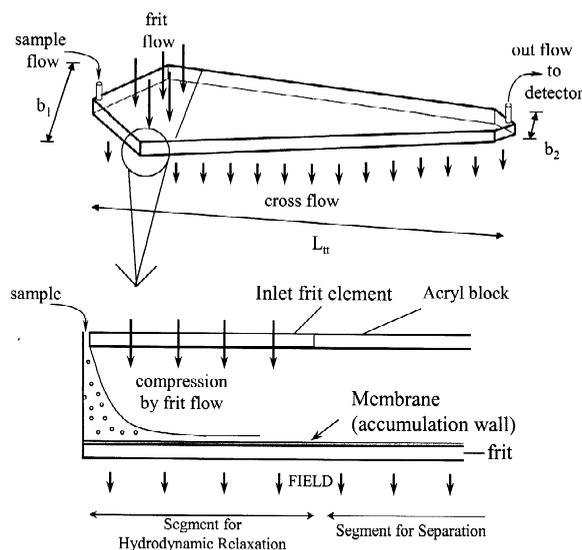


Fig. 1. Schematic diagrams showing hydrodynamic relaxation in a frit inlet asymmetrical flow FFF channel system.

2. Experimental

2.1. Materials

HDL standard and a few protein standards were purchased from Sigma (St. Louis, MO, USA). Protein standards used for the evaluation of the flow FFF system are carbonic anhydrase (M_r 29 000), alcohol dehydrogenase (M_r 150 000), apoferritin (M_r 443 000), and thyroglobulin (M_r 670 000). For lipoprotein studies, human blood plasma were directly used with the following staining method. For staining lipid components in lipoproteins, 25 μ l of plasma sample was mixed with 200 μ l of Lipoprint loading gel containing Sudan Black B obtained from Quantimetrix (Redondo Beach, CA, USA) and photopolymerized for 30 min. Plasma samples from healthy persons and from patients having CAD, proven angiographically, were obtained from Seoul National University Hospital (Seoul, Korea). Three patients (two males and one female) with CAD, as documented by coronary angiography due to recent myocardial infarction or angina, were selected. None of the selected CAD patients was on therapy to lower lipid levels at the time of sampling. The three controls (three males) were obtained from healthy persons who were selected by health-screening at the same hospital in order to screen out those who had a history of chest pain, diabetes, hypertension and general illness. Hypertension was defined as a diastolic blood pressure >90 mmHg. Blood samples were obtained after fasting for 12 h, to exclude the influence of diet on LDL size. Samples were placed in EDTA tubes and stored at -70°C .

2.2. Flow field-flow fractionation

The type of channel used for flow FFF runs in this study was a frit inlet asymmetrical flow field-flow fractionation (FI-AFIFFF) channel that was built in-house and the construction of the channel was described elsewhere [20–24]. The depletion wall of the frit inlet asymmetrical channel was made with a Plexiglass block, with a small inlet frit implanted at the injection end, as shown in Fig. 1. The channel space was made by cutting a 254- μm thick Mylar spacer in a ribbonlike shape. The channel had a tip-to-tip length, L_{tt} , of 27.2 cm, and an initial

breadth, b_1 , of 2.0 cm that trapezoidally decreased to the final breadth, b_2 , 1.0 cm. The membrane was layered over the porous frit wall mounted on the accumulation wall block, and it was YM-30, a regenerated cellulose with a M_r cut-off of 30 000, from Amicon (Beverly, MA, USA). Actual channel thickness was calculated as 259 μm by using the protein standards mentioned above, according to the method reported in an earlier report [22]. The actual channel thickness was slightly increased due to the use of silicon glue layered on the back side of the edge of the membrane sheet, to keep the channel from leaking, and the use of glue compensated for a possible decrease of channel thickness that was normally observed due to the compression of the membrane at the contact area with the plastic channel spacer. For the carrier solution used for separation of proteins and plasma samples in the FI-AFIFFF channel, Tris-HCl buffer ($I=0.10\text{ M}$) solution, adjusted to pH 7.8, was prepared with deionized ultrapure water. The carrier solution was filtered with membrane filters having a pore size of 0.45 μm prior to use. For the delivery of carrier liquid to the FI-AFIFFF channel, two HPLC pumps were used for the sample flow and the frit flow: a Tosoh Model CCPD from Style Electronics (Tokyo, Japan), and a Dynamax Model SD-200 from Rainin Instrument (Woburn, MA, USA).

Sample materials were injected to the channel via a Rheodyne 9125 loop injector from Rheodyne (Cotati, CA, USA). Injected amounts were about 2–4 μg for protein standards, about 24 μg for HDL standards, and about 10 μl for each plasma mixture with Sudan Black B. Eluted sample components were monitored by a Model S-3710 UV detector from Soma Optics, (Tokyo, Japan), at wavelengths of 280 nm for proteins and of 610 nm for stained lipoproteins. Detector signals were recorded by AUTOCHROWIN data acquisition software from Young-Lin Scientific (Seoul, Korea).

3. Results and discussion

The separation capability of the FI-AFIFFF channel system used in this study was demonstrated using protein standard mixtures. It was obtained at a flow-rate ratio of sample flow to frit flow of $\dot{V}_s/\dot{V}_f=0.07/$

3.20 in ml/min, and a ratio of outflow to cross-flow of $\dot{V}_{\text{out}}/\dot{V}_c=0.30/2.97$ in ml/min. Fig. 2 shows a typical separation of four different protein standards obtained with the stopless injection at the FI-FIFFF. The separation was achieved within 30 min, including the small shoulder peak eluted after thyroglobulin. According to an earlier report, the small peak that appeared at around 25 min was presumed to be the elution of apoferritin trimers based on the calibration of retention times vs. M_r . The field strength used in Fig. 2 was not sufficient to baseline resolve all components, however it was appropriate not to lose resolution in separating lipoproteins of size range somewhat larger than the sample components shown in Fig. 2. The Stoke's diameter scale marked at the top of Fig. 2, is calculated by rearranging Eq. (1) as

$$d_s = \frac{2kT}{\pi\eta w^2 \dot{V}_c} \cdot \frac{V^0}{t^0} \cdot t_R \quad (2)$$

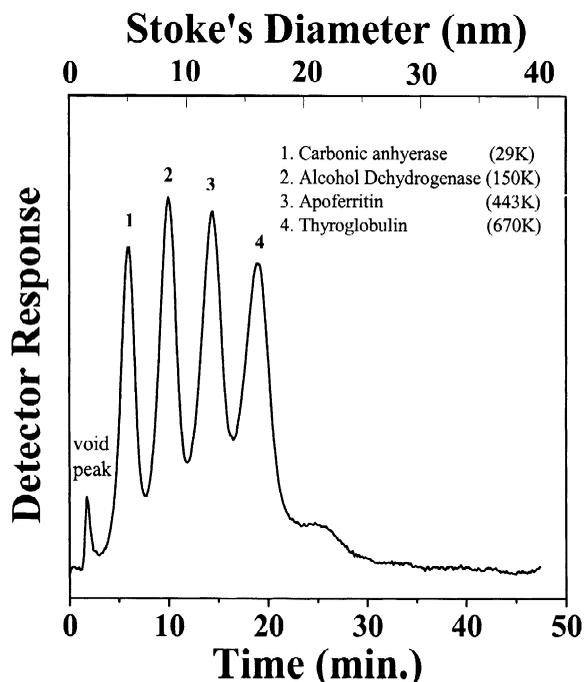


Fig. 2. Separation of protein standards by FI-AFIFFF. Flow-rate conditions were \dot{V}_s (sample flow)=0.070 ml/min, \dot{V}_f (frit flow)=3.20 ml/min, \dot{V}_{out} (outflow)=0.30 ml/min and \dot{V}_c (cross flow)=2.97 ml/min. The wavelength used for detection was 280 nm.

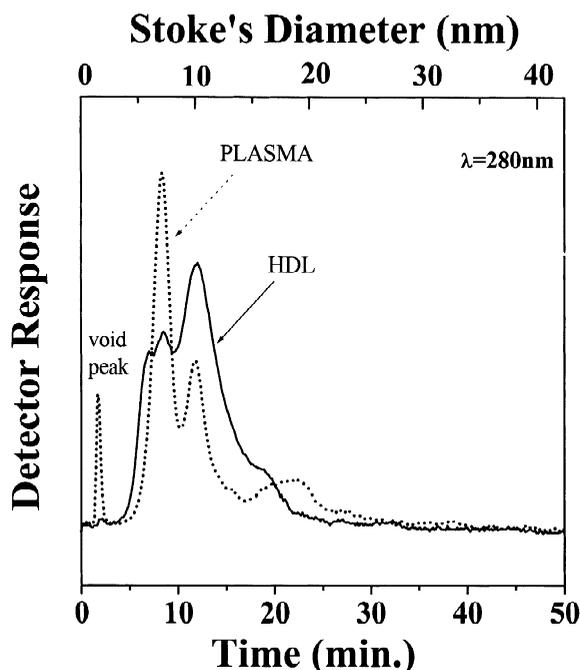


Fig. 3. Separation of a HDL standard and a plasma sample obtained by FI-AFIFFF. Run conditions were as in Fig. 2. Detection was made at 280 nm.

Fig. 3 illustrates the fractionation of a human blood plasma sample by FI-AFIFFF, superimposed with the fractogram of a HDL standard under the same run condition utilized in Fig. 2. The UV detection in Fig. 3 was made at $\lambda=280$ nm. Since human blood plasma contains lipoproteins and a number of protein components, the fractogram shown with the dotted line represents the peaks of components that can be detected at 280 nm. The large peak of the plasma eluting at $t_R=8.4$ min was presumed to be albumin (67 000), which was a characteristic peak observed in earlier studies on lipoproteins with flow FFF [17,19]. Among the three distinct peaks of the plasma sample shown in Fig. 3, the second peak eluting at 11.9 min (dotted line) was presumed to be HDL, when compared with the elution profile of the HDL standard (solid line), it agrees well with the observations made by Madorin et al. [19]. According to the diameter scale shown at the top axis of Fig. 3, the hydrodynamic diameter of the second peak maximum was about 10.1 nm,

which is similar to the mean value (9.86 ± 0.8) listed for HDL in the literature [9]. However, the fractogram of the HDL standard also showed a distribution of HDL subfractions, by showing a peak at about 8.5 min and a corresponding Stoke's diameter equivalent to 7.2 nm. This has not been demonstrated in the earlier studies. All of the size values fell in the size range of the HDL standard provided by the manufacturer. Since low levels of HDL are also known to be associated with CAD, separation of HDL subfractions was itself a challenge; however it was not further focused on in this work. Further assessment of LDL, or VLDL components for the elution peaks of the plasma sample, could not be made here due to the relatively small signals observed. As shown in Fig. 3, it was not straightforward to observe all of the lipoprotein components such as HDL, LDL and VLDL at the typical UV detection of $\lambda=280$ nm.

Treating lipoproteins with Sudan Black B can be a powerful method to detect lipoproteins exclusively, if it is utilized before the separation by flow FFF. Since it stains only the lipoprotein components, selective detection of stained lipoproteins can be made at $\lambda=610$ nm, and this will resolve the detector signals of lipoprotein particles from those of co-eluting protein components. Fig. 4 shows the detector signals for stained plasma samples with Sudan Black B, monitored at two different wavelengths: $\lambda=610$ nm (solid line) and $\lambda=280$ nm (dotted line). By comparing signals obtained at two different wavelengths, it was clearly shown that the peak intensity of stained LDL particles observed at 25.2 min was greatly increased at $\lambda=610$ nm, while it was relatively very small or nearly undetected at $\lambda=280$ nm. Since the plasma sample used in Fig. 4 was obtained from a patient with CAD, the LDL particle size had decreased as is often observed in this disease. This was further investigated by comparing the retention profiles of CAD patients with those of healthy persons. In addition, it was found from the experiments that the two distinct peaks at around 10 min were related to HDL components since the stained lipoprotein sample showed a characteristic peak at 8.5 min even at $\lambda=610$ nm. If the first peak observed at 8.5 min originated totally from proteins, it should have disappeared or significantly decreased when detection was attempted at $\lambda=610$ nm. Moreover, the relative peak intensities

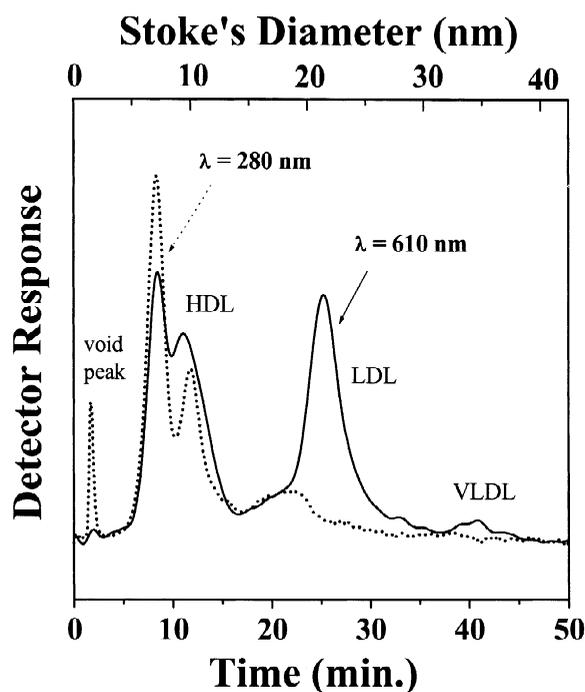


Fig. 4. FI-AFIFFF fractograms of a plasma sample detected at two different wavelengths.

of two HDL peaks were similar for the peaks observed at $\lambda = 610$ nm, while they were very much different at $\lambda = 280$ nm. The relative peak intensities of the HDL and LDL peaks obtained at $\lambda = 610$ nm were similar, while those observed at $\lambda = 280$ nm were significantly different. This supported the conclusion that the detection at 610 nm was due to stained lipoproteins mostly and was not much influenced by the elution of protein components that could be left by chance in the stained plasma sample. Therefore, the relatively large ratio of the first to the second peak detected at $\lambda = 280$ nm showed that there was a considerable amount of proteins eluting together with HDL subfractions. The small peak at 22.4 min ($\lambda = 280$ nm) became relatively smaller than the LDL peak. This can be assigned to α_2 -macroglobulin, as has been noted in the literature [19]. In addition, the void peak signal was significantly reduced when it was detected at $\lambda = 610$ nm, which also provided support for the advantage

of Sudan Black B in excluding impurities other than lipid containing materials. The separation of lipoprotein particles and the selective detection by staining demonstrated in Fig. 4 was an outstanding combination that can be applied to the clinical application of the flow FFF technique in determining lipoprotein profiles, without being influenced by plasma protein components, and without pretreatment of plasma samples to isolate lipoproteins. In the case of sample concentration needed for the flow FFF analysis, it was extremely small compared to that required for conventional centrifuge techniques.

The combined method was applied for the characterization of the LDL pattern of blood plasma for three healthy persons and for three patients with CAD. All samples were stained before the FFF analysis. The three fractograms at the top of Fig. 5 were obtained from plasma control samples of three healthy persons. Three stained lipoprotein samples showed a nearly identical elution profiles, except for the LDL peak of the control-02 sample, which was somewhat shifted toward a shorter time scale (27.7 from 28.6 min). Since the decrease of retention time originated from a decrease of hydrodynamic diameter calculated by flow FFF theory (23.5 from 24.2 nm), it can be thought that the control-02 contained smaller LDL particles than the others. From the repeated experiments, the difference in the LDL pattern of the control-02 from the others was believed to originate from personal differences. The LDL particle sizes for the two control samples (01 and 03) were nearly the same at 24.2 nm. When plasma samples obtained from patients with CAD were tested, it was shown that the LDL peaks shifted toward a shorter time scale (down to 25.5 min) with a reasonable reproducibility. The decrease of LDL particle size ranged from 24.2 to 21.6 nm. This phenomenon agreed with reports that dense, smaller LDL particles were associated with an increased risk of developing CAD [3–6]. Based on this fact, it may be surmised that the control-02 sample was in the process of developing CAD. Fig. 5 also showed that the relative amount of the secondary peak of HDL with CAD was greatly decreased while the particle size of HDL did not appear to change. The latter was also consistent with the fact that low levels of HDL cholesterol have a strong association with CAD.

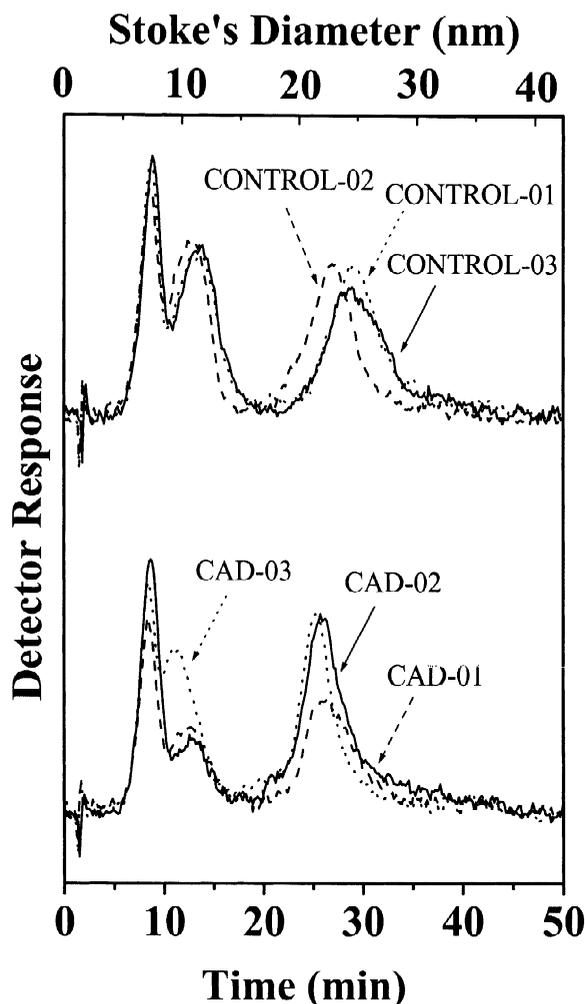


Fig. 5. FI-AFIFFF separation of human plasma lipoproteins obtained from (a) healthy persons and (b) patients with CAD. Wavelength for detection was $\lambda=610$ nm and the flow-rate conditions were the same as in Fig. 4.

4. Conclusions

Frit inlet asymmetrical flow FFF was applied to the separation of LDL particles according to their hydrodynamic diameters, and the selective detection of lipoprotein components was combined with preliminary staining of the lipoprotein particles with Sudan Black B. The method introduced here showed the great potential of using the flow FFF technique as

a diagnosis tool in CAD, by characterizing the decrease of LDL particle sizes compared to those of healthy LDL particles. Since the present study dealt with lipoprotein samples from three healthy persons and three patients, it would be desirable to examine more cases in a systematic way. However, the current technique has the unique advantages of a direct sample injection after a simple staining of blood plasma without ultracentrifugation, of using a negligibly small amount of sample for analysis, and of a fast analysis time. Sample recovery optimization needs to be further investigated since channel contamination is often observed and it requires thorough cleaning. Since the injected sample contains dyes and other impurities, it often causes a fast deterioration of the surface of the channel membrane. This can be solved by examining a proper carrier solution with the current flow FFF channel system, or by utilizing a disposable flow FFF system, such as the hollow fiber flow FFF (HF FIFFF) [25,26]. However, the flow FFF system utilized in this work, the frit inlet asymmetrical flow field-flow fractionation channel, has an advantage of injecting and separating sample components continuously, without having a separate stoppage of migration flow, which gives a simple operating system and a reduced chance of sample adhesion to the channel wall.

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