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Fractionation of human serum lipoproteins and simultaneous enzymatic determination of cholesterol and triglycerides

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ABSTRACT

A method based on Asymmetric Flow Field-Flow Fractionation (AF4) was developed to separate different types of lipoproteins from human serum. The emphasis in the method optimization was on the possibilities to characterize the largest lipoprotein fractions (LDL and VLDL), which is usually not possible with the size-exclusion chromatography methods applied in routine analysis. Different channel geometries and flow programs were tested and compared. The use of a short fractionation channel was shown to give less sample dilution at the same fractionation power compared to a conventional, long channel. Different size selectivities were obtained with an exponential decay and a linear cross flow program. The ratio of the UV absorption signal to the light scattering signal was used to validate the relation between retention time and size of the fractionated particles.

An experimental setup was developed for the simultaneous determination of the cholesterol and triglycerides distribution over the lipoprotein fractions, based on enzymatic reactions followed by UV detection at 500 nm. Coiled and knitted PTFE tubing reactors were compared. An improved peak sharpness and sensitivity were observed with the knitted tubing reactor. After optimization of the experimental conditions a satisfactory linearity and precision (2–3% rsd for cholesterol and 5–6% rsd for triglycerides) were obtained. Finally, serum samples, a pooled sample from healthy volunteers and samples of sepsis patients, were analyzed with the method developed. Lipoprotein fractionation and cholesterol and triglyceride distributions could be correlated with the clinical background of the samples.

1. Introduction

Coronary heart disease (CHD), one of the major causes of death in the world, is strongly related to the human lipoprotein metabolism [1,2]. Lipoproteins are complex agglomerates of different sizes, composed of a variety of different types of molecules in their core and on their surface. Triglycerides, lipids and cholesteryl esters form the hydrophobic core of the spherical lipoprotein particles; this core is surrounded by an amphiphilic shell of free cholesterol, phospholipids and apoproteins. Lipoproteins can be classified according to their density and size into high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) and into various subclasses of these. HDL and LDL, being considered as anti-atherogenic and atherogenic in nature, have drawn most attention of researchers. LDL consists of relatively large particles, rich in cholesterol that transport triglycerides and cholesterol from the liver to all cells and tissues. HDL on the other hand removes cholesterol from cell membranes and the walls of blood vessels and delivers it to the liver for excretion. It has been well established that an increase in total cholesterol, LDL-cholesterol and triglycerides in human blood are positive risk factors for CHD, while an increase in HDL-cholesterol is considered as an inverse risk factor [3]. Hence, the quantitative analysis of cholesterol and triglycerides associated to the various (sub)classes of lipoproteins is extremely important in clinical laboratories, because of their predictive association not only with CHD [4] but also with sepsis [5], liver dysfunction and cancer [6].

Since decades assays using enzymatic reagents are considered to be the most reliable methods for the selective and quantitative determination of total cholesterol and triglycerides in serum samples [4]. The enzymatic assays for the two clinically important analytes can be carried out in a single cuvette sequentially [7,8] and automated assays through flow-injection analyses (FIA) have been proposed [9–14]. However, the total serum levels of cholesterol and triglycerides are nowadays regarded as indicators with a limited diagnostic value, and data on the distribution of the lipids over the lipoprotein (sub)fractions are preferred [4]. Originally, fractionation was performed by ultracentrifugation, the technique that provided the classification system for lipoproteins [15]. However, ultracentrifugation is a lengthy and laborious method. A method for the fractionation of lipoproteins based on size-exclusion chromatography (SEC) or gel permeation chromatography was developed that

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could separate HDL, LDL and VLDL in less than 60 min [16]. The cholesterol and triglyceride distribution over the lipoprotein fractions could be determined with enzymatic methods applied on collected fractions, but on-line quantification of one of the analytes associated to the different (sub)fractions of lipoproteins after their separation by SEC is also possible with a post-column enzymatic reaction setup [17,18]. Finally, Usui et al. showed that both the cholesterol and the triglyceride content of the separated fractions can be determined on line, by splitting of the column effluent and using two post-column reaction systems simultaneously [19].

Although the method of SEC with post-separation enzymatic determination of cholesterol and triglycerides eliminated laborious and time consuming procedures, there are also some limitations. First, the selectivity of the SEC separation is limited, especially at the high-MW (VLDL) side, even when multiple columns in series are used [16]. Moreover, undesired interaction between proteins and the stationary phase may result in adsorption or entanglement, which reduces recovery and separation [20]. As an alternative separation method for lipoproteins Flow Field-Flow Fractionation (F4) has been proposed [21–25]. F4 is a separation technique that enables the separation of (biological) macromolecules without mechanical or shear stress on their native conformational structures [26]. In F4 macromolecules are separated based on their diffusion coefficients by the combined action of an axial flow stream of a carrier liquid in a thin channel and a cross flow applied in the perpendicular direction [27]. In the asymmetrical variant (AF4) one of the walls of a flat channel is permeable for the carrier liquid and the cross flow is created as part of the inlet flow [28–30]. Compared to the original symmetrical system (with two porous walls), AF4 offers the advantages of a simplified instrumental setup and diminished sample dilution [31].

In the work presented here, we studied the coupling of AF4 for the size-based fractionation of lipoproteins to the dual enzymatic system for the simultaneous determination of the triglycerides and cholesterol distribution over lipoproteins fractions. For the fractionation different cross flow programs were tested and different channel geometries were compared. The emphasis in the method optimization was on the possibilities to characterize the largest lipoprotein fractions (LDL and VLDL), which is usually not possible with the SEC methods applied in routine analysis. For optimization of the enzymatic reaction systems kinetic parameters were studied such as the reaction temperature, time, flow rates and geometries of reactor tubing. Finally, the optimized system was tested by analyzing serum samples of healthy persons and of patients of the university hospital.

2. Experimental

2.1. Chemicals and solutions

All reagents used were of analytical grade. HDL (25.8 mg protein per mL) and LDL (5.0 mg protein per mL) standards were purchased from Sigma Aldrich (Saint Louis, USA). Phosphate buffer saline (PBS, 138 mM sodium chloride, 2.7 mM potassium chloride, and 10 mM phosphate buffer salts at pH 7.4) was used as carrier solution. It was prepared in doubly distilled water and filtered with 0.22 μm GV Millipore filters before use. Cholesterol CHOD-PAP and Triglycerides GPO kits (Biolabo SA, Maizy, France) were used for the quantitative determination of cholesterol and triglycerides. The enzymatic reagents were prepared by mixing an enzymes solution (R2) with a buffer solution (R1). For the cholesterol measurements R2 contained cholesterol oxydase ($\geq$100 U L^{-1}), cholesterol esterase ($\geq$170 U L^{-1}), peroxydase ($\geq$1200 U L^{-1}), 4-amino antipyrine (PAP, 0.25 mmol L^{-1}), and PEG 6000 (167 μmol L^{-1}). The buffer solution R1 contained phosphate buffer (100 mmol L^{-1}), 4-chlorophenol (4-CP, 5 mmol L^{-1}), sodium chloride (2.3 mmol L^{-1}) and Triton X 100 (1.5 mmol L^{-1}). For triglyceride measurements R2 contained lipase ($\geq$1000 U L^{-1}), peroxydase ($\geq$1700 U L^{-1}), glycerol-3-phosphate oxydase ($\geq$3000 U L^{-1}), glycerol kinase (GK) $\geq$660 U L^{-1}, PAP (0.5 mmol L^{-1}) and sodium adenosine triphosphate (ATP, 1.3 mmol L^{-1}). Buffer R1 contained PIPES (100 mmol L^{-1}), magnesium chloride (9.8 mmol L^{-1}) and 4-CP (3.5 mmol L^{-1}). The reagents were stored at 2–5 °C in the dark after preparation. Standard solutions of cholesterol and triglycerides were prepared by diluting in carrier solution.

A pooled serum sample of healthy donors (80 volunteers, male and female) and samples from two sepsis patients were obtained from the university hospital (AMC, Amsterdam). The samples had been collected under non-fasting conditions. Samples were stored at −20 °C before analysis.

2.2. Apparatus

An Agilent 1100 series degasser and a 1200 HPLC series isocratic pump were coupled with an Eclipse2 AF4 separation system (Wyatt Technology Europe GmbH, Dernbach, Germany) to carry out fractionation. Separation channels with 350-μm spacers of trapezoidal shape of different dimensions were used, and a regenerated cellulose membrane with a molar mass cut off of 10 kDa. Samples were injected with a 6-port valve with a 20 or 100 μL loop. A DAWN-DSP MALS detector (Wyatt) was used in series with UV detector (Applied Biosystems, USA). ASTRA software (Wyatt Technology) version 4.9 was used to handle signals from the detectors.

For the simultaneous enzymatic determination of lipoprotein–cholesterol and lipoprotein–triglycerides the main channel flow of 0.6 mL min^{-1} was split into two lines by a Micro-Splitter P-460 (Upchurch Scientific Inc., Oak Harbor WA, USA). The enzymatic reagents were pumped in the separate lines using two HPLC pumps (Applied Biosystems, USA) with a flow rate of 0.2 mL min^{-1}. The reagents were placed in amber glass bottles immersed in melting ice. PTFE tubing of 0.5 mm ID (Supelco, USA) of lengths 5.1 and 10.2 m was used to make coiled and knitted enzymatic reactors. Different lengths of reactors correspond to different reaction times, i.e., 2 and 4 min. Reactors were placed in thermostated bath at 37 °C. Two Spectroflow 757 UV detectors (Applied Biosystems, USA) were used at 500 nm for detection. The complete system is shown schematically in Fig. 1.

3. Results and discussion

3.1. Optimization of the cholesterol and triglycerides measurements

Determination of cholesterol and triglycerides by the CHOD and GPO methods involves different sequences of enzymatic reactions as shown in Table 1. In the final step of both sequences, hydrogen peroxide reacts with PAP and 4-CP to form an imine derivative

Table 1
Enzymatic reactions for cholesterol and triglycerides determination.

Cholesterol

1. Cholesterol esters $\xrightarrow{\text{cholesteryl esterase}}$ cholesterol + free fatty acids
2. Cholesterol + O_2 $\xrightarrow{\text{cholesteryl oxidase}}$ cholestenone + H_2O_2
3. H_2O_2 + 4-CP + PAP $\xrightarrow{\text{peroxidase}}$ quinoneimine (pink) + H_2O

Triglycerides

1. Triglycerides $\xrightarrow{\text{lipase}}$ glycerol + free fatty acids
2. Glycerol + ATP $\xrightarrow{\text{glycerol kinase}}$ glycerol phosphate + ADP
3. Glycerol phosphate + O_2 $\xrightarrow{\text{glycerol phosphate oxidase}}$ dihydroxyacetone phosphate + H_2O_2
4. H_2O_2 + 4-CP + PAP $\xrightarrow{\text{peroxidase}}$ quinoneimine + H_2O