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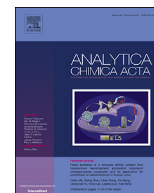

A novel method for serum lipoprotein profiling using high performance capillary isotachophoresis

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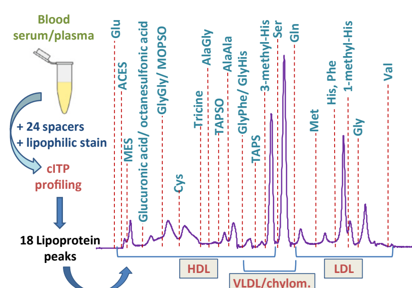
HIGHLIGHTS

- New isotachophoresis method for lipoprotein separation with higher particle coverage.
- 18 lipoprotein peaks could be separated using a fine-tuned mixture of 24 spacers.
- Highly reproducible separations achieved with a new stable doubly-coated capillary.
- Capillary isotachophoresis results were compared with those of a NMR-based method.
- Subparticle lipoprotein changes found in a high cholesterol atherosclerosis model.

GRAPHICAL ABSTRACT



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ABSTRACT

A new capillary isotachophoresis (cITP) method for lipoprotein profiling with superior lipoprotein coverage compared to previous methods has been developed, resolving twice as many lipoprotein species (18 peaks/fractions) in serum or plasma in less than 9.5 min. For this, a novel mixture of 24 spacers, including amino acids, dipeptides and sulfonic acids, was developed and fine-tuned, using predictive software (PeakMaster) and testing of spiked serum samples. Lipoprotein peaks were identified by serum-spiking with reference lipoproteins. Compatibility with common lipophilic stains for selective lipoprotein detection with either UV/Vis or laser-induced fluorescence was demonstrated. A special new capillary with a neutral coating (combining water-compatible OV1701-OH deactivation and methylation) was used for the first time for electrodriven separations, allowing very stable separations in a pH 8.8–9.4 gradient system, being functional for more than 100 injections. Excellent reproducibility was achieved, with coefficients of variation lower than 2.6% for absolute migration times. Comparison was performed with human plasma samples analyzed by NMR, leading to similar results with cITP after multivariate statistics, regarding group-clustering and lipoprotein species correlation. The new cITP method was applied to the analysis of serum samples from a LDL receptor knock-out mice model fed either a normal diet or a western-type diet. Differences in the lipoprotein levels and in the sublipoprotein types were

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detected, showing a shift to more atherogenic particles due to the high cholesterol diet. In summary, this novel method will allow more detailed and informative profiling of lipoprotein particle subtypes for cardiovascular disease research.

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1. Introduction

Lipoproteins are heterogeneous blood particles delivering or uptaking specific lipids within the body, thus playing an essential role in lipid metabolism [1]. In addition, they have important roles in signalling and regulation. These particles present a superficial hydrophilic monolayer of phospholipids (PL), including apolipoproteins, and unesterified free cholesterol (FC); and a hydrophobic core, comprising cholesterol esters (ChoE) and triglycerides (TG). Based on their density, size and/or protein composition, lipoproteins are divided into five main classes: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) -mainly apoB-containing lipoproteins-; and high density lipoproteins (HDL) -mainly apoA1-containing lipoproteins. Within these classes, different subtypes with diverse lipid and protein composition, relating to different biological functions, are found. LDL has the physiological role of providing cells with cholesterol and is therefore its major carrier. High levels of LDL and, to a lesser extent, VLDL particles are associated with the development of atherosclerosis, the main cause of cardiovascular disease (CVD) [2]. Especially, small, dense LDL and modified electronegative particles such as oxidized LDL, release and accumulate cholesterol in the arterial intima, triggering plaque formation [3,4]. HDL particles, in contrast, protect against the development of atherosclerosis [5] through reverse cholesterol transport (mainly pre-β HDL and α-3-HDL), removing cholesterol from the arterial intima to the liver [6] and through their antioxidant properties [7].

Among the different approaches for atherosclerosis risk and progression prediction, routine determination of total cholesterol and TG in serum or cholesterol associated to the whole HDL or LDL fractions have been shown not to fully serve, whereas the number of atherogenic lipoprotein particles instead is better correlated [8]. Alternatively, lipoprotein subfractionation and subsequent biochemical profiling would be very useful to improve prediction of atherosclerosis risk and progression and the outcome of therapeutic approaches, since each lipoprotein subtype has a different composition and role in atherosclerosis [8]. Indeed, lipoprotein profiles have been used in computational modeling for prediction of CVD [9]. This could also help to understand the role of lipoproteins in more detail.

Diverse separation methods have been described for lipoprotein fractionation, giving rise to different classifications of lipoprotein subfractions depending on the analytical separation mechanism [10]. These techniques include: (density gradient) ultracentrifugation [11], size exclusion chromatography (SEC) [12] gel electrophoresis [13], capillary zone electrophoresis [14], isotachophoresis [15], immunoaffinity chromatography [16], field-flow fractionation [17], precipitation [18], nuclear magnetic resonance (NMR) [19] or ion mobility mass spectrometry [20]. However, difficulties are encountered in most of the methods, including: high sample amount requirements, alterations in the original lipoprotein composition during the analytical procedure, limited resolution of particle subtypes, lack of robustness, lengthy procedures, or the need to use mathematical calculations for data transformation, like in NMR. In addition, several methods do not allow collecting subfractions of interest. Table S-1 summarizes the most important characteristics (separation resolution, automation, fraction collection, possible degradation, required sample volume) of reported lipoprotein separation techniques. Ultracentrifugation is considered as the "gold standard" for lipoprotein separation, allowing fractionation of lipoprotein particle types and also subclasses according to density, which is the classical classification criteria for lipoproteins. However, its technical demands make this method non suitable for routine clinical use, although being especially useful for producing lipoprotein standards [8]. Capillary isotachophoresis (cITP) is a very powerful alternative for lipoprotein profiling due to its ability to separate several lipoprotein subparticle types in a few-minute single run. Moreover, very small sample volumes can be analyzed with cITP, also offering easy automation and on-line coupling possibilities, in contrast to some of the alternative techniques. Separation of ions takes place in a discontinuous system, where the sample is injected between a leading electrolyte (LE), with the highest mobility in the system, and a terminating electrolyte (TE), with lower mobility than those ions of interest in the sample. Such a system ensures isotachophoresis, where concentrating and self-sharpening effects take place [21]. Once equilibrium is reached, analytes are separated into adjacent zones arranged according to their electrophoretic mobilities. The analyte concentrations can be described according to the Kohlrausch regulating function [21]. So-called spacer molecules, possessing intermediate mobilities in comparison to those of the analyte zones, can be added to the sample in order to separate analytes into discrete peaks, thus greatly improving the separation resolution.

With respect to cITP of lipoproteins, a method developed by Schmitz et al. [22], and further improved [15,23,24] was applied routinely in dozens of studies related to cardiovascular disease [3,25,26]. These cITP methods employ anionic mode separation in the presence of 9 spacer molecules [15] achieving 8 discrete lipoprotein peaks. For selective detection of lipoproteins, lipophilic staining reagents are available: NBD-C6-Ceramide [15,27] (for laser induced fluorescence (LIF) detection), and Sudan Red 7B [24], or Sudan Black B [27] (for UV/vis detection). Lipoprotein ITP separations are challenging as they should be performed in anionic mode at basic pH and coated capillaries are required for prevention of lipoprotein adsorption and for EOF suppression [28]. However, long-term stability of capillary coatings at basic pH can be critical [24,29]. However, in order to consolidate cITP as a reference technique for lipoprotein profiling, a significant improvement in separation resolution and therefore in sublipoprotein fractionation is still needed, which would have tremendous benefits for clinical analysis and diagnostics.

In conclusion, there is still a pressing demand for improved separation methods to enable more detailed fractionation of sublipoprotein populations in serum with the possibility to subsequently apply another biochemical analysis. Herein, a new cITP method is reported for higher resolution and reproducibility of lipoprotein profiling in serum/plasma than reported before [15]. A novel 24 spacers mixture was fine-tuned using software-based (Peakmaster) [30] prediction of electrophoretic behavior and tested on human serum. The separation of 18 lipoprotein peaks was

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