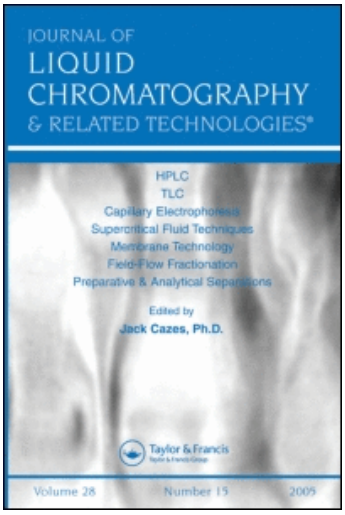

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Separation of Apolipoprotein A-I from Human Plasma by On-Line Two Dimensional Liquid Chromatography

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Abstract: On-line two dimensional liquid chromatography (2D LC) was used to determine apolipoprotein A-I (apo A-I) in human plasma without sample pretreatment. The water soluble apolipoproteins were separated from other plasma proteins and lipids by reversed phase (RP) LC in the first dimension using acetonitrile/20 mM aqueous ammonium acetate as mobile phase. A strong anion exchange (SAX) column was subsequently employed in the second dimension to separate the apo A-I in the apolipoprotein fraction using pH gradient elution with simple buffer mixtures of ammonium acetate and ammonium formate as mobile phase. Compared to other methods requiring separation of high density lipoprotein (HDL) fraction by ultracentrifugation and delipidation of apolipoproteins with organic solvents, loss of apo A-I is avoided in the on-line 2D approach. The concentration of apo A-I in plasma, determined by using the calibration curve for commercial apo A-I, was found to be 1.12 mg/mL. The identity of apo A-I isolated from human plasma was confirmed by trypsination and peptide mass fingerprinting (PMF).

Keywords: Apolipoprotein A-I, pH Gradient anion exchange liquid chromatography, Two dimensional liquid chromatography, Human plasma, Isoelectric point (pI), High density lipoprotein

INTRODUCTION

The high density lipoproteins (HDL) are comprised of proteins known as apolipoproteins and non-covalently bound lipids that play a central role in reverse cholesterol transport.[1] High plasma levels of HDL cholesterol are inversely correlated to the development of coronary artery disease (CAD).[2] Recent

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studies have suggested that some of the apolipoproteins, including the major apolipoprotein constituent of HDL, apolipoprotein A-I (apo A-I) which constitutes 70% of the HDL protein content,[3] are better discriminators of CAD risk than the total HDL cholesterol levels.[4,5] An inverse relationship between CAD and apo A-I levels has also been shown to exist.[5,6]

HDL from human plasma/serum is typically isolated by ultracentrifugation and delipidated by liquid-liquid extraction (LLE) with an organic solvent to remove the lipids from the apolipoproteins. Following delipidation, the protein composition of HDL can be analyzed by electrophoresis, immunoassay, anion exchange liquid chromatography (LC) or reversed phase (RP) LC. The ultracentrifugation followed by organic delipidation is time consuming and can result in partial loss of apolipoproteins.[7,8] A rapid procedure for measuring apo A-I directly in plasma without ultracentrifugation and LLE delipidation is, therefore, needed. There are only a few studies about direct measurement of apo A-I in plasma/serum. Liebich et al.[9] used capillary electrophoreses (CE) to measure apo A-I directly from human serum by utilizing Bio-Rad LLV buffer. In another study, Atwal et al.[10] used gel filtration LC to quantitate apo A-I directly from guinea pigs plasma after precipitating the very low density lipoproteins (VLDL) and low density lipoproteins (LDL) with a standard precipitating reagent.

In addition to RP,[11–14] anion exchange is another LC based separation technique used for separation of apolipoproteins.[15–18] Generally, a salt gradient is applied for separation of proteins using anion exchange LC. However, as shown in our previous study,[19] the use of pH gradient elution instead of salt gradient elution may provide better separation. The separation of proteins during pH gradient elution is predominantly based on the isoelectric point (pI) values of the proteins.[19]

The focus of this study was to develop a simple technique for determination of apo A-I directly in human plasma, omitting prefractionation steps as HDL isolation by ultracentrifugation and delipidation by LLE. A two dimensional (2D) LC technique was developed employing RP in the first dimension and pH gradient anion exchange in the second dimension. The separation of apolipoproteins from other plasma proteins and lipids was performed during the first dimension RP analysis. A strong anion exchange (SAX) column was subsequently employed in the second dimension to separate the apo A-I in the apolipoprotein fraction, using pH gradient elution with simple buffer mixtures of ammonium acetate and ammonium formate as mobile phase.

EXPERIMENTAL

Chemicals and Materials

The apolipoproteins A-I (apo A-I) and A-II (apo A-II) and high density lipoprotein fraction (HDL) were purchased from Calbiochem (part of Merck

Biosciences Darmstadt, Germany). Diethyl ether, human serum albumin (HSA), human transferrin, hydrochloric acid (HCl), and ion pair chromatography grade (≥99.0%) sodium dodecyl sulphate (SDS) were obtained from Sigma-Aldrich (Steinheim, Germany). HPLC grade (≥99.0%) ammonium acetate and ammonium formate, analytical grade ≥99.0% ammonium bicarbonate and sodium phosphate, HPLC grade formic acid (FA) and trifluoroacetic acid (TFA), and HPLC gradient grade water and methanol were obtained from Fluka (Buchs, Switzerland). HPLC grade acetonitrile (ACN) was purchased from Rathburn Chemicals Ltd. (Walkerburn, UK). Nitrogen (99.99%) was purchased from AGA (Oslo, Norway). Polyimide coated fused silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA). The 1.0 i.d. × 150 mm strong anion exchanger PL-SAX analytical column (5 μm, 1000 Å), the 2.0 id × 20 mm PL-SAX trap column (5 μm, 1000 Å), and the 1.0 i.d. × 5 mm Kromasil C18 trap column (5 μm, 100 Å) were purchased from G&T SepTech (Kolbotn, Norway). The 4.6 i.d. × 50 mm polymeric based PLRP analytical column (5 μm, 300 Å) was obtained from Polymerlabs (MA, USA). The 0.3 i.d. × 150 mm Jupiter Proteo column (4 μm, 90 Å) for peptide separation was purchased from Phenomenex (Torrance, CA, USA).

Preparation of Buffers, Standard Protein Mixtures, HDL, and Plasma Samples

A stock solution of 500 mM ammonium acetate and 500 mM ammonium formate was prepared by dissolving appropriate amounts of each buffer in 500 mL HPLC gradient grade water. The stock solution was filtered through 0.45 μm Minisart-plus filters from Sartorius (Göttingen, Germany) and stored at 4-5°C for no more than two weeks. Working buffer solutions were prepared by appropriate dilution of the stock solution with water to desired buffer concentrations, and then titrated with 3.0 M HCl to obtain the desired pH. The working buffer solutions were degassed with 99.998% helium from AGA before use. Blood was drawn after at least a 12 hr fast, into tubes containing EDTA and immediately placed on ice. Plasma was separated by centrifugation at 2000 g for 10 min at 4°C. Prior to injection, plasma samples were passed through a 0.45 μm low protein adsorption regenerated cellulose filter from Alltech (Deerfield, IL, USA), and 2 fold diluted with water containing 20% acetonitrile and 0.1% TFA. For anion exchange LC, standard solutions of apo A-I, apo A-II, HSA and transferrin were prepared by dilution with start buffer (A) to a final concentration of 0.2 mg/mL. For RP LC, standard solutions of the above proteins were prepared by dilution with solvent A (water and 0.1% TFA). The HDL was prepared as described under the following section. All solutions were stored at 4°C.

Delipidation of Commercial HDL

Delipidation Using Methanol-Diethyl Ether Liquid-Liquid Extraction (LLE)

HDL was delipidated according to the procedure described by Osborne.[20] Briefly, HDL (200 μL) was delipidated with methanol (1.5 mL) and diethyl ether (3.5 mL). The precipitated proteins retained in the methanol fraction were dried under vacuum at room temperature and then dissolved in 20 mM ammonium bicarbonate buffer, pH 8. The protein solution was filtered through a 0.45 μm low protein adsorption regenerated cellulose filter (Alltech), prior to injection.

Delipidation Using Temperature and Sodium Dodecyl Sulphate (SDS)

HDL was also delipidated according to the procedure described by Kinoshita.[21] Briefly, HDL (40 μL) was added to 1 mL of 0.1 M phosphate buffer (pH 7.0) containing 0.1% SDS. The mixed solution was incubated at 60°C for 5 min using a Mistral 880 oven from Spark Holland Instrument (Emmen, The Netherlands).

Delipidation Using RP LC

HDL was separated into apolipoproteins and lipids according to the procedure described by Watkins et al.,[7] with small modifications: we used a 5 cm long polymeric based RP column (instead of a C_{18} cartridge), TFA instead of formic acid and the separation was monitored using an UV-detector at 280 nm. Forty μL HDL was added to 1 mL of water containing 0.1% TFA and 20% ACN, and 10 μL of this solution were loaded on the RP column. The apolipoproteins were separated from the lipids using isocratic elution: 40% of solvent A (water and 0.1%TFA) and 60% of solvent B (ACN and 0.1% TFA). The HDL lipids were eluted from the column with 100% of solvent C (isopropanol and 0.1% TFA) according to.[7]

2D LC Chromatographic System

Two Agilent Series 1100 capillary gradient pumps (Palo Alto, CA, USA) with an incorporated on-line vacuum degasser were used to deliver the mobile phases. A 6-port Valco Cheminert C2 injection valve from Valco Instruments (Houston, TX, USA) with a 10 μL external injection loop was used for manual injections, and a 6-port Valco cheminert C2 switching valve, with a trap column, was used to interface the two dimensions. The detection was performed at 280 nm using a Wellchrom K-2600 UV-detector from Knauer

(Wissenschaftliche Geratebau, Germany) (first dimension) and a Waters 486 tunable UV-detector (Milford, MA, USA) (second dimension). The separation profile was monitored using Science Workshop Data Studio software from PASCO (Rosewille, CA, USA).

2D LC: RP × Anion Exchange LC of Plasma Proteins

Off-Line 2D LC

The first dimension separation of plasma proteins was performed on a PLRP column (4.6 i.d. × 50 mm) using the following mobile phases: water and 0.1% TFA (solvent A), ACN and 0.1% TFA (solvent B), and isopropanol and 0.1% TFA (solvent C). The RP separation gradient profile (%B) used was: 35% − 45% B in 5 min, 45% B in 5 min, then, 45-80% B in 5 min following lipid elution with 100% of solvent C at a flow rate of 0.6 mL/min. The isolated apolipoprotein fraction was manually collected into 1.5 mL polypropylene tubes, the solvents were evaporated under a gentle nitrogen stream, and the residue redissolved in the start buffer and loaded on the second dimension strong anion exchange (SAX) analytical column for further separation of apolipoproteins according to pI values. The SAX analytical column (1.0 i.d. × 150 mm) was initially presaturated for 30 min with the start buffer (A) (pH 6.6) containing a mixture of ammonium acetate and ammonium formate at 40 mM concentrations. The elution step was performed with a low pH elution buffer (B), adjusted to pH 3.5 with 3M HCl, having the same buffer composition and concentration as (A). The elution gradient profile (% B) used was: 0-100% B in 25 min and 100% B for 10 min, followed by 30 min equilibration with 100% A. The flow rate was 25 μL/min.

On-line 2D LC

For the on-line 2D LC separations, TFA in solvent A and B (preceding section) was replaced with 20 mM ammonium acetate, pH 6.6, in order to focus the apolipoproteins on the SAX trap column. The separated apolipoprotein fraction (first dimension) was trapped on the SAX trap (2.0 i.d. × 20 mm) column and back flushed from the trap column with the same gradient as the one used during the separation on the second dimension SAX analytical column in the off-line 2D LC approach.

Tryptic Digestion

The separated apo A-I was collected into 1.5 mL polypropylene tubes for the purpose of tryptic digestion, and subsequent mass spectrometry (MS) analysis

using electrospray ionisation time-of-flight (ESI TOF). Immediately after separation, the collected apo A-I fraction was evaporated under a gentle nitrogen stream and the residue was redissolved in 80 μL 50 mM ammonium bicarbonate buffer, pH ~ 7.8. Twenty μL of 0.1 μg/μL sequential grade porcine trypsin (Promega) was added to the 80 μL protein sample, making a final sample volume of 100 μL. The mixture was incubated at 37°C for 2 h. The trypsination was stopped by adding 10 μL of 5% formic acid (FA). The digestion of the commercial Apo A-I (0.1 mg/mL) was performed using the same procedure.

Column Switching Capillary LC ESI TOF MS of Tryptic Peptides

The tryptic digests were analyzed by column switching capillary LC ESI TOF MS. The column switching system used for enrichment and separation of tryptic peptides consisted of an LC isocratic loading pump (L-7100, Hitachi, Tokyo, Japan) and an Agilent Series 1100 capillary gradient pump. The valves used were a 6-port Valco cheminert C2 injection valve with external loop for sample introduction, and a 6-port Valco Cheminert C2 switching valve with a precolumn for sample enrichment. Fifty microliters of the tryptic digests were loaded and enriched on the Kromasil C18 trap (1.0 i.d. × 5 mm) column using a 50 μL sample loop. The loading mobile phase consisted of water and 0.1% FA. Back flushing from the trap column was performed with the same gradient as the one used during the separation on the Jupiter Proteo analytical (0.3 i.d. × 150 mm) column. The mobile phases used were: HPLC gradient grade water with 0.1% FA (solvent A) and ACN with 0.1% FA (solvent B), at a flow rate of 5 μL/min. The gradient profile used for solvent B was as follows: 3-15% B in 3 min, 15-30% B in 25 min, 30-70% B in 5 min, and 70% B for 4 min, followed by 15 min equilibration with 3% B. Peptide analysis was performed with a Micromass LCT TOF MS (Micromass, Manchester, UK). The TOF MS was equipped with a Z-spray atmospheric pressure ionization source for ESI, which was modified to handle flow rates in the low μL/min range. Ionization was performed in positive mode. The following voltages were used: 3.0 kV on the capillary, 20 V on the sample cone, and 3 V on the extraction cone. In order to obtain a stable spray performance and aid solvent vaporization, nebulizer gas and desolvation gas were applied at 50 L/hour and 250 L/hour, respectively. Nitrogen was used as nebulizer and desolvation gas. The TOF MS instrument was controlled by MassLynx v4.0 software, and mass spectra were acquired in the m/z range 300–2000. The Mr of the tryptic fragments were measured as monoisotopic masses. Doubly and triply charged peptides were manually deconvoluted to obtain singly protonated molecular ions $[MH]^+$ for database search.

Protein Identification

Protein identification was accomplished using the Mascot software from Matrix Science (London, UK). Peptide mass fingerprinting (PMF) was performed using experimental monoisotopic masses from ESI TOF MS experiments to search the Swiss Prot protein database. The Mascot search parameters were held constant for the data generated in this study. These include the variable modification, i.e., oxidation of methionine, cleavage by trypsin, one missed cleavage, and no restriction on the pI value or Mr. The peptide mass tolerance was set to 0.2 Da. Positive protein identification was based on standard Mascot criteria for PMF.

RESULTS AND DISCUSSIONS

Generally, identification and quantitation of apolipoproteins in serum or plasma involves tedious sample preparation steps, e.g., density ultracentrifugation and delipidation using organic solvents, which can result in partial loss of apolipoproteins.[7,8] The aim of this study was, therefore, directed towards determination of apolipoprotein A-I (apo A-I) directly in human plasma using a simple and fast sample preparation procedure.

Separation of Commercial Apo A-I and A-II by pH Gradient Anion Exchange LC

The major apolipoproteins of HDL apo A-I and A-II constitute approximately 70 and 25% of the HDL protein content, respectively.[3] Although RP LC[11–14] has been used to separate apolipoproteins from the HDL fraction, separation of the isoforms of apo A-I and A-II has been difficult to achieve.[11,13] Separation of apolipoproteins has been successfully performed on anion exchange column materials using salt gradient elution.[15–18] In our previous study,[19] we have shown that resolution of proteins can be improved when a pH gradient is employed during elution instead of a salt gradient. The pH gradient separation of proteins is mostly according to their isoelectric point (pI) values. Thus, based on the pI values of the major isoforms of apo A-I and A-II, 5.6 and 4.9,[22] respectively, it was reasonable to believe that separation could be achieved on a strong anion exchange (SAX) column using pH gradient elution. Ammonium acetate and ammonium formate buffers were used to generate gradient pH elution in the pH range from 6.6 (buffer A) to 3.5 (buffer B). Figure 1 shows the separation of the commercial apo A-I and A-II using pH gradient elution with linear gradient elution (0-100% B in 25 min). The use of volatile buffers, such as ammonium acetate and formate was favourable, since the sample was to be subjected for further analysis, e.g., tryptic digestion.

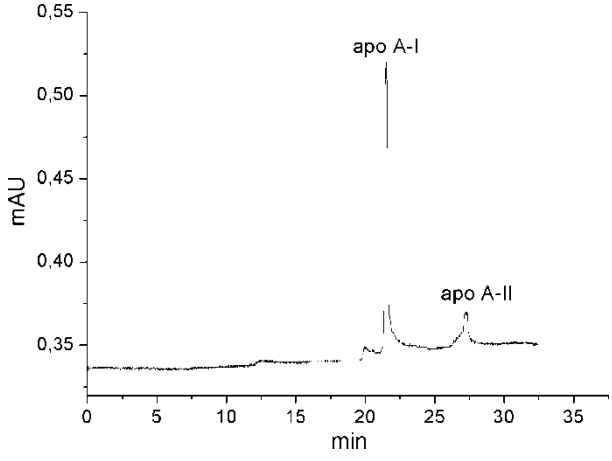

Figure 1. Separation of commercial apo A-I and A-II on the 1.0 i.d. × 150 mm SAX column. The SAX column was initially presaturated for 30 min with the start buffer (A) (pH 6.6) containing a mixture of ammonium acetate and ammonium formate at 40 mM concentrations. The elution step was performed with a low pH elution buffer (B) (pH 3.5), having the same buffer composition and concentration as start buffer (A). The elution gradient profile (% B) used was: 0–100% B in 25 min, and 100% B for 10 min at a flow rate of 25 μL/min.

Delipidation of Commercial HDL

Generally, identification and quantiation of apo A-I in plasma is performed after HDL isolation by ultracentrifugation, and HDL delipidation using liquid-liquid extraction (LLE) with organic solvents. In this study, four extraction procedures were investigated in order to find the best extraction conditions for apo A-I from the commercial HDL fraction: (1) the conventional delipidation method using methanol-ether LLE (Figure 2A), (2) undelipidated HDL (Figure 2B), (3) delipidation using temperature and SDS (Figure 2C), and (4) delipidation using RP LC (Figure 2D). The best extraction was obtained using the latter procedure. Elution of apolipoproteins from RP columns is commonly observed in the range of 50–55% ACN.[11–14] Based on these studies, and in order to be certain that apolipoproteins were completely eluted from the RP column, 60% ACN was used during their elution. The differences in the retention factors of the apolipoproteins and the lipids enabled separation of the apolipoproteins from the lipids under the elution conditions. The strongly retained lipids were eluted from the RP column with acidified (0.1% TFA) isopropanol, according to Watkins et al.[7] In addition to the differences in the retention factors, delipidation of apolipoproteins was further enhanced by the use of ACN and TFA in the mobile phase. The non-covalent protein-lipid bonds are

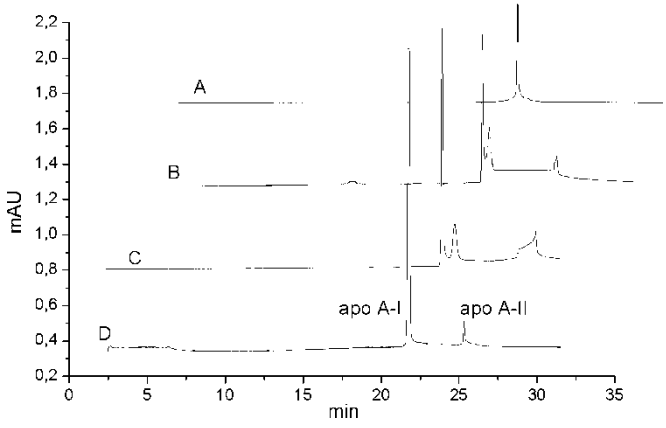

Figure 2. Analysis of commercial HDL: (A) Delipidation using methanol-diethyl ether LLE, (B) dilution with start buffer, (C) delipidation using temperature and SDS and (D) delipidation using RP LC. Separation of the apolipoproteins was performed on the 1.0 i.d. × 150 mm SAX column and the chromatographic conditions were the same as in Figure 1.

cleaved and HDL is dissociated into apolipoproteins and lipids by the combination of TFA and ACN.[23,24]

Isolation of Apo A-I from Human Plasma by 2D LC: RP × pH Gradient Anion Exchange LC

Off-line 2D LC

Initially off-line 2D LC, employing RP in the first dimension and pH gradient anion exchange chromatography in the second dimension, was performed for separation of apo A-I in plasma. Plasma proteins were separated from lipids on the RP column using the isocratic elution profile developed for commercial HDL (preceding section). However, this simple protein lipid separation procedure could not be directly adapted for plasma samples. This is due to the complexity of plasma, and the fact that two of the major plasma proteins, i.e., human serum albumin (HSA) and transferrin have pI values close to that of apo A-I. We assumed that HSA (pI 5.8[25] for the major isoform) and/or transferrin (pI 5.4[26] for the major isoform) probably would interfere during the second dimension separation of apo A-I on the SAX column. This was examined by analyzing commercial standards of transferrin, HSA and apo A-I (data not shown), and it was found that HSA and apo A-I coeluted, while transferrin was separated from both. Consequently, a preseparation of apolipoproteins from HSA by RP chromatography (first dimension) was included. In order to optimize the separation conditions, a

mixture of commercial standards of apo A-I and HSA was used. Due to their differences in retention factors, separation of apo A-I from HSA in the first dimension RP column was easily achieved (Figure 3A). Plasma apolipoproteins were readily separated from other proteins (Figure 3B), using the same elution conditions. Immediately after RP separation, the apolipoprotein fraction was collected on ice, evaporated under a gentle nitrogen stream, and the residue was redissolved in start buffer and injected on the second dimension SAX column. The separation of the apolipoprotein fraction obtained on the SAX column is shown in Figure 4. The chromatogram shows the apo A-I peak in addition to apo A-II and some proteins eluting in the front (probably apo C variants).

Quantitation of Apo A-I

The concentration of apo A-I in human plasma was measured using a calibration curve made from analysis of standard solutions of commercial apo A-I. The peak area of the commercial apo A-I standard was linearly related to concentration over the range 0.125-2.0 mg/mL ($R^2 = 0.998$). The concentration of apo A-I in human plasma, determined using the calibration curve, was found to be 0.68 ± 0.091 mg/mL ($n = 6$). The measured concentration is lower than the actual concentration of apo A-I in plasma (1.0-1.5 mg/mL),[23] and was in accordance with the low recovery $62 \pm 11\%$ ($n = 4$)

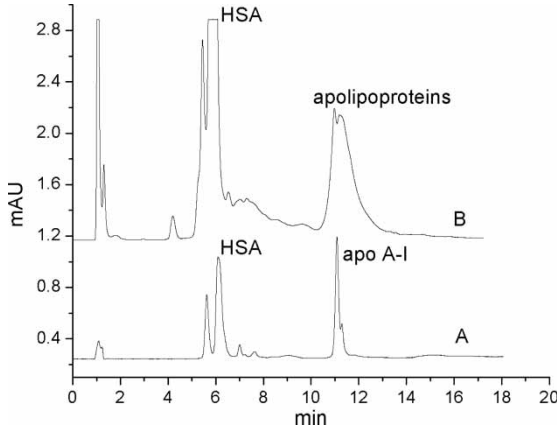

Figure 3. First dimension RP separation using the off-line 2-D LC setup: (A) separation of HSA and apo A-I standards and (B) separation of 2-fold diluted plasma. The separation was performed on a PLRP column (4.6 i.d. × 50 mm) using water and 0.1% TFA (solvent A), ACN and 0.1% TFA (solvent B) and isopropanol and 0.1% TFA (solvent C). The gradient profile (% B) used was: 35%-45% B in 5 min, 45% B in 5 min then 45-80% B in 5 min following lipid elution with 100% of solvent C (isopropanol and 0.1% TFA). The flow rate was 0.6 mL/min.

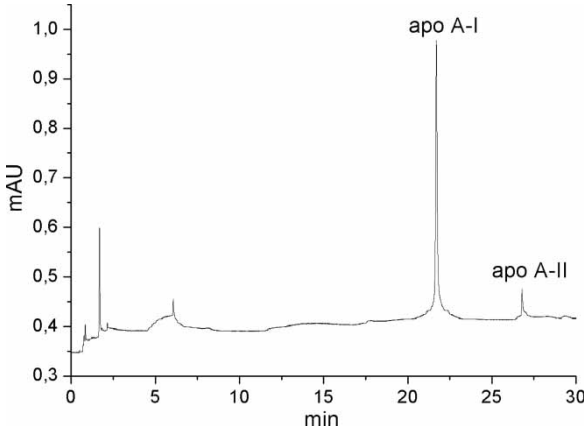

Figure 4. Second dimension SAX separation of apo A-I using the off-line 2D setup. The chromatographic conditions were the same as in Figure 1.

obtained with the off-line method using commercial apo A-I. The recovery was measured by analysis of commercial apo A-I (0.5 mg/mL) subjected to the same off-line procedure as plasma samples. The low recovery obtained with the off-line 2D LC system is most probably due to sample loss during the sample transferring steps, i.e., collection, evaporation, etc.

On-Line 2D LC

In order to minimize sample loss during analysis and, hence to increase the method recovery, on-line 2D LC was investigated. However, the acidic mobile phase (containing 0.1% TFA) used in first dimension in the off-line 2D LC method, could not be adapted for the on-line 2D LC. The pH of the mobile phase during separation in the first dimension RP column had to be 6.6 or higher, in order to get sufficient retention on the SAX trap column. Hence, TFA was replaced with various buffers (ammonium acetate, phosphate, ammonium bicarbonate) which were tested to optimize resolution. The separation of the plasma proteins obtained with different buffers was essentially the same (data not shown). Ammonium acetate buffer (pH 6.6) was, however, chosen since it was already used in the second dimension. Plasma samples were 2-fold diluted (in water containing 20% ACN and 0.1% TFA) and loaded on the first dimension RP column, and the apolipoprotein fraction was eluted as illustrated in Figure 5A. Subsequently, this fraction was trapped on the SAX trap column, back flushed from the trap column and separated on the second dimension SAX analytical column (Figure 5B). The capacity of the SAX trap column used in the on-line 2D LC system was examined by injecting 10 μL of commercial HDL (containing approx. 50% apolipoproteins and 50% lipids) at three different concentrations: 5, 10, and

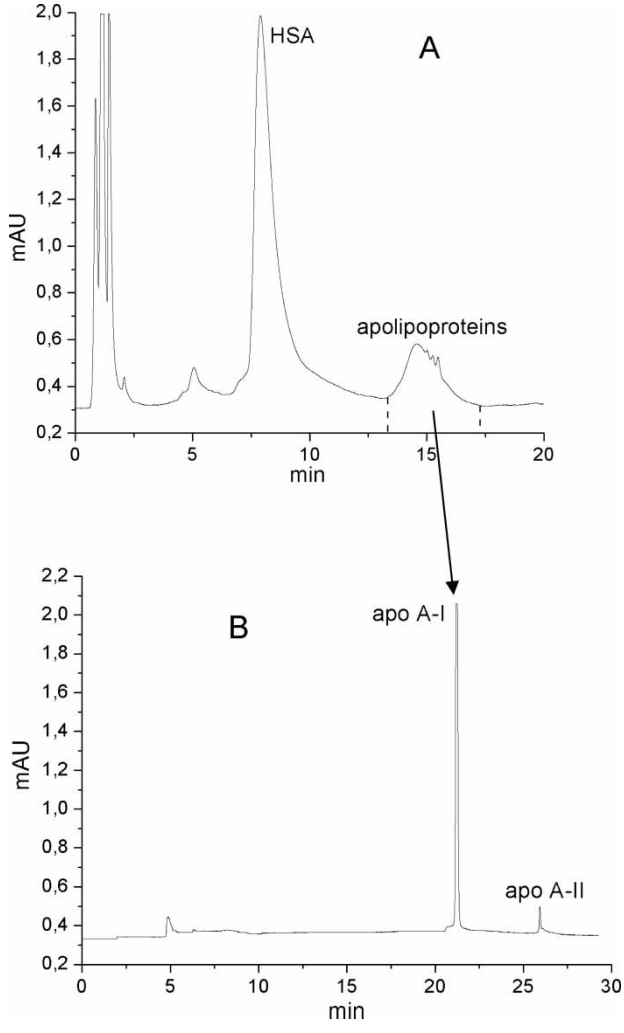

Figure 5. (A) First dimension RP separation of 2-fold diluted human plasma using the on-line 2D LC setup. The chromatographic conditions were the same as in Figure 3, except that TFA in solvent A and B was replaced with 20 mM ammonium acetate, pH 6.6. (B) Second dimension SAX separation of apo A-I using the on-line 2D LC setup. The chromatographic conditions were the same as in Figure 1.

20 μg/μL (apolipoprotein concentration was approx: 2.5, 5 and 10 μg/μL, respectively). No breakthrough was observed for these concentrations when the first dimension mobile phase was run for 15 min. The capacity of the trap column was, therefore, satisfying with regard to the actual concentration of apolipoproteins in plasma (2.5-3.5 μg/μL)[22] and the practical trapping time (less than 5 min). The second dimension separation of the apolipoprotein

fraction, obtained on the SAX column (Figure 5B) using the on-line 2D LC system, was identical with the one obtained during the off-line 2D LC system (Figure 4), except that of the peak height of apo A-I, which was approx. two times higher with the on-line 2D LC system. The concentration of apo A-I in human plasma, determined using the calibration curve obtained for commercial apo A-I, was found to be 1.12 ± 0.077 mg/mL ($n = 6$). The measured concentration was within the actual concentration of apo A-I in plasma (1.0-1.5 mg/mL),[23] and in accordance with the recovery $97 \pm 8\%$ ($n = 4$) obtained with the on-line 2D LC method using commercial apo A-I. The recovery was measured by analysis of commercial apo A-I (0.5 mg/mL) subjected to the same on-line procedure as plasma samples. The method is selective for apo A-I determination and the total analysis time is less than one hour.

Identification of Apo A-I Isolated from Human Plasma Using 2D LC

In order to verify the identity of apo A-I isolated from human plasma, using this 2D LC method, the fraction containing apo A-I was tryptic digested and analyzed by ESI TOF MS, and compared to tryptic peptides derived from commercial apo A-I. In order to inject large sample volumes and thus facilitate detection of low abundance peptides, a column switching capillary RP LC system was used for the analysis of the tryptic peptides. Satisfying separation of tryptic peptides was achivied on the Proteo RP column, as illustrated by the base peak intensity (BPI) chromatogram in Figure 6. Figure 6A shows

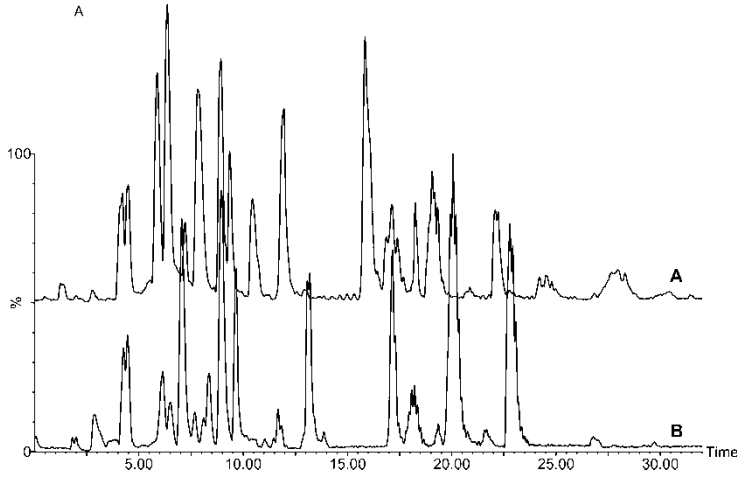

Figure 6. Base peak intensity (BPI) chromatogram of tryptic peptides from commercial apo A-I (A) and apo A-I isolated from plasma (B) obtained with column switching capillary LC ESI TOF MS.

the BPI chromatogram of tryptic peptides obtained from commercial apo A-I, while 6B shows the BPI chromatogram of tryptic peptides obtained from apo A-I isolated from plasma. When triply and doubly charged tryptic peptides, obtained from apo A-I isolated from plasma, were deconvoluted to singly charged peptides and searched against the Swiss Prot protein database, 17 peptides matched the human apo A-I (Accession no. P02647) with 55% protein sequence coverage and a Probability Based Mowse Score (Mascot) of 210. The number of peptides matching the sequence coverage, and the Mascot score obtained for apo A-I isolated from plasma, was highly satisfying with regard to unambiguous identification. In addition, the results obtained for apo A-I isolated from human plasma were highly comparable with results obtained for the commercial apo A-I (21 matching peptides, 65% sequence coverage and a Mascot score of 239).

CONCLUSIONS

An on-line 2D LC method for selective determination of apo A-I in human plasma without sample pretreatment has been developed. The apolipoprotein fraction was isolated from human plasma in the first RP dimension followed by apolipoprotein separation in the second dimension employing a strong anion exchange (SAX) column subjected to a descending pH gradient. Compared to other methods requiring separation of HDL by ultracentrifugation and delipidation of apolipoproteins by LLE, no loss of apo A-I is observed using the on-line 2D LC method. The method is selective for apo A-I determination and the total analysis time is less than one hour. This on-line 2D LC system can readily be automated.

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