A Streamlined Method for Quantification of Apolipoprotein A1 in Human Plasma by LC-MS/MS

To the Editor:

Apolipoprotein A1 (apoA1) is the major protein component of high-density lipoprotein particles in blood, and its concentration in serum and plasma is a marker of atherosclerotic cardiovascular diseases. Clinical laboratories commonly use immunometric methods to measure apoA1; however, an alternative approach, LC-MS/MS, has been proposed (1–3). Uptake of these LC-MS/MS methods for routine testing in a clinical setting has been challenging owing to complex and time-intensive sample preparation work flows. Overnight digestion has been previously applied for quantification of apoA1 by use of proteolytic peptides VQPYLDDFKQ, DYVSQFEGSALGK, and THLAPYSDELR (1, 2) and a 3-h digest using peptide VQPYLDDFKQ (3). Toward the design of a streamlined workflow for implementation in a clinical laboratory, we simplified sample preparation by using only additive steps and eliminating the use of chemical denaturants, reduction, and alkylation. Herein we demonstrate that such a simple and rapid work flow, targeting a fast-forming proteolytic peptide, can be used to develop a quantitative apoA1 LC-MS/MS assay in agreement with an established immunonephelometric method.

Our optimized protocol generated several proteotypic peptides suitable for apoA1 quantification by using a brief 20-min digestion without reduction or alkylation steps or the use of chemical denaturants. On the basis of several factors including the 24-h digestion profile (4), THLAPYSDELR was selected for further development. To generate the external 6-point calibration curve, the unlabeled THLAPYSDELR peptide was synthesized, with purity determined by high-performance LC and amino acid analysis (New England Peptide). The lyophilized peptide stocks were initially solubilized in a 5% acetonitrile and 0.1% formic acid solution and further diluted with a solution of PBS and rat plasma (ratio of 2:43:5), generating 6 peptide calibration solutions ranging from \(6.52 \times 10^{-7}\) to \(9.75 \times 10^{-15}\) mol/L, based on the manufacturer-determined mass and purity, corresponding to 20–300 mg/L of apoA1 protein. The internal standard, stable isotopically-labeled THLAPYSDELR* peptide (C-terminal \(^{13} \text{C}^{15} \text{N}-\text{Arg}\)), was prepared by dissolving in 5% acetonitrile and 0.1% formic acid, and a total of 7 pmol of labeled peptide was added to all samples [i.e., quality controls (QCs), calibrators, and patient samples] before denaturation and trypsin digestion.

Rat plasma was selected as the surrogate matrix for the calibrators because (a) the THLAPYSDELR sequence was not found in the rat proteome and (b) we observed no cross-reactivity of endogenous apoA1 in rat plasma with the immunonephelometric Siemens BNII N Antiserum to Human Apolipoprotein A-I assay. Peptide calibration concentrations were assigned with EDTA plasma specimens by immunonephelometry. Briefly, plasma specimens with apoA1 concentrations determined by immunonephelometry (range, 0.29–2.38 g/L) were run as standards by LC-MS/MS and the to-be peptide calibrators were analyzed as unknown samples. The immunonephelometry-assigned values of the 6 peptide calibrator solutions corresponded to 18–516 mg/L of apoA1 protein; these peptide calibrators were used in subsequence experiments. A limitation of peptide- vs protein-based calibrators and internal standards is the potential for bias associated with incomplete protein digestion and peptide decay during the, traditionally, lengthy digestion phase (5). However, by developing a reproducible and rapid digestion process and assigning peptide calibrators against protein standards, a peptide-based approach can achieve comparable analytical performance characteristics.

QC material was prepared by pooling human EDTA plasma with target means determined by LC-MS/MS: QC1 = 0.45, QC2 = 1.26, QC3 = 1.77 g/L. Patient samples were prepared as follows: 5 µL of EDTA human plasma was diluted in 45 µL of PBS. A volume of 10 µL of the PBS-diluted plasma and 10 µL of the internal standard solution (0.7 pmol/µL) were added to the digestion buffer (490 µL of 50 mmol/L ammonium bicarbonate in water). After addition of the internal standard, all samples (i.e., QCs, calibrators, and patient samples) were denatured by heating at 99 °C for 10 min with shaking at 1000 rpm on an Eppendorf ThermoMixer C. After denaturation, samples were left to cool at room temperature, and 30 µL of 5 µg/µL N-tosyl-l-phenylalanine chloromethyl ketone–treated trypsin was added. Samples were incubated at 37 °C for 20 min, with the process quenched by the addition of 310 µL of buffer composed of 3% acetonitrile, 1% formic acid, and 0.5% acetic acid. A volume of 12 µL of the quenched solution was injected into the LC-MS/MS (Phenomenex Aeris Peptide 3.6-μm XB-C18 column, Shimadzu LC 20AD, and SCIEX Triple Quad 5500) and run on a 10-min gradient at a flow rate of 0.25 mL/min. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in acetonitrile. The LC gradient was 5% B from 0 to 1 min, 5%–56% B from 1 to 4 min, 56%–95% B from 4 to 4.5 min, 95% B from 4.5 to 8 min.
The lower limit of the measuring interval, characterized by a CV /H11021/20% and signal-to-noise ratio /H11022/10, was 0.01 g/L. Precision was assessed by analyzing 5 replicates of each QC level on 5 different days. CVs were calculated as per the CLSI EP5 guideline: the intraassay CVs were 9.5% (QC1), 6.6% (QC2), and 7.5% (QC3); the interassay CVs were 9.3% (QC1), 5.0% (QC2), and 11.1% (QC3); and total CVs were 13.3% (QC1), 8.3% (QC2), and 13.3% (QC3).

A method comparison of the LC-MS/MS method with the Siemens BNII assay was performed with 61 human EDTA plasma samples. The analysis was performed over 3 days, whereby individual samples were analyzed with both methods on the same day. The comparison revealed the following by least squares regression: LC-MS/MS = 1.00 × immunonephelometry + 0.07, R² = 0.9031, CI_slope = 0.912, 1.082 (Fig. 1).

While traditional multiday, bottom-up proteomics work flows are tailored to maximize sequence coverage and protein identifications, targeted quantitative clinical assays have different objectives and constraints, and thus, a different approach to sample preparation is warranted. By using cost-effective reagents, streamlining sample preparation, and optimizing conditions of heat denaturation and digestion, we have demonstrated that a simple and rapid work flow can be used for quantification. The design of such clinically tailored work flows aims to increase the feasibility of implementing quantitative protein LC-MS/MS assays in clinical laboratories.

### Author Contributions

All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.

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